Universidade do Minho
Escola de Ciências

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Biochemical and molecular approaches to study the effect of calcium-based supplements on grape berry (*Vitis vinifera L.*) quality

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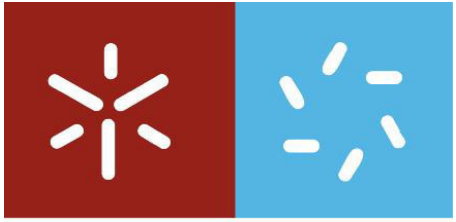

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Biochemical and molecular approaches to study the effect of calcium-based supplements on grape berry (*Vitis vinifera L.*) quality

Dissertação de Mestrado

Biologia Molecular, Biotecnologia e Bioempreendedorismo em Plantas

Trabalho efetuado sob a orientação da

Doutora Viviana Maria Varajão Martins

e do

Professor Doutor Hernâni Varanda Gerós

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BIOCHEMICAL AND MOLECULAR APPROACHES TO STUDY THE EFFECT OF CALCIUM-BASED SUPPLEMENTS ON GRAPE BERRY (*VITIS VINIFERA L.*) QUALITY

Grapevine is one of the most important crops worldwide. The wine sector is of utmost relevance for national economies and the most dynamic of agriculture export in several countries. Grapevines require very specific conditions to grow appropriately; however, a large proportion of vineyards, such as those in the Mediterranean basin, are located in regions where soil and atmospheric water deficits, and high temperatures, may exert large constraints on yield and quality. Calcium-based supplements have been widely used to improve fruit firmness and prevent cracking, being good candidates to ameliorate grape berry mechanical properties and then resistance to abiotic and biotic stresses. However, the effect of calcium (Ca^{2+}) supplementation on grape berry metabolism still requires further investigation. In this study, molecular and biochemical approaches were combined to investigate the effect of Ca^{2+} in secondary metabolism of grape cells, using cultures of Gamay Fréaux var. Teinturier as model. Cells were elicited with 10 mM $CaCl_2$, alone or in combination with 20 µM MeJA or 20 µM ABA, which are two strong inducers of secondary metabolism in fruits. The expression of genes that encode for key enzymes of secondary metabolism pathways was studied by real-time PCR, including phenylalanine ammonia lyase (PAL), from phenylpropanoid pathway, stilbene synthase (STS) from stilbene pathway and UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT) that catalyzes a major limiting step in anthocyanin biosynthesis. In addition, genes encoding anthocyanin transporters (AM1 and ABCC1) were also investigated, as well as the expression and activity of the cell wall enzymes pectin methyl esterase (PME) and polygalacturonases (PG1 and PG2). In general, cells elicited with calcium exhibited an increased expression of most of the target genes. In contrast, gene expression was downregulated by Ca^{2+} in cells elicited with MeJA. Spectrophotometric studies demonstrated that the activity of UFGT and PG was significantly reduced by the presence of Ca^{2+} in all treatments. Altogether, the results showed that Ca^{2+} regulates enzyme activity at both gene expression and protein levels. Field experiments were performed in vineyards of *cv.* Vinhão to test the effect of the exogenous application of Ca^{2+} on biochemical and physical parameters of the fruit. Results showed that important biochemical parameters, including berry fresh weight, berry water content, °Brix, pH, titratable acidity (TA), were not significantly different between control and Ca^{2+}-treated berries. However, berry firmness was significantly modified in response to Ca^{2+} because the force needed to compress or perforate the berries was significantly higher when grapevines were treated with $CaCl_2$. Altogether, both *in vitro* and field experiments showed that Ca^{2+} plays important roles in fruit development and fruit physical properties, and open good perspectives for its application in the field as an efficient supplement to improve fruit quality and resistance against pathogen attack and abiotic stress.

ABORDAGENS BIOQUÍMICAS E MOLECULARES PARA O ESTUDO DO EFEITO DE SUPLEMENTOS DE CÁLCIO NA QUALIDADE DO BAGO DE UVA (*VITIS VINIFERA L.*)

A videira é uma das plantas perenes com maior importância ao nível mundial. O setor vitivinícola é de extrema relevância para as economias nacionais, com um peso elevado ao nível do setor exportador de muitos países. A videira requer condições muito específicas para crescer adequadamente; no entanto, o rendimento e qualidade do fruto de uma grande proporção de vinhas, como as cultivadas na bacia do Mediterrâneo, são afetados negativamente por condições ambientais adversas, como o stresse hídrico e temperaturas elevadas. Suplementos à base de cálcio (Ca^{2+}) têm sido testados para melhorar a firmeza dos frutos e evitar o rachamento, sendo bons candidatos para melhorar a resistência mecânica do bago de uva e, consequentemente, a resistência aos stresses biótico e abiótico. No entanto, são necessários estudos mais aprofundados para se elucidar o efeito da suplementação de Ca^{2+} no metabolismo do bago de uva. Neste estudo foram combinadas abordagens bioquímicas e moleculares para se elucidar o efeito do Ca^{2+} no metabolismo secundário das células do bago, tendo-se recorrido a culturas de células pigmentadas obtidas a partir da variedade Gamay Fréaux var. Teinturier. As células foram tratadas com 10 mM de $CaCl_2$ ou com 10 mM de $CaCl_2$ em combinação com 20 μM de MeJA ou 20 μM de ABA, duas hormonas vegetais que estimulam o metabolismo secundário. Abordagens de PCR em tempo real permitiram estudar a expressão de genes que codificam enzimas-chave de vias do metabolismo secundário, incluindo a fenilalanina amónia liase (PAL), da via dos fenilpropanóides, a estilbeno sintase (STS), da via dos estilbenos, e a UDP-glicose:flavonóide 3-O -glucosiltransferase (UFGT), que catalisa um passo limitante na biossíntese das antocianinas. Foi ainda estudada a expressão dos genes que codificam os transportadores de antocianinas (AM1 e ABCC1), bem como a expressão e atividade das enzimas da parede celular, pectina metil-esterase (PME) e poligalacturonases (PG1 e PG2). Genericamente, os resultados mostraram que o tratamento com Ca^{2+} estimula a expressão da maioria dos genes alvo. Em contraste, o tratamento com Ca^{2+} e MeJA reprimiu a expressão da maioria dos genes em estudo. As atividades bioquímicas da UFGT e da PG foram significativamente reduzidas na presença de Ca^{2+}. Os resultados obtidos mostraram que o Ca^{2+} regulada a atividade enzimática ao nível da expressão genética e ao nível da pós-tradução. Foram realizados estudos de campo em vinhas da casta Vinhão para testar o efeito da aplicação exógena de Ca^{2+} em parâmetros bioquímicos e físicos do bago. Os resultados mostraram que parâmetros bioquímicos importantes, incluindo o peso fresco e o teor de água do bago, o grau de Brix, o pH, a acidez titulável (TA), não foram significativamente diferentes em vinhas tratadas com Ca^{2+} relativamente ao controlo. No entanto, a firmeza do bago foi significativamente modificada em resposta ao Ca^{2+} porque a força necessária para comprimir ou perfurar os bagos foi significativamente maior quando as videiras foram tratadas com $CaCl_2$. No seu conjunto, os estudos in vitro bem como as experiências de campo mostraram que o Ca^{2+} desempenha papéis importantes no desenvolvimento do fruto e influencia a suas propriedades físicas, abrindo excelentes perspetivas para a sua aplicação no campo como um suplemento eficiente para melhorar a qualidade do fruto e estimular as defesas contra o ataque de agentes patogénicos e condições ambientais adversas.

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Abreviations And Acronyms

ABA	Abscisic acid
ABC	ATP binding cassette
ABCC1	ATP binding cassette, subfamily C
AM1	*Antho*MATE1
ATP	Adenosine triphosphate
cDNA	Complementary DNA
CHS3	Chalcone synthase
coA	Coenzyme A
CTAB	Cetrimonium bromide
cv.	Cultivar
Cyt	Cytosolic
DF	Dilution factor
DFR	Dihydroflavonol-4-reductase
DNA	Deoxyribonucleic acid
DOC	Controlled Designation of Origin
EDTA	Ethylenediaminetetraacetic acid
FLS	Flavonol synthase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Reduced glutathione
HGA	Homogalacturonic acid
MATE	Multidrug and toxic extrusion
MeJA	Methyl jasmonate
mRNA	Messenger RNA
MW	Molecular weight
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
PG	Polygalacturonase
PME	Pectin methylesterase
PVP	Polyvinylpyrrolidinone
qPCR	Quantitative real-time PCR
RNA	Ribonucleic acid

r.p.m	Rotation per minute
SD	Standard deviation
STS	Stilbene synthase
TA	Titratable acidity
Tris	Tris(hydroxymethyl)aminomethane
UFGT	UDP-glucose: flavonoid 3-O-glucotransferase
Var.	Variety
Vv	*Vitis vinifera*
x*g*	Relative centrifugal force (to Earth's gravitational acceleration)

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1 Introduction

1.1 Economic and social value of the grape berry

Grapevine is considered one of the most valuable horticultural crops in the world and grape berries are the second most produced fruit, being cultivated worldwide. In 2014, the estimated surface area of grape cultivations was about 7.5 million ha, with 75 million tonnes produced. It is one of the oldest cultivated plants in a variety of environments, and thus very adaptive (Giribaldi and Giuffrida 2010). Its economic impact is mostly due to its various uses. Almost 50% of grapes are used to make wine, about one third is used as fresh product and the rest is dried, consumed as juice or used to make liquor and raisins. Due to the versatility of grapes and the size of the global grape crop, the grape market has a substantial role in global food consumption (Gang 2013).

In the last few years, the interest in products derived from grapes has increased, in the same extend that also increased the attention related to the benefits of the biologically active dietary components present in this fruit. Processed or freshly consumed grapes possess nutritional value and health benefits for humans making them desirable in human diet (Gang 2013). The main contributors for their nutritional value are the secondary metabolites, including the famous antioxidant resveratrol (Giribaldi and Giuffrida 2010). Furthermore, wine, one of the most important grape berry derived product, has a cultural and social connotation. For instance, in both the Greek and Roman traditions as well as the Christian and Jewish ones, wine has always been considered a symbol of life and of prosperity. Beyond that, wine is a product which people often associate with festive and convivial moments in their lives (Ferrarini et al. 2010).

Due to the importance of grape berry and its derived products, efforts have always been made in the study of berry development and ripening. The improvement of cultivation practices and increase of yield and quality of the final product is a priority for producers.

1.2 Grape berry in a challenging environment

Grape berry quality includes a complex combination of factors that are related with berry chemical composition, including sugars, acids, phenolics, and many others. Several factors like environmental, endogenous or management practices can lead to a change in the composition and concentration of these compounds during berry development (Mirás-Avalos and Intrigliolo 2017). In recent years, the knowledge about the influence of the environment on grape berry growth and development has increased considerably.

The most studied factors affecting grape berry include biotic and abiotic stresses. Most of grapevine varieties are susceptible to biotic agents such as phytoplasma, bacteria, fungi, oomycetes, viruses and nematodes. The responses of plants to these agents are characterized by their ability to recognize pathogen components, transduce the stress signal and induce a defence response. Depending on the type of pathogen, different responses are originated on the plant. Some of these agents feed on living tissue, maintaining the viability of the host to obtain metabolism products, and others can secret lytic enzymes and phytotoxins, promoting necrosis in the host plant (Armijo et al. 2016).

Common abiotic stresses, which can be defined as environmental conditions that reduce growth and yield below optimum levels, include drought, salinity, temperature, and acid soils (Cramer et al. 2011). The effects of water stress on grapevine have been widely studied, once water availability has a large impact on grape production, quality, and economic value (Mirás-Avalos and Intrigliolo 2017). The increase of global temperature and the intensification of climatic changes such as drought, heat waves or heavy rains, makes water stress effects on grapevine one of the main concerns for viticulture (Medrano et al. 2015). On the other hand, irrigation water and soils are becoming gradually more saline, mainly in semi-arid areas. A high concentration of solutes in the soil, which causes a water deficit stress, and altered K^+/Na^+ and NO^{3-}/Cl^- ratios are the main responsible for stress in plants caused by salinity (Mohammadkhani 2013). These stress agents have a wide range of effects on the process of grape berry ripening, affecting the content of total soluble solids (fructose, glucose, sucrose) and flavonoids (particularly the flavonols and anthocyanins), among others. Additionally, water stress can also affect abscisic acid (ABA) levels and transcripts of cell wall enzymes (Kuhn et al. 2014). Furthermore, these environmental conditions are known as major reasons for the incidence of microscopic cracks in the cuticle membrane of berries.

The cuticle forms the outer surface of grape berry epidermal cell walls (see below) and plays a critical role in the susceptibility of grape berry to pathogen, since they act as a barrier for pathogen defence (Becker and Knoche 2012; Herzog, et al 2015). Previous studies have shown that cracks facilitate the penetration of *Botrytis cinerea* (Commenil et al 1997). Thus, genotypes with thick berry cuticles are more resistant to cracking than genotypes with thin cuticles, making more difficult to *B. cinerea* penetrate the berries (Herzog et al 2015). Physical resistance to infection also depends of the cell wall structure and composition of the cells of the grape berry skin (Deytieux-Belleau et al. 2009). Moreover, in other fruits, like sweet cherry, rain-induced cracking before harvest is the most important crop loss in many producing areas, seriously compromising fruit quality (reviwed by Balbontín et al. 2013).

The constrains posed by the stress agents mentioned above, require a deeper knowledge of plant responses to these factors to further adapt new management techniques in order to decrease the negative impacts on grape berry yield and quality.

1.3 Grape berry development and composition

The grape berry is composed by three major types of tissue: skin, flesh and seed (figure 1). The skin, in particular, which comprises an outer epidermis, covered by a cuticle and wax, and collenchymatous hypodermal cell, has a crucial role in the protection and support of grape berry (Considine and Knox 1979). The chemical composition of the tissues that constitute grape berry strongly influences the final quality of the fruits.

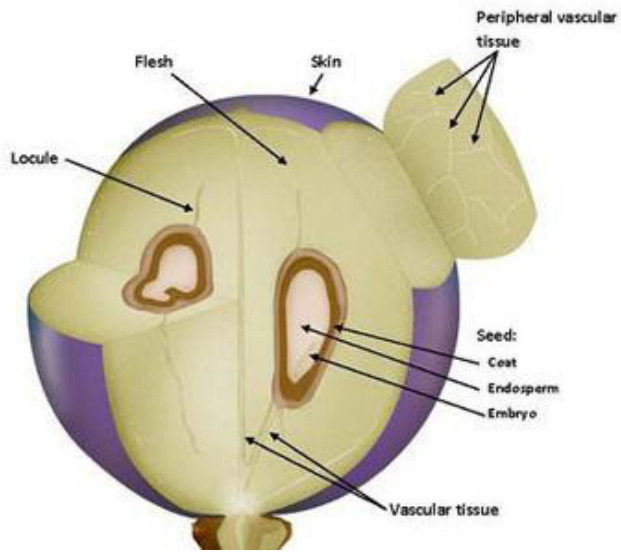


Figure 1 – Structure of a grape berry (Mcglynn 2012) .

The growth pattern of grape berries exhibits two successive sigmoid cycles with distinctive biophysical and biochemical characteristics. The two phases of rapid growth are separated by a lag phase, characterized by little or no growth, as illustrated in figure 2 (Davies et al 1997). The first phase, which mainly corresponds to berry formation, largely determines the size and shape of the berry. This phase begins with a pericarp cell division, followed by cell expansion, that later slows as the first sigmoid cycle ends (Coombe 1992). In this stage, several compounds are accumulated such as the organic acids malic, tartaric and hydroxycinnamic. Tannins, including the monomeric catechins, are also accumulated during this period and several phenolic compound precursors are synthetized. Hence, at this period, berries are small, hard, green and with little sugar (Coombe and McCarthy 2000; Kuhn et al. 2014)

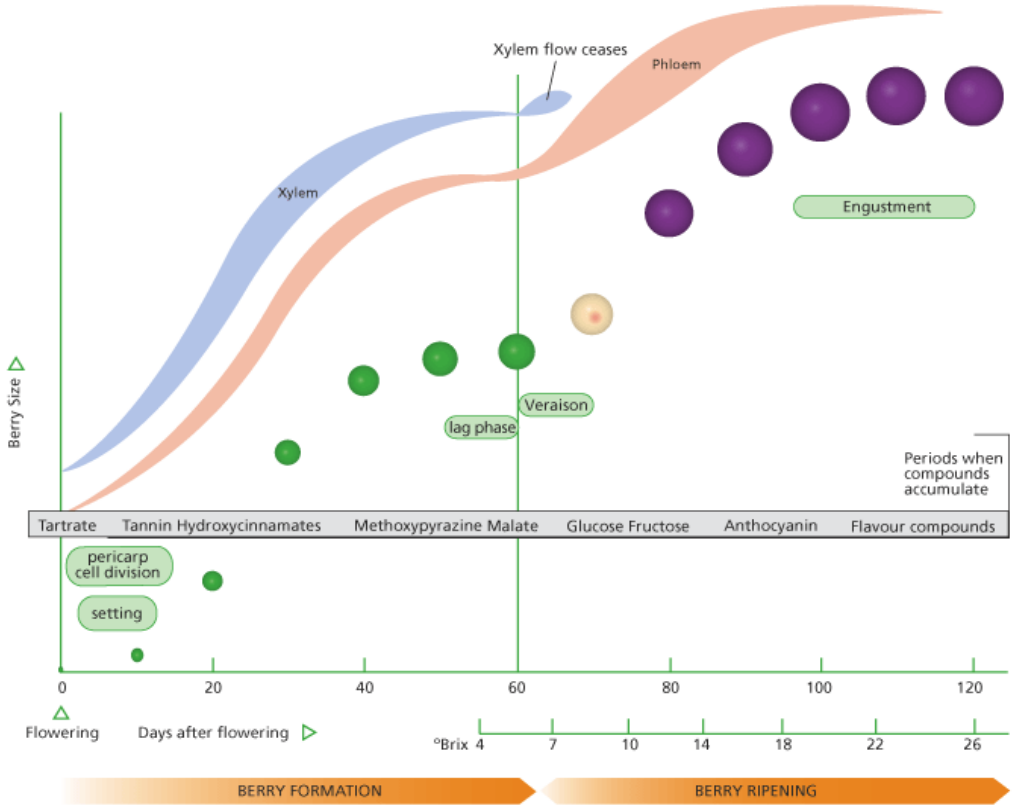


Figure 2 – Diagram of grape berry development and ripening (adapted by Mpelasoka et al. 2003).

During the lag phase the concentration of acids reaches its highest levels. This phase immediately precedes the véraison stage, a French word coined to describe the change in berry skin colour (Conde et al. 2007; Letchov and Roychev 2017).

After véraison berries continue to grow, as a consequence of the enlargement of mesocarp cells that accumulate large amounts of water. During this phase, the most significant changes in berry composition take place. The acidity decreases, mostly due to degradation of malic acid, making tartaric acid the predominant one. This period is also characterized by berry softening and accumulation of sugars, such as glucose and fructose, which reach, at this stage, their maximum levels. As ripening continues, phenolic compounds such as anthocyanin pigments, responsible for berry colour of red cultivars, and aromatic and flavour compounds are accumulated (Coombe and McCarthy 2000; Letchov and Roychev 2017).

1.3.1 Mineral elements in the grape berry

The role of water, carbon and mineral supply has been studied over the years due to its undeniable role on plant development. In grape berry, the import of mineral nutrients is extremely important since they are involved in the development and physiology of the fruit, being essential to sustain its growth and equilibrium. Macro and micronutrients such as nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), sulfur (S), magnesium (Mg), copper (Cu), iron (Fe), among others, are crucial for proper metabolic functioning. Some of the major macronutrients associated to growth, yield and berry quality have been a target of study because of the wide range of functions they play in the cell. Elements such as K have essential roles in enzyme activation, membrane transport and osmotic regulation. N determines grape berry growth, being responsible for the formation of proteins, enzymes and coenzymes, nucleic acids, chlorophyll, vitamins and hormones. Among all the other macronutrients indispensable for plant survival, Ca is the one with the most outstanding role in the structure of grape berry, being also a central element in cell signalling. Given the importance of Ca in fruit integrity, a better understanding of its role in grape berry metabolism and composition is of considerable importance not only for scientists, but also for grape growers and winemakers (Martins et al. 2012). Hence, a special emphasis will be given to this macronutrient in the following sections.

1.3.1.1 Transport and accumulation of Ca^{2+} in the grape berry

In plants, Ca^{2+} is known phloem immobile, being taken up from the soil by the root system. Then, Ca^{2+} is translocated to the aerial parts of plants by symplastic and apoplastic transport via the xylem system (White 2001). The high dependence of the transpiration rates for the uptake of Ca^{2+} frequently results in low levels of this ion, leading, for instance, to membrane breakdown and/or cell wall (Hocking et al. 2016; White 2001). Ca^{2+} - deficiency symptoms in plants include poor root development, leaf necrosis and curling, blossom end rot in tomatoes, bitter pit in apples and increased susceptibility to fruit cracking, observed in fruits like tomato, cherry and grape berry among others (Simon 1978; White and Broadley 2003).

In grape berry, the Ca^{2+} content ranges depending on the cultivar and climacteric conditions. Even within the same cultivar, Ca^{2+} levels may change due to fluctuations in xylem flow.

The evolution of Ca^{2+} content in berry compartments is cultivar dependent. Ca^{2+} content in the seeds increased throughout the development of the berries, including ripening. In the flesh and skin calcium is mainly accumulated during the first stage of growth until véraison, which is in accordance with is

structural role in grape berry (Cabanne and Donèche 2003). Several studies show that after this phase Ca^{2+} accumulation may change, depending on the cultivar.

In *cv.* Chaunac, Ca^{2+} accumulation ceased at the onset of ripening (Hrazdina, et al 1984) while in Cabernet Sauvignon, Ca^{2+} accumulation did not cease but slowed temporarily at the onset of ripening (Ollat and Gaudillère 1996). Yet, in Shiraz berries showed a continuous accumulation of Ca^{2+} from set to harvest (Rogiers et al. 2000).

In grape berry cells, the movement of Ca^{2+} requires several transporters. The tonoplast contains at least one vacuolar Ca^{2+} pump (Ca^{2+} - ATPase), Ca^{2+}/H^{+} antiporters responsible for Ca^{2+} uptake and channels catalysing the efflux of Ca^{2+} from the vacuole. While this Ca^{2+} vacuolar pump mediates high-affinity Ca^{2+} transport, the Ca^{2+}/H^{+} antiporters exhibit lower affinity but higher capacities for Ca^{2+} transport (Martins et al. 2012). Since a high concentration of Ca^{2+} in the cytosol is cytotoxic, these transporters ensure that a micromolar $[Ca^{2+}]_{cyt}$ is maintained.

1.3.1.2 Ca^{2+} as a central messenger of cell growth and development

Since the beginning of the sixties that Ca^{2+} started to be noticed as a central cellular regulator in many organisms. In particular, Ca^{2+} attracted special attention because of its central role as a second messenger in plant cell growth and development.

Plants are able to respond to various stimuli by eliciting a change in the concentration of Ca^{2+}. In biotic or abiotic stress conditions there is a perturbation in $[Ca^{2+}]_{cyt}$ caused by a rapid influx of Ca^{2+}, triggering cellular responses. Studies show that an exposure of plant to low temperatures causes an instantaneous influx of Ca^{2+} ions caused by the opening of Ca^{2+} channels rather than a general cold-induced increased membrane permeability (Rincon and Hanson 1986). Several studies suggest that this elevations in $[Ca^{2+}]$ are responsible for triggering several mechanisms of cold tolerance and cold acclimation. For instance, Ca^{2+} induce the accumulation of proline, which is correlated with improved tolerance of osmotic stress and high temperature (De et al1996).

Moreover, Ca^{2+} controls membrane structure and function, having a key role in the stabilization of lipid bilayers and controlling membrane permeability (reviewed by Hepler 2005)

1.3.1.3 The role of Ca^{2+} in cell wall structure

As pointed out in several studies, Ca^{2+} levels determine cell wall structure and strength. Low concentrations of Ca^{2+} seem to promote softening of fruit flesh, which is characteristic of berry ripening. This process is related to changes of the structure and composition of pectin and hemicellulose polysaccharides of the cell wall. Pectins are a family of covalently linked galacturonic acid-rich plant polysaccharides. Pectins are abundant in walls that surround growing and dividing cells, walls of cells of soft plant tissues, and in the middle lamella and cell corners. The most abundant pectic polysaccharide is homogalacturonic acid (HGA), which is a highly methylesterified polymer that is transported to the cell wall, where it is de-esterified (figure 3).

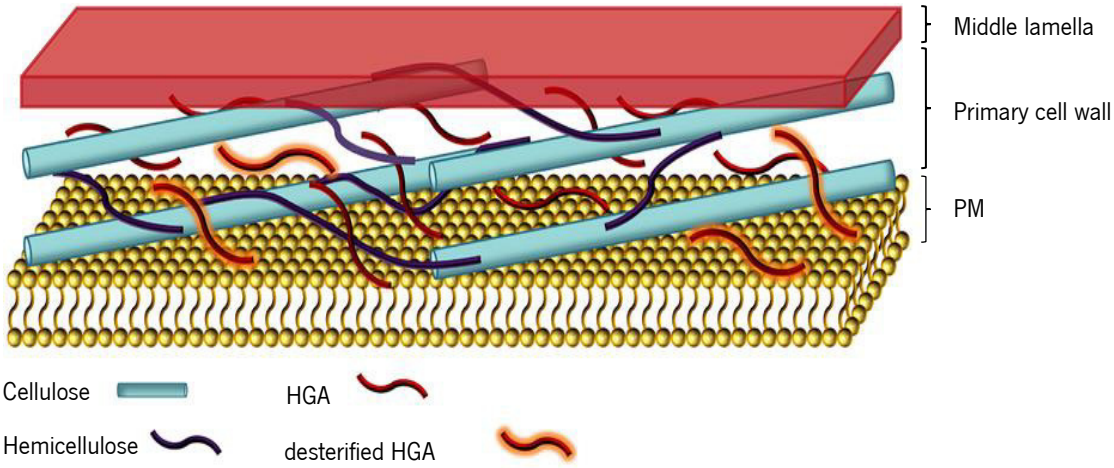


Figure 2 - Structure of middle lamella, primary cell wall (with cellulose, hemicellulose, homogalacturonic acid (HGA) and its de-esterified form) and plasma membrane (PM) (adapted by Malinovsky, 2014).

The process of de-esterification is carried out by the wall-localized enzyme pectin methylesterase (PME) (Mohnen 2008). The de-esterified HGA backbone is then susceptible to the activity of the pectin-degrading enzyme polygalacturonase (PG), thus causing fruit softening. Otherwise, the removal of methylester groups leaves a negatively-charged carboxylic acid group available for Ca^{2+} binding, forming a tight complex with pectin ("egg box" model) (Brummell 2006) (figure 4).

Increasing the number of Ca^{2+} bridges between HGA chains leads to a decrease in polygalacturonase accessibility to the polymer, thus maintaining fruit firmness. However, the mechanisms whereby Ca^{2+} reduces the rate of polygalacturonic acid breakdown are still unclear (Cabanne and Donèche 2002)

Nonetheless, low concentrations of Ca^{2+} seem to make the cell wall more pliable and easily ruptured, whereas high concentrations rigidify the wall and make it less plastic (Hepler 2005). This essential role

of Ca^{2+} in the cell walls of fleshy fruits may render protection against both abiotic and biotic stress, by thickening the cell wall. In fact, several fungi including *B. cinerea,* are able to produce endogenous PG during infection, to facilitate penetration of the fruit exocarp and colonization of the fruit surface. Thus, the high stability of the complex formed by the plant pectins cross-linked by Ca^{2+} is essential to decrease the vulnerability of the plant, by limiting the action of fungal PG (Cabanne and Donche 2002).

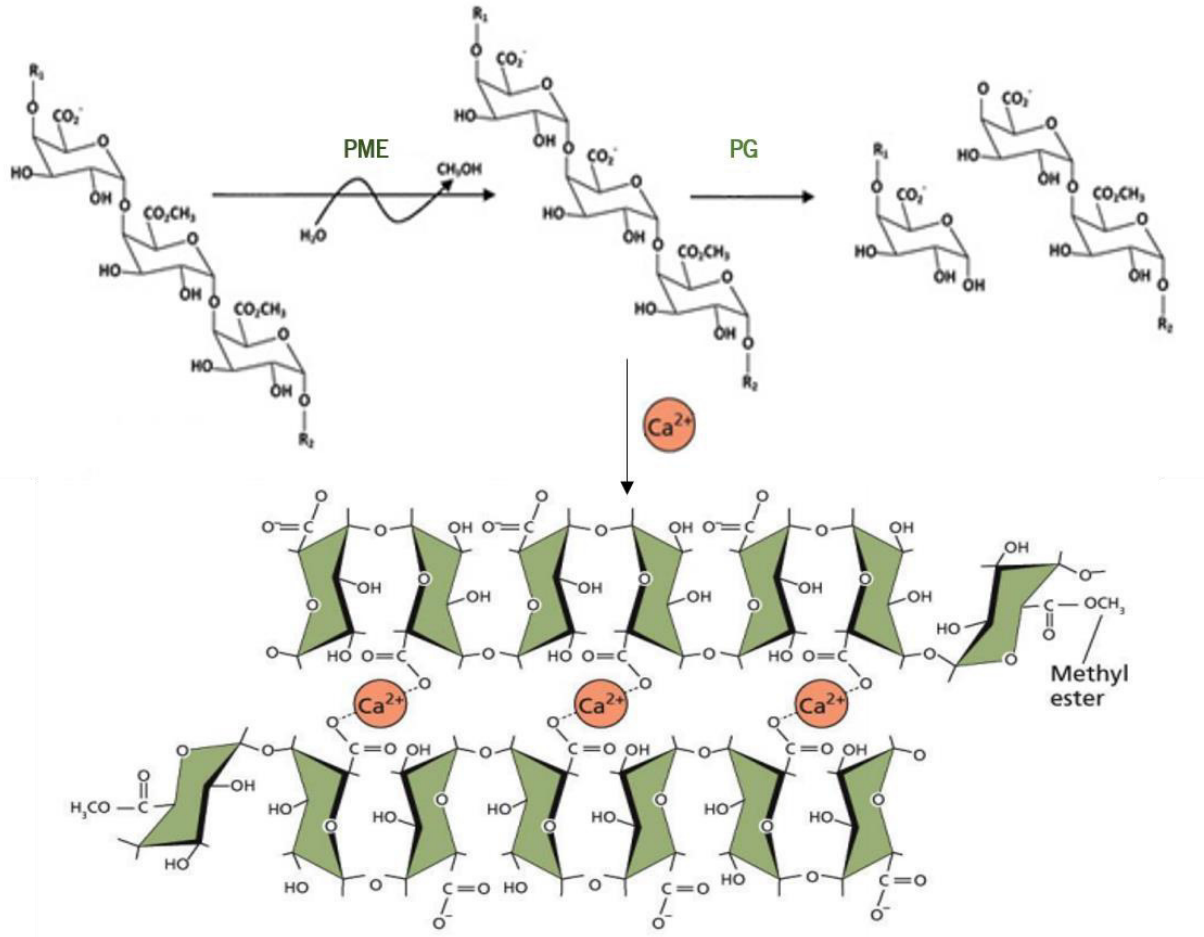


Figure 3 - De-esterification of homogalacturonic acid by pectin methylesterase (PME) and posterior cross-link with Ca^{2+} or degradation by polygalacturonase (PG) (adapted from Jolie, 2010; Taiz, 2010).

1.3.2 Phenolic compounds in the grape berry

Phenolic compounds are secondary metabolites which share a structure of a benzene ring with one or more hydroxyl groups attached. In the grape berry, phenolic compounds are present largely in skins and seeds and their concentrations depend on the grapevine variety, being influenced by viticultural and environmental factors. Many of these compounds are involved in plant protection as biologically active growth inhibitors of other living systems. They are also responsible for the colour and flavour of berry and

many of them are known for their strong antioxidant activity (reviewed by Conde et al. 2007)). Grape phenolics can be divided into two major groups: non-flavonoids (hydroxybenzoic and hydroxycinnamic acids and stilbenes) and flavonoids (anthocyanins, flavonols and flavanols).

1.3.2.1 Biosynthetic pathways underlying the formation of phenolic compounds

Phenolic compounds share the same initial biosynthetic pathway, the phenylpropanoid pathway, which comprises a complex series of branching biochemical reactions, via phenylalanine ammonia lyase (PAL) (figure 5) (Weisshaar and Jenkinst 1998)

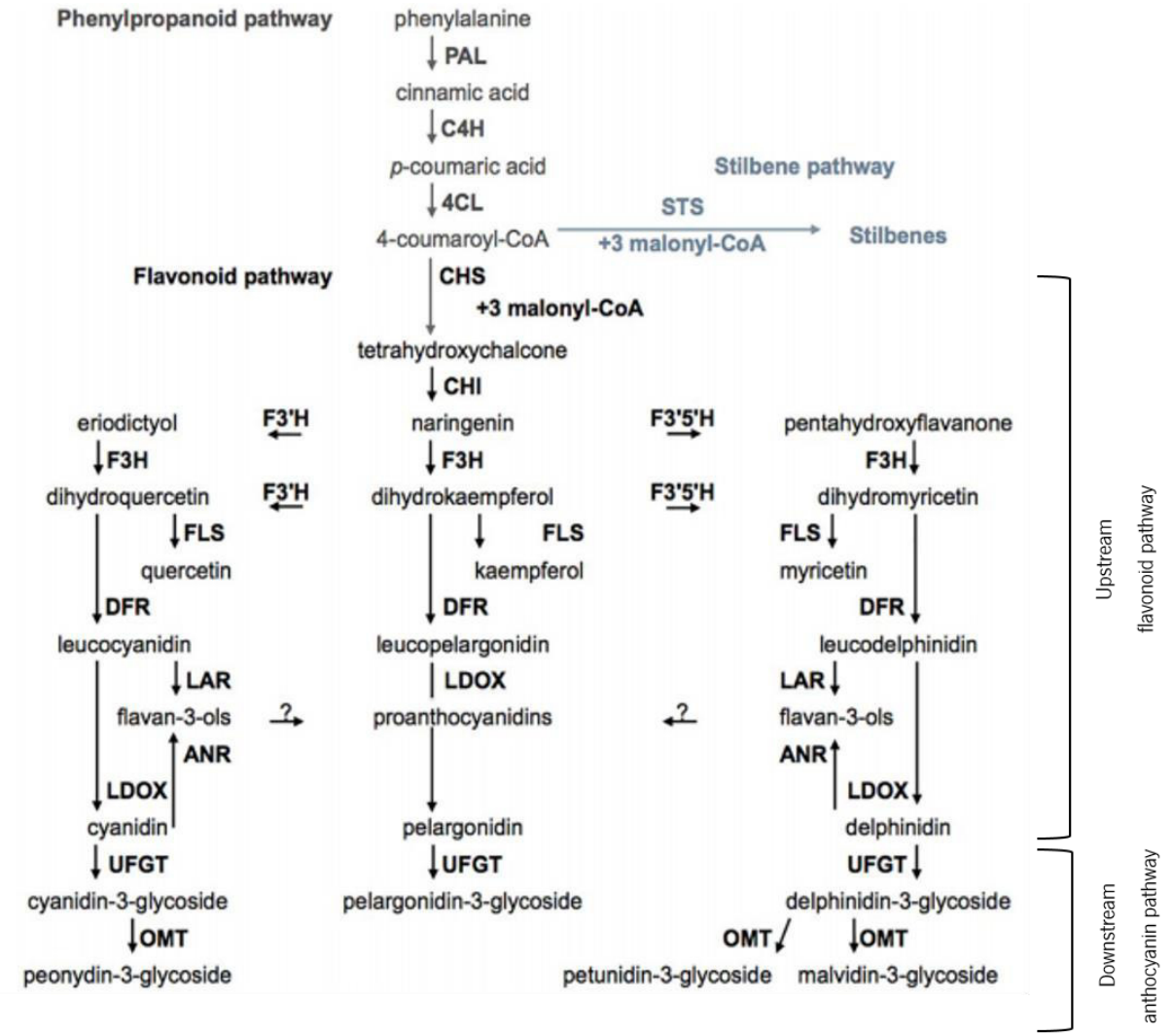


Figure 5 - Schematic representation of the phenylpropanoid pathway with emphasis on flavonoid pathway and biosynthesis of anthocyanins (adapted from Teixeira et al. 2013).

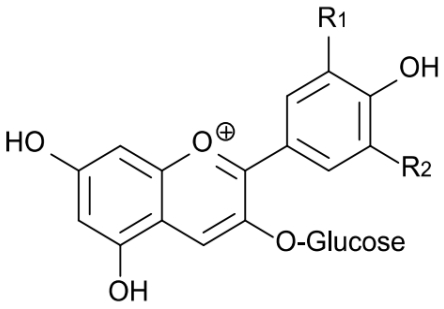
This enzyme uses phenylalanine, a product of the shikimate pathway, as substrate to produce cinnamic acid. After a few transformations, this compound leads to the formation of precursors of several simple phenolics like phenolic acids, lignin precursors, among others. Some complex phenolic compounds like flavonoids and stilbenes are also formed in the first steps of this pathway. Chalcone synthase (CHS) and stilbene synthase (STS), respectively, are the two main enzymes responsible for the production of these compounds, and compete for the same substrate: 4-coumaroyl-CoA (reviewed by Teixeira et al. 2013)). STS leads to the production of resveratrol, a stilbene that attracted significant attention due to its anti-oxidant, anti-inflammatory, cardioprotective, and anti-cancer properties (Ko et al. 2017). Otherwise, CHS catalysis the first committed step of one of the most important branches of phenylpropanoid pathway, that leads to the production of flavonols, tannins, and anthocyanins. In the first section of these branch, which is known as the flavonoid upstream pathway, the enzymes dihydroflavonol 4-reductase (DFR) and flavonol synthase lead to the production of two distinct flavonoids: anthocyanins and flavonols, respectively. Given the role of anthocyanins in grape berry defence against several stress agents and their importance as health promoting metabolites, special attention will be given to these phenolic compounds in the next section.

1.3.2.2 Structure and Functional roles of anthocyanins

Flavonoids are the most abundant phenolic compounds in grapes and wines and possess a common C15-skeleton, composed of three rings (A, B, C). They comprise different sub-categories, including flavones, flavonols, flavanones, flavanols and anthocyanins, differing by the ring C (Lorrain et al. 2013).

Anthocyanins represent the largest class of flavonoids and are specific to red grape varieties. These water-soluble vacuolar pigments may appear red, purple, or blue depending on the pH, which has a dramatic effect on their colour (Ananga et al. 2013). They execute several important physiological functions in plant cells as protective compounds in response to abiotic and biotic stresses, such as UV radiation, cold, drought, osmotic stress, microbial and viral attacks, but also to attract pollinators. Nevertheless, studies show that the positive effects of anthocyanins are not restrict to the plant kingdom. Several authors reported its benefits in human health, as being involved in the prevention of the onset and development of many diseases (Ananga et al. 2013; Francisco et al. 2013). Some health promoting effects of anthocyanins include stimulation of visual acuity and reduction of retinal damage, decreased expression of inflammatory biomarkers, diminished risk of type-2 diabetes mellitus, reduced weight gain and anti-cancerogenic activity (reviewed by Passeri et al 2016).

The accumulation of anthocyanins in the skin of red grape berry varieties, and in the flesh of Teinturier varieties, begins at véraison, and is one of the most commonly recognized features of berry ripening. Structurally, they are glucosylated derivatives of five aglycones or anthocyanidins: cyanidin, peonidin, petunidin, delphinidin and malvidin (figure 6) (Lorrain et al. 2013).



Anthocyanins

	R1	R2
cyanidin-3-*O*-gluc	OH	H
delphindin-3-*O*-gluc	OH	OH
paeonidin-3-*O*-gluc	OCH_3	H
petunidin-3-*O*-gluc	OCH_3	OH
malvidin-3-*O*-gluc	OCH_3	OCH_3

Figure 6 – Basic structure of anthocyanins (adapted from Lorrain, 2013).

The limiting step in the biosynthesis of this pigment is catalysed by the enzyme UDP-glucose: anthocyanidin: flavonoid glucosyltransferase (UFGT), which defines the second section of flavonoid pathway: the specific anthocyanin downstream branch. This enzyme is responsible for the glycosylation of anthocyanidins, which are inherently unstable under physiological conditions, leading to the production of anthocyanins (Ananga et al. 2013).

1.3.2.3 Transport of anthocyanins in the grape berry

After being synthesized in the cytosol, like many other secondary metabolites, anthocyanins are transported and stored into the cell vacuole. Scientific evidence supports two mechanisms by which anthocyanins are transported into the vacuole: membrane vesicle- and membrane transporter–mediated transport (Grotewold and Davies 2008; Zhao et al 2010). Concerning the transporter-mediated model, biochemical, molecular, and genetic evidence support the involvement of both multidrug and toxic extrusion (MATE) and the ATP binding cassette (ABC) proteins in the transport of anthocyanins (reviewed by Francisco et al. 2013) (figure 7). Recent studies showed that AM1, a protein from MATE family, is localized in the tonoplast and transports acylated anthocyanins, but not glucosylated ones. This acylated form of anthocyanins is the predominant form that accumulate in berries of most red varieties. Their transport into the vacuole by AM1 is H^+-dependent (antiport) (Gomez et al. 2009). Unlike AM1, ABCC1,

is involved in the transport of glucosylated anthocyanins, which strictly depends on the presence of GSH, that is co-transported with the anthocyanins (Francisco et al. 2013)

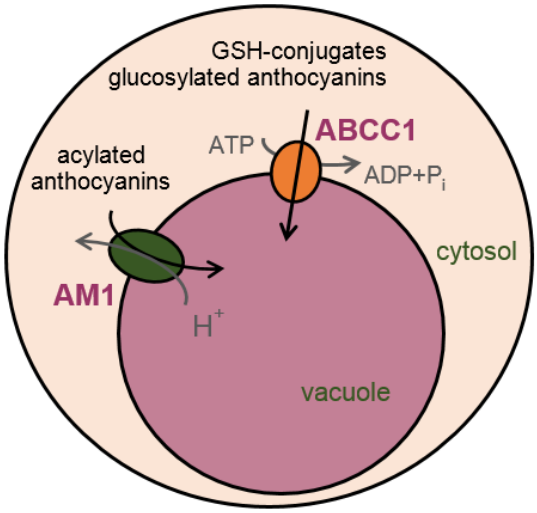


Figure 7 - Schematic representation of the transport of anthocyanins to the vacuole by ABCC1 and AM1 transporters.

1.3.3 Hormonal regulation of grape berry development

Berry ripening involves the coordination of a large number of events which are controlled by multiple regulatory pathways and occurs in an organized and developmentally timed manner. Interactions between transcriptional regulators and plant hormones regulate several processes including the initiation and progression of fruit ripening (Blanco-Ulate et al. 2017).

Different fruit species are traditionally categorized as being either climacteric or non-climacteric, depending on the physiological differences in their development (Tucker 1993). Those that show a rapid increase in respiration are known as climacteric fruits and include tomatoes, bananas, apples, pears, mangoes, and papayas. These fruits also show an enhanced production of the plant hormone ethylene at the onset of ripening. In non-climacteric fruits such as grape berry, the respiratory burst and rise in ethylene production are absent (Karlova et al. 2014) Hence, the activation of ripening events does not depend primarily on ethylene signalling (Blanco-Ulate et al. 2017).

Growth and ripening of grape berry are controlled by other plant hormones such as auxins, abscisic acid (ABA), steroids, methyl jasmonate (MeJA), among others.

It is well acknowledged that ABA is the signal that triggers berry ripening, since there is a strong increase in berry ABA content at the end of the colour turning period and during the initial stages of

ripening. Its concentration increases and peak at the onset of ripening and then declines toward harvest (Gagne et al. 2006; Kuhn et al. 2014). Beyond the stimulation of ripening processes, the application of ABA in the field specifically stimulates anthocyanin accumulation but does not seem to affect anthocyanin composition (Peppi et al 2007). Furthermore, several studies indicate that ABA effects are mediated in concert with other hormones, since ABA treatments up-regulated genes related to ethylene biosynthesis and repress putative auxin response genes (Kuhn et al. 2014). Besides modulating the processes underlying grape berry development and ripening, ABA is involved in adaptive responses to stresses, including drought, seed desiccation tolerance and dormancy (reviewed by Villalobos-González et al. 2016).

Along with ABA, MeJA - a methyl ester derivate of jasmonic acid - is also involved in plant growth regulation. MeJA participates in several processes in plants, being an important cellular regulator involved in diverse developmental processes, such as seed germination, root growth, fertility, fruit ripening, and senescence. MeJA signalling can be induced as a plant defence mechanism in response to several biotic and abiotic stresses like pathogens, drought, low temperature, and salinity (reviewed by Cheong and Choi 2003). In grape berries, several studies show that this plant growth regulator has a salient role in grape secondary metabolism. MeJA is capable of inducing the activation of several plant defence response genes, including those encoding enzymes of the phenylpropanoid pathway, modulating the biosynthesis of several compounds such as anthocyanins, flavonols, and stilbenes (reviewed by Creelman and Mullet 1997; Repka 2001; Ruiz-García et al. 2012). Importantly, MeJA acts synergistically or antagonistically with other phytohormones, such as ethylene, salicylic acid, auxin, and abscisic acid in the responses to abiotic and biotic stress (reviewed by Cheong and Choi 2003).

1.4 Objectives

The main goal of this work was to address the hypothesis that Ca^{2+} plays important roles in grape berry development and physical properties, and secondary metabolism. Two biological models were used in this study: the pigmented cell line Gamay Fréaux for *in vitro* studies and *cv.* Vinhão for field studies. Molecular and biochemical approaches, including the expression of key genes like, for instance, *VvPAL*, *VvUFGT, VvAM1* and *VvABCC1* were performed with the cell lines to understand the effect of Ca^{2+} on the production and accumulation of anthocyanins. To understand the effects of Ca^{2+} on cell wall composition and fruit firmness the activity of the key enzymes PME and PG were also evaluated. Since hormones play a major role in grape berry growth and development, the effect of ABA and MeJA and their combined effect with Ca^{2+} were also evaluated in this study.

At the field level, a solution of $CaCl_2$ was exogenously applied (sprayed) to vines *cv.* Vinhão to study the effect of Ca^{2+} on biochemical and physical parameters of the mature grape berry. The combination of *in vitro* and field studies contributed to elucidate the importance of Ca^{2+} in grape berry firmness. Moreover, this study provided a deeper understanding of the possible advantages of the exogenous application Ca^{2+} aiming at the production of berries and wines with improved quality.

2 MATERIAL AND METHODS

2.1 Cell suspension cultures and growth conditions

Suspension cells of *V. vinifera* L. *cv.* Gamay Fréaux var. Teinturier, gently provided by Prof. Serge Delrot from UMR 1287 laboratory of University of Bordeaux (France), were grown in liquid medium containing Gamborg B5 nutrients and vitamins. The medium was supplemented with 88 mM sucrose, 250 mg L^{-1} of casein hydrolysate, 0.1 mg L^{-1} 1-naphthaleneacetic acid and 0.2 mg L^{-1} kinetin. Cell suspensions were maintained in 250 mL flasks on a rotatory shaker at 100 r.p.m., under a 200 µmol photons m^{-2} s^{-1} light regime, at 24°C and 10 mL aliquots were transferred weekly into 30 ml of fresh medium.

2.2 Stimulation of secondary metabolism in Gamay cultures and elicitation

To study the effect of calcium on secondary metabolism and its possible interactions with hormones associated to fruit ripening and stress response, cells cultivated for three days in a medium supplemented with 2 mM $MgSO_4$, 2 mM NH_4SO_4, and 2.2 mM $Na_2H_2PO_4$ (induction medium) (Larronde et al. 1998), were elicited for 12 h with: 1) 10 mM $CaCl_2$; 2) 20 µM ABA; 3) 20 µM MeJA; 4) 10 mM $CaCl_2$ + 20 µM ABA; and 5) 10 mM $CaCl_2$ + 20 µM MeJA. Cells were collected by vacuum filtration, ground in liquid nitrogen and stored at -80 °C for further experiments.

2.3 Expression studies by real-time PCR

2.3.1 RNA extraction and cDNA synthesis

The extraction of the total RNA was performed according to the method described by Reid et al. 2006, with a buffer containing 300 mM Tris-HCl (pH 8), 2% CTAB, 2% PVP, 25 mM EDTA, 2 M NaCl, 2% β-mercaptoethanol. RNA was purified with GRS total Plant RNA Kit according to the manufacturer's instructions. Total RNA was quantified by Nanodrop and RNA integrity was analyzed in an agarose gel. The conversion of mRNA to cDNA was performed by reverse transcription with Omniscript® RT Kit and oligodT primers (Qiagen), using 3 µg of total RNA.

2.3.2 Real-time PCR

Real-time PCR was performed to study the expression of several genes involved in secondary metabolism pathways including anthocyanin biosynthesis and cell wall composition, using a BioRad CFX96 qPCR Instrument. The Luminaris HIGR qPCR Master Mix kit was used following the manufacturer's instructions, using 1 µL of diluted (1:10) cDNA, 5 µl Master Mix, 300 nM of each primer (forward and reverse) and nuclease-free water in a final reaction mixture volume of 10 µl. All reactions of qPCR were made in triplicate for each biological sample. Amplification was performed under the following conditions: 15 min at 95 °C and 45 cycles of 15 s at 94 °C for denaturation, 30 s to 1 min at 55 °C for annealing and 30 s at 72 °C for extension.

Specific primers were used to anneal with each gene (Table I) and glyceraldehyde-3-phosphate dehydrogenase gene (*VvGAPDH*; NCBI/Genbank Database accession no. XM_002263109) was selected as standard (housekeeping gene).

Table I - Primers forward (F) and reverse (R) used in qPCR.

NAME	SEQUENCE (5' – 3')	TM (°C)	%GC	LENGTH	REFERENCE
VvPAL1	**F** CCGAACCGAATCAAGGACTG	55	60.5	20	Boubakri et al. (2013)
	R GTTCCAGCCACTGAGACAAT	50	58.4	20	
VvUFGT	**F** TGCAGGGCCTAACTCACTCT	55	60.5	20	Conde et al. 2016
	R GCAGTCGCCTTAGGTAGCAC	60	62.5	20	
VvSTS	**F** CGAAGCAACTAGGCATGTGT	57	50	20	Boubakri et al. (2013)
	R CTCCCCAATCCAATCCTTCA	55	50	20	
VvCHS3	**F** TCGCATCACAAATAGCGAAC	55	45	20	Ageorges et al. (2006)
	R CAGGGAAGCTGCCATGTATT	56	50	20	
VvFLS1	**F** CAGGGCTTGCAGGTTTTTAG	55	50	20	Downey et al. (2003)
	R GGGTCTTCTCCTTGTTCACG	56	55	20	
VvDFR	**F** GGCTTTCTAGCGAGAGCGTA	58	55	20	Bogs et al. (2006)
	R ACTCTCATTTCCGGCACATT	56	45	20	
VvPME1	**F** GGATACCAGGACACACTGT	54	53	19	Barnavon et al. (2001)
	R CTTCCATGGCCTACCGAGGTA	59	57	21	
VvPME_00730	**F** TGGACCACTTTCCAATCGGG	56	53	19	Lionetti et al. (2015)
	R AAGCGGCACAGGTAGAAGAC	58	55	20	
VvPMEI1	**F** GTCCTGGCATCCACATATCC	56	55	20	

	R CAGTGCGAGGATCAGACTT	55	53	19	Lionetti et al. (2015)
VvPG1	**F** GAATCAAGACATGGCAGG	51	50	18	Deytieux-Belleau et al. (2008)
	R TAATCCTAGCTTCCATGCA	52	42	19	
VvPG2	**F** GAATCAAGACATGGCAGG	51	50	18	Deytieux-Belleau et al. (2008)
	R ATCACCTTCATTACACTAC	47	37	19	
VvABCC1	**F** AATTCAAAGATTGGAAGC	50	33	18	Francisco et al. (2013)
	R GCACTGATTTTGAATAGAA	50	32	19	
VvAM1	**F** CATCATGGAGACGCCGCTG CTCAACAG	67	59	27	Gomez et al. (2009)
	R GACATCATCACTCGTCTTC TT	57	43	21	
VvGAPDH	**F** TTCCGTGTTCCTACTGTTG	55	48	21	Gainza-Cortés et al. (2012)
	R CCTCTGACTCCTCCTTGAT	54	53	19	

2.4 Enzyme activity assays

2.4.1 Protein extraction

Total protein extraction from Gamay cells powder was performed according to the method described by Deytieux-Belleau et al. 2008, with some modifications. Protein was extracted from 300 mg of sample in 800 µl of ice-cold buffer containing 0.3 M Tris-HCl pH 7, 13 mM of EDTA, 5 mM dithiothreitol (DTT), 1 M NaCl, 1% polyvinylpyrrolidone (PVP) (w/v), 20% glycerol (v/v) and 1% Triton X-100. The mixture was vortexed vigorously and then centrifuged at 9000 g for 20 min, at 4 °C. The supernatant was maintained at - 20 °C until further analysis.

Quantification of total protein was performed by the Bradford method (Bradford 1976), using bovine albumin serum as standard. Absorbance measurements were performed in a Shimadzu UV-1700 Spectrophotometer.

2.4.2 Phenylalanine ammonia lyase (PAL) activity

The activity of PAL was assessed as described by Conde et al. 2016, with some adaptations. The reaction mixture contained 100 mM Tris-HCl pH 8.9, 4.3 mM NaCl, 19.2 mM L-phenylalanine as substrate, and 50 µL of protein extract, in a final volume of 1 mL. The reaction was started by the addition

of the extract. The production of trans-cinnamic acid was followed at 39 °C for 15 min at 290 nm. Results were presented in mmol trans-cinnamic acid $min^{-1}mg^{-1}$ protein.

2.4.3 UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT) activity

UFGT activity was determined according with the method described by Mori et al 2005, with some modifications. The assay mixture consisted of 20 mM sodium phosphate buffer pH 8.0, 50 µM of quercetin, 0.5 mM of UDP-glucose and 50 µL of protein extract in a final volume of 2 mL. The mixture was incubated at 30°C under shaking, and the reaction was started by addition of protein extract. The production of quercetin-3-glucoside was recorded at 350 nm during 30 min. Results were expressed as pmol quercetin 3-glucoside min^{-1} mg^{-1} protein, using the extinction coefficient of quercetin-3-glucoside (ε = 21877M/cm).

2.4.4 Polygalacturonase (PG) activity

PG biochemical activity was accessed by a colorimetric assay as described by Lohani et al 2004. The reaction mixture contained 60 mM sodium acetate buffer pH 4.5, 20 mM NaCl, 1% (w/v) polygalacturonic acid (PGA) and 100 µL of protein extract, in a final volume of 1 mL. The mixture was incubated at 37 °C for 15 min and heated in a boiling water bath for 5 min to stop the reaction. Next, 100 µL of 3,5-dinitrosalicylic acid (DNS) was added to 100 µL of reaction mixture and incubated at 100 °C for 15 min. The formation of reducing sugar equivalents was measured at 540 nm and determined by interpolation with a D-glucose calibration curve. Results were expressed in µmol reducing sugars min^{-1} $µg^{-1}$ protein.

2.4.5 Pectin methylesterase (PME) activity

The activity of PME was assayed following an adaptation of the method of Hagerman and Austin 1986. The reaction mixture contained 700 µL of 0.5% pectin solution (w/v), 50 µL of 0.01% bromothymol blue (w/v) and 200 µL of protein extract. The reaction occurred at 25°C and absorbance was recorded at 620 nm, at two-time points: 30 s and 3 min. Enzyme activity was determined using a standard curve with HCl as described by Hagerman and Austin. Results were expressed as µmol acid min^{-1} $µg^{-1}$ protein.

2.5 Vineyard treatments

Field experiments were performed with three-year old grapevines *cv.* "Vinhão" from a commercial vineyard in the north of Portugal, in the DOC region of "Vinhos Verdes". Grapevines used in this study were cultivated under the same microclimate and applications were performed as follows: grapevines treated with a solution containing 2% $CaCl_2$ and 0.1% Silwet L-77 that acted as surfactant agent; control grapevines were sprayed with a solution containing surfactant only, as described in previous studies (Saftner et al 1997). The first treatment was performed at the green stage, the second during véraison and the last one at mature stage, one week before harvest. Six grapevines were used for each treatment, and three bunches were randomly collected per grapevine for further analysis.

2.5.1 Determination of biochemical parameters

Twenty-four grape berries of each treatment were individually weighted for determination of berry fresh weight, and placed on a drying oven at 60°C for 3 days for determination of berry water content. Juice extracted from another set of berries was used to determine °Brix, titratable acidity and pH, using a digital wine refractometer (Hanna HI 96813) and a multi-parameter analyzer (Consort C-860), respectively.

Titratable acidity (TA) was determined by adding a solution of 0.1 N NaOH to each sample, until the ending point of pH 8.2 was reached (Martins, 2015). Titratable acidity as tartaric acid equivalents was calculated as TA (%) = (NaOH volume used × 0.1 × 100) / (sample volume×1000).

2.5.2 Anthocyanins quantification

The total anthocyanin content was determined by the differential pH method (Nicoué et al 2007). The extraction of total anthocyanins was performed by adding 1 mL of acetone (100%) to 40 mg of berry fresh weight. The mixture was homogenized on a shaker for 10 min and centrifuged at 18,000 x *g* for 20 min and, finally, sonicated for 10 min. Two dilutions of the same sample were prepared, one in 25 mM KCl (pH 1.0) and sodium acetate 25 mM (pH 4.5). Total anthocyanin quantification was evaluated spectrophotometrically at 520 nm and 700 nm. The following equation was used to determine the total anthocyanin concentration:

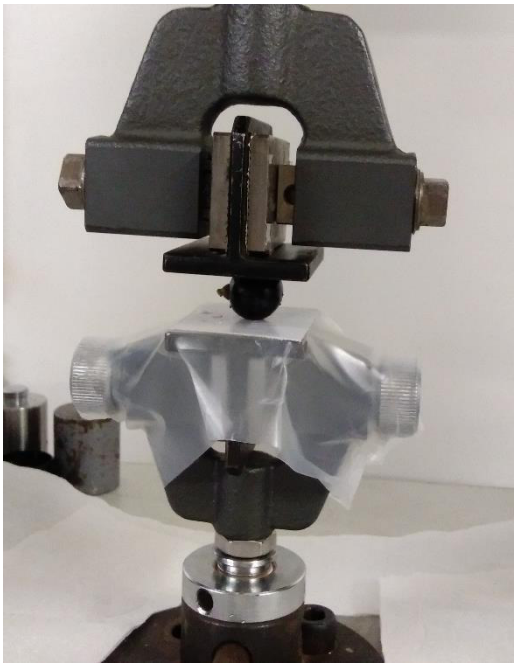
$$[\text{total anthocyanins}]\ (\text{mg/L}) = \frac{\Delta A \times MW \times 1000 \times DF}{\varepsilon},$$

where ΔA is the absorbance variation between different pH values and wavelengths [ΔA= pH 1 (A_{520}-A_{700}) - pH 4.5 (A_{520}-A_{700})], MW is the malvidin-3-O-glucoside molecular weight, DF is the dilution factor and ε is the molecular absorbance coefficient of malvidin-3-O-glucoside, used as reference (Nicoué et al 2007).

2.5.3 Determination of grape berry firmness compression and perforation tests

Fruit firmness was assessed through analysis of compression force and perforation force, corresponding to the force necessary to cause fruit cracking or skin perforation, respectively. Tests were conducted with a Shimadzu Autograph (AG-IS) equipped with a 50 N load cell, in the Department of Physics of University of Minho. Compression tests were performed by compressing the berries between a pair of rigid plates of 16 cm^2 at a loading rate of 5 mm min^{-1} (figure 8A). Perforation tests were performed at the same loading rate, using a 1 mm diameter needle (figure 8B). Force-stroke plots were recorded in Trapezium 2.0 Software, and the force necessary to cause fruit cracking (compression force) or skin perforation (perforation force) was determined.

A



B

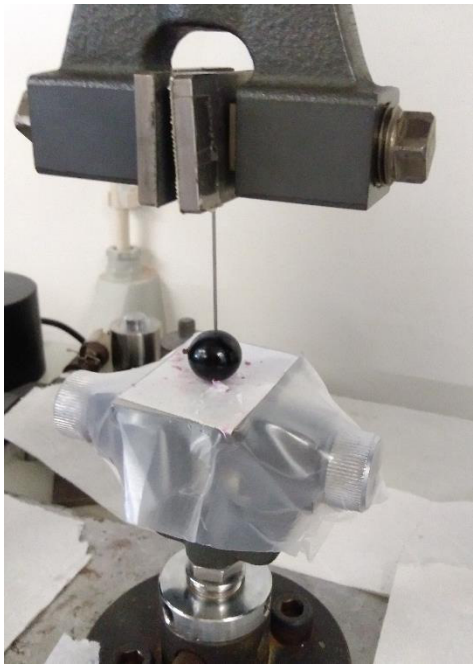


Figure 8 – Experimental setup for performing compression **(A)** and perforation **(B)** tests in mature berries *cv.* Vinhão, using a Shimadzu Autograph (AG-IS).

2.6 Statistical analysis

The results were statistically analyzed using the tools t-test and one-way ANOVA by Prism®6 (GraphPad Software, Inc.). Significant differences among the columns in each graph were marked with asterisks (**P < 0.01), or different letters (a, b, c, d, e), respectively.

3 Results

3.1 Gamay cell line as a model to study grape berry secondary metabolism at a biochemical and molecular level

Suspension cells of *V. vinifera* L. *cv.* Gamay Fréaux var. Teinturier are characterized by a large production of anthocyanins, which accumulate in the large central vacuole (figure 9A). In the present work, the effect of Ca^{2+} was tested in cells suspensions elicited with two hormones that play a major role in fruit ripening and stress response: ABA and MeJA, both at a concentration of 20 µM. Results showed that cells treated with 10 mM $CaCl_2$ alone or in combination with ABA or MeJA, were less pigmented than control cells and cells elicited with ABA or MeJA alone. (figure 9B). This pigmentation pattern was correlated with total anthocyanin content as confirmed in previous experiments performed in our group (Martins et al. 2016).

A

B

Figure 9 – **(A)** Gamay cell suspensions observed under a bright-field microscope, after 7 days of growth. **(B)** Colour of grown cells submitted to treatments with 10 mM $CaCl_2$, 20 µM ABA, 20 µM MeJA, and combinations of Ca^{2+} to hormones, or with no treatment – C.

3.1.1 Effect of Ca^{2+} on the expression and activity of secondary metabolism enzymes

To evaluate the effect of Ca^{2+} on the secondary metabolism of grape berry, expression studies made by qPCR were performed with genes that encode for key enzymes of the phenylpropanoid pathway.

PAL is the enzyme that catalyses the first step of this pathway and, therefore, is the starting point for the biosynthesis of phenolic compounds. The highest expression of *VvPAL1* was detected in cells elicited with MeJA. Once cells were treated with Ca^{2+} in the presence of this hormone, the expression levels decreased significantly. In contrast, in control cells and in cells elicited with ABA, the presence of Ca^{2+} led to an increased gene expression (Figure 10A).

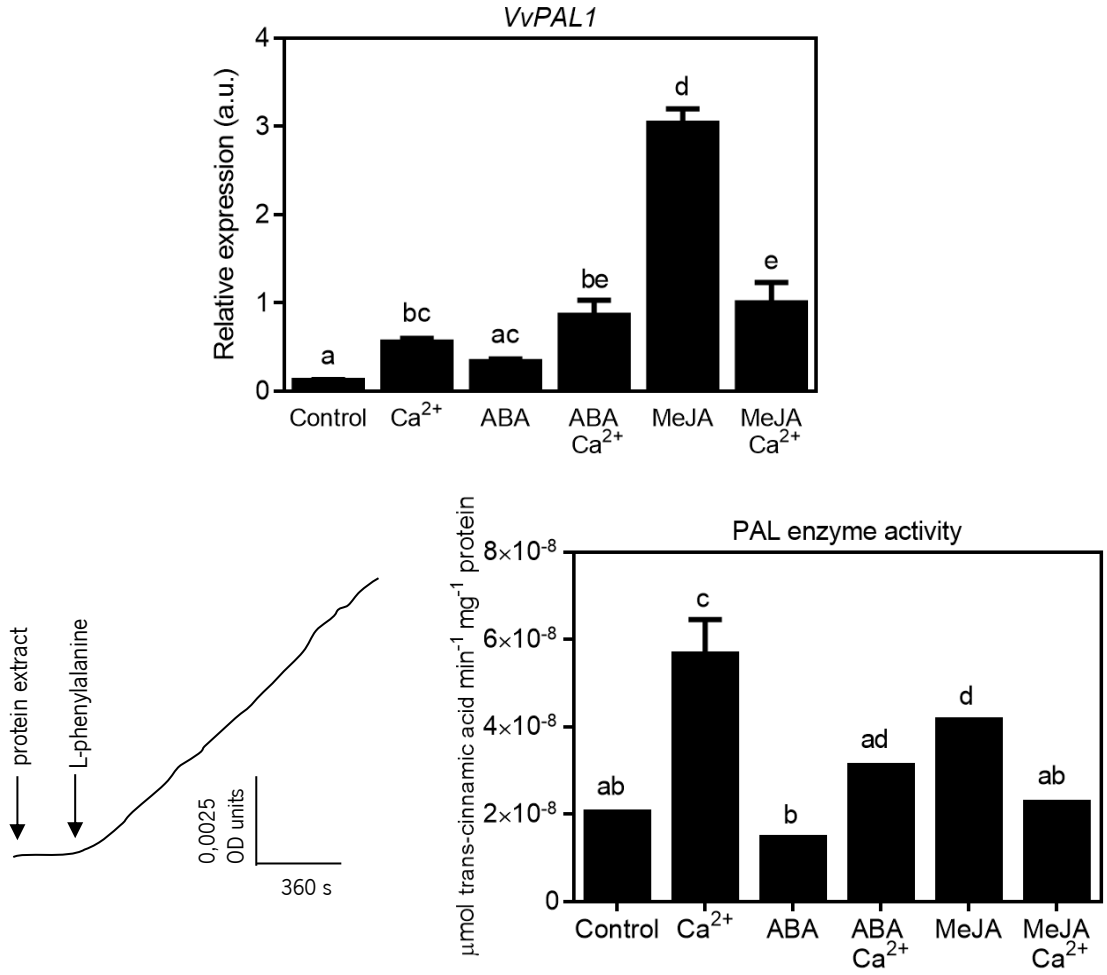


Figure 10 - (A) Expression of *VvPAL1* and PAL enzyme activity in grape cells *cv.* Gamay elicited for 12 h with different combinations of calcium and hormones: [Ca^{2+}] = 10 mM, [ABA] = 20 µM and [MeJA] = 20 µM. Gene expression was normalized to the transcript levels of *GAPDH* (internal standard). **(B)** Activity of PAL was evaluated through continuously monitorization of trans-cinnamic acid production, assessed spectrophotometrically at 290 nm. **(C)** PAL activity, determined as V_{max}, was expressed as µmol trans-cinnamic acid min^{-1} mg^{-1} protein. Results indicate mean ± SD of values obtained for three replicates. In bars, different letters indicate significant differences.

To study PAL activity in protein extracts, the reaction started by the addition of the substrate phenylalanine and activity was assessed through a continuous measurement of the absorbance at 620

nm (figure 10B). The profile obtained for total PAL activity (figure 10C), showed that the highest PAL activity was observed when cells were treated with Ca^{2+} only. Also, the presence of Ca^{2+} led to higher activity of this enzyme in cells elicited with ABA, but decreased PAL activity in cells elicited with MeJA.

The expression of two genes that encode key enzymes of the first step of stilbenes and flavonoids biosynthesis was next evaluated, namely *VvSTS* and *VvCHS3* (figure 11).

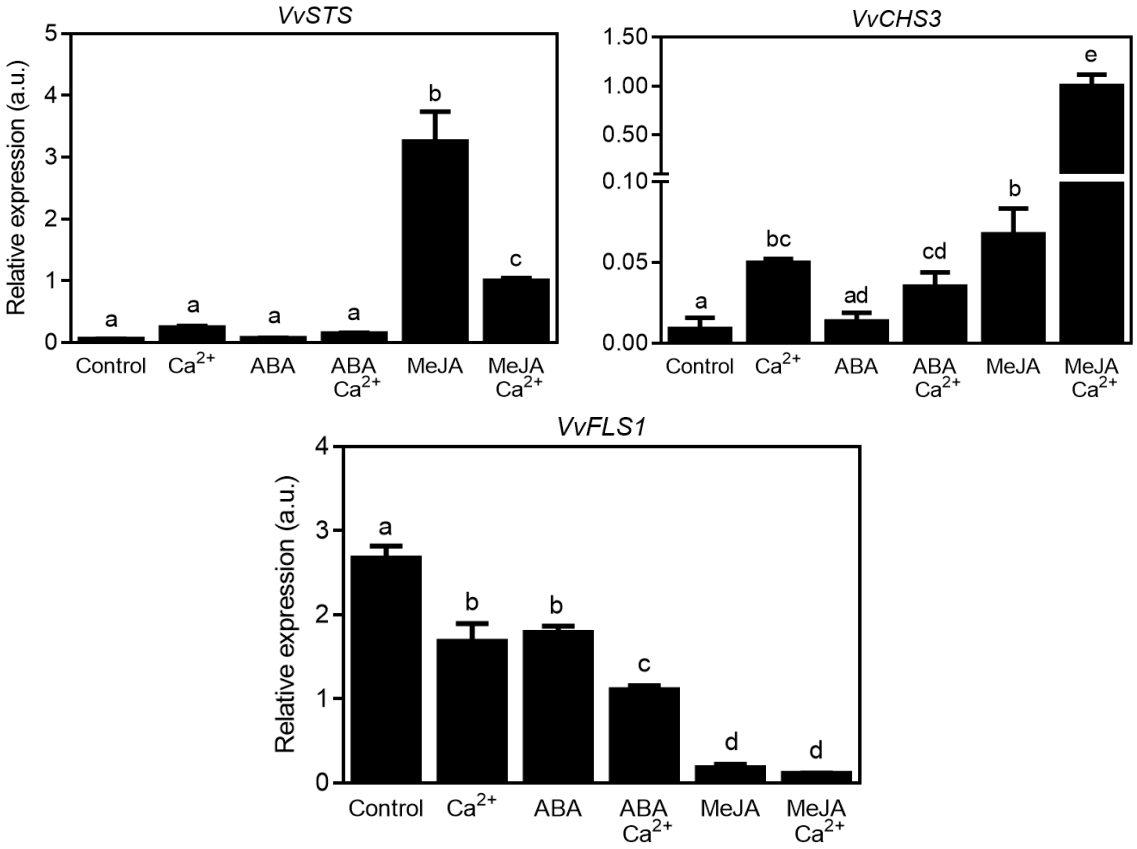


Figure 11 - Expression of *VvSTS, vVCHS3* and *VvFLS1* in grape cells *cv.* Gamay elicited for 12 h with different combinations of calcium and hormones: [Ca^{2+}] = 10 mM, [ABA] = 20 µM and [MeJA] = 20 µM. Gene expression was normalized to the transcript levels of *GAPDH* (internal standard). Results indicate mean ± SD of values obtained for three replicates. In bars, different letters indicate significant differences.

Results showed that *VvSTS* transcript levels were similar in control cells, and in cells treated with Ca^{2+}, ABA, or ABA + Ca^{2+}. In contrast, *VvSTS* was strongly upregulated by the elicitation with MeJA, that was prevented by the presence of Ca^{2+}.

In parallel, expression studies of *VvCHS3* demonstrated that this gene was upregulated in the presence of Ca^{2+} in all conditions tested. Transcriptional studies of *VvFLS1* encoding an isoform of the first

enzyme of flavonols biosynthesis showed that the highest expression levels of this gene were detected in control cells and its expression was repressed by Ca^{2+} alone, by ABA and by MeJA.

3.1.2 Effect of Ca^{2+} in the pathways involved in anthocyanin biosynthesis and accumulation

After demonstrating that *VvCHS3* was upregulated in cells treated with Meja+Ca^{2+}, while *VvFLS1* transcript levels decreased abruptly under the same treatment, genes involved in another branch of flavonoids pathway were studied – the ones that culminate in the biosynthesis of anthocyanins. The pPCR analysis of *VvDFR*, the gene which codes for the enzyme that catalyses the first step of this pathway, revealed that the presence of Ca^{2+} differentially regulated *VvDFR* transcript levels depending on the hormonal treatment. For instance, transcript levels increased in the presence of Ca^{2+}, while in cells upon treatment with MeJA + Ca^{2+} a significant decrease was recorded (figure 12).

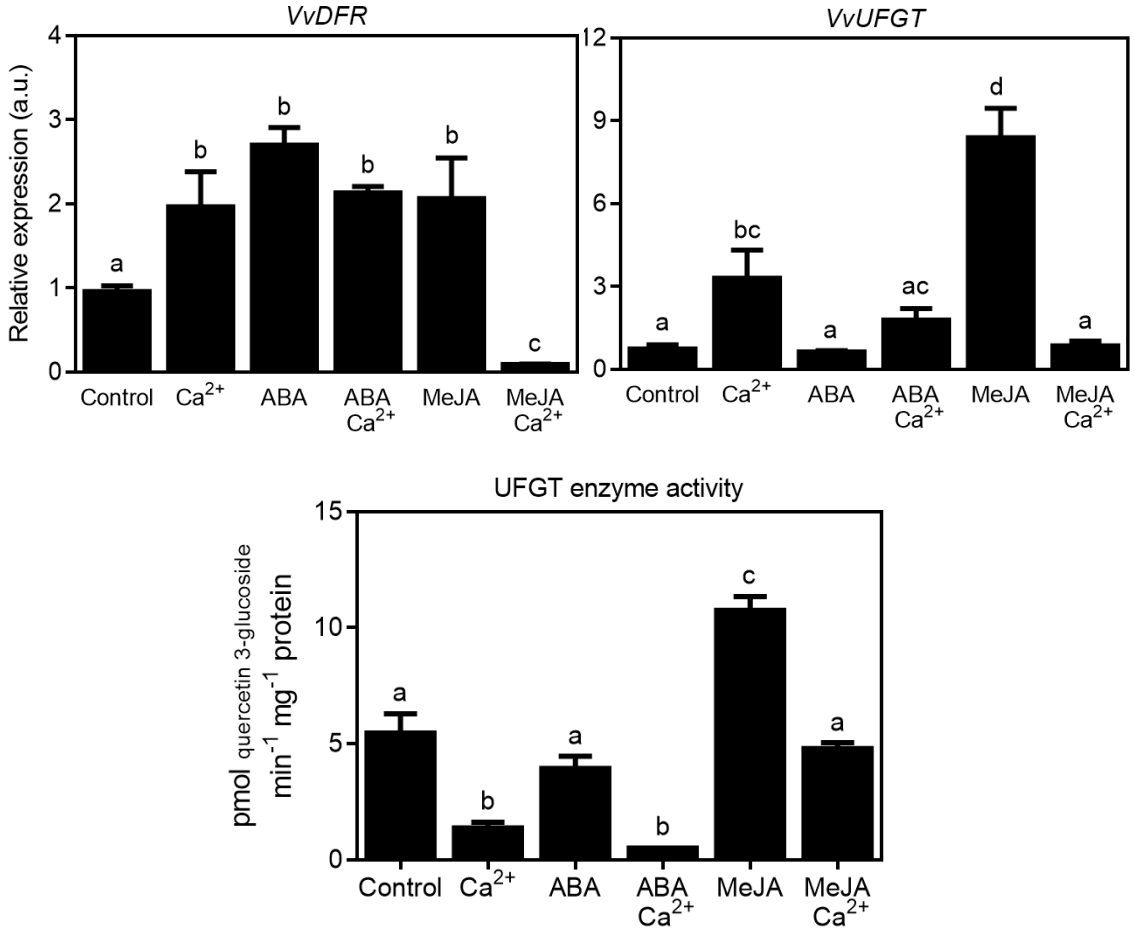


Figure 12 - Expression of *VvDFR* and *VvUFGT*, and UFGT enzyme activity in grape cells *cv.* Gamay elicited for 12 h with different combinations of calcium and hormones: [Ca^{2+}] = 10 mM, [ABA] = 20 µM and [MeJA] = 20 µM. Gene expression was normalized to the transcript levels of *GAPDH* (internal standard). Results indicate mean ± SD of values obtained for three replicates. In bars, different letters indicate significant differences.

Lastly, in cells elicited with ABA, which also upregulated *VvDFR* expression, the presence of Ca^{2+} did not affect gene expression.

Another key gene, which encodes the enzyme responsible for the last and limiting step of anthocyanins biosynthesis, UFGT, was also a target of study. As shown in figure 12, the highest transcript levels of *VvUFGT* were observed in cells treated with MeJA. However, when cells were treated with MeJA + Ca^{2+}, the expression of this gene reached levels similar to the ones observed in control cells and in cells elicited with ABA, which correspond to the lowest expression levels detected. In these cells, the treatment with Ca^{2+} significantly upregulated *VvUFGT* expression.

To complement these studies, the activity of UFGT was evaluated in these cells (figure 12). Results confirmed that the highest UFGT activity was detected in cells treated with MeJA, and showed that the presence of Ca^{2+} significantly downregulated UFGT, at protein activity level, in all conditions tested.

The expression of *VvAM1*, which encodes a MATE-type protein that mediates the antiport of acylated anthocyanins with H+ across the vacuole membrane (Gomez et al. 2009) is depicted in figure 13.

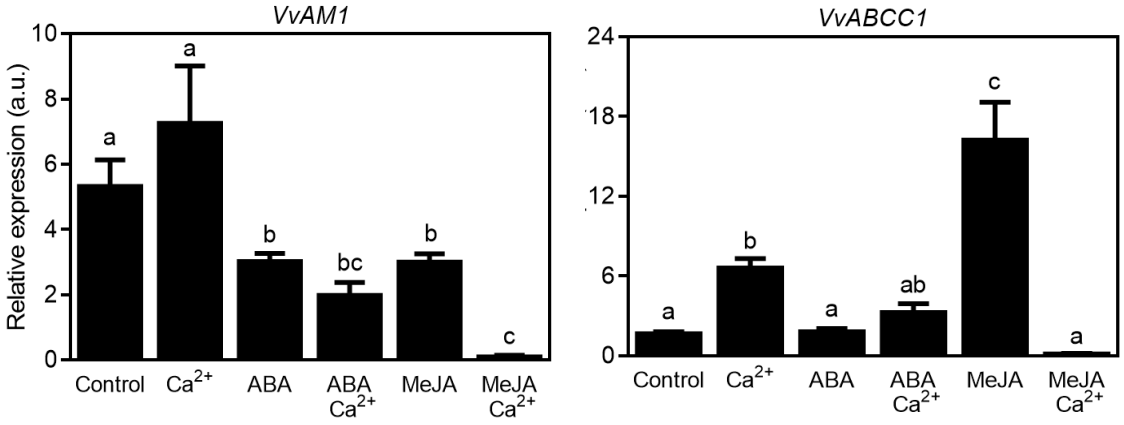


Figure 13 - Expression of *VvAM1* and *VvABCC1* in grape cells *cv.* Gamay elicited for 12 h with different combinations of calcium and hormones: [Ca^{2+}] = 10 mM, [ABA] = 20 µM and [MeJA] = 20 µM. Gene expression was normalized to the transcript levels of *GAPDH* (internal standard). Results indicate mean ± SD of values obtained for three replicates. In bars, different letters indicate significant differences.

The elicitation of the cells with ABA or MeJA downregulated the expression of *VvAM1*, but while Ca^{2+} did not affect the expression of *VvAM1*, neither when applied alone nor in cells elicited with ABA, it strongly decreased the steady-state transcript levels in cells treated with MeJA. The expression pattern of VvABCC1, encoding an ATP-binding cassette-type protein, which transports glucosylated anthocyanidins dependent on the presence of reduced glutathione -GSH (Francisco et al. 2013) is depicted in figure 13.

Calcium alone was able to stimulate the VvABCC1 transcription, much like when cells were elicited with MeJA alone, while the elicitation with ABA alone did not affect gene expression. The presence of calcium in cells elicited with MeJA prevented the upregulation of gene observed in cells elicited with MeJA alone.

3.1.3 Effect of Ca^{2+} on the expression and activity of cell wall enzymes

Pectin methylesterase (PME), one of the enzymes involved in cell wall structure was one of the targets of this study. This enzyme generates hydrolysable sites in pectin polysaccharides, that become susceptible to the action of polygalacturonase (PG), another cell wall enzyme that its responsible for softening of the cell wall and which was also a target enzyme in this work due to its competition with Ca^{2+} for the binding to these sites. Expression studies involving *VvPME1* showed that the highest transcript levels were detected in cells elicited with MeJA, although there was no significant difference between this treatment and MeJA + Ca^{2+} (figure 14).

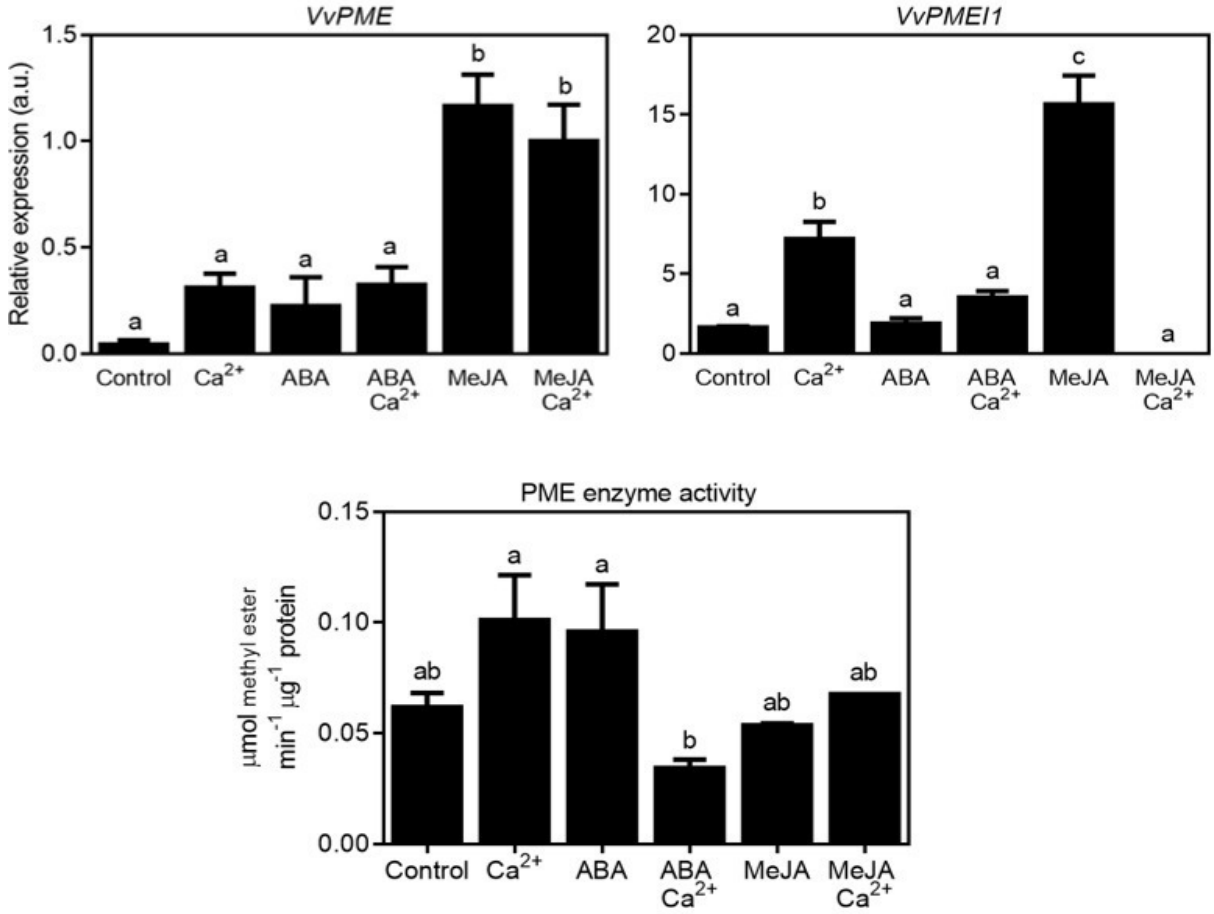


Figure 14 - Expression of *VPME1* and *VvPMEI1*, and PME enzyme activity in grape cells *cv.* Gamay elicited for 12 h with different combinations of calcium and hormones: [Ca^{2+}] = 10 mM, [ABA] = 20 µM and [MeJA] = 20 µM.

Gene expression was normalized to the transcript levels of *GAPDH* (internal standard). Results indicate mean ± SD of values obtained for three replicates. In bars, different letters indicate significant differences.

The other conditions did not exhibit significant differences in transcript levels. Transcriptional levels of *VvPMEI1*, encoding an inhibitor of some PMEs, were the highest in cells elicited with MeJA. However, when cells were treated with MeJA + Ca^{2+}, *VvPMEI1* transcripts were not detected. Finally, cells treated with Ca^{2+} exhibited a higher expression of *VvPMEI1* when compared to control cells, and no differences were observed between the treatments with ABA and ABA + Ca^{2+}. Studies regarding PME activity (figure 14), showed that there were no significant differences between control and Ca^{2+}-treated cells, and between MeJA and MeJA + Ca^{2+}-treated cells. However, a substantial decrease was observed when cells were treated with ABA + Ca^{2+}, compared to cells treated with ABA only.

In the present study, expression of two genes encoding two different isoforms of PG were evaluated – *VvPG1* and *VvPG2 (figure 15).*

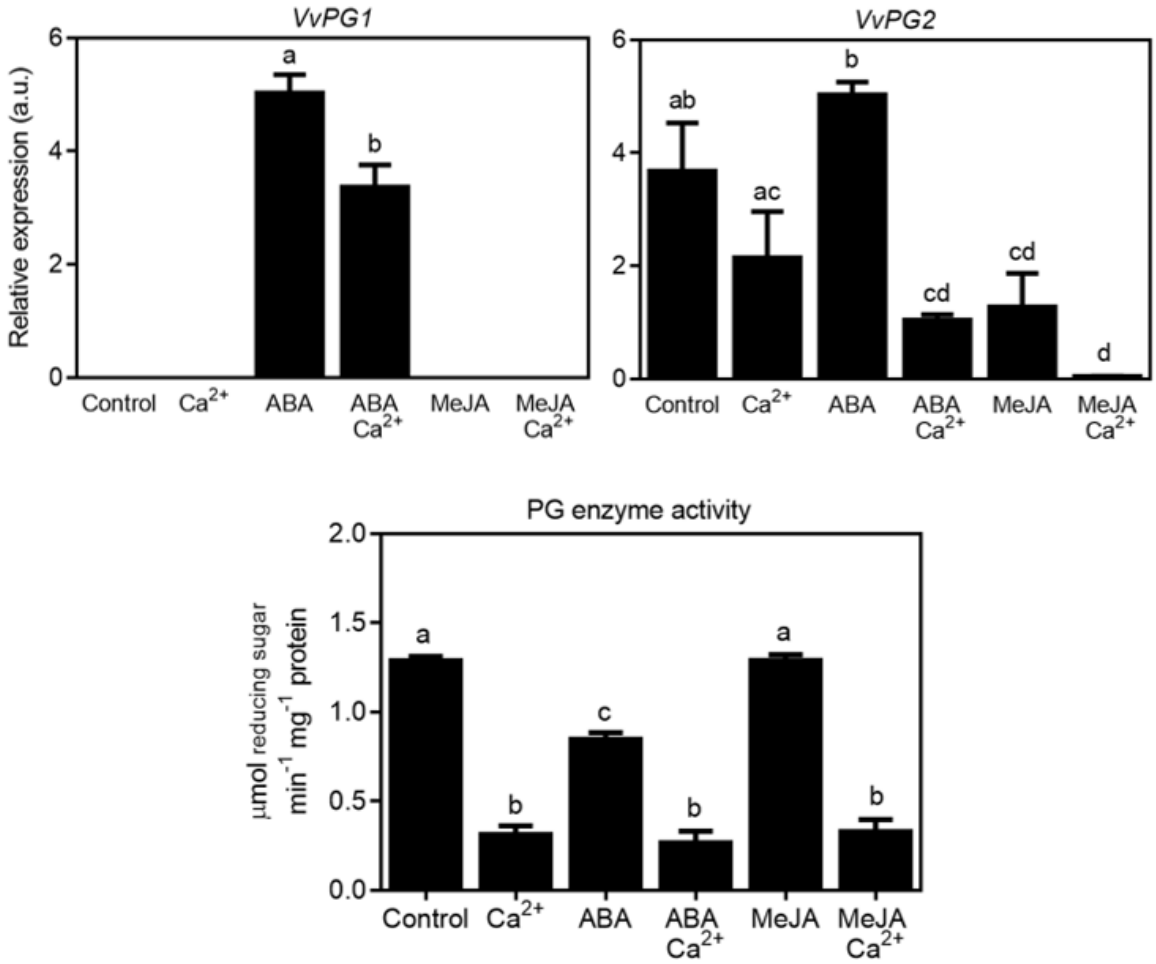


Figure 15 - Expression of *VvPG1* and *VvPG21*, and PG enzyme activity in grape cells *cv.* Gamay elicited for 12 h with different combinations of calcium and hormones: [Ca^{2+}] = 10 mM, [ABA] = 20 µM and [MeJA] = 20 µM. Gene

expression was normalized to the transcript levels of *GAPDH* (internal standard). Results indicate mean ± SD of values obtained for three replicates. In bars, different letters indicate significant differences.

Transcription of *VvPG1* was only detected in the presence of ABA and ABA + Ca^{2+}, and the presence of Ca^{2+} downregulated gene expression. In contrast to results observed for *VvPG1, VvPG2* transcripts were detected in all conditions tested.

Like *VvPG1,* qPCR analysis showed that transcription of *VvPG2* tended to increase when cells were elicited with ABA, although there were no statistical differences between this condition and the control. In general, the presence of Ca^{2+} seemed to decrease *VvPG2* expression in all conditions tested, however, statistical significance was only found between ABA and ABA + Ca^{2+} treatments. In accordance to these results, enzyme activity of PG (figure 15) showed that, in all cases, the presence of Ca^{2+} visibly decreased PG enzyme activity.

3.2 Field studies to evaluate the effect of Ca^{2+} on biochemical and physical parameters of grape berries

To evaluate the effects of Ca^{2+} in the biochemistry and firmness of grape berries grown in field conditions, grapevines *cv.* Vinhão were sprayed with a surfactant solution containing 2% $CaCl_2$. Fruits treated with Ca^{2+} or with surfactant only (control) were collected at mature stage (figure 16) and parameters such as berry weight, berry water content, °Brix, pH and titratable acidity were assessed.

Figure 16 – Grape berry clusters at mature stage subjected to surfactant treatments with (right) or without (left) $CaCl_2$ throughout development, in three-year-old grapevines *cv.* "Vinhão" located in the DOC region of "Vinhos Verdes".

As shown in table I, the fresh weight in control fruits ranged between 2.05 g and 2.53 g, while fruits treated with Ca^{2+} weighed on average 2.51 ± 0.16 g, and no significant differences were observed regarding this parameter. Concerning berry water content, there were also no significant differences between control fruits and fruits treated with Ca^{2+}; however, it is noteworthy that the latter presented higher variability in this parameter (66,33 ± 25,91%), whereas in control fruits there was more homogeneity (73,14 ± 1,84%). °Brix was also assessed and no significant difference was observed between control fruits and the ones subjected to treatment with Ca^{2+}. The same was verified for the parameters pH and titratable acidity.

Table II – Biochemical parameters of mature grape berries from grapevines *cv.* Vinhão subject to surfactant treatments with $CaCl_2$ (Ca^{2+}) or without (control) throughout development. Results are expressed as mean ± SD.

	Berry fresh weight (g)	Berry water content (%)	°Brix	pH	Titratable acidity (%)
Control	2.29 ± 0.24	73.14 ± 1.84	20.97 ± 0.71	3.15 ± 0.12	0.60 ± 0.06
Ca^{2+}	2.51 ± 0.16	66.33 ± 25.91	21.13 ± 0.12	3.18 ± 0.02	0.61 ± 0.04

Given the strong effect of Ca^{2+} in anthocyanin content observed *in vitro*, the level of these pigments was quantified in whole fruits. As shown in figure 17, the Ca^{2+} treatment in the field did not affect the content of anthocyanins in grape berries.

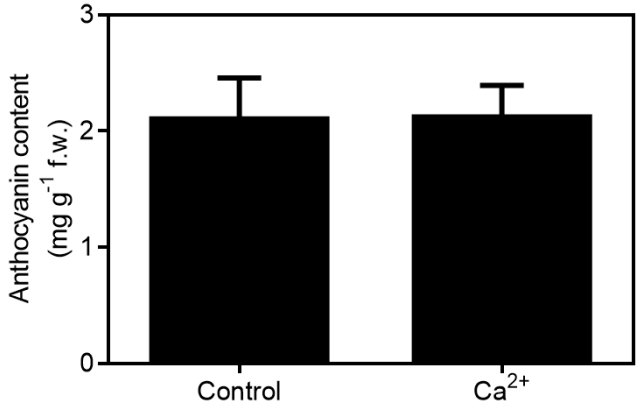


Figure 17 – Effect of Ca^{2+} on total anthocyanin content in mature berries from grapevines *cv.* Vinhão subject to surfactant treatments with $CaCl_2$ (Ca^{2+}) or without (control). Results indicate mean ± SD.

Given the role of Ca^{2+} in the regulation of cell wall enzymes, demonstrated *in vitro*, the effect of Ca^{2+} in the firmness of grape berries from the field was assessed. For this purpose, two parameters were studied – compression force and perforation force. The first test was performed to evaluate the overall fruits consistency, and the second one was performed to analyse the skin resistance. In each test, the force necessary to cause fruit cracking or skin perforation was recorded, respectively. These measurements were extracted from force-stroke plots performed for each berry individual (figure 18A).

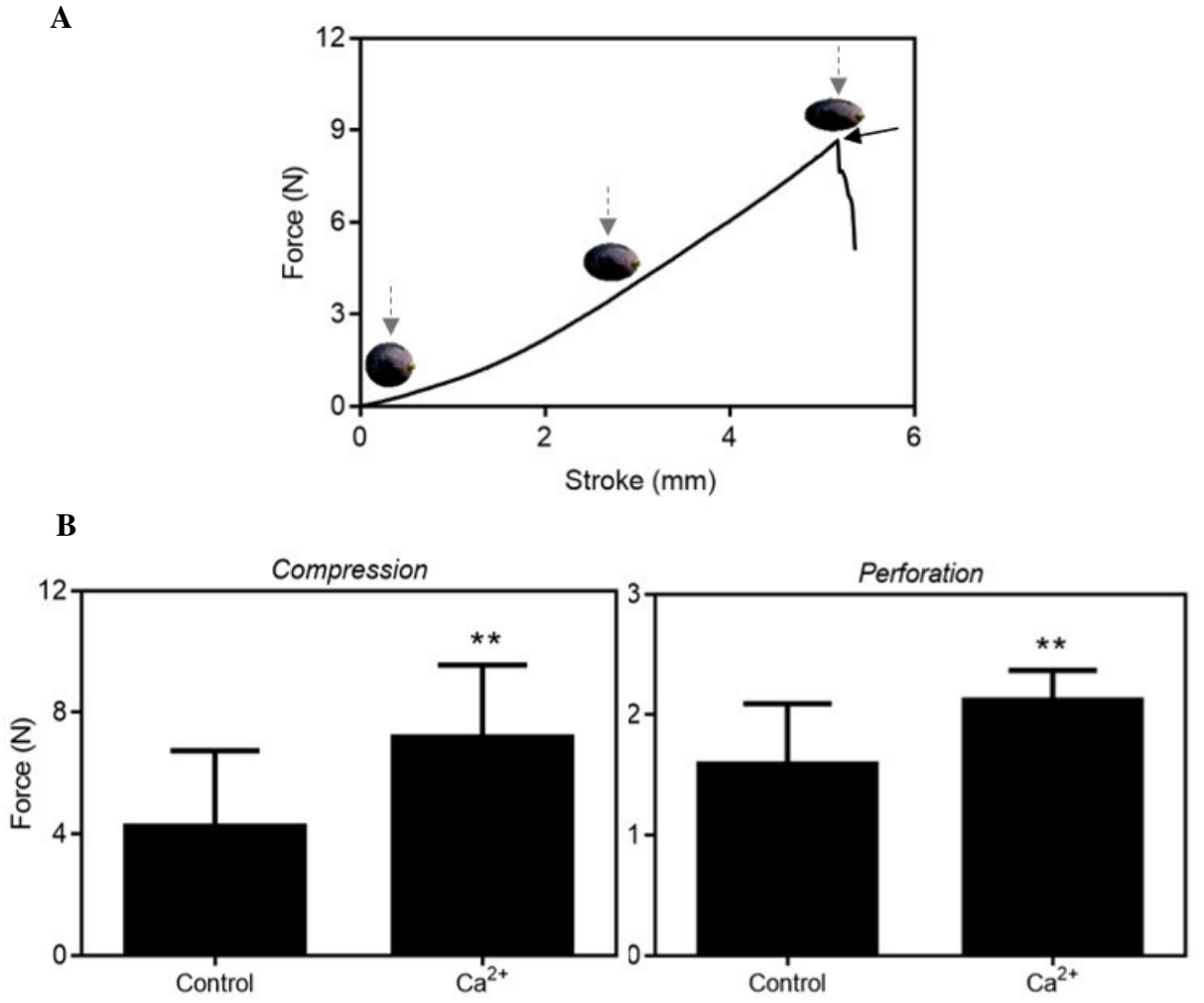


Figure 18 – Effect of Ca^{2+} on the firmness of mature berries from grapevines *cv.* Vinhão subject to surfactant treatments with $CaCl_2$ (Ca^{2+}) or without (control). **(A)** Typical force–stroke plot obtained for one grape berry. The *dashed arrows* indicate the direction of the force and the *black arrow* indicates the break point where the compression/perforation force was recorded. **(B)** Force needed to cause fruit cracking (compression test) or skin perforation (perforation test). Results indicate mean ± SD of 13 biological replicates for each treatment, and asterisks indicate statistical significance (Student's *t*-test; **P < 0.01).

As shown in the graph, the force exerted initially increases as the berry presents mechanical resistance to compression/perforation, and reaches a maximum which corresponds to the highest force that each fruit can withstand. Right after that maximum value is achieved, the fruit is disrupted, and the force decreases dramatically, as fruit integrity is severely compromised. These analyses were performed in several control and Ca^{2+}-treated fruits and results are shown in figure 18B. As depicted, the necessary force to compress or to perforate the control fruits was lower than that necessary to disrupt berries treated with Ca^{2+}.

4 Discussion

One of the main goals in grape berry research has always been the investigation of berry development and ripening, since producers are interested in improving cultivating practices and in increasing yield and quality of the final product (Giribaldi and Giuffrida 2010). In the last few years, Ca^{2+} has been elucidated as a major regulator of growth and development in plants and several researchers highlighted its structural role and its importance in cell signalling (reviewed by Hepler 2005).

In the present work, molecular, biochemical and physical approaches were used to study the role of Ca^{2+} in grape berry. In a first stage, *in vitro* studies were performed using the model cell line *V. vinifera* L. *cv.* Gamay Fréaux var. Teinturier producer of anthocyanins (Mewis et al. 2011), to understand the effect of Ca^{2+} in genes encoding the enzymes and transporters involved in the biosynthesis and accumulation of phenolic compounds, and specifically anthocyanins. Given the importance of Ca^{2+} in berry integrity, its effect on cell wall enzymes modulating pectin organization was also a target of study. In a second approach, field studies were performed using grapevines *cv.* Vinhão to evaluate the effects of Ca^{2+} on the biochemical and physical parameters of the grape berries.

4.1 Ca^{2+} modulates the biosynthetic pathways leading to anthocyanin biosynthesis at gene expression and enzyme activity levels

Transcriptional analysis of the target genes in Teinturier cell suspensions, encoding enzymes of the phenylpropanoid pathway, showed that, in general, cells treated with Ca^{2+} exhibited a higher expression of the genes involved in this pathway when compared to control cells. This effect is perceptible early in the first steps of this pathway, starting with the expression of *VvPAL1*. Ca^{2+} showed to be responsible for a strong upregulation of this gene, exhibiting also a major effect in PAL activity. Further along the phenylpropanoid pathway, results showed a downregulation of *VvSTS* and upregulation of *VvCHS3* in cells under Ca^{2+} treatment. At this point, these findings suggested that flavonoids pathway was being favoured in detriment of stilbenes pathway. Expression studies of *VvFLS1* showed a downregulatory effect of this gene in the presence of Ca^{2+}. Knowing that this ion is an important regulator having crucial roles in a wide range of signalling pathways, it is tempting to speculate that Ca^{2+} is favouring the production of other flavonoids. An upregulation of *VvDFR* in the presence of Ca^{2+} suggested that Ca^{2+} directed the flavonoid pathway towards the biosynthesis of anthocyanins. Although transcriptional studies of *VvUFGT* corroborate this hypothesis, studies of enzyme activity showed that Ca^{2+} repressed UFGT activity. The transcriptional studies of the anthocyanin transporters ABCC1 and AM1 suggested that Ca^{2+} stimulates the accumulation of anthocyanins. Interestingly, Ca^{2+} seems to stimulate at a much higher level the

expression of *VvABCC1* when compared with *VvAM1.* These results suggest that in the presence of Ca^{2+}, the transport of glucosylated anthocyanins may be favoured, although these hypothesis needs to be tested.

Once anthocyanins accumulation in grape berry is also known to be influenced by several hormones, the combined effect of Ca^{2+} with ABA and MeJA was also evaluated in the present work. ABA regulates many processes in berry development and appears to be involved in the regulation of anthocyanin biosynthesis and its accumulation in the skin of grapes (Kataoka et al. 1982). The results obtained for the expression of the genes encoding for the enzymes of the phenylpropanoid pathway when cells were treated with ABA + Ca^{2+} were very similar to the ones described above for cells treated with Ca^{2+}. Although in most cases significant statistical differences were not found, the elicitation with ABA + Ca^{2+} showed a tendency to stimulate gene expression.

In parallel, almost all the genes encoding for enzymes of the phenylpropanoid pathway and anthocyanin transporters showed high expression levels in the presence of MeJA. However, when cells where treated with Ca^{2+} and elicited with MeJA the expression levels decreased significantly, being even lower than the expression levels observed when cells are only treated with Ca^{2+}. The same profile was often observed in enzyme activity assays. The high expression of the genes and activity of the enzymes in study was already expected since several studies reported that the role of MeJA in plant defence is closely related with an increase of PAL activity, leading to the production of many compounds which confer protection to the plant (Akbari-Vafaiiet al 2014; Pérez-Díaz et al. 2016). A study performed in Chinese bayberry showed that MeJA-induced resistance in pathogen-infected plants was found to involve the stimulation of the phenylpropanoid pathway (Wang et al. 2014). It is not easy to explain why the combination of MeJA with Ca^{2+} was responsible for such lower gene expression and enzyme activity levels because of the wide range of functions that this hormone plays in plants. The crosstalk of MeJA with many other signalling pathways makes MeJA signalling pathway a complex regulatory network, becoming very difficult to understand the interactions between this hormone and several other compounds (reviewed by Cheong and Choi 2003). Some evidences suggest that MeJA signalling process changes the concentration of free Ca^{2+} in the cytosol (Saito et al. 2009).

Overall, *in vitro* studies confirmed that Ca^{2+} and its combined effect with hormones may have a substantial influence on fruit secondary metabolism. Few studies on strawberry and grape suggested a link between calcium and anthocyanins, where Ca^{2+}, calmodulin, sugars, and protein kinases/phosphatases are key players, possibly by modulating anthocyanin biosynthetic genes (Peng et al. 2016; Vitrac et al. 2000; Xu et al. 2014).

In this work, field studies were performed to gain additional information about the effect of Ca^{2+} in grape berry through determination of anthocyanins content and study of biochemical parameters of the fruit. The *cv.* Vinhão was chosen for these experiments because it a very common grape Teinturier variety founded in DOC region of "Vinhos verdes". No significant differences were found between Ca^{2+}-treated berries and control regarding the biochemical parameters of the berry nor regarding water content. Moreover, no differences were observed in the total anthocyanin content between control berries and berries treated with Ca^{2+}, contrarily to what was observed in *in vitro* cultures. Overall these results may suggest that the exogenous application of Ca^{2+} does not affect, at least in the conditions tested, the quality/palatability of the grape berry. Nonetheless, these findings do not exclude the hypothesis that maybe different types of anthocyanins or other phenolics are being accumulated.

Further studies that are in progress in our laboratory will elucidate what particular changes at the gene expression/protein activity levels are induced by Ca^{2+} in fruit tissues regarding secondary metabolism.

4.2 Ca^{2+} improves grape berry firmness by regulating enzymes involved in pectin organization in the cell wall

The role of Ca^{2+} on expression and activity of important cell wall enzymes, which play a main role in grape berry integrity was studied. Ca^{2+} did not significantly stimulate the expression of *VvPME1* and activity of the enzyme. Contrarily, previous findings suggesting that PME is needed for the de-esterification of pectins, enabling the cross-link between Ca^{2+} and cell wall pectins (Jolie et al. 2010). The results obtained for *VvPMEI1* are very similar to the ones obtained for *VvPME1*, possibly because this inhibitor may not be specific for this gene.

Contrarily to the results of transcriptional studies obtained for *VvPME1*, Ca^{2+} repressed the expression of *VvPG1* and *VvPG2* and, concordantly, PG activity also decreased in the presence of this ion. PG is responsible for the softening of the fruits cell wall and it is negatively regulated by the formation of the Ca^{2+} bridges (Balic et al. 2014). These results, together with previous studies performed in tomato, suggest that Ca^{2+} may be required to inhibit premature fruit softening, what is extremely important during the first phases of berry growth (Wills and Rigney 1979). In papaya Ca^{2+} was responsible for a reduction in fungal infection, apparently due to the stabilization of cell wall structure and protection against pectinolytic enzymes secreted by the pathogen (Madani et al. 2016).

Recent results suggest that ABA can stimulate fruits softening (Conde et al. 2007). Concordantly, in the present study, *VvPG1* is only expressed in the presence of this hormone. Previous studious in tomato showed that ABA directly participates in the cell wall catabolism involved in fruit ripening via the upregulation of the expression of a suite of important genes, including *PG* (Sun et al. 2012). However, in the present work, when cells were elicited with ABA in the presence of Ca^{2+} a downregulation of *VvPG1* and *VvPG2* was observed. Also, the levels of PG activity decreased significantly in these conditions. The previously described inhibitory role of Ca^{2+} on cell wall softening enzymes may account for these observations.

The most outstanding result was observed in transcriptional studies of *VvPG2* when cells were treated with MeJA + Ca^{2+}. The transcript levels of this gene under MeJA + Ca^{2+} treatment was significantly lower when compared with the remaining treatments. Furthermore, cells elicited with MeJA showed a lower expression level when compared with the control. However, results of PG activity showed that, although a decrease of enzyme activity in cells treated with MeJA + Ca^{2+} was observed, the profile is very similar to the one obtained in the remaining treatments. Some studies suggest that besides being inhibited by Ca^{2+}, as shown previously, PG may also be inhibited by MeJA. PG activity during normal ripening of tomatoes was inhibited upon MeJA treatment and this effect apparently caused by a stimulatory effect of MeJA on PG degradation (Saniewski et al 1987).

Besides transcriptional studies, field studies also provided important information about the role of Ca^{2+} in fruit firmness or integrity. The susceptibility of grape berry to environmental cues is major topic of debate once it is responsible for many injuries such as berry cracking, compromising berry quality. The firmness of berries from the field was evaluated using a test to evaluate fruits consistency (compression test) and another one to evaluate skin resistance (perforation test).

Results showed that a lower force was needed to compress or to perforate the control fruits when compared with the necessary force to disrupt berries treated with Ca^{2+}. These findings demonstrate that berries treated with Ca^{2+} presented higher consistency and skin resistance, respectively. These findings are in agreement with the *in vitro* studies regarding the activity of PG. These results also suggest that Ca^{2+} is capable of increasing berry firmness maybe through the inhibition of the activity of cell wall degrading enzymes. Such hypothesis was already tested is fruits like sweet cherries where $CaCl_2$ sprays reduced decay and cuticular fractures (Vangdal et al. 2008). Another experiment, of $CaCl_2$ in apple fruits, showed that preharvest treatment of $CaCl_2$ may not necessarily always lead to firmer fruits at the time of harvest but may result in better retention of firmness during storage (Siddiqui and Bangerth 1995).

Altogether*, in vitro* and field results emphasised the role of calcium in the biosynthesis of anthocyanin and in grape berry firmness. Although in cells cell suspensions several modifications of the secondary activity metabolism were observed, these were noticed in field studies, so Ca^{2+} did not apparently modify the quality parameters in mature berries, but, instead, Ca^{2+} improved the physical properties of the fruit. This, could open good perspectives for its regular utilization in the field to protect berries from fungal attack, particularly those with thin skin, like *B. cinerea* and from cracking under heavy rains. Moreover, additional studies are needed to clarify hormone-Ca^{2+} interplay.

5 Conclusion and future perspectives

The present work aimed to understand the role of Ca^{2+} on grape berry secondary metabolism pathways that lead to the production and accumulation of anthocyanins. Given the well-known role of this ion on cell wall structure, its effect on expression and activity of cell wall enzymes was also a target of study. Transcriptional studies in cultured cells showed that Ca^{2+} is responsible for changes in the expression of genes and activity of enzymes of the phenylpropanoid pathway, particularly the ones that lead to the production and accumulation of anthocyanins, emphasizing the regulatory role of Ca^{2+}. However, in field studies, differences on anthocyanins content between control and treated berries were not observed. Curiously, results obtained of the combined effect of MeJA with Ca^{2+} also highlighted the strong link between the Ca^{2+} and MeJA signaling pathways.

Studies involving PG showed that Ca^{2+} is responsible for a repression in the expression and activity of this enzyme, which reinforced the idea that Ca^{2+} is responsible for strengthening the cell wall. Interestingly, these findings are in agreement with the results obtained from field studies showing that Ca^{2+}-treated berries are more resilient to crushing or perforation. Altogether these results suggest that Ca^{2+} improves berries firmness and that Ca^{2+} treatments could be an effective practice to improve the quality of fruits and wine, preventing the incidence of diseases and fruit cracking.

This study added important insights about the role of Ca^{2+} on grape berry secondary metabolism and on cell wall structure, but so far results have essentially of basic science dimension. Thus, important steps remain to be done, especially at the field level.

1) Additional biochemical and molecular analysis should be performed in berries from grapevines treated in the field to see how Ca^{2+} promotes a reprograming of gene expression, particularly regarding those genes coding for enzymes of the secondary metabolism. These grape samples are stored in laboratory ad will be used for these experiments in a short term.

2) An accurate analysis of the secondary metabolites by HPLC in Ca^{2+}-treated berries is already in progress in our laboratory, although no changes in total anthocyanins were observed in treated berries.

3) Studies of the berry skin by scanning electron microscopy will allow to obtain more solid information about the role of Ca^{2+} on the physical properties of the fruits observed in the present study.

4) We also intend to quantify the levels of Ca^{2+} in the pulp and in the skin of treated berries, and then in wines, to evaluate if Ca^{2+} levels may affect berry organoleptic characteristics and wine quality.

5) Fermentations at the laboratory scale with grapes treated with calcium and control are still in progress, preliminary results were obtained recently showing that endogenous fermentation is a bit delayed when Ca-treated berries were used, possibly because the microbiome of the berry is affected by Ca^{2+}. This could have a positive dimension to the producers, as they could inoculate musts with selected yeast strains for the production of specific volatile compounds.

6) Thus, the study of the microbiome of Ca^{2+} treated and control berries should be carefully evaluated, because figure 16 showed interesting differences of the surface of the fruits, possible because Ca^{2+} treated fruits lacks pruine, which may affect skin microbiome.

7) Finally, field studies should be repeated in two more seasons in order to strength all the above conclusions, and, eventually, to propose this approach to the stakeholders as a sustainable and eco-friendly treatment that may protect vines from diseases and from cracking.

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