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The study of the reprogramming of metabolism of Trincadeira grapes upon infection with *Botrytis cinerea*

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Resumo

As uvas (espécie *Vitis*) são, a nível económico, o fruto mais importante à escala mundial. No entanto, as videiras são suscetíveis a várias doenças, sendo que os fungos são a principal causa para a redução de qualidade em uvas e do seu respetivo rendimento. A *Trincadeira* é uma variedade de videira muito importante em Portugal, pois dá origem a excelentes vinhos em alguns anos. No entanto, é também extremamente suscetível a doenças derivadas de fungos como o bolor cinzento causado por *Botrytis cinerea*, que é uma das doenças mais perigosas para as uvas. Esta tese de mestrado tem como objetivo aumentar o conhecimento relativo à forma como o metabolismo das uvas, bem como a qualidade do vinho, é afetado pela infeção com *B. cinerea*, complementando estudos recentes. A iniciação dos mecanismos de defesa contra patógenos necrotróficos, biotróficos e hemibiotróficos está já bem documentada no estudo de tecidos vegetativos, enquanto outros órgãos como os frutos (neste caso, a uva) ainda não foram bem estudados nesse aspeto. Aquando a infeção, o metabolismo hormonal das uvas é reprogramado graças ao envolvimento putativo de jasmonatos, ácido abcísico, auxinas e outras fito-hormonas, ao passo que o ácido salicílico não aparenta estar envolvido neste processo. Como tal, estamos interessados em estudar a forma como o metabolismo hormonal é regulado em uvas infetadas com *B. cinerea*, especialmente tendo em conta o papel desempenhado por estas hormonas tanto no amadurecimento do fruto como na resposta a *stress* biótico. Com este trabalho, temos como objetivo revelar nova informação relativamente às respostas das uvas durante interações patógeno-hospedeiro. Isto será feito através de uma análise combinada de perfis metabólicos de bagos infetados com análise da expressão de alguns genes envolvidos no metabolismo de hormonas. Pretendemos comparar as mudanças observadas no metabolismo hormonal aquando infeção com *B. cinerea* entre uma variedade suscetível (*Trincadeira*) e uma variedade resistente (*Syrah*). Por outro lado, o estudo da forma de como o aroma das uvas é influenciado por este tipo de infeção (em particular, o estudo do metabolismo de terpenos), e quais as consequências na qualidade do vinho são aspetos importantes neste contexto.

De modo a conseguir estes resultados, bagos de uva controlo e infetados em três estágios de amadurecimento (green, EL32; *veraison*, EL35; harvest, EL38) foram colhidos para podermos efetuar quantificação de hormonas, análise de expressão de genes envolvidos no metabolismo hormonal e identificação de voláteis. Para fazer a análise de voláteis, foram usadas amostras liofilizadas a -40ºC. No entanto, foi concluído que, através do uso desta abordagem, muito poucos compostos conseguem ser detetados no processo e que uma grande quantidade inicial de material é necessária, de preferência proveniente de amostras frescas. Relativamente ao metabolismo de hormonas, os resultados obtidos mostram que os jasmonatos (OPDA e JA-Ile) estão envolvidos na resposta das uvas contra *stress* biótico proveniente da infeção. Estas hormonas já tinham sido caracterizadas como estando envolvidas no amadurecimento através do crescimento e do desenvolvimento de coloração dos bagos. Estudos prévios indicam que os jasmonatos são também responsáveis pela regulação de respostas de defesa como respostas contra *stress* oxidativo e stress por dessecação. Neste caso, as uvas da variedade *Syrah* apresentaram um elevado conteúdo basal de jasmonatos, ao passo que as uvas da variedade *Trincadeira* apresentaram um grande aumento na concentração de JA-Ile após estarem infetadas. As mudanças observadas na expressão dos genes envolvidos na biossíntese de JA-Ile (genes que codificam para as proteínas *allene oxide synthase* e *12-oxophytodienoate reductase 1*) apoiam este aumento observado na quantidade de jasmonatos, já que uma baixa expressão destes genes a nível basal significa que não são necessários nas primeiras fases de crescimento devido ao já elevado conteúdo em jasmonatos. O metabolismo relativamente ao ácido abcísico apoia o já conhecido papel desta hormona no amadurecimento. É sabido de estudos anteriores que o ácido abcísico tem um papel importante no amadurecimento das uvas, sendo responsável por processos importantes na acumulação

de açúcares e aumento da coloração dos bagos. Estes dados foram apoiados pelo aumento de ABA que observámos durante o início do amadurecimento. Para além do amadurecimento, os resultados que obtivémos sugeriram ainda que esta hormona possa estar também envolvida na defesa das uvas contra a infeção por *Botrytis cinerea*. Esta observação advém do facto de que o teor em ácido abcísico tende a aumentar após a infeção e de que se verificou uma maior expressão dos genes envolvidos na síntese e na sinalização desta hormona (genes que codificam para a *9-cis-epoxycarotenoid dioxygenase* e para o *ABA receptor PYL4 RCAR10*, respetivamente). No entanto, observou-se também um conteúdo basal em ABA baixo na variedade *Syrah*, que é resistente contra a infeção. Como tal, futuras investigações terão de ser feitas relativamente ao papel do ácido abcísico aquando a infeção. Relativamente ao papel das auxinas, sabe-se que estas hormonas estão presentes em baixas concentrações no início do amadurecimento, pois níveis elevados de auxinas atrasam a acumulação de açúcares que é essencial nesta fase. Estes dados estão de acordo com os resultados que obtivemos relativamente à expressão génica. No que toca ao seu papel na defesa, foram observados níveis basais elevados em uvas da variedade *Syrah* (que podem sugerir a existência de uma resposta acelerada quando infetadas) e aumentos de concentração em uvas da variedade *Trincadeira* após estas serem infetadas. Ambos estes fatores podem ser interpretados como indicadores de que as auxinas estão envolvidas na resposta das uvas contra o patógeno. Foram ainda observadas mudanças na expressão de genes envolvidos na síntese, sinalização e transporte de auxinas (genes que codificam para a *IAA-amido synthetase* e para o *auxin-responsive SAUR29*), em especial durante o início do amadurecimento. Estas mudanças sugeriram que as auxinas têm um papel importante tanto no crescimento como no amadurecimento das uvas. No que toca à importância do ácido salicílico, esta hormona tinha sido, devido a estudos anteriores, associada apenas à resposta contra fungos biotróficos. Estudos prévios estudaram o seu envolvimento na resposta contra fungos necrotróficos e foi colocada a hipótese de que, aquando a infeção, um fungo como *Botrytis cinerea* ativa alguns dos mecanismos de defesa da uva mas inibe as vias relacionadas com o ácido salicílico. No entanto, ao contrário do sugerido, também aparenta estar envolvida na resposta à infeção por fungos deste tipo, devido aos resultados obtidos em que a variedade de vinha resistente apresentou um alto conteúdo basal de ácido salicílico, o que pode significar uma resposta rápida contra a infeção. A expressão de genes envolvidos na sinalização mediada por ácido salicílico (genes que codificam para a proteína *enhanced disease susceptibility 1* e para o seu co-regulador, *phytoalexine deficient 4*) mostrou ter uma progressão que, inicialmente, apoiava estes resultados. No entanto, foi observado que esta expressão decresceu em uvas da variedade *Syrah* durante os primeiros estágios de crescimento, o que não está de acordo com os resultados obtidos pela quantificação de hormonas. Desta forma, serão precisos estudos mais desenvolvidos no que toca ao papel do ácido salicílico na defesa contra patógenos necrotróficos. Em conclusão, este trabalho permitiu efetuar uma análise de um modo mais detalhado do papel desempenhado por algumas fito-hormonas tanto no crescimento e amadurecimento de uvas como na defesa relativamente a *stress* biótico. Permitiu também a sugestão de mecanismos, tanto moleculares como metabólicos, que podem estar envolvidos na regulação de ambos os processos.

Palavras-chave: *Botrytis cinerea*, videira, metabolismo de hormonas, amadurecimento, resposta ao *stress*

Abstract

Grapes (*Vitis* species) are economically the most important fruit crop worldwide. However, grapevines are prone to several diseases, with fungi being the major cause of reduction in grape quality and yield. *Trincadeira* is a very important Portuguese grapevine cultivar, giving rise to unique and excellent wines in certain years. However, it is extremely susceptible to fungal diseases such as grey mold caused by *Botrytis cinerea*, which is one of the most dramatic diseases for grapes. This master thesis aims at further elucidating how the metabolism of grapes, as well as wine quality, is affected upon infection with *Botrytis cinerea*, complementing recent studies. The initiation of defense mechanisms against necrotrophic, biotrophic and hemibiotrophic pathogens has been documented mostly for vegetative tissues, while organs like the fruits have not been well studied in that regard. Upon infection, hormonal metabolism is reprogrammed with the putative involvement of jasmonates, abscisic acid, auxins and other phytohormones, whereas salicylic acid does not seem to play a role in the process as assessed by microarray. As such, we are interested in studying how hormonal metabolism is regulated in infected grapes given the role of hormones in both fruit ripening and biotic stress response. With this work, we aim at bringing novel insights into the responses of grapes during a pathogen-host interaction by a combined metabolic profiling analysis of infected fruits together with an analysis of the expression of genes involved in hormonal metabolism. We want to compare the changes in hormonal metabolism in a susceptible (*Trincadeira*) and a resistant (*Syrah*) variety upon infection with *Botrytis cinerea*. Furthermore, we want to study how the aroma of grapes is influenced by the infection, in particular the metabolism of terpenes, with consequences on wine quality. To this end, control and infected berries at the green, *veraison* and harvest stages were collected for hormonal quantification, analysis of gene expression regarding the metabolism of hormones and volatile identification. Regarding volatile analysis using samples lyophilized at -40ºC, it was concluded that few compounds can be detected using this approach and that a great quantity of starting material is needed and preferably fresh samples. In what concerns hormonal metabolism, our results showed that jasmonates were heavily involved in biotic stress response, as not only did *Syrah* grapes have a high basal content in jasmonates, but *Trincadeira* grapes displayed a significant increase in JA-Ile concentration upon infection. Changes in the expression of genes involved in JA-Ile biosynthesis accompanied this increase in quantity. Abscisic acid metabolism, while mostly cementing its role in ripening, also suggested an involvement in grape defense against *B. cinerea*, mainly due to increases in ABA content upon infection, as well as heightened expression of genes involved in its synthesis and signaling. However, due to the low basal content in ABA in the *Syrah* variety, further investigation will be pursued. Regarding auxins, the high basal levels in *Syrah* grapes and increased concentration in infected *Trincadeira* grapes were indicators of the hormone's involvement in response to the pathogen. Changes in the expression of genes involved in IAA synthesis, signaling and transport, especially during the onset of ripening, also suggested an important role of auxin regulation during grape growth and ripening. Salicylic acid, which had only been previously suggested to be involved in response against biotrophic fungi, also appears to play a part during *Botrytis cinerea* infection due to elevated basal SA content in the resistant cultivar. Expression of genes involved in SA-mediated signaling, although in accordance with these results at first, decreases in *Syrah* grapes during earlier stages, which does not support the obtained results from hormonal quantification. As such, further studies might be needed concerning the role of salicylic acid in response to necrotrophic pathogens. In conclusion, this work allowed a more detailed analysis of the role played by hormones in both grape ripening and defense and suggested molecular and metabolic mechanisms that might regulate both processes.

Keywords: *Botrytis cinerea*, grapevine, hormonal metabolism, ripening, stress response

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1. INTRODUCTION

1.1. Grape ripening

Grapevine is a fruit species of worldwide economic importance, mainly due to its use for vinification and distillation. It is classified as a non-climacteric fleshy fruit and is made up of three tissue layers and its seeds (Kuhn *et al*., 2014). Grapevine growth and development has been extensively studied, with the berries displaying changes in composition, size, color, texture and susceptibility against biotic stress during the process (Conde *et al*., 2007). It is a complex process, normally represented by a double sigmoid growth curve that results from the existence of two different stages of growth separated by a lag phase, during which berry growth is slow or nonexistent.

The first phase of berry development, which according to the BBCH identification system (COOMBE, 1995) encompasses the stages EL31 to EL34, is a phase marked mainly by rapid berry growth through cell division and enlargement. No further cell division occurs during berry development. While the berries remain green in color due to the presence of chlorophyll and sugar levels remain low, there is a large accumulation of organic acids in the vacuoles and precursors of other important compounds, such as phenols, are synthesized (Dokoozlian, 2000; Kuhn *et al*., 2014). The lag phase, whose duration depends on grape variety and climacteric conditions, is where the berries' chlorophyll content begins to drop. It is also marked by accumulation of organic acids. After the lag phase, the second phase of rapid berry growth begins with a short period known as *véraison* (stage EL35 in the BBCH identification system), characterized by sugar accumulation and pigmentation of berries by anthocyanins. The stages EL36 to EL38 makes the phase of berry development known as ripening, where red pigments accumulate further, the berries lose chlorophyll and compounds related to the aroma are created (Lund and Bohlmann, 2006). During this phase of development, sugars begin to accumulate, organic acids concentration declines and berries also become softer in texture and resume a rapid growth rate through cell enlargement (Dokoozlian, 2000). The amount of accumulated sugar and organic acids will ultimately influence the taste and other characteristics of the grape berries after the harvest stage (Conde *et al*., 2007).

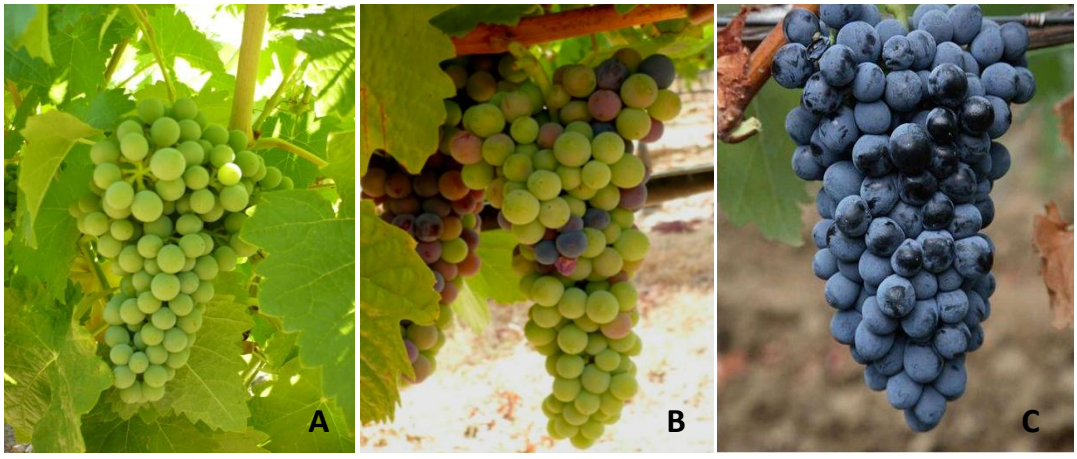


Figure 1.1 – Grape clusters of the *Trincadeira* cultivar through different stages of development: green (stage EL32; A), *veraison* (stage EL35; B) and harvest (stage EL38; C).

While the first rapid growth period contributes to the final quality of the grape berry, ripening is the most important phase for its development do to it containing massive increases in important compounds such as glucose and fructose, and it is heavily influenced by grape variety, crop level and

the environment. Temperature, water content, light conditions and pathogen intervention are the most important environmental factors, being able to enhance or delay ripening. Low temperatures, moderate water deficit and certain types of radiation such as UV-B can enhance the ripening process, while high temperatures or seasonal drought, lack of sunlight and biotic stress derived from pathogens are inhibitors of ripening (Kuhn *et al*., 2014). For example, a slight water deficit in earlier stages of berry development usually results in the increase of the concentration of anthocyanins and sugars, and some metabolites and transcripts are positively under these conditions (Deluc *et al*., 2009). On the other hand, light exclusion causes significant decreases in anthocyanin content through down-regulation of genes related to its biosynthesis (Matus *et al*., 2009).

Environmental conditions are also associated with changes in overall hormone content in grape berries, which is directly affects the process of ripening. One such example is abscisic acid, as its content increases in conditions of water deficit, which is beneficial for berry ripening.

1.2. Hormonal metabolism during grape ripening

Hormones play a key role in the development and ripening of grapes and other fleshy fruits. Depending on the phase of growth of a given fruit, different hormones can act either independently or in cooperation, by means of a complex hormonal crosstalk, to ensure the regulation of the fruit's development through all of its growth phases.

Across berry development, the synthesis of different hormones is controlled depending on the phase of growth and climacteric conditions, with some of them promoting the process of ripening and others inhibiting it. Ethylene synthesis, for example, is essential for several types of fruits known as climacteric fruits, being the main driving force behind the ripening process (Alkan and Fortes, 2015). During grape ripening, however, the peak of ethylene production observed in climacteric fruits does not happen. Instead, enzymes involved in ethylene biosynthesis have increased expression before *veraison*, and this hormone is suggested to influence berry diameter and anthocyanin accumulation (Chervin *et al*., 2004).

Abscisic acid (ABA) is a key hormone during in the maturation of both climacteric and non-climacteric fruits, but more importantly during grape ripening. Abscisic acid accumulation peaks at *veraison*, at the same time as berry softening and skin coloration, suggesting that it may help controlling these processes related to the ripening stage (Kuhn *et al*., 2014). Increases in the level of ABA also influence the accumulation of sugars by enhancing their uptake and storage, which can be stimulated earlier on by exogenous ABA application before *veraison* (C. Davies and Böttcher, 2009). Treating grape berries with ABA at *veraison* can also help improving anthocyanin levels and the biosynthesis of certain polyphenol compounds (Lacampagne, Gagné, and Gény, 2010).

Brassinosteroids are helpful for overall plant maturation and act towards the promotion of ripening in grapes and other non-climacteric fruits. During a previous study, genes involved in brassinosteroid synthesis revealed increased expression levels at the beginning of grape ripening, which was consistent with a larger content in the hormone at that stage. The application of a brassinosteroid isolate, epibrassinolide (Epi-BL), resulted in berry coloration and higher levels of sugar, leading to enhanced berry ripening. On the other hand, an inhibitor of brassinosteroid synthesis caused ripening to be delayed (Symons *et al*., 2006).

Auxins have an important role in fruit growth, but act as inhibitors of ripening in both climacteric and non-climacteric fruits (Kumar, Khurana, and Sharma, 2014). Indole-3-acetic acid (IAA), an important compound within the auxin class, has been observed as being present in higher amounts during early developmental stages of grape berries and in lower amounts in latter stages, including during berry ripening (X. Zhang *et al*., 2003). The onset of berry ripening is associated with

low levels of auxin, since this hormone causes delays in the accumulation of sugars, anthocyanins and in the increase of berry diameter. Low levels of auxin are directly connected to the inactivation of IAA by conjugation, which is realized by specific enzymes involved in IAA regulation mostly during grape ripening of several different cultivars (Fortes *et al*., 2011). The regulation of this hormone is also controlled by auxin response factors (ARFs), transcription factors that regulate the expression of responses mediated by auxin and that have an important role during *veraison* and initiation of grape berry ripening (Gouthu and Deluc, 2015).

Like auxins, cytokinins are involved in berry growth, but decrease in concentration during *veraison* and further maturation, because of which they act as promoters of early development but inhibitors of ripening (Böttcher *et al*., 2015). Treatment with cytokinins is often used as a way to reduce anthocyanin content in vineyards (Ferrara *et al*., 2014). Accordingly, previous studies have reported the down-regulation at stage EL36 of genes coding for cytokinin dehydrogenase 5, involved in cytokinin degradation, and for a purine permease involved in cytokinin transportation (Agudelo-Romero *et al*., 2013).

Gibberellins follow a similar pattern, being essential for early plant development. This hormone is often applied on plants due to its function as a regulator of cell division and expansion. Due to this, active gibberellins are found in higher concentrations during early stages but in lower concentration during further berry development (X. Zhang *et al*., 2003), and their abundance is regulated by the abundance of gibberellins oxidase transcripts. A previous study showed that, during ripening, some genes coding for these oxidases were up-regulated when compared to stage EL32, but others were down-regulated, which makes the metabolism of gibberellins harder to understand (Fortes *et al*., 2011).

In non-climacteric fruits, jasmonic acid levels are higher during early development and decrease during ripening, which appears to enable the beginning of this stage. This observation was supported by the lesser amount of jasmonic acid biosynthesis-related mRNAs during and after *veraison* (Fortes *et al*., 2011). Jasmonates are also involved in the synthesis of methyl jasmonate, which when applied in grapevine increases the production of a compound that acts during plant defense against pathogens (Almagro *et al*., 2014).

The role of polyamines during ripening is harder to classify due to lack of information. Polyamines have been suggested to be inducers of flowering and fruit set in grapes during earlier developmental stages (Aziz, Brun, and Audran, 2001), and their content has been shown to decrease at the beginning of ripening, because of which this hormone's role has been exclusively associated with early fruit development. However, previous studies have shown that genes coding for enzymes involved in polyamines synthesis, such as arginine decarboxylase, increased their expression during the ripening stage (Fortes *et al*., 2011). Products originated from the catabolism of polyamines, such as γ-aminobutyric acid (GABA), have also been observed as being accumulated during grape ripening (Agudelo-Romero *et al*., 2014). By further analysis of the polyamine pathway, it was noticed that, indeed, the content in free polyamines decreased during ripening and was accompanied by an increase in activity of polyamine catabolic enzymes, namely polyamine oxidase and diamine oxidase. This increase in polyamine catabolism leads directly to a larger content in hydrogen peroxide. Polyamine catabolism can then be seen as a way to produce reactive oxygen species that are important in plant defense against stress during ripening (Fortes, Teixeira, and Agudelo-Romero, 2015) Grape ripening is, therefore, controlled by crosstalk between these hormones. The study of hormonal crosstalk has revealed several hormonal interactions, such as the one between ethylene and abscisic acid. Through an experiment based on blocking the synthesis and the signaling of ethylene, Sun *et al*. (2010) discovered that the effects of ethylene inhibition vary according to the berry's developmental stage and that both ethylene and ABA are important for starting berry ripening, which is achieved through hormonal interplay.

Auxins, on the other hand, appear to inhibit ripening processes induced by ABA. A previous study revealed that the treatment of berries with naphthaleneacetic acid delayed ripening onset, which was confirmed by transcriptomic analyses. The expression of genes related to ABA biosynthesis also decreased as a consequence of this delay, as opposed to ethylene biosynthetic genes, which had their expression increased (Ziliotto *et al*., 2012). Treatment of berries with artificial auxins also caused delays in the increase of ABA content (Christopher Davies, Boss, and Robinson, 1997).

Application of gibberellins promotes several interplays with different hormones such as brassinosteroid, ethylene, cytokinin and ABA. In this study, crosstalk between gibberelins and ABA was observed in the down-regulation of genes related to ABA signaling and synthesis, while in the case of ethylene it was observed in the regulation of the amount of ethylene transcription factors (Chai *et al*., 2014).

1.3. Hormonal metabolism during grape defense

Another factor that plays a part in the process of growth and ripening of fleshy fruits is stress. Stress can be caused by environmental conditions, availability of nutrients or infections from organisms such as bacteria or fungi.

One such fungal pathogen is grey mold, caused by *Botrytis cinerea*, which, in grapevines, is the main cause of infection, damage and loss in grape quality (Agudelo-Romero *et al*., 2015). *Botrytis cinerea* is a necrotrophic fungus to which several varieties of grapevine are susceptible, and it achieves a drop in grape quality by penetrating its host with specialized enzymes that degrade the cell wall and by using compounds such as reactive oxygen species to kill the cell (Tatsuki *et al*., 2013).

In fruit-pathogen interactions such as the one between tomato fruit and *B. cinerea*, stress hormones, prone to changes in their relative expression, act as regulators and heavily influence the fruit's susceptibility to infection. The influence of ethylene regarding fruit ripening is especially important in these scenarios because it contributes to fruit ripening, a state in which it becomes more susceptible to infection (Cantu *et al*., 2009). Ethylene-related signaling components, such as receptors for ethylene, and the way they react to the presence of the hormone are also of note, as some fruits that have mutations for reduced receptor sensitivity are less susceptible to infection by *B. cinerea* (Cantu *et al*., 2009).

Experiments were conducted involving the exposure of ethylene receptors to specialized inhibitors: high inhibitor levels were able to halt the function of ethylene receptors and, therefore, the plant's resistance mechanisms, showing that plant responses to ethylene depend on the receptors' ability to perceive it during tomato fruit infection (Blanco-Ulate *et al*., 2013). Moreover, ethylene synthesis in the host can be caused by pathogen infection, which was suggested by an augmented expression of two genes related to the ripening of tomato, after the microarray analysis of fruits infected with *B. cinerea*. Pathogens such as *B. cinerea* can also synthesize it independently in order to promote earlier ripening and facilitate the infection (Cantu *et al*., 2009). Salicylic acid is also an important hormone, and tomato lines that do not accumulate this hormone have been shown to be more susceptible to infection than regular tomato lines, proving that salicylic acid-mediated plant responses contribute to fruit resistance (Blanco-Ulate *et al*., 2013).

Based on microarray analysis by Blanco-Ulate *et al*. (2013), the role of jasmonic acid during tomato fruit infection is not clear due to the lack of changes in expression of important components from the jasmonic acid signaling pathway, which may suggest the existence of different signaling pathways through which jasmonic acid-related responses in fruit occur. It is known, however, that the signaling pathways of jasmonic acid and salicylic acid are generally antagonistic, and that the interplay between them, mainly influenced by hormone concentration, allows plants to regulate their

defense mechanisms and adapt to the pathogen in question (Pieterse *et al*., 2009). However, pathogens like *B. cinerea* can bypass this interplay and inactivate specific genes necessary for plant resistance.

Abscisic acid synthesis is mainly related to increased fruit susceptibility. During infection, certain plant pathogens can directly produce ABA or induce its synthesis in the host in order to accelerate fruit ripening (Siewers, Smedsgaard, and Tudzynski, 2004). Overall perception and synthesis of different stress hormones, along with their relative concentrations, are the main driving forces behind plant resistance against pathogens.

A research was conducted by Agudelo-Romero *et al*. (2015) in order to obtain information concerning the way *B. cinerea* affects grape metabolism and overall functioning. It was revealed that *B. cinerea* causes not only a decrease in berry weight and an increase in pigmentation, but it also influences grape metabolism to a point where several metabolites are present in different contents when comparing between control berries and infected berries. For example, some organic acids such as tartaric and malic acid decreased in infected berries.

On the other hand, adding to the increase in pigmentation due to anthocyanin, the content in certain sugars such as fructose and glucose also increased upon infection. In this study, it was also seen that the infection by *B. cinerea* activates influences the reprogramming of metabolism regarding the activation of defense mechanisms in different growth phases of the berries, with some examples being the oxidative stress response and the activation of heat-shock proteins. Other types of suppression of the infected berries' activated defenses were the downregulation of resistance genes involved in pathogen recognition, genes related to the organization of the cytoskeleton and the metabolism of lipids, showing that *B. cinerea* does indeed reprogram the metabolism of its host in order to facilitate infection.

1.4. Volatiles and grape quality

Directly tied to the development and ripening of berries is the production of volatile compounds, which is one of the most important factors influencing the aroma development of grape cultivars and, consequently, the characteristics and quality of wine (Palomo *et al*., 2007). Even though most of these volatile organic compounds exist in odorless and inactive forms (Hjelmeland and Ebeler, 2015), some grape compounds such as terpenes, mainly those belonging to the monoterpenes, sesquiterpenes and norisoprenoids sub-families, are the ones more directly linked to wine aroma, even if still present in trace amounts (Dunlevy *et al*., 2009). In grapevine, important volatile compounds that have been identified include varietal aroma-responsible monoterpenes and rotundone, which belongs to the sesquiterpenes sub-family (Siebert *et al*., 2008). Several other volatile organic compounds belong to different chemicals groups, and the proportions in which these are present is largely dependent on the grape variety and berry developmental stage (Slegers *et al*., 2015), while environmental factors have a lesser impact.

Besides terpenes, the volatile compounds that are most relevant to aroma development are aldehydes, ketones, alcohols of various kinds, including unsaturated alcohols and aromatic alcohols, esters of acetic acid, chlorophylls, carotenoids and compounds derived from amino acids or fatty acids (Rambla *et al*., 2016). All these aroma-related compounds have been reported to be present in both free and glycosylated non-volatile forms, with monoterpenes being the most abundant ones included in this group (Agudelo-Romero *et al*., 2013).

Volatiles derived from amino acids are mostly synthesized from phenylalanine, and a large amino acid profile has been established as being directly connected with higher alcohol content and, consequently, wine aroma (Hernández-Orte, Cacho, and Ferreira, 2002). Their biosynthesis begins with the enzyme phenylalanine ammonialyase and latter steps involve catabolyzation by an O-

methyltransferase (Mageroy *et al*., 2012). Amino acid-derived volatiles fall into the categories of aldehydes, esters or alcohols. Volatile aldehydes and phenols, with phenylacetaldehyde being an example of the latter, are responsible for distinct aromas of some grape varieties (Wang and Kays, 2000).

Those derived from fatty acids have a poorly understood process of biosynthesis, although it is known to involve the catabolization of free fatty acids through the lipoxygenase pathway. Common lipidic precursors used catabolized during this pathway are linoleic and linolenic acids (Rambla *et al*., 2016). Metabolites resulting from this pathway can also be reduced to alcohols through the activity of alcohol dehydrogenases (Tesniere *et al*., 2006).

Terpenes, most importantly monoterpenes and sesquiterpenes, play a major role in influencing the overall aroma of grape varieties, and are synthesized by means of two distinct pathways known as the isoprenoid pathways. These pathways are sources of the five-carbon precursor isopentenyl diphosphate and its isomer, dimethylallyl diphosphate, which are then used to create volatile terpenoid compounds (Rambla *et al*., 2016), including the aforementioned monoteprenes and sesquiterpenes, and others such as sterols, phytols and carotenoids (Agudelo-Romero *et al*., 2013). Volatile terpenes like norisoprenoids can also be derived from the carotenoids formed during these pathways, an action that is carried out by the activity of carotenoid cleavage dioxygenases.

Terpene evolution throughout berry development can be studied by comparing terpene profiles from different developmental stages, with the most relevant ones being early development, pre-*veraison*, *veraison* and post-*veraison*, which takes into account the harvest stage (Zhang *et al*., 2016). A study conducted by Zhang et al. (2016) has shown that terpene concentration was lower during the *veraison* stage when compared to both pre-*veraison* and post-*veraison*. During early development, a high production of terpenes is present in grape berries, be it in concentration or variety. The early developmental stage shows the highest concentration of volatile compounds out of all relevant stages of berry development, both when it comes to overall terpene and, more specifically, monoterpene and sesquiterpene concentrations.

A significantly different profile can be observed during the pre-*veraison* stage, as the grape berries show a large decrease in terpene concentration. This happens mostly due to a much lower concentration of volatiles from the sesquiterpene sub-family, while monoterpene concentration was only slightly reduced. Norisoprenoid concentration, however, reaches its peak in this stage.

A lower concentration of total volatile compounds is observed at *veraison*, during which the monoterpene and sesquiterpene sub-families reach their lowest concentration levels and several compounds were unable to be detected (Coelho *et al*., 2007). A less drastic decrease in concentration is observed for the norisoprenoid sub-family, which contributes for most of the total terpene concentration in the berries during this stage.

During a post-*veraison* stage where the grape berries display intermediate ripeness, overall terpene concentration increases, even though the monoterpene and sesquiterpoene concentrations remained at low levels, which coincided with the observations from a previous study by Coelho *et al*. (2006).

At harvest, berries showed a very different terpene profile when compared to the previous developmental stage. Overall terpene concentration increases, mainly due to a significant rise in sesquiterpene concentration, since monoterpenes and norisoprenoids decrease during this period. Therefore, the late period of ripening appears to be crucial to the development of sesquiterpenes at harvest. Previous observations made by Kalua and Boss (2009) have confirmed similar patterns in terpene profiles in all relevant developmental stages. These compounds belong in the group of secondary metabolites and are distributed through the pulp and skin of the berry, with several studies showing volatile compound storage predominantly in the skin tissue (Perestrelo *et al*., 2011).

Harvesting at the correct growth stage is, then, important to optimize the overall quality of the grape wine aroma.

1.5. Impact of *Botrytis cinerea* infection in grape quality

Most previous studies have focused on the mechanisms of volatile compounds and how they affect aroma development and grape quality, which are directly related to primary metabolites such as amino acids or polyphenols (López-Rituerto *et al*., 2010). Because of this, the study of how infection by *Botrytis cinerea* affects the production of primary metabolites in grapes can greatly help the understanding of how fungal infection is related to alterations in grape and wine quality (Hong *et al*., 2011).

B. cinerea infection of grape berries happens with greater intensity from the beginning of ripening until the stage of harvest, and leads to reductions in grape and wine quality by influencing the chemical composition of the berries. A previous study was focused on analyzing metabolic variations of pulp and skin metabolites upon infection, comparing healthy and infected grape clusters (Hong *et al*., 2012). In infected clusters it was observed a greater content in several aminoacids such as fructose and glucose in berry skin, while phenolic compounds were not detected when compared with healthy clusters. Regarding the berry pulp, infected clusters showed larger concentrations of GABA and arginine, for example. Healthy berries present in infected clusters were also analyzed, containing a greater content in a larger number of metabolites when compared to those of healthy clusters in both berry skin and pulp. These metabolic variations ultimately show that *B. cinerea* does affect the metabolome of both infected berries and even healthy berries when present in an overall infected grape cluster.

The study of Hong *et al*. (2012) also allowed the analysis of the accumulation of several primary metabolites related to plant defense strategies, both on healthy berries collected from infected bunches and infected berries collected from infected bunches. Proline accumulation in infected grape berries resulted from its involvement in strengthening the cell-wall during plant defense against pathogen infection (Haudecoeur *et al*., 2009). The accumulation of arginine is a result of the need to synthesize polyamines that conjugate with phenolic compounds and are helpful in the defense response against *B. cinerea*. Arginine acts as a precursor to these polyamines, and the resulting products of conjugation have also been shown as accumulated in infected plants. In conjunction with proline and arginine accumulations, another compound that accumulated in grape berries was glutamate, which serves a precursor to both these stress-related metabolites and GABA (Forde and Lea, 2007). Accumulation of alanine in scenarios of biotic stress has also been observed, although its exact role is unclear. GABA accumulation accompanies that of alanine, since the catabolism of the former by means of a GABA transaminase leads to the formation of the latter (Hong *et al*., 2012).

Alterations of the overall metabolome were reported in a study, conducted by Hong *et al*. (2011), regarding champagne base wines when influenced by *B. cinerea*. Differences in the metabolites were seen between wines based on healthy berries and botrytized ones, therefore allowing to assess not only the grape quality but also the quality of the wine. Increased concentrations in gluconic acid were observed in infected grapes and wines, as well as higher pH values when compared to those of healthy samples. These results were in accordance with a previous study, demonstrating that gluconic acid content can be seen as an indicator of *B. cinerea* infection and that a lower pH value is directly linked to degradation of organic acids by the pathogen (Cilindre *et al*., 2007).

Variations regarding glucose levels in wine were also analyzed, by comparing glucose contents between base wines derived from healthy berries and botrytized berries at different levels of infection. Due to glucose and infection levels not being linearly correlated, it was concluded that the

amount glucose consumed during the process of fermentation was not influenced by *B. cinerea* infection of grape berries.

Succinate, an important component for grape and wine quality, was found at lower levels in botrytized base wines when compared to wines derived from healthy grapes. This can be explained by the fact that, in a previous study, *B. cinerea*-infected grape berries showed a reduction in overall amino acid content, since amino acids contribute to the formation of succinate through fermentation (BELL and HENSCHKE, 2005). Reduced succinate levels in infected base wines are directly related to a flawed tricarboxylic acid cycle when compared to healthy base wines. This was seen as an indicative that *B. cinerea* inhibits alcoholic fermentation since succinate is a major component of this process, therefore causing a quality loss in wine (Hong *et al*., 2011). This research showed that metabolome analysis is crucial do the understanding of metabolic modifications in infected grape berries, and therefore in overall grape and wine quality.

1.6. Scope and objectives of the study

In the same segment of the work realized by Agudelo-Romero *et al*. (2015), this study has the objective of exploring how the metabolism of grape berries is affected by infection with the necrotrophic fungus *Botrytis cinerea*, and how these changes affect not only grape quality, but also wine quality.

Besides assessing grape and wine quality, it is also known that how defense mechanisms in plants are triggered when infected by necrotrophic, biotrophic and hemi-biotrophic pathogens has mostly been documented for vegetative tissues (Blanco-Ulate *et al*., 2013). As such, this work will allow for novel insights into the response of other organs, namely fruits, during an interaction between host and pathogen. Previously, a combined analysis of the transcriptome and metabolome of infected grape berries was conducted and suggested that hormonal metabolism play an important role in the interaction (Agudelo-Romero *et al*., 2015). In this thesis, the expression of genes related to hormonal metabolism and signaling, as well the analysis of their content was carried out. It is known from previous studies that hormones play an important role during fruit berry ripening and as a response to biotic stresses such as infection by *B. cinerea* (Blanco-Ulate *et al*., 2013). Genes related to aroma development will also be analyzed in the future, mainly those related to the biosynthesis of volatile compounds during grape development. The latter study will allow to deepen the understanding of how grape infection can be directly linked to wine quality and, consequently, its production worldwide.

To this end, analyzing a susceptible variety (*Trincadeira*) and a resistant variety (*Syrah*) of grape is essential, allowing to deepen the understanding of hormone regulation and aroma development. Studies of both varieties were conducted with control samples and samples infected with *Botrytis cinerea*, and these samples were distributed across three time points according to the BBCH identification system (COOMBE, 1995): green stage (EL32), *véraison* (EL35) and harvest stage (EL38).

2. MATERIALS & METHODS

2.1. Infection of berries and sample collection

Infections of grapevine berries with *Botrytis cinerea* were made in June 2016 at the Instituto Superior de Agronomia, University of Lisbon, Portugal. The infections were made by spraying berry clusters with a conidial suspension at the developmental stage of peppercorn size (stage EL29), following the procedure by Agudelo-Romero *et al*. (2015). Collection of *Trincadeira* and *Syrah* samples was performed at three different stages of development: green (stage EL32), *veraison* (stage EL35) and harvest (stage EL38) (COOMBE, 1995). Five to six replicates were obtained for each stage of development and for each treatment (control and infected), and all berry clusters were transported to the laboratory in dry ice and then conserved at -80°C until further use.

2.2. Sample preparation and conservation

With the use of a mortar and pestle, berries were smashed in liquid nitrogen, the seeds were removed and frozen samples were grinded into a fine powder, organized according to their stages of development and transferred to *falcon* tubes, which were then kept at -80°C. During grinding, liquid nitrogen was carefully used in order to consistently keep the samples in a frozen state. The materials used in the grinding and transference of samples were sterilized by leaving them in contact with sodium hydroxide overnight, followed by extensive washing in tap water and DEPC water (previously prepared by adding 0,1% of DEPC to Mili-Q ultrapure water) and further sterilized in the oven at 180°C for a minimum of 2 hours.

2.3. Hormone quantification

Stock solutions of each original phytohormone standard were prepared at 1 mg/mL in MeOH at Max Planck Institute for Chemical Ecology (Jena, Germany). For deuterated compounds, stock solutions were prepared in acetonitrile at 100 µg/mL. Working solutions of original phytohormones standards were prepared by diluting stock solutions in MeOH:water (7:3), at different concentration for each phytohormone depending on the range of the calibration curve: ABA and IAA at 100 µg/mL; JA and SA at 200 µg/mL; OPDA at 50 µg/mL; and JA-Ile at 40 µg/mL. The internal standard stock solutions (d5-JA, d6-ABA, d4-SA, and d5-IAA) were combined and diluted in MeOH:water (7:3) ratio, resulting in the extraction solution. The final concentrations were 10 ng/mL for both d4-SA and d5-IAA, and 20 ng/mL for both d5-JA and d6-ABA.

The samples (5 to 6 biological replicates) were transferred to *Falcon* tubes, which then contained grounded plant material up to the 25mL mark, and freeze dried at -40°C for three days. *Eppendorf* tubes containing approximately 25mg of material were sent to Max Planck Institute for Chemical Ecology. 1mL of extraction solution containing the internal standards (d5-JA, d6-ABA, d5-IAA, and d4-SA), prepared as described previously, was directly added. The samples were briefly mixed with a vortex, and spiked with phytohormones standards as described in Trapp *et al*. (2014). The spiked samples were shaken for 30 minutes and centrifuged at 16000g and 4°C for 5 minutes. The supernatant was transferred into a new micro-centrifuge tube and dried in speed vac. After drying, 100µL of MeOH were added to each sample, which were then mixed with a vortex and centrifuged at

16000g and 4°C for 10 minutes. The supernatant was analyzed by HPLC-MS/MS (high performance liquid chromatography-mass spectrometry).

2.4. DNA extraction

DNA extraction was done according to Lodhi *et al*. (1994). In order to isolate DNA, 5 mL of DNA extraction buffer (20 mM sodium EDTA, 100 mM Tris-HCl at pH 8.0, 1.4 M NaCl, CTAB 2.0% w/v, β-mercaptoethanol 0.2%) were added to each sample, which were then incubated at 60°C for 25 minutes. After letting the samples cool down at room temperature, an extraction with 6 mL of chloroform:octanol 24:1 (v/v) was performed, right before a centrifugation at 5000 g for 20 minutes at 4°C. The supernatant was recovered to a new tube, and 1/2 volume of 5 M NaCl was added and thoroughly mixed. DNA precipitation was performed by the addition of 2 volume of ethanol 95% at -20°C, after which the samples were incubated at -20°C for 10 minutes, followed by approximately 1 hour at 4°C. Two 4-minute centrifugations at 4°C were performed next, at 3000 g and 5000 g respectively. The supernatant was discarded and the pellet was washed with 10 mL of cold ethanol 76%. This was followed by a centrifugation at 5000 g for 5 minutes at 4°C, after which the samples were incubated at 37°C for 25 minutes in order to completely remove the ethanol. Approximately 250 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA) at pH 8.0 were added, and the samples are incubated at 4°C overnight. The samples were transferred to *eppendorf* tubes and 1/10 volume of 2 M potassium acetate was added, which was followed by an 45-minute incubation on ice (this procedure was not included in the initial protocol). After incubation, a centrifugation at 10000 g for 10 minutes at 4°C was performed, and then the supernatant was recovered. RNase A at 1 µL/100 µL was added to the pellet, and the *eppendorf* tubes were incubated at 37°C during 15 minutes. A second extraction with chloroform:octanol 24:1 (v/v), now with 1 volume being added, was performed, after which the tubes were centrifuged at 10000g for 10 minutes. The largest amount of sample possible was transferred into a new *eppendorf* tubes and a new precipitation step occurred with the addition of 1/10 volume of sodium acetate and 2 volumes of ethanol 100%. After 1 hour at -20°C, the tubes were centrifuged at 10000g during 10 minutes. The supernatant was then discarded and the pellet washed by adding approximately 200 µL of ethanol 76% at -20°C, followed by another 5-minute centrifugation at 10000g. The ethanol was discarded and, after incubating the tubes for 15 minutes at 37°C, 15 µL of TE buffer were added before leaving the *eppendorf* tubes conserved at 4°C. After the extraction, a 0,8% agarose gel was used to verify the quality of the DNA. The DNA purity and concentration were measured using a spectrophotometer (Appendices, Table 1)

2.5. RNase A preparation

The RNase A solution used in the extraction of DNA was prepared according to the Ribonuclease A from bovine pancreas preparation protocol from Sigma-Aldrich, with minor alterations. A 10 mg/mL stock solution in 10 mM of sodium acetate buffer was prepared for 50 mL, and the pH was adjusted to 5.2 with the addition of HCl 37%. The solution was heated to 100°C during 15 minutes and then allowed to cool down to room temperature. The pH was adjusted to 7.4 by adding 1 M Tris-HCl at pH 7.4, after which the solution was aliquoted and stored at 20°C.

2.6. Assessment of *Botrytis cinerea* infection with PCR

Detection of samples infected with *Botrytis cinerea* was made by visual inspection and through PCR. After DNA extraction and quantification, the PCR reaction was prepared by mixing 3 μL of DNA:water (containing 100 ng of DNA) with 22 μL of a previously prepared master-mix: 16,76 μL of DEPC water, 0,6 μL of a dNTP mix, 0,14 μL of DreamTaq DNA polymerase, 2,5 μL of the appropriate buffer (10x) and 2 μL of *forward* and *reverse* primers specific for amplification of fungal DNA. Primers for species-specific amplification of *B. cinerea* DNA were previously reported (Rigotti *et al*., 2002) and are as follows: C729+: AGCTC-GAGAGAGATCTCTGA; C729-: CTGCAATGTTCT-GCGTGGAA.

The samples were submitted to a PCR program (35 cycles with an initialization step at 95°C for 3 minutes, a denaturation step at 95°C for 30 seconds, an annealing step at 55°C for 45 seconds, an extension step at 72°C for 45 seconds and a final elongation step for 10 minutes at 72°C for the extension of any remaining single-strand) and then loaded in a 1,2% agarose gel in order to evaluate the level of infection.

Using this approach, it was possible to confirm the samples that were infected and those that would serve as controls without natural infections (Appendices, Figure 1). It is noteworthy that *Botrytis cinerea* was only slightly detected in Syrah grapes at green and *veraison* stages. Certain samples were later pooled so that, for RNA extraction, 3 biological replicates would be used.

2.7. RNA extraction

Small scale RNA extraction was performed following the RNeasy Plant Mini Kit protocol. A different extraction buffer (300 mM Tris-HCl at pH 8.0, 25 mM EDTA, 2 M NaCl, CTAB 2% w/v, PVPP 2% w/v, β-mercaptoethanol 2%) was used for this extraction, and it was prepared beforehand and incubated at 60°C for 20 minutes in agitation. The samples had a maximum of 100 mg of tissue powder, and 450 μL of the extraction buffer were added to each one. The samples were then vigorously agitated. The resulting lysate was transferred to a lilac QIAshredder spin column in a 2 mL collection tube, which was centrifuged at full speed during 2 minutes. The supernatant of the flow-through was transferred to a new tube, to which 0.5 volume of highly concentrated ethanol (96% to 100%) was added and mixed by pipetting. The resulting sample was transferred in its entirety to an RNeasy Mini pink spin column in a 2 mL collection tube. The tube was centrifuged at 10000 g for 15 seconds and the flow-though was discarded. 700 μL of Buffer RW1 (wash buffer) were added to the RNeasy spin column, and the tube was centrifuged at 10000g during 15 seconds before discarding the flow-through. The same procedure was done for the addition of 500 μL of Buffer RPE (buffer for washing membrane-bound RNA; requires the addition of ethanol). A third centrifugation at 10000g was done, after adding another 500 μL of Buffer RPE, but this time for 3 minutes in order to dry the membrane. The column was placed in a new 1.5 mL collection tube and 30 μL of RNase-free water were directly added to the column membrane, which was then centrifuged at 10000g for 1 minute to elute the RNA. The extracted RNA quantification was conducted using a spectrophotometer.

A large scale RNA extraction protocol was used for larger samples. A new extraction buffer (1 M Tris-HCl at pH 9.0, SDS 1%, PVP 0.8%, β-mercaptoethanol 5%) was used, and approximately 12 mL were added to each *falcon* tube containing powder tissue up to the 16.5 mL mark. Using the vortex, the samples were agitated for 1 minute and then put on ice. An extraction with chloroform:isoamyl alcohol was performed, after which the tubes were centrifuged at 7300 rpm for 10 minutes. Approximately 20 mL of the aqueous phase of each sample were transferred to a new set of

falcon tubes and 1.6 mL of 2 M potassium chloride were added for protein precipitation. The tubes were mixed and left on ice during 1 hour. After a centrifugation at 9800 rpm for 15 minutes, the supernatant was recovered to *Corex* tubes. 1/10 volume of 3M sodium acetate and 0.8 volume of cold isopropanol were added to each sample to precipitate nucleic acids, followed by mixing the tubes thoroughly and a centrifugation at 10000 rpm during 15 minutes. The supernatant was discarded and two washes with 70% ethanol were performed, after which the tubes were left drying in the fume chamber for 15 minutes. The *pellet* was then dissolved in approximately 1.4 mL of DEPC water, and all the samples were transferred to *eppendorf* tubes. 1/10 volume of 2 M potassium acetate was added to aid in the precipitation of polissacharides, and the tubes were left on ice for 60 minutes, followed by a centrifugation at 10000g for 15 minutes. The supernatant of each sample was transferred to two separate *eppendorf* tubes, with 1 volume of 4 M of lithium chloride being added to each tube. The samples were left overnight at 4°C for precipitation, after which they were centrifuged at 10000g for 15 minutes. The supernatants were discarded. Two washes using 70% ethanol were performed and the tubes were left drying for 30 minutes in the fume chamber. The *pellet* was later dissolved in 50 μL of DEPC water and stored at -20°C for a short period. The extracted RNA quantification was conducted using a spectrophotometer. RNA integrity was verified through agarose gel electrophoresis (Appendices, Figure 2).

2.8. Sample purification

In order to purify the extracted samples, a treatment with DNase was conducted. To new *eppendorf* tubes, 24,5 μL of each RNA sample were transferred, along with 1,5 μL of RNase inhibitor, 3 μL of buffer (10x) and 1 μL of Turbo DNase Ambion. The samples were mixed and then diluted in 100μL of DEPC water. Then the Spectrum™ Plant Total RNA Kit was used, and 250 μL of binding solution were added, after which the samples were mixed with a pipette. The mixture was transferred to a binding column in a collector tube, which was centrifuged at 10000g for 1 minute. 500 μL of wash solution 1 were added to the mixture, followed by another centrifugation at 10000g for 1 minute. Two more identical centrifugations were executed after adding 500 μL of wash solution 2. After each centrifugation, the binding column was removed and the collector tube was dried. The column was then dried by centrifuging during 1 minute at 10000g, after which it was put in a new collector tube. 50 μL of elution solution were carefully added to the center of the membrane, followed by 1 minute at room temperature and a short centrifugation at 10000g to elute the sample. After the purification step, a 1,2% agarose gel was used to verify the quality of the RNA. The quantification of the newly purified RNA samples was conducted using a spectrophotometer.

2.9. Reverse transcriptase protocol

First-strand cDNA was synthesized from 2μg of total RNA as described previously in Ana M Fortes *et al*. (2011). The Thermo Scientific RevertAid H Minus Reverse Transcriptase protocol was followed in order to obtain cDNA from the extracted RNA. For each sample, 11,5 μL of RNA with DEPC water and 1 μL of oligo(dT) primers were added to a sterile *eppendorf* tube. The tubes were mixed gently, briefly centrifuged at 10000g and incubated at 65°C during 5 minutes, after which they were put on ice and centrifuged a second time. 4 μL of reaction buffer (5x) were added to each tube, as well as 0,5 μL of RNase inhibitor, 2 μL of a dNTP mix and 1 μL of RevertAid H Minus reverse

transcriptase. The tube were mixed gently, centrifuged at maximum speed and incubated at 42°C during 60 minutes, after which they were stored at -20°C.

2.10. Real-time quantitative PCR

Samples to be analyzed were diluted either 40x or 80x according to the level of expression. Before the final qPCR essays, all primer pairs were tested using two samples of each cultivar and a negative control. A dilution series was also prepared, with either a 1:10 dilution factor or, alternatively, 1:5 (for less expressed genes). Plates with 48 wells were used: the first wells were used for the dilution series (1:1, 1:10, 1:100, 1:1000 or 1:1, 1:5, 1:25, 1:125, 1:625), with each one having three replicates; the remaining wells were used for the samples, with three biological replicates for each time point and grape variety, and for the negative control. Additionally, each qPCR essay was repeated in a separate plating, yielding two technical replicates.

A master-mix was prepared for a total of 51 reactions to avoid pipetting errors, so that each well would contain 10 μL of Sybr Green, 0,7 μL of each primer (*forward* and *reverse*, diluted to a stock solution of 100 μM), 4,6 μL of ultra-pure water and 4 μL of the appropriate cDNA sample. In these essays, the preparation of the master-mix and its distribution were done inside a UV chamber.

Data were normalized using the expression of the actin gene (VIT_04s0044g00580) and elongation factor 1α gene (VIT_06s0004g03220), which are the most stable genes according to NormFinder software (Agudelo-Romero *et al*., 2015). The quantitative PCR program used in these essays consisted of 42 cycles with an initialization step at 95°C for 10 minutes, a denaturation step at 95°C for 15 seconds, an annealing step for 40 seconds whose temperature was based on the calculated melting temperature of the primers being used (generally one degree lower), an extension step at 95°C for 15 seconds and an elongation step at 60°C for 1 minute. All primer pairs used in the qPCR were designed using the PrimerSelect software (Appendices, Table 2). qPCR essays were carried out using the StepOne Real-Time PCR System (Applied Biosystems).

2.11. Identification of volatiles

In order to identify volatile compounds, approximately 1-2g of two harvest samples, one control and one infected, of each cultivar were used, and divided by two smaller tubes. A solution of 0,2 g NaCl/1 mL was prepared, and 500 μL were added to each tube. After adding the solution, each tube was left in a water bath at 50°C for 30 minutes to stimulate the emission of volatiles. For another 30 minutes, the volatiles compounds were analyzed using solid-phase microextraction and gas chromatography-mass spectrometry (GC-MS). Lastly, the enzyme β-glycosidase was added and left to act overnight at 37°C, and then analyzed again.

3. RESULTS

3.1. Optimization of RNA extraction protocol

With the initial RNA extractions following the RNeasy Plant Mini Kit protocol, we were unable to get the desired results in order to proceed to reverse transcription (Appendices, Table 3). Due to the use of spin columns in the essay, large amounts of sample could not be employed, which in turn lead to a lower yield. Different types of extraction buffer were also used in the process: but to no better results. By using an alternative, large scale RNA extraction protocol, we were able to use larger amounts of each sample, which allowed us to attain better yields at the end of the process.

Some changes were made to the large scale RNA extraction protocol in order to further increase its effectiveness. In the original protocol, the addition of potassium acetate for polyssacharide precipitation was done after the *overnight* RNA precipitation with lithium chloride. This procedure would mean an additional *overnight* precipitation and an additional set of two washes with ethanol. With the modified protocol, we were able to effectively reduce the duration of the RNA extraction without skipping important steps during the process. Further RNA purification allowed us to obtain purified RNA samples (Table 3.1) and proceed to reverse transcription.

Table 3.1 – RNA quantification after extraction and purification. RNA was obtained from two grape cultivars (*Trincadeira*, susceptible; *Syrah*, resistant) across three development stages: green (EL32), *veraison* (EL35) and harvest (EL38). C = control sample; I = infected sample.

Sample name	Concentration (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
EL32 Trincadeira C 1	297,7	2,06	2,27
EL32 Trincadeira C 2	326	2,05	2,29
EL32 Trincadeira C 3	403	2,02	2,3
EL32 Trincadeira I 1	224,9	2,09	2,41
EL32 Trincadeira I 2	629,5	2,14	2,33
EL32 Trincadeira I 3	304,9	2,04	2,28
EL32 Syrah C 1	1291,7	2,1	2,41
EL32 Syrah C 2	947,9	2,1	2,36
EL32 Syrah C 3	1045,4	2,1	2,41
EL32 Syrah I 1	1233	2,11	2,44
EL32 Syrah I 2	739	2,1	2,41
EL32 Syrah I 3	1100,3	2,09	2,37
EL35 Trincadeira C 1	564,2	2,12	2,46
EL35 Trincadeira C 2	140,4	2,04	2,28
EL35 Trincadeira C 3	321,6	2,03	2,21
EL35 Trincadeira I 1	322,2	2,04	2,25
EL35 Trincadeira I 2	272,8	2,06	2,25
EL35 Trincadeira I 3	259,2	2,06	2,42
EL35 Syrah C 1	240,3	2,06	2,3
EL35 Syrah C 2	152,8	2,06	2,26
EL35 Syrah C 3	245,6	2,05	2,29
EL35 Syrah I 1	170,5	2,07	2,3
EL35 Syrah I 2	225,4	2,05	2,34
EL35 Syrah I 3	199,6	2,08	2,39
EL38 Trincadeira C 1	275,4	2,03	2,22
EL38 Trincadeira C 2	180,6	2,05	2,1

EL38 Trincadeira C 3	353,6	2,03	2,26
EL38 Trincadeira I 1	318,5	2,06	2,34
EL38 Trincadeira I 2	391	2,02	2,3
EL38 Syrah C 1	331,3	2,05	2,33
EL38 Syrah C 2	337,1	2,05	2,24
EL38 Syrah C 3	221,2	2,06	2,35
EL38 Syrah I 1	1021,8	2,09	2,39
EL38 Syrah I 2	754,9	2,1	2,29
EL38 Syrah I 3	576,9	2,1	2,31

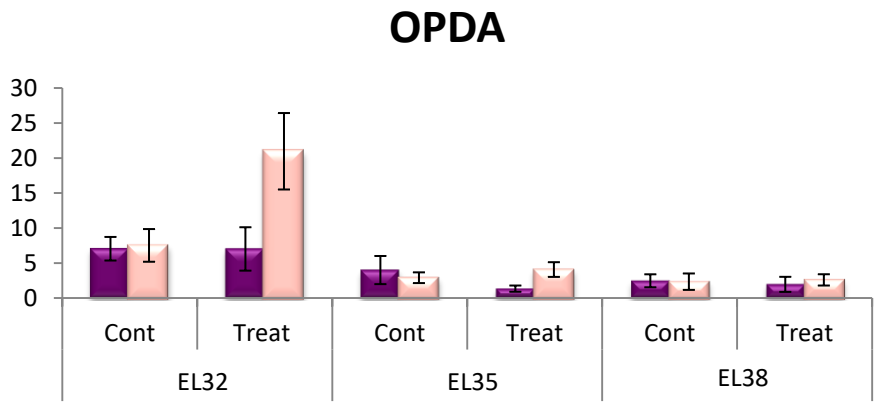
3.2. Hormonal metabolism

3.2.1. Metabolism of jasmonates

3.2.1.1. Content in oxo-phytodienoic acid and jasmonic acid

Our results (Figure 3.1) showed that, in what concerns grape ripening, the amount of oxo-phytodienoic acid (OPDA), a precursor to jasmonic acid, was equivalent in both cultivars at EL32 (annotation according to the BBCH identification system (COOMBE, 1995)), and started decreasing with grape ripening until it reached its lowest value at EL38. The concentration of jasmonic acid had its peak at EL32 in both cultivars, and was especially abundant in Syrah grapes. The quantity of jasmonic acid decreased with grape ripening, reaching its lowest amount at the harvest stage.

Regarding infected samples, during the green stage (EL32), OPDA levels (Figure 3.1) in *Syrah* were maintained after infection, while in the susceptible variety they increased drastically. During the *veraison* and harvest stages, the concentration of OPDA decreased in *Syrah* grapes when infected and increased in *Trincadeira* grapes when infected, albeit in a much smaller amount than in stage EL32. Jasmonic acid concentrations followed a similar pattern, as their highest level was at stage EL32. Similarly to OPDA, the larger increases in JA levels were observed in *Trincadeira*, although a high variance was also observed.



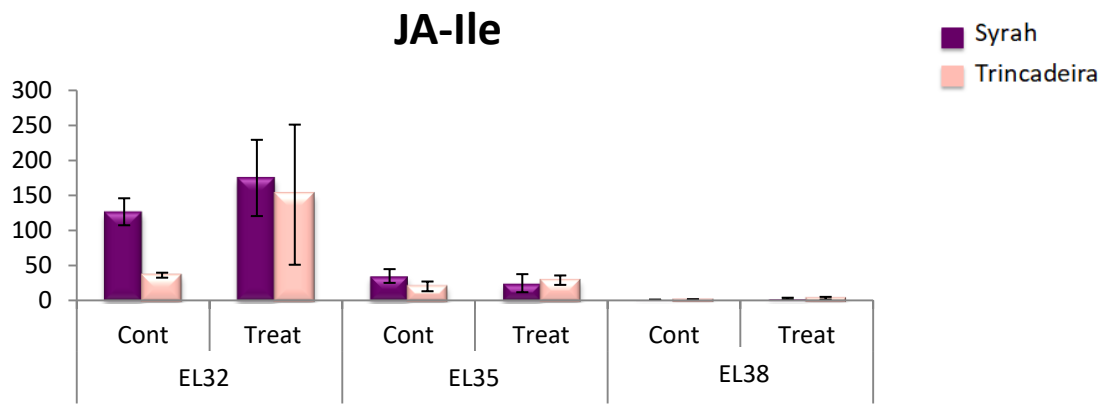


Figure 3.1 – Hormonal quantification of jasmonates, namely OPDA and JA-Ile, in *Trincadeira* and *Syrah* across three development stages: green (EL32), *veraison* (EL35) and harvest (EL38). Cont = control sample; Treat = sample treated with *B. cinerea* infection.

3.2.1.2. Expression of allene oxide synthase and 12-oxophytodienoate reductase 1

During the green stage, levels of allene oxide synthase (AOS) expression (Figure 3.2) were very low, possibly due to the already high basal concentrations of jasmonic acid in *Syrah* grapes. AOS content at *veraison* is in agreement with the hormonal alterations for both jasmonates and OPDA (Figure 3.1). However, AOS has its largest expression at EL38, while jasmonic acid (JA) and OPDA concentrations peak at EL32.

12-oxophytodienoate reductase 1 (OPR1) expression (Figure 3.2) peaks at the green stage, which is in accordance with the concentration of jasmonic acid at that stage. Particularly, it illustrates the increase in jasmonic acid concentration in *Trincadeira* grapes upon infection. OPR1 decreases at *veraison*, in a similar way as the content in jasmonic acid. Both genes present relatively high levels of expression at EL38, which was not reflected in the hormone analysis for either jasmonic acid or OPDA (Figure 3.1).

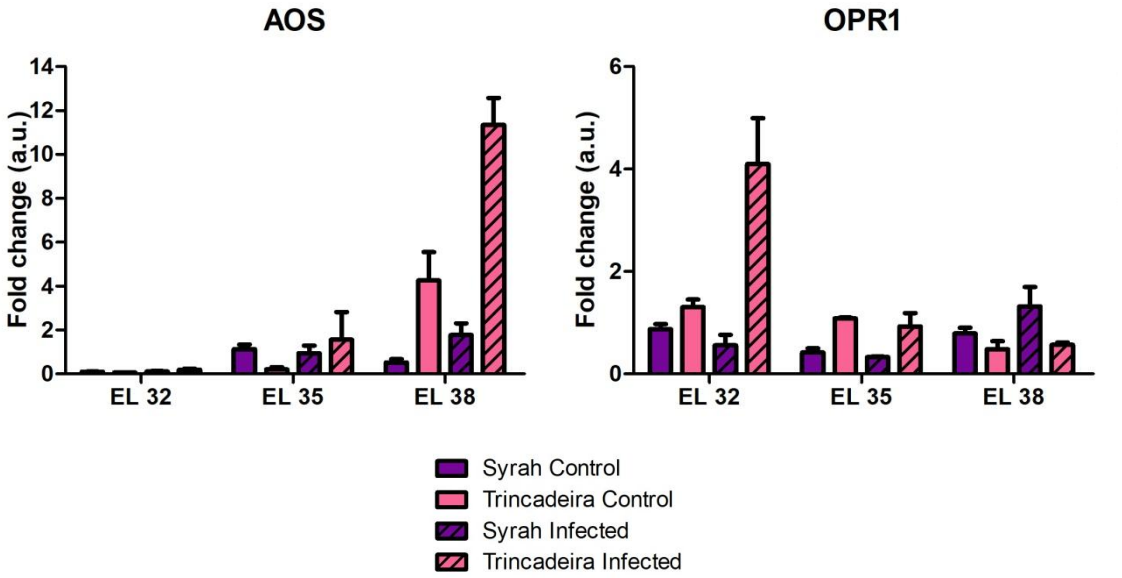


Figure 3.2 – Gene expression of AOS (allene oxide synthase) and OPR1 (12-oxophytodienoate reductase 1) in *Trincadeira* and *Syrah* across three development stages: green (EL32), *veraison* (EL35) and harvest (EL38).

3.2.2. Metabolism of abscisic acid (ABA)

3.2.2.1. Content in ABA

In what concerns grape ripening, abscisic acid (ABA) concentration (Figure 3.3) increased drastically at *veraison* in both *Trincadeira* and *Syrah*, with the latter having greater amount. At harvest, the amount of ABA decreased and reached its lowest point in *Syrah* grapes. In *Trincadeira* the decrease was less pronounced and a large amount of abscisic acid was still present at stage EL38.

Regarding infected samples, ABA also displayed larger increases in concentration (Figure 3.3) in the *Trincadeira* cultivar upon infection, with the largest increase being during stage EL32. In this stage, infection lead to an increase in ABA content only in *Trincadeira* grapes, while during EL35 an increase in concentration was observed in both *Trincadeira* and *Syrah*. However, changes concerning ABA concentration in *Syrah* grapes were generally less pronounced across all time points when compared to *Trincadeira*.

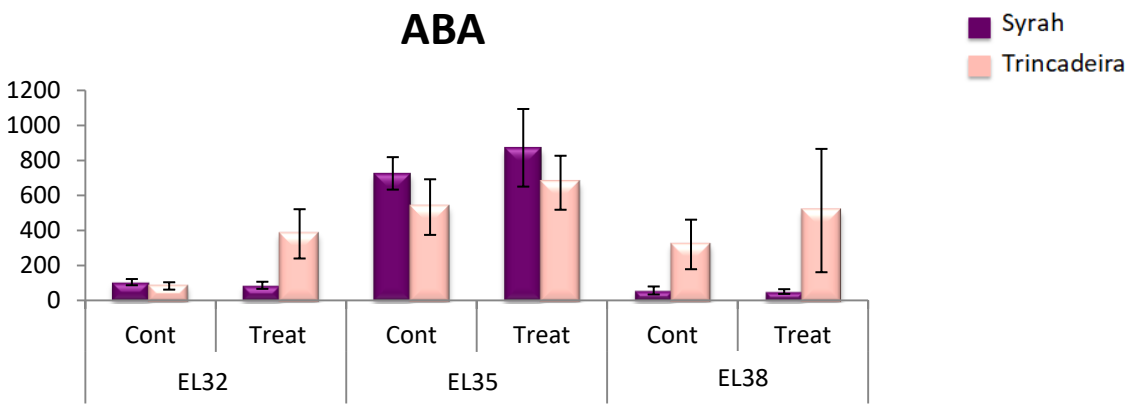


Figure 3.3 – Hormonal quantification of ABA in *Trincadeira* and *Syrah* across three development stages: green (EL32), *veraison* (EL35) and harvest (EL38). Cont = control sample; Treat = sample treated with *B. cinerea* infection.

3.2.2.2. Expression of 9-*cis*-epoxycarotenoid dioxygenase and ABA receptor

Our results showed that the levels of 9-*cis*-epoxycarotenoid dioxygenase (Figure 3.4) were relatively high during the grape development cycle, having large increases at the green stage of *Trincadeira* grapes and *veraison* in both cultivars. These data are in accordance with the variations in ABA content showed in the hormone quantification (Figure 3.3).

The expression of the gene coding for ABA receptor PYL4 RCAR10 (Figure 3.4) was at its highest during the green stage, especially in *Syrah* grapes, meaning that the abscisic acid signaling was more intense during this stage in the development. The peak of ABA concentration, however, happened at *veraison*, both for *Syrah* and for *Trincadeira* grapes (Figure 3.3). ABA receptor expression was still noticeable in *Trincadeira* grapes at harvest, possibly related to an increase in concentration of abscisic acid in this cultivar.

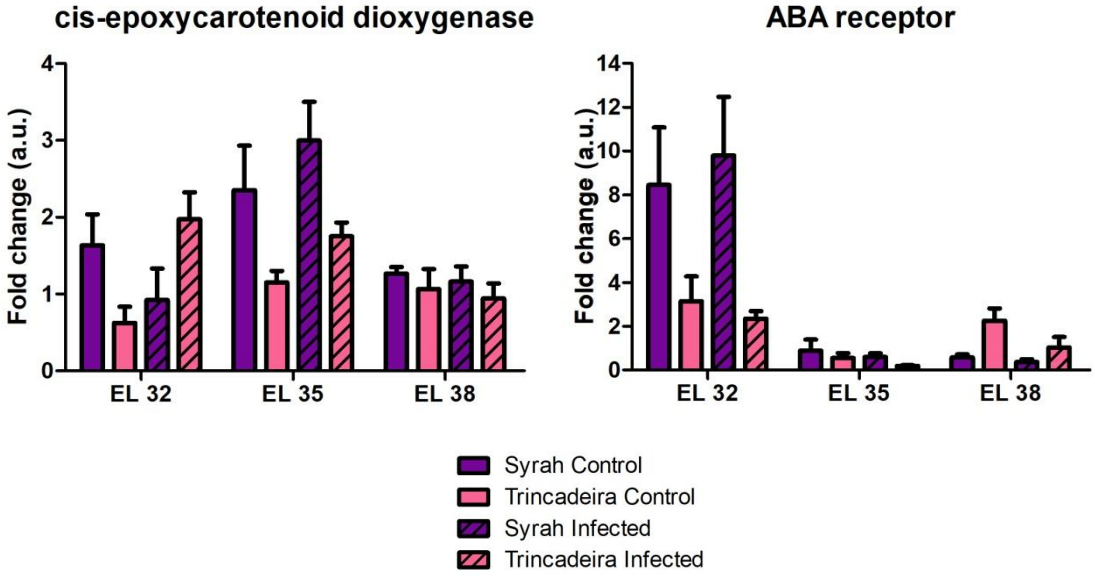


Figure 3.4 – Gene expression of 9-*cis*-epoxycarotenoid dioxygenase (NCED) and ABA receptor PYL4 RCAR10 in *Trincadeira* and *Syrah* across three development stages: green (EL32), *veraison* (EL35) and harvest (EL38).

3.2.3. Metabolism of auxins

3.2.3.1. Content in auxins

In what concerns ripening, *Syrah* grapes showed a constant decrease in auxin concentration (Figure 3.5). In *Trincadeira*, this concentration increased slightly from stage EL32 to stage EL35. However, in both cultivars, the amount of auxins reached its lowest point at the harvest stage.

Regarding infected samples, *Trincadeira* grapes also showed increases in concentration (Figure 3.5) during the green and *veraison* stages, and during harvest the amount of IAA was maintained. The level of auxins in *Syrah* upon infection increased at stages EL32 and EL38, but suffered a slight drop at stage EL35.

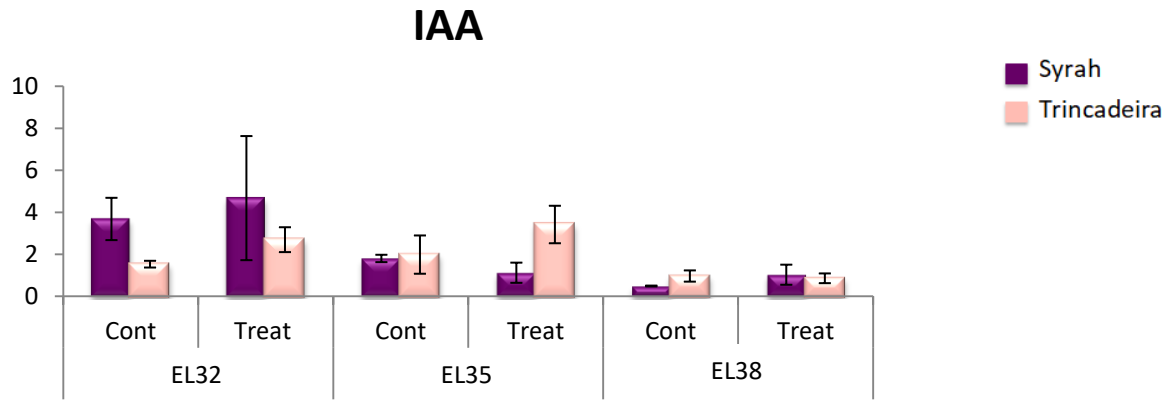


Figure 3.5 – Hormonal quantification of indole-3-acetic acid (IAA) in *Trincadeira* and *Syrah* across three development stages: green (EL32), *veraison* (EL35) and harvest (EL38). Cont = control sample; Treat = sample treated with *B. cinerea* infection.

3.2.3.2. Expression of IAA-amido synthetase GH3.2 and auxin-responsive SAUR29

The highest expression levels of the gene coding for IAA-amido synthetase (Figure 3.6) were at stage EL32, with only minor increases in both cultivars upon infection. Concordantly, the concentration of auxins in both *Syrah* and *Trincadeira* grapes during the green stage reflects these results (Figure 3.5). IAA-amido synthetase expression lowers with grape development, although some expression was still seen in the *Trincadeira* cultivar at *veraison*, which was reflected in the higher content in auxins manifested during this stage (Figure 3.5).

Some members of the auxin-responsive SAUR gene family act as negative regulators of auxin biosynthesis (Kant *et al*., 2009), being at lower levels of expression (Figure 3.6) during stage EL32 and in accordance with the peak of auxin content observed in both cultivars during this stage (Figure 3.5). As the expression of the auxin-responsive SAUR29 increased, auxin concentration decreases, until the gene reached its peak of expression at stage EL38. Concordantly, auxin concentration in both cultivars was very low at harvest (Figure 3.5).

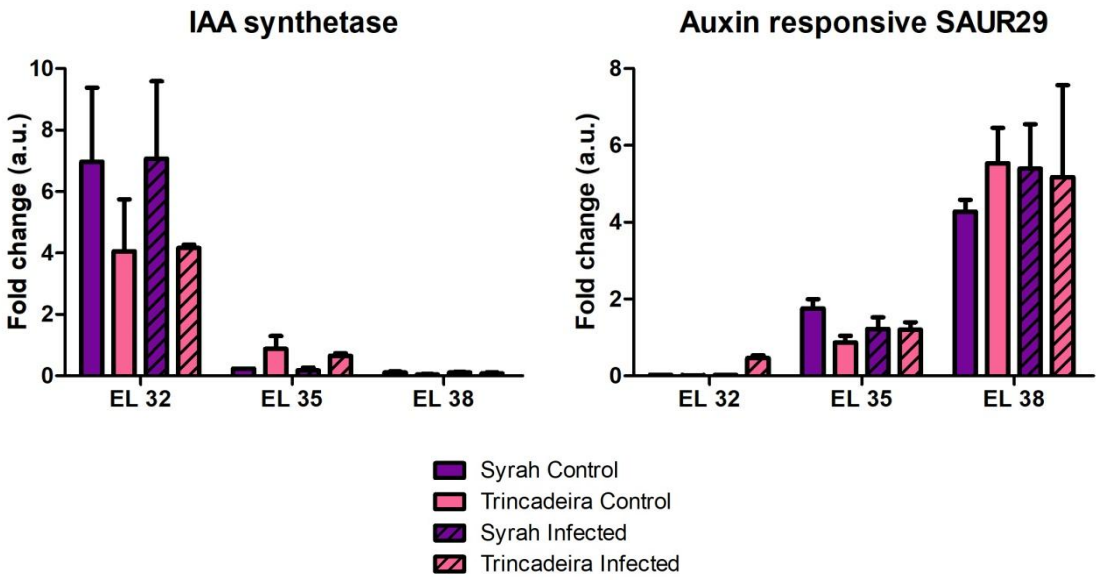


Figure 3.6 – Gene expression of IAA-amido synthetase GH3.2 and auxin-responsive SAUR29 in *Trincadeira* and *Syrah* across three development stages: green (EL32), *veraison* (EL35) and harvest (EL38).

3.2.4. Metabolism of salicylic acid (SA)

3.2.4.1. Content in SA

In what concerns grape ripening, salicylic acid (SA) concentrations (Figure 3.7) steadily decreased across the various stages of ripening, both in *Syrah* and in *Trincadeira* grapes. Out of the two cultivars, *Syrah* displays higher basal levels of SA.

Regarding infected samples, salicylic acid was shown to accumulate more in Syrah grapes upon infection, especially at *veraison* (Figure 3.7). During this stage, *Trincadeira* displayed levels of salicylic acid that suffered no changes in response to the pathogen. At the harvest stage, SA content stayed relatively the same in both grape cultivars after being infected.

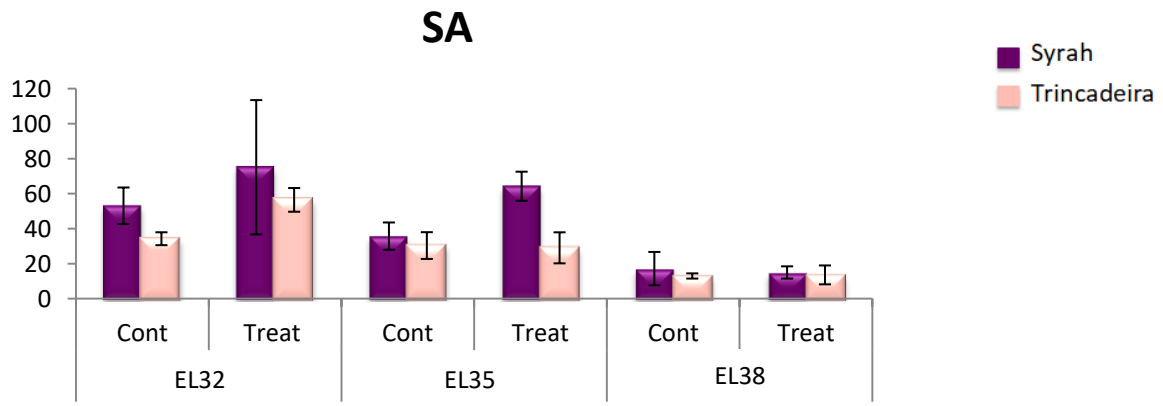


Figure 3.7 – Hormonal quantification of SA in *Trincadeira* and *Syrah* across three development: green (EL32), *veraison* (EL35) and harvest (EL38). Cont = control sample; Treat = sample treated with *B. cinerea* infection.

3.2.4.2. Expression of PAD4 and EDS1

The green stage was marked by high levels of expression (Figure 3.8) of the gene coding for enhanced disease susceptibility (EDS1) in infected *Tricandeira* grapes, as the gene is involved in signaling pathways associated with salicylic acid. Accordingly, SA was at its overall highest concentration during stage EL32, with *Trincadeira* grapes showing a larger increment of the hormone upon infection (Figure 3.7). EDS1 expression levels were low at *veraison* and at harvest.

The gene coding for phytoalexine deficient 4 (PAD4) displayed similar high levels of expression (Figure 3.8) in *Trincadeira* upon infection during the green stage (EL32). PAD4 expression remained noticeable in both cultivars throughout grape development, particularly upon infection. This pattern was also seen in the hormonal analysis of salicylic acid (Figure 3.7), which increased in concentration at stage EL35 in both cultivars and at stage EL38 in *Trincadeira*.

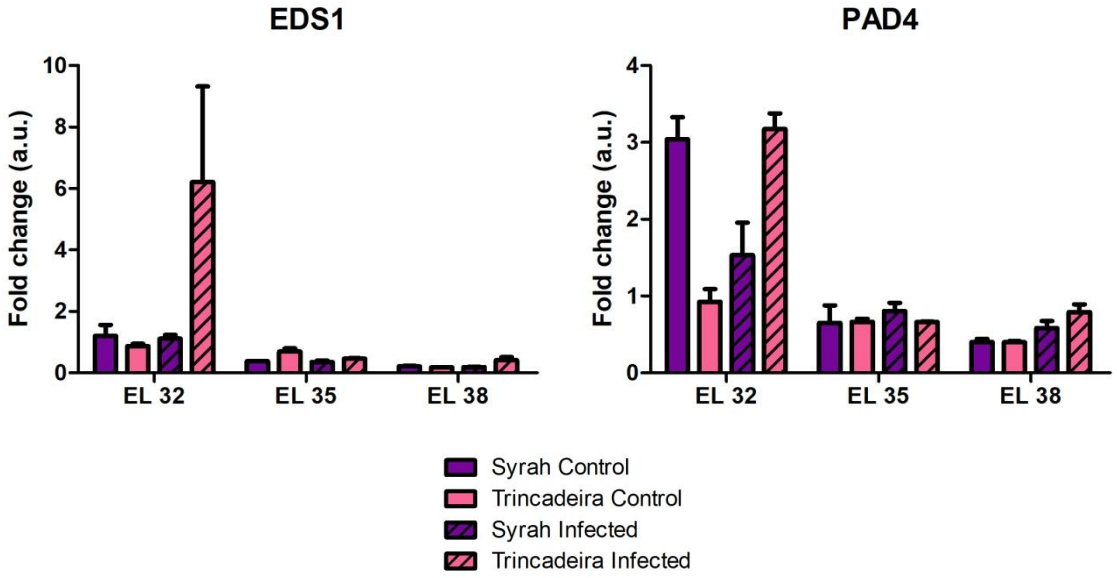


Figure 3.8 – Gene expression of EDS1 (enhanced disease susceptibility 1) and PAD4 (phytoalexine deficient 4) in *Trincadeira* and *Syrah* across three development stages: green (EL32), *veraison* (EL35) and harvest (EL38).

3.3. Identification of volatiles

Due to the low amount (3-4g) of each sample available to be used in the process, the identification of volatiles could not be properly finished. Some examples of the results obtained can be found in the Appendices section (Appendices, Figure 3). It is planned to carry out the quantification of volatiles in fresh samples, with a minimum of 10g of grape material, instead of using samples frozen in liquid nitrogen.

4. DISCUSSION

Hormones play an important role in plant development and stress responses. Disclosing the role played by hormones in grape ripening and grape defense against major fungal pathogens will enable improvement in fruit traits and productivity. Hormonal metabolic pathways regarding jasmonates, abscisic acid, auxins and salicylic acid can be found in the Appendices section (Appendices, Figure 4).

4.1. Role of jasmonic acid and oxo-phytodienoic acid (OPDA)

Jasmonic acid has been suggested to play a part in grape fruit development and ripening by influencing the coloring and softening of the fruit (Jia *et al*., 2016). A volatile form of JA, methyl jasmonate, is responsible for promoting red coloring and increase of anthocyanin content when exogenously applied on fruit (Rudell *et al*., 2002). In our results, jasmonyl-isoleucine (JA-Ile, which is the biologically active form of jasmonic acid (Fonseca, Chico, and Solano, 2009)) content dropped significantly between the green stage and the onset of ripening.

Jasmonic acid has also been characterized as being involved in stress responses of grape berries against necrotrophic pathogens, such as *B. cinerea* (Agudelo-Romero *et al*., 2015). Previous studies have also established that this hormone mediates defense responses such as oxidative stress and desiccation stress responses (Vannini and Chilosi, 2013). This is supported by our results in hormonal quantification, which show a large increase in JA-Ile content in *Trincadeira* grapes upon infection, especially in earlier stages of development. On the other hand, *Syrah* grapes display high basal levels of JA-Ile, which suggest a more accelerated stress response against the infection, therefore supporting the innate resistance of this cultivar when infected with *B. cinerea*.

The initial increase in JA-Ile content in the *Trincadeira* variety upon infection is in accordance with the increase in the expression of *OPR1*, which leads to the biosynthesis of jasmonic acid. Moreover, the expression of this gene is heavily related to the amount of OPDA available, which makes sense due to the fact that it is a precursor to jasmonic acid. *OPR1* and *AOS* expression in *Syrah* is not essential due to the already high amount of the hormone during the green stage (EL32)

Microarray analysis conducted by Agudelo-Romero *et al*. (2015) revealed that a gene coding for AOS was differentially expressed in *Trincadeira* at *veraison* (EL35), showing an increase upon infection. Our results support this data, showing a slight increase in AOS expression at that stage. However, the expression of this gene is not in accordance with JA-Ile and OPDA content at EL38, because of which the gene may be coding for an isoenzyme related to methyl jasmonate production (Seo *et al.*, 2001). If so, it does make sense that gene expression starts to increase at *veraison*, as methyl jasmonate plays a role in grape ripening (Rudell *et al.*, 2002).

4.2. Role of abscisic acid

Abscisic acid plays an important part in berry ripening. It has been suggested that increases in the level of ABA at *veraison* are probably responsible for controlling several ripening-related processes such as sugar accumulation and increased skin coloration of the berries, which ultimately leads to berry susceptibility during these later stages (Agudelo-Romero *et al*., 2015; Blanco-Ulate *et al*., 2013). Certain pathogens, including *Botrytis cinerea*, can generate abscisic acid upon infection or induce its synthesis in the host, thus acceleration ripening and making it easier for the infection to proceed (Verena Siewers *et al*., 2006).

Our results show that ABA levels increase in both cultivars at the onset of ripening, which supports the claim that it does in fact have a role in the process. ABA levels also increase in both cultivars at *veraison* as a result of infection, which may suggest the hormone's involvement in the defense of berries against pathogens, possibly through interaction with other growth regulators.
However, the presence of a high content in ABA in the *Trincadeira* variety throughout ripening may suggest its involvement in berry susceptibility, due to the ease of infection in this cultivar being naturally higher than in *Syrah*.

In fact, it has been studied that ABA deficient mutants of tomato have increased resistance against *Botrytis cinerea*, and that application of exogenous ABA leads to more susceptibility towards the fungus (Asselbergh *et al*., 2007; Audenaert, De Meyer, and Höfte, 2002). As such, depending on the host species and crosstalk with other hormones, abscisic acid can either be involved in pathogen resistance or susceptibility.

The expression of 9-*cis*-epoxycarotenoid dioxygenase supports the effect of ABA in ripening, but may also be an indicator of ABA's role in stress response and/or susceptibility, due to its increase in *Trincadeira* at EL32 and EL35 upon infection. However, the increases in abscisic acid content are not as pronounced as the ones showed by gene expression. The resistant *Syrah* variety also did not show high basal levels of ABA. As such, further investigation might be needed concerning the effects of ABA in grape defense, particularly relating to the mechanism of tolerance and susceptibility.

ABA receptors help in the up-regulation of ABA signaling by inhibiting specific phosphatases which, through a cascade reaction, can lead to the activation of several target proteins and transcription factors (Pilati *et al*., 2017). In turn, this process of inhibition depends and is induced by the availability of abscisic acid (Saavedra *et al*., 2010; Santiago *et al*., 2012). Interestingly, previous studies have shown that exogenous treatment of developing grape berries with ABA can affect ABA mediated processes through signaling, as some ABA receptors were down-regulated in the process while phosphatases were induced (Pilati *et al*., 2017). Our results showed higher expression of ABA receptor PYL4 RCAR10 in *Syrah* when compared to *Trincadeira*, especially during the green stage, meaning that ABA-mediated processes are more activated in *Syrah* grapes. This might be another indicator of ABA's role in response against biotic stress. Later in development, however, *Trincadeira* grapes show higher expression of ABA receptor PYL4 RCAR10.

4.3. Role of auxins

It has been suggested that auxins are present in low concentration at the onset of ripening in grapes, due to the fact that high auxin levels delay the sugar accumulation that is essential during ripening (Davies, Boss, and Robinson, 1997). This is in accordance with the obtained results for the expression of the IAA-amido synthetase GH3.2, which leads to the inactivation of indole-3-acetic acid by promoting the formation of its conjugated form (Böttcher *et al*., 2010). The expression of this gene peaks during earlier stages, allowing the formation of conjugated IAA to regulate free IAA levels before the onset of ripening.

Besides berry ripening, auxins are also crucial when it comes to plant defense against necrotrophic pathogens. Previous studies have shown that treatment of plants with inhibitors for auxin transport lead to an increase in necrotrophic infection (Llorente *et al*., 2008). Moreover, mutants with either decreased auxin influx or auxin-mediated signaling have increased susceptibility against *Botrytis cinerea* (Korolev, Rav David, and Elad, 2008). It is also of note that *B. cinerea* has been shown to be able to synthesize auxin, though its use upon infection has not been fully investigated (Sharon *et al*., 2007).

Concerning the results we obtained, auxin basal levels are high in *Syrah* grapes, which might

suggest that it is indeed involved in grape defense. Auxin concentration also increases in *Trincadeira* upon infection even though the largest increase happens during *veraison*. This is not in accordance with the usual defense responses, which are normally suggested by substantial increases of hormonal concentration in *Trincadeira* at EL32. The expression of auxin-responsive SAUR29 rises with the onset of ripening, reaching its peak at EL38.

Proteins of the SAUR family have been suggested to be involved in different processes of plant growth and development, with one hypothesis being auxin-regulated cell-expansion (Ren and Gray, 2015). SAUR proteins are also responsible for modulating IAA signaling and transport, which is essential at the onset of ripening (Fortes, Teixeira, and Agudelo-Romero, 2015). Because of this, it does make sense that gene expression starts to become noticeable at *veraison* (Davies, Boss, and Robinson, 1997).

4.4. Role of salicylic acid

Previous studies have highlighted the role of salicylic acid in responses against biotrophic pathogens through signaling processes (Glazebrook, 2005). It does not, however, appear to be involved in the defense against necrotrophic pathogens such as *B. cinerea*, with one possibility being that, upon infection, this pathogen activates some defense mechanisms but suppresses the salicylic acid response (Agudelo-Romero *et al*., 2015). Interestingly, salicylic acid signaling has been suggested to have an antagonistic relationship with auxin signaling: for example, auxins can inhibit plant defenses that are dependent on SA, while SA-deficient plants display a higher content in IAA (Wang *et al*., 2007; Abreu and Munné-Bosch, 2009).

Our results show that resistant *Syrah* grapes have a higher basal content in SA when compared to *Trincadeira* grape and also a higher amount at *veraison*. A high basal concentration in the resistant cultivar suggests a rapid response upon infection, which in turn may suggest that salicylic acid is indeed involved in grape defense against *Botrytis cinerea*.

The low expression of *EDS1* in *Syrah* grapes is not in accordance with the elevated basal concentration of salicylic acid. This protein, along with its co-regulator PAD4, contributes to SA-mediated signaling and, under some conditions, further salicylic acid accumulation (Agudelo-Romero *et al*., 2015; Glazebrook 2005) due to the activation of specific salicylic acid biosynthetic enzymes (Seyfferth and Tsuda, 2014). Because of this, the increase in salicylic acid in infected *Trincadeira* grapes at EL32 is in accordance with the increases in expression of both these genes, even though the latter are more pronounced. This might hint at a slow response against infection.

PAD4 activity changes drastically between cultivars during earlier stages: while it does increase in *Trincadeira* grapes upon infection, the already high *PAD4* expression verified in healthy *Syrah* grapes decreases in response to the pathogen. The latter does not translate to the results we obtained in hormonal quantification. Overall expression of the genes coding for EDS1 and PAD4 do not appear to be related to the content in salicylic acid, which can be explained by the fact that these genes, besides SA-dependent defense pathways, are also involved in SA-independent pathways (Cui *et al.*, 2017).

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APPENDICES

Appendices, Table 1 – Examples of RNA quantification before protocol optimization and without the purification step. RNA was obtained from two grape cultivars (*Trincadeira*, susceptible; *Syrah*, resistant) across three development stages: green (EL32), *veraison* (EL35) and harvest (EL38). C = control sample; I = infected sample.

Sample name	Concentration (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
EL32 Trincadeira C 1	330,2	2,02	1,69
EL32 Trincadeira C 3	455,5	1,99	1,52
EL32 Trincadeira C 3	233,3	2,04	1,77
EL32 Trincadeira I 1	738,0	1,93	1,35
EL32 Trincadeira I 2	176,0	2,04	1,77
EL32 Trincadeira I 3	834,8	1,97	1,48

Appendices, Table 2 – Primer pairs designed for qPCR. Primer sequences are ordered from 5' to 3'. A set of rules was taken into account when designing the primers: primer size should be between 18 and 25 nucleotides; the melting temperature for each primer should be between 58°C and 61°C; GC content of each primer should be between 40% and 60%; amplicon size should be between 80 and 200 base pairs. Fw = forward primer; Rev = reverse primer.

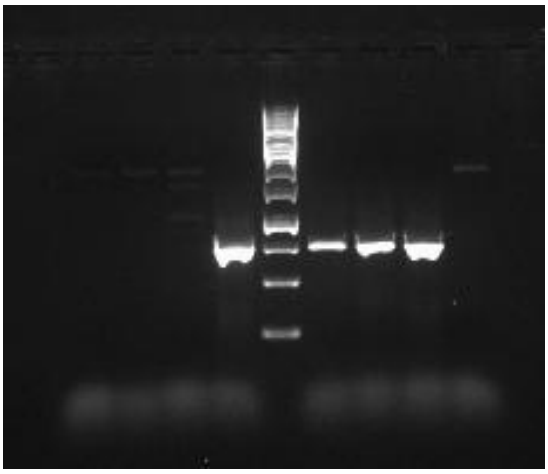
Gene coding for:		Primer sequence	$T_{melting}$ (°C)
Allene oxide synthase (AOS)	Fw	GCTTTACCGCGCCTTTTATGC	57,7
	Rev	TCCTGCTGAGCCAACCCACTT	58,3
12-oxophytodienoate reductase 1 (OPR1)	Fw	CCCCGGGTATATGGACAAAAGA	56,6
	Rev	CCACATGCCAAAGCTGACAAAT	56,4
9-*cis*-epoxycarotenoid dioxygenase (NCED)	Fw	CGCTCGCCTCCTCCTCTTCTAT	57,9
	Rev	AGGGCTTGATTCGCACTTGGTA	57,5
ABA receptor PYL4 RCAR10	Fw	TGCCGCCGCGAATAACCATA	60,7
	Rev	GACGGCGGAGCAGCATTGATT	60,9
IAA-amido synthetase GH3.2	Fw	GAGGCCATTCTTTGCGTTGACT	57,1
	Rev	CGACTCGGAGGACTTCTTTGTG	55,4
Auxin-responsive SAUR29	Fw	GGGGAGGAGCAGCAGAGGTTTG	60,9
	Rev	GGCTGTGGTGGTGGTGGGACTT	61,7
Enhanced disease susceptibility 1 (EDS1)	Fw	GAGCTTCCGGTGTCTTCTGATG	58,1
	Rev	TTTCGCTTCTCCAACTCTCCTG	60,1
Phytoalexine deficient 4 (PAD4)	Fw	GGCTAGCTGGGCAGGAGTCAA	55,3
	Rev	AGGTGTGGCGGTAACGGATTCA	55,2

Appendices, Table 3 – Quantification of extracted DNA. DNA was obtained from two grape cultivars (*Trincadeira*, susceptible; *Syrah*, resistant) across three development stages: green (EL32), *veraison* (EL35) and harvest (EL38). C = control sample; I = infected sample.

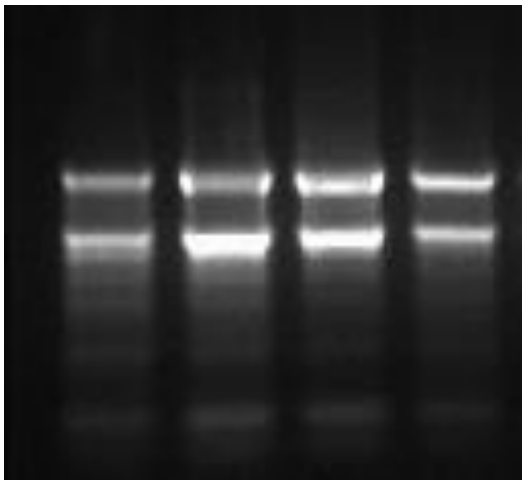
Sample name	Concentration (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
EL32 Trincadeira C 1	219,7	1,85	1,89
EL32 Trincadeira C 2	72,2	1,66	1,21
EL32 Trincadeira C 4	-	-	-
EL32 Trincadeira C 5	16,4	1,74	1,24

EL32 Trincadeira I 1	261,8	1,92	2,39
EL32 Trincadeira I 2	168,0	1,92	2.39
EL32 Trincadeira I 3	11,8	1,81	-0,37
EL32 Trincadeira I 4	122,0	1,76	1,37
EL32 Trincadeira I 5	120,2	1,94	3,21
EL32 Syrah C 1	129,5	1,98	2,27
EL32 Syrah C 2	174,1	1,91	1,95
EL32 Syrah C 3	189,2	1,83	1,01
EL32 Syrah C 4	160,9	1,88	1,58
EL32 Syrah C 5	148,8	2,12	3,76
EL32 Syrah I 1	33,2	1,62	1,11
EL32 Syrah I 2	437,5	1,69	1,04
EL32 Syrah I 3	147,4	1,87	1,54
EL32 Syrah I 4	159,5	1,66	1,22
EL35 Trincadeira C 1a	6,7	1,61	-0,36
EL35 Trincadeira C 1b	18,2	1,61	-1,37
EL35 Trincadeira C 2	200,8	1,79	1,40
EL35 Trincadeira C 3	289,4	1,80	1,32
EL35 Trincadeira C 4	207,3	1,64	1,11
EL35 Trincadeira C 5	78,6	2,04	-5,44
EL35 Trincadeira I 3	49,6	1,44	0,71
EL35 Trincadeira I 4	174,8	1,95	2,52
EL35 Trincadeira I 5	309,2	1,55	0,92
EL35 Trincadeira I 6	114,7	1,48	0,65
EL35 Trincadeira I 7	350,2	1,71	1,14
EL35 Trincadeira I 8	159,5	1,91	2,80
EL35 Syrah C 1	252,5	1,83	1,24
EL35 Syrah C 2	215,1	1,94	2,20
EL35 Syrah C 3	188,9	1,92	1,25
EL35 Syrah C 4	128,0	1,81	1,12
EL35 Syrah C 5	109,7	1,69	1,55
EL35 Syrah I 1	200,2	1,96	2,20
EL35 Syrah I 2	169,7	2,02	2,89
EL35 Syrah I 3	170,6	1,92	1,82
EL35 Syrah I 5	207,4	1,88	1,34
EL35 Syrah I 6	283,2	2,00	2,14
EL38 Trincadeira C 1	232,9	1,76	1,17
EL38 Trincadeira C 2	33,1	1,96	-2,62
EL38 Trincadeira C 3	153,9	1,81	1,64
EL38 Trincadeira C 4	207,5	1,69	0,94
EL38 Trincadeira C 5	83,2	1,77	2,22
EL38 Trincadeira C 6	29,1	2,70	-0,67
EL38 Trincadeira I 3	85,2	1,42	0,53
EL38 Trincadeira I 4	104,4	1,66	0,89
EL38 Trincadeira I 5	63,4	1,69	1,94
EL38 Trincadeira I 6	114,2	1,71	1,20
EL38 Trincadeira I 8	68,6	1,91	2,32
EL38 Syrah C 1	336,4	1,81	0,99
EL38 Syrah C 2	241,2	1,92	1,45
EL38 Syrah C 3	5,5	0,92	0,29
EL38 Syrah C 4	129,4	1,53	0,69

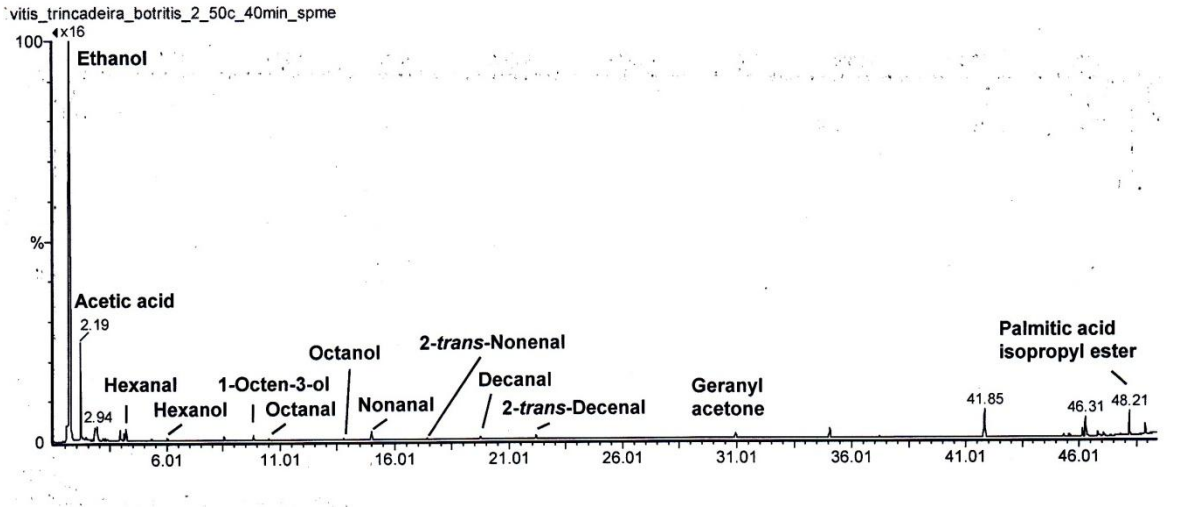
EL38 Syrah C 5	161,0	1,87	1,76
EL38 Syrah C 6	154,9	1,70	0,90
EL38 Syrah I 2	203,7	1,78	1,83
EL38 Syrah I 3	255,6	1,46	0,63
EL38 Syrah I 4	280,8	1,56	0,77
EL38 Syrah I 5	-	-	-
EL38 Syrah I 6	-	-	-
EL38 Syrah I 8	171,1	1,95	2,42



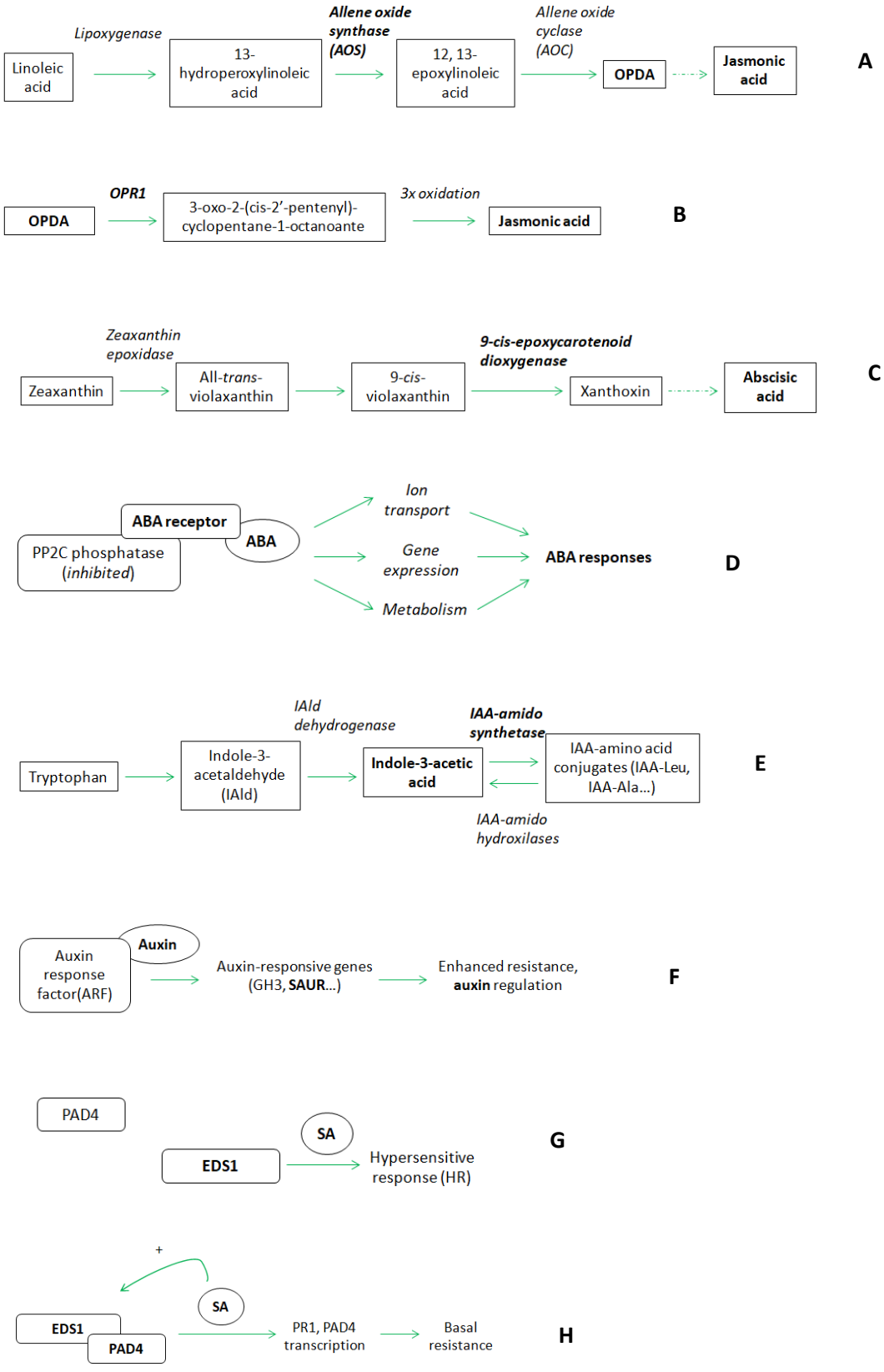
Appendices, Figure 1 – Assessment of *B. cinerea* infection in grapes using a 1.2% agarose gel. The PCR procedure was able to amplify the 0.7-kb *B. cinerea* fragment from 100 ng of DNA. Samples on the left represent controls, samples on the right represent samples that have been infected and presented visible symptoms. One control sample presented natural infection despite the negative results from preliminary visual inspection and was removed from further analyses.



Appendices, Figure 2 – Assessment of RNA integrity (from left to right: EL32 *Trincadeira* control, EL32 *Trincadeira* infected, EL32 *Syrah* control, EL32 *Syrah* infected) by agarose gel electrophoresis, using a 1.2% agarose gel. The 18S and 28S ribosomal RNA bands are clearly visible in the RNA sample.



Appendices, Figure 3 – Example of the results obtained in identification of volatile compounds. Volatiles such as hexanol and octanol have been identified though at low quantity.



Appendices, Figure 4 – Hormonal pathways of jasmonates (A, B), abscisic acid (C, D), auxins (E, F) and salicylic acid (G, H).