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Dissertação de Mestrado
Biologia Molecular, Biotecnologia e
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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

Botrytis cinerea é um fungo necrotrófico responsável pela "podridão cinzenta", uma doença que causa enormes perdas e diminuição na produtividade em culturas de videira. No entanto, em condições microclimáticas particulares, este fungo pode causar "podridão nobre", um tipo de infeção que leva à produção de vinhos doces de alta qualidade. Durante a podridão nobre, várias alterações morfológicas, metabólicas e moleculares acontecem no bago da uva. Nos bagos infetados, a concentração de vários metabolitos, tais como açúcares e ácidos orgânicos, sofre grandes alterações, diminuindo também o conteúdo em água dos bagos da uva infetados. Estas alterações nos solutos existentes no bago de uva resultam tanto de modificações no metabolismo do bago da uva como da ação do metabolismo da *Botrytis*.

No presente trabalho pretendeu-se estudar os efeitos da podridão nobre no perfil de açúcares e polióis do bago da uva, assim como nos mecanismos moleculares envolvidos no transporte e metabolismo dos polióis. Para tal, várias abordagens bioquímicas e moleculares foram usadas, tais como a quantificação de solutos por HPLC, análises transcricionais por qPCR e a determinação de atividades enzimáticas do metabolismo dos polióis.

Observamos uma diminuição na concentração de sacarose, glucose e frutose em bagos de uva infetados por *Botrytis*, juntamente com uma pequena diminuição no rácio glucose/frutose. Contrariamente, a concentração dos polióis manitol e sorbitol aumentou em bagos infetados. Juntamente, observamos um aumento da abundância dos transcritos do *VvMTD1* (gene que codifica para a manitol desidrogenase, enzima que cataboliza, reversivelmente, o manitol) juntamente com uma diminuição muito acentuada do *VvPLT1* (gene que codifica para um transportador de manitol da membrana plasmática). Determinamos ainda um aumento da atividade enzimática da VvMTD no sentido do catabolismo do manitol. Conjuntamente, os resultados apontam para um aumento do catabolismo do manitol e para uma diminuição da capacidade de transporte deste para as células, sugerindo uma ação defensiva por parte da VvMTD na resposta da planta ao agente patogénico.

Palavras chave: *Botrytis cinerea*, defesa da planta, manitol, *Vitis vinifera*

Abstract

Botrytis cinerea is a necrotrophic fungus responsible for the grapevine gray mold, a disease that causes great productivity losses. However, in certain microclimate conditions, this fungus can cause noble rot, a type of infection that leads to the production of high-quality sweet wines. During noble rot, several morphological, metabolic, and molecular alterations occur in the grape berry. In infected berries, the concentration of several metabolites, such as sugars and organic acids, suffer great alterations. Additionally, grape berry water content diminishes. These modifications result both from variations in the grape berry metabolism and by the metabolism of *Botrytis*.

In the present work, we studied the effects that noble rot causes in the profile of grape berry sugars and polyols, as well as in the molecular mechanisms involved in polyol transport and metabolism. For this, different biochemical and molecular approaches were used, such as the quantification of solutes by HPLC, transcriptional analyses by qPCR and the determination of polyol metabolism enzyme activities.

We observed a slight reduction in the concentration of sucrose, glucose, and fructose in infected grape berries, and also a reduction of the glucose/fructose ratio. Contrarily, the concentration of the polyols mannitol and sorbitol increased in infected grape berries. Moreover, we observed an increase in the transcript abundance of *VvMTD1*, a gene that encodes for a mannitol dehydrogenase, an enzyme that reversibly catabolizes mannitol, together with a sharp decline in the expression profile of *VvPLT1* (that encodes for a plasma membrane mannitol transporter). Furthermore, an increase in the enzymatic activity of VvMTD towards mannitol catabolism was observed. Together, our results indicate an increase in the catabolism of mannitol and a reduction in the uptake capacity of this polyol towards the cell, suggesting a defensive action by VvMTD in the plant-response against the pathogen agent.

Keywords: *Botrytis cinerea*, mannitol, plant defense, *Vitis vinifera*.

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List of abbreviations and acronyms

AgMaT1	Plasma membrane celery mannitol transporter 1
AgMaT2	Plasma membrane celery mannitol transporter 2
BA	Beerenauslese
BIS-TRIS propane	1,3-Bis[tris(hydroxymethyl)methylamino]propane
CTAB	Hexadecyltrimethylammonium bromide
cv.	Cultivar
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
ha	Hectare
HPLC	High-performance liquid chromatography
HR	Hypersensitive response
L.	Carl Linnaeus
M6PR	NADPH-mannose-6-phosphate reductase
MOPS	3-(N-morpholino)propanesulfonic acid
MTD	Mannitol dehydrogenase
OeMAT1	*Olea europea* mannitol carrier 1
PDA	Potato dextrose agar
PLT or PMT	polyol or monosaccharide transporter proteins
PMSF	Phenylmethylsulfonyl fluoride
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
qPCR	Quantitative polymerase chain reaction
RH	Relative humidity
ROS	Reactive oxygen species
SAR	Systemic acquired resistance
SWEET	Sugars Will Eventually be Exported Transporters
TBA	Trockenbeerenauslese
TRIS HCL	Tris(hydroxymethyl)aminomethane hydrochloride
Triton x-100	Polyethylene glycol tert-octylphenyl ether
UV	Ultraviolet
VvACT1	*Vitis vinifera* actin 1

VvMTD	*Vitis vinifera* mannitol dehydrogenase
VvMTD1	*Vitis vinifera* mannitol dehydrogenase 1
VvPLT1	Plasma membrane *Vitis vinifera* polyol transporter 1
VvPMT1 to *VvPMT5*	*Vitis vinifera* polyol and monosaccharide transporter 1 to 5
VvSDH	*Vitis vinifera* sorbitol dehydrogenase

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1. INTRODUCTION

1.1. The grapevine (*Vitis vinifera* L.)

Vitis vinifera L., of the Vitaceae family, is a creeper or trailing plant that is divided in two subspecies: the domesticated (*V. vinifera* subsp. *sativa*) and the wild (*V. vinifera* subsp. *silvestris*) (Soneji and Nageswara-Rao, 2011; Garfi *et al.*, 2013). The wild form is dioecious and produces black, small berries in loose bunches compared to cultivated varieties (Figure1) (Hardie, 2000; Marrano *et al.*, 2018).

Figure 1 - Wild form of *Vitis vinifera* in its most common habitat in Portugal, woodlands along the banks of a stream of a river, also known as riparian woodlands. Adapted from Cunha et al. (2013).

The origins of the grapevine (*Vitis vinifera L.*) are suggested to be dated around 65 million years ago (This, Lacombe and Thomas, 2006). This species evolved in Eurasia, its ancestor possibly being of a bushy aspect that occupied open, sunny habitats (Hardie, 2000).

Grapevine is the most widely cultivated fruit crop in the world, occupying 7,463,908 ha in 2016, especially in Europe along the Mediterranean region (*OIV Advanced Search on Database*, 2019). Viticulture is the cultivation and harvesting of grapes for their direct consumption as table grapes, winemaking, and also for raisins production. This practice probably originated in north Turkey's Anatolian

region and/or Transcaucasia, a geographical region near Turkey on the border of eastern Europe and western Asia (Jackson, 2016). Later, around 1000 BC, viticulture was introduced across most of the Mediterranean Basin, and into central Europe under Roman occupation. In the Iberian region, it was introduced by trading between Phoenicians and Greeks and continued during Roman command (Cunha *et al.*, 2013). During the last centuries, grape cultivation reached most of the remaining parts of the world. The majority of grape berry harvest is aimed at the production of wine. Wine has great importance in the Mediterranean lifestyle and culture, being present across human history for more than 8000 years. Greek and Roman gods, Dionysus and Bacchus, respectively, were dedicated to wine which was seen as a gift to humankind (Charters, 2006). Additionally, the health benefits of the grapevine by-products are a key factor in the importance of this plant species. These benefits include decreasing chronic disease risk (some types of cancer and cardiovascular disease), the inhibition of cancer cell development, and the suppression of platelet aggregation. The presence of antioxidant activity in phenolic compounds, anthocyanin pigments and procyanidins, especially in grape seeds, can also bring health gains (Yang and Xiao, 2013; Ma and Zhang, 2017).

Of the *Vitis* genus, *Vitis vinifera* is the only species extensively used in winemaking (This, Lacombe and Thomas, 2006). Modern cultivars originated by selection from wild individuals and also by artificial breeding. Grapevine cultivars are grouped according to their morphological features and geographic source, being divided into three recognized ecological groups: Pontica, Occidentalis, and Orientalis (Hardie, 2000; Bessis, 2007).

Around 15,000 cultivated varieties can be found worldwide, 343 of which are officially recognized and authorized for wine production in Portugal (Cunha *et al.*, 2013; Jackson, 2016). Despite that, in 80% of the country's newly created vineyards, only 25 cultivars of the 343 mentioned are used. One of the reasons for the dwindling number of planted cultivars is the globalization of the wine market and how wine is currently marketed. Another motive is the devastation of many of Europe's grapevine crops by mildews and phylloxera in the 19th century which greatly reduced diversity. To remediate this problem other species of the *Vitis* genus were brought to Europe to breed disease-resistant hybrids or to be used as rootstock (This, Lacombe and Thomas, 2006).

1.2. The grapevine fungal pathogen *Botrytis cinerea*

Grapevine is very susceptible to several diseases, mainly grey mold, powdery mildew, and downy mildew which causes several problems to farmers worldwide. *Botrytis cinerea*, the causal agent of grey

mold disease, can infect several tissues of the grapevine at different developmental stages and negatively affects grape berry production and quality (Gomès and Coutos-Thévenot, 2009; Walker and Leroux, 2015). Nevertheless, it can also cause noble rot, a disease that only develops under specific climatic and edaphic conditions, leading to the production of exceptionally sweet wines (Magyar, 2011; Vannini and Chilosi, 2013; Jackson, 2020).

Botrytis cinerea has a necrotrophic lifestyle as they invade and kill host cells, feeding on the remains of the dead tissue (Laluk and Mengiste, 2010; van Kan, Shaw and Grant-Downton, 2014; Elad *et al.*, 2016). This fungus stays dormant for extended periods of time, until favorable environmental and plant physiological conditions are present. This characteristic is quite problematic with agricultural products that are transported over long distances as damage only occurs upon arrival or when the product is ready for sale (Williamson *et al.*, 2007; Dewey and Grant-Downton, 2016). However, infection can also begin during plant development, as early as the seedling stage in some hosts such as sunflower, leading to productivity losses before harvest (Williamson *et al.*, 2007).

In the grape berry, this fungus generally develops during berry ripening, when acidity and antifungal compound levels decline (Magyar, 2011). Before ripening, grape berries are more resilient to *B. cinerea* infection due to the presence of constitutive phenolic compounds and polymeric proanthocyanidins in the berry skin, and the poor penetration capacity of the fungus (Vannini and Chilosi, 2013). *B. cinerea* manages to infect the plant through spontaneous wounds and micro-fissures. These are formed near stomata due to fruit enlargement and on the cuticle owing to a reduction of its thickness during ripening. The presence of water on the surface of the grape berry and a temperature of 18°C are ideal conditions for the germination of sclerotia, chlamydospores, and the growth of mycelia. Conidial germination is possible in the temperature range between 10°C and 25°C (Ribéreau-gayon *et al.*, 2006).

However, if certain microclimatic conditions are present, infection of the mature grape berry by *Botrytis* can cause noble rot, which can lead to the production of excellent sweet wines (botrytized wines). For noble rot to develop there is a need for a cycle of alternating dry and rainy periods for 10 to 15 days. Humid nights, in conjunction with dew and morning fog, promote *B. cinerea* growth while windy, dry afternoons limit excessive development of the fungus (Ribéreau-gayon *et al.*, 2006; Magyar, 2011; Jackson, 2014; Magyar and Soós, 2016). Additionally, factors like grape cultivars (cv.) with ripening times that coincide with late fall – the time of the year in which said microclimatic conditions are more likely to occur – along with thinner berry skins, closely packed grape bunches, and lower phytoalexin production facilitate *B. cinerea* infection (Magyar, 2011; Vannini and Chilosi, 2013; Magyar and Soós, 2016; Jackson, 2020). The intersection between those microclimatic conditions and these factors are only

gathered in a few areas of the world, like Sauternes in France, Schloss Johannisberg in Germany, Tokaj in Hungary, and Alijó, in Vila Real, Portugal. Noble rot infection leads to grape berry dehydration and, consequently, to an increase in sugar concentration which limits the expansion of *B. cinerea* and contains the pathogen to the outer layers of the epidermis (Figure 2) (Ribéreau-gayon *et al.*, 2006; Vannini and Chilosi, 2013; Magyar and Soós, 2016).

Of note, noble rot, and grey mold diseases, while displaying different symptoms, share the same specific *B. cinerea* populations in each vineyard. The microclimate and the environmental conditions are the most important factors for noble rot symptoms to develop (Fournier, Gladieux and Giraud, 2013).

Figure 2 – Image on the left is a *Botrytis cinerea* conidiophore on the surface of a berry infected by noble rot taken by scanning electron microscopy (Magyar and Soós, 2016). The image on the right is a botrytized grape bunch from the Hungarian Tokaj region (Magyar, 2011).

1.2.1. Grape berry physiochemical changes caused by noble rot

Both noble rot and grey mold induce different physical and biochemical changes in the infected grape. Modifications caused by noble rot in grape berries from different cultivars are detailed in Table 1. The modifications to the physical properties and metabolite composition during noble rot infection mainly result from the increase in the concentration of several grape sugars and acids due to grape berry water loss in conjunction with *B. cinerea* metabolism (Magyar, 2011; Magyar and Soós, 2016; Jackson, 2020). During infection, grape berry dehydration is caused by the activity of *Botrytis* secreted hydrolytic enzymes that degrade the components of plant cell walls, causing the death of cells adjacent to the pedicel, interrupting the connection between the berry and the pedicel (Jackson, 2020). Also, *B. cinerea* uses grape sugars to produce biomass, energy, and several metabolites. In the initial stages of infection, glucose is catabolized to gluconic acid and glycerol leading to the accumulation of these compounds (Ribéreau-gayon *et al.*, 2006; Magyar, 2011). Like glycerol, other polyols like arabitol, mannitol, erythritol, D-sorbitol, and inositol also increase during noble rot infection (Loescher, 1987; Ribéreau-gayon *et al.*, 2006; Magyar, 2011). During this developmental phase, the fungus develops under the grape skin. Due to lower access to oxygen, the growth of the fungus is limited (Ribéreau-gayon et al., 2006; Magyar, 2011).

In infected grapes, sugar concentration increases despite the high consumption of sugars by the fungus (50% of the initial total sugar content is absent in botrytized wines). The concentration of sugars in infected berries compared to healthy grapes can reach values between 2 and 5 fold (Ribéreau-gayon *et al.*, 2006; Magyar, 2011). It has been reported that *Botrytis cinerea* favors the metabolism of glucose compared to fructose leading to high fructose/glucose ratios in infected berries (Magyar, 2011; Jackson, 2020). Nevertheless, *B. cinerea* metabolism favors sucrose consumption over glucose and fructose (Dulermo *et al.*, 2009; Hong *et al.*, 2012). The profile of other sugars like arabinose, rhamnose, galactose, mannose, xylose, and galacturonic acid is also altered during noble rot due to the fungus' degradation of polysaccharides and pectins (Magyar, 2011; Jackson, 2020).

In infected berries, total acidity varies according to dehydration levels (Magyar, 2011). During the initial stages of infection, *Botrytis* favors the consumption of tartaric acid relatively to malic acid, however at later stages of infection malic acid is preferred by the fungus due to a strong resource demand for conidia development (Ribéreau-gayon *et al.*, 2006; Magyar, 2011; Jackson, 2020). Gluconic acid, only present in botrytized grapes can be used as a noble rot indicator. However, the fact that acetic acid bacteria on the berry skin also produce this acid must be taken into account when looking at this compound's levels (Magyar, 2011; Magyar and Soós, 2016; Jackson, 2020). Commonly, *B. cinerea*

doesn't use other organic acids as much, leading to an increase in their concentration especially at the later stages of infection (Ribéreau-gayon *et al.*, 2006).

As mentioned above, noble rot causes berry dehydration leading to shriveling and changes in the physical properties of the grape, including color and berry skin characteristics (Ribéreau-gayon *et al.*, 2006; Magyar, 2011; Magyar and Soós, 2016; Jackson, 2020). Besides using chemical changes as an evaluation of noble rot infection and disease progression, there is also the possibility of using physical markers. On this subject, a study on botrytized grapes from the Chenin blanc cv. from Carbajal-Ida *et al.* in 2016 found that the best tests to evaluate and differentiate microscopical changes in disease progression seem to be puncture in conjunction with compression and color tests. Furthermore, a decrease in the mechanical resistance of berries' skin was observed during infection.

Table 1-Physical and chemical parameters of non-infected and noble rotted grapes and juice. Beerenauslese (BA) in the table refers to grapes from a type of wine from which its grapes are collected later after the specified harvest date. Trockenbeerenauslese (TBA) refers to grapes from a class of wines that are made from thoroughly selected, overripe grapes. The berries analyzed under Tokaj (wines from the Hungarian Tokaj region) have the same description; however, each column refers to a different vintage from Tokaj wines. This table is adapted from a book section by Magyar in 2011.

	Sauternes		Germany			Tokaj	
Constituent	Healthy berry	Noble-rotted berry	Healthy berry	Noble-rotted berry BA	Noble-rotted berry TBA	Noble-rotted berry (Essencia)	Noble-rotted berry (Essencia)
Weight per 100 berries (g)	202	98	209	85	36	-	-
Sugar (g/l)	247	317	182	295	500	685	708
Glucose/fructose ratio	-	-	0,98	0,80	0,94	0,98	0,88
Glycerol (g/l)	-	-	0,09	8,00	20,67	24,3	30,77
Total acidity (g/l)	9,23[a]	8,40[a]	11,8	15,2	20,8	16,55	14,7
Tartaric acid (g/l)	5,33	2,48	7,3	2,6	2,4	4,81	4,44
Malic acid (g/l)	5,43	7,84	4,2	8,00	10,1	5,82	7,42
Citric acid (g/l)	0,17	0,22	0,19	0,20	0,24	0,11	0,99
Acetic acid (g/l)	0,32	0,41	0,00	0,45	0,13	-	0,49
Gluconic acid (g/l)	0	2,08	0,02	1,5	2,17	3,20	3,88
Galacturonic acid (g/l)	-	-	0,1	0,6	1,1	-	-
Galactaric acid (g/l)	-	-	0,1	1,00	1,2	-	-
Mannitol (mg/l)	-	-	12	516	2132	-	-
Arabitol (mg/l)	-	-	0	463	818	-	-
Inosit (mg/l)	-	-	148	335	634	-	-
Sorbitol (mg/l)	-	-	30	371	362	-	-
Total polyphenols (mg/l)	-	-	-	-	-	986	1080
Ammonium (mg/l)	85	56	-	-	-	-	-
Amino acids (mg/l)	1282	1417	-	-	-	-	-
Protein (mg/l)	2815	3795	-	-	-	-	-

[a] Concentrations of total acidity organic acids were converted from milliequivalent to gram per litre

1.3. Polyols in grapevine

Polyols, also known as sugar alcohols, are the reduced forms of aldose or ketose sugars. Polyols have several roles, such as protection against biotic and abiotic stresses, and as storage compounds. Polyols can be found in many organisms, ranging from plants to algae and fungi. In angiosperms, the most common polyols are galactitol, sorbitol and mannitol (Nathalie Noiraud, Maurousset and Lemoine, 2001).

Mannitol is widely spread, being the most prevalent polyol in plants being present in more than 100 vascular plants in 70 different families (Stoop, Williamson and Masonpharr, 1996; N. Noiraud, Maurousset and Lemoine, 2001). Mannitol has several functions in plants including as a source of carbon and energy and as a protector against osmotic and heat-induced stress (Stoop, Williamson and Masonpharr, 1996; Zhifang and Loescher, 2003; Conde *et al.*, 2015). In the mature leaves of vascular plants, mannose-6-phosphate is converted to mannitol-1-phosphate by a NADPH-mannose-6-phosphate reductase (M6PR). This step is followed by dephosphorylation of mannitol-1-phosphate by a phosphatase to synthesize mannitol. After its synthesis, mannitol is generally translocated to sink organs where it is either stored as a reserve carbohydrate or catabolized (Stoop, Williamson and Masonpharr, 1996; Loescher and Everard, 2000; N. Noiraud, Maurousset and Lemoine, 2001; Parvaiz and Satyawati, 2008; Conde *et al.*, 2015). Mannitol is transformed by mannitol dehydrogenase (MTD) to mannose in celery and olive and fructose in grapevine (Conde *et al.*, 2015).

The translocation of mannitol or sorbitol in plants is controlled by sugar transporters designated as polyol or polyol/monosaccharide transporter proteins (PLTs or PMTs), which belong to the Major Facilitator Superfamily (MFS) (Nathalie Noiraud, Maurousset and Lemoine, 2001; Afoufa-Bastien *et al.*, 2010; Tian *et al.*, 2017; Shakya and A. Lal, 2018).

The first reported mannitol transporter was AgMaT1 in celery. Its function was demonstrated by yeast cells heterologously expressing *AgMaT1* that were capable of growing on mannitol supplemented medium and to transport radiolabeled mannitol (N. Noiraud, Maurousset and Lemoine, 2001). Later, AgMaT2, a second mannitol transporter was also characterized in celery as a plasma membrane H^+/mannitol cotransporter expressed in phloem cells (Juchaux-Cachau *et al.*, 2007). A study by Conde *et al.*, in 2011 suggested that olive trees (*Olea europaea*) respond to water deficit and salt stress by adjusting mannitol metabolism and transport. More specifically, in drought and salt-stressed cells, an increase in mannitol levels was observed together with the increase of *OeMAT1* (mannitol transporter) gene expression and protein levels.

In grapevine, Afoufa-Bastien *et al.*, in 2010 identified 5 putative polyol transporter genes by *in silico* analysis in the plant's genome which were named *Polyol and Monosaccharide Transporters* (*VvPMT*). Of this family, *VvPMT5,* also known as *VvPLT1* (designation adopted by Conde et al., 2015), is the most expressed gene in vegetative organs. This gene is also highly expressed in the grape berry, especially in the fruit set phase. Recently, VvPLT1/VvPMT5 was characterized as a plasma membrane, H^+-dependent polyol transporter, with a high affinity for sorbitol and mannitol. Competitive inhibition studies suggested that VvPLT1 might also transport other polyols and even monosaccharides (Conde *et al.*, 2015). The same study also associated an increase in *VvPLT1* expression and a decrease in mannitol catabolism with water deficit stress, clearly hinting at an important role of mannitol and other polyols transport and accumulation during drought stress.

Like mannitol, sorbitol is produced in mature leaves, in vascular plants. Sorbitol is produced from glucose-6-phosphate through the enzyme aldose-6-P-reductase and a sorbitol-6-P phosphatase (Conde *et al.*, 2015). Sorbitol is present in several members of the Rosaceae family such as pears, apples, and peaches. In these plants, sorbitol is used as the main translocated photosynthetic product. Sorbitol is also associated with a role in salt stress tolerance and as a detoxifier under stress (Nosarzewski *et al.*, 2012; Conde *et al.*, 2015).

1.3.1. Mannitol synthesis and metabolism in *Botrytis cinerea*

Mannitol is also present in the spores, fruiting bodies, and mycelia of diverse fungi species (Solomon, Waters and Oliver, 2007; Meena *et al.*, 2015; Patel and Williamson, 2016). It functions as a carbohydrate reservoir, protector against osmotic and oxidative stress, coenzyme regulator, and as a storage for reducing power, even though its functions are not as well defined as in plants (Dulermo *et al.*, 2010; Williamson *et al.*, 2013; Meena *et al.*, 2015; Patel and Williamson, 2016). Additionally, during plant fungal infection, mannitol appears to have a significant role, quenching reactive oxygen species (ROS) that are produced by plants as an early defense mechanism (Voegele *et al.*, 2005; Calmes *et al.*, 2013; Meena *et al.*, 2015; Patel and Williamson, 2016).

Mannitol metabolism in filamentous fungi occurs as a cyclic process, involving four enzymes (Hult and Gatenbeck, 1978; Hult, Veide and Gatenbeck, 1980; Meena *et al.*, 2015). According to these authors, mannitol-1-phosphate dehydrogenase (M1PDH) converts fructose-6-phosphate to mannitol 1-phosphate which is then dephosphorylated into mannitol by mannitol 1-P-phosphatase. Subsequently, mannitol would be oxidized to fructose, catalyzed by MTD. More recent studies have questioned this cyclic model

of mannitol metabolism, suggesting the existence of other reactions involved in this process (Solomon, Waters and Oliver, 2007; Dulermo *et al.*, 2010; Patel and Williamson, 2016). For example, *B. cinerea* double mutants (*Δmtd/Δm1pdh*) still produce mannitol, suggesting the existence of a different pathway for mannitol synthesis (Dulermo *et al.*, 2010). The same authors suggested that *B. cinerea Δm1pdh* mutants produced mannitol 1-phosphate from a different phosphorylation pathway.

1.3.2. The role of mannitol in plant-fungal interactions

Besides the previously mentioned roles of mannitol in plants and fungi, this polyol also has a crucial role during plant-pathogen interactions (Voegele *et al.*, 2005; Calmes *et al.*, 2013; Meena *et al.*, 2015; Patel and Williamson, 2016). Generally, fungi attacks trigger a release of plant-generated ROS to combat infection. ROS also have a role in cross-linking plant cell wall proteins and in lignification which constrains pathogen spreading to near plant cells (Patel and Williamson, 2016). Additionally, ROS can trigger both the hypersensitive response (HR) and the systemic acquired resistance (SAR) mechanisms. HR precedes programmed cell death which is another way to contain pathogen proliferation (Meena *et al.*, 2015; Patel and Williamson, 2016). During infection, *B. cinerea* produces mannitol (Dulermo *et al.*, 2010), and also its own ROS, consequently inducing a HR. *B. cinerea*, as a necrotrophic pathogen, benefits from the HR as it can feed on dead tissue. In fact, in *Arabidopsis thaliana*, *B. cinerea* infection was facilitated by HR induction (Govrin and Levine, 2000). Therefore, the production of mannitol by *B. cinerea* can have a role in protecting the fungus against the initial burst of ROS induced by the plant and by the fungi itself (Meena *et al.*, 2015; Patel and Williamson, 2016).

1.4. Aim and objective of the study

Our scientific group currently studies plant-environmental interactions and plant stress biology. Recently, we focused on grapevine source to sink interactions in response to environment and key biochemical and molecular events occurring during fruit development and ripening. As previously discussed in the Introduction section, the polyols mannitol and sorbitol have recognized roles in resistance to abiotic stresses such as high salinity and heat. Additionally, these compounds can have a key role in the interaction between fungal pathogens and their plant hosts.

Therefore, in the present study, we aimed to study the transcriptional and biochemical changes in polyol transport and metabolism caused by artificial *Botrytis* infection in the grape berry, to shed more

light on these mechanisms' possible relation with the infection process. Different strategies were used to achieve our scientific objectives. Firstly, we established an artificial *Botrytis* infection of healthy grape berries in a *terroir* that has the potential to develop noble rot. After that, a set of physiological, biochemical, and molecular approaches were combined to address the influence of a mild *Botrytis* infection on the metabolic and transcriptomic processes of polyol metabolism, determining its concentration by analytical methods and studying the gene expression and biochemical activities of key enzymes related to polyol metabolism.

In this work, we took advantage of a recent collaboration between our group and a "wine company", which allowed us to perform field experiments. Also, we benefited from collaborations with different national and international research groups and from funding provided by different ongoing R&D projects: ("MitiVineDrought"- PTDC/BIA-FBT/30341/2017 and POCI-01-0145-FEDER-030341; "BerryPlastid" - POCI-01-0145-FEDER-028165 and PTDC/BIA-FBT/28165/2017; "GrapeInfectomics" - TDC/ASPHOR/28485/2017; "CherryCrackLess" - PTDC/AGR-PRO/7028/2014).

2. MATERIALS AND METHODS

2.1. Plant material

Field experiments were performed in grapevines of the cultivar Sémillon in a commercial vineyard "Quinta do Casal da Granja" located at Alijó, Portugal. Alijó is situated in a plateau with the microclimatic conditions needed for noble rot infection to develop (*Grandjó Late Harvest*, 2019).

For artificial infection, *B. cinerea* was isolated from diseased vines and maintained in potato dextrose agar (PDA) at 5 °C. For conidia production, petri dishes were exposed to fluorescent light at 24 °C and after 14-18 days of treatment, conidia were collected from Petri dishes by rubbing the culture with phosphate buffer (0.03 M KH_2PO_4). The suspension was then filtered with cheesecloth to remove residual mycelia and its concentration was adjusted to 10^5 conidia mL^{-1}. Grape clusters were sprayed in well-established and standardized conditions according to Agudelo-Romero *et al.*, (2015) and Coelho *et al.*, (2019) with the conidia solution. Phosphate buffer (0.03 M KH_2PO_4) was sprayed on control berries. After the inoculation, each cluster was enclosed by a plastic bag during one week to maintain 100% relative humidity (RH).

During the late-harvest season and 71 days after inoculation, several grape clusters, control ones, and infected, were randomly and representatively collected. For each treatment (control and infected), three biological replicates were collected at around 10 a.m., each one constituted by a composite pool of at least 12 berries collected from different clusters from three different plants. The collected samples were frozen in liquid nitrogen and stored at -80 °C for RNA extraction and sugar quantification. The seeds of each of the three sampled biological replicates were removed and the remaining tissues were ground in liquid nitrogen to a fine powder.

2.2. Major sugars extraction and quantification by HPLC

The extraction of sugars from grape berry samples was performed following a method described by Eyéghé-Bickong *et al.*, (2012) and modified by Conde *et al.*, (2018). 800 µl of ultrapure water and a small amount of Polyvinylpolypyrrolidone (PVPP) were added to 80 mg of ground sample material. 800 µl of chloroform were added and the mixture was vortexed for 5 minutes followed by incubation in a plate shaker for 30 minutes. Next, the mixture was centrifuged in a Centrifuge 5418 R (Eppendorf) for 10 mins at 16,900 x g and the aqueous phase collected. Subsequently, the resulting solution was filtered with a 0,2 µm nylon filter and injected in a Rezex RCM–Monosaccharide Ca^{2+} (8%) column (Phenomenex), appropriate for mono and disaccharides, and polyols separation. HPLC runs lasted for 30 min at 60 °C with a flow rate of 0,6 mL min^{-1}, using ultrapure water as the mobile phase.

Extracted samples were injected in a high-performance liquid chromatographer, namely an Auto Sampler L-2200 Elite LaChrom elite model (Hitachi) connected to a Refractive Index detector. Sugars concentrations on each sample were determined by comparison of the peak area and retention time with previously established calibration curves of each analyzed compound: fructose, glucose, sucrose, sorbitol, and mannitol.

2.3. Grape berry water content

Control and infected berries were completely dried during one week at 60 °C. For each treatment, three biological replicates were used, each constituted by a composite pool of 3 randomly picked berries collected from different clusters. The fresh and dry weights were measured on an analytical scale.

2.4. RNA extraction and cDNA synthesis

The following RNA extraction protocol steps were based on the work of Reid *et al.* in 2006, with some modifications. 1 mL of extraction buffer [2% Hexadecyltrimethylammonium bromide (CTAB), 300 mM Tris HCl (ph 8.0), 25 mM EDTA, 2 M NaCl and 2% PVP] was added to 100 mg of grounded grape berry tissue. Subsequently, the mixture was incubated for 10 minutes at 65 °C. Chloroform-isoamyl alcohol (24:1), was then added in a 1:1 ratio and the mixture was centrifuged (3500 x g) for 15 minutes at 4°C followed by the collection of the aqueous phase. This step was repeated. Then, 0,1 volumes of 3 M sodium acetate (pH 5,2) and 0,6 volumes of isopropanol were added, and the mixture was stored at -80°C for 25 minutes. Afterward, the mixture was centrifuged at 16900 x g for 30 minutes at 4 °C. The supernatant was discarded, and the RNA was dissolved in 1X TE buffer (10 mM Tris; 1 mM EDTA). The RNA was then extracted by in column purification using the "GRS Total RNA Kit – Plant" (GRISP) following the manufacturer steps.

RNA sample concentration was determined with the NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific). Following, to assess RNA integrity, a gel electrophoresis assay was performed.

After isolation and verification of RNA purity, treatment with DNase I (Qiagen) was performed, and cDNA was synthesized from 1 µg of total RNA using the Xpert cDNA Synthesis Mastermix Kit (GRISP). Subsequently, the cDNA obtained was stored at -20 °C for posterior use in qPCR.

2.5. Gene Expression Analysis by qPCR

The expression profile of *VvPLT1* and *VvMTD1* genes in all the studied samples (control and infected grape berries) was analyzed by real-time qPCR performed using cDNAs obtained from RNAs extracted from each of the three composite pools of grape berry samples. Real-time qPCR was performed with Xpert Fast SYBR Blue (GRISP) using 1 µL of diluted cDNA (1:10) in a total of 10 µL of reaction mixture per well. qPCR thermal cycling conditions were already optimized and consisted of an enzyme activation and cDNA denaturation initial step of 95°C for 3 minutes, followed by 40 cycles of 95 °C for denaturation for 5 seconds, 60°C for annealing during 20 seconds, and finally, 72 °C for extension during 20 seconds. As a reference gene, *VvACT1* (actin) was used, as it is considered an extremely adequate reference gene for gene expression normalization purposes in qPCR analyses in grapevine (Reid et al., 2006). Specific primers used for each studied gene are listed in Table 2. Melting curve analysis was performed for specific gene amplification confirmation. The stability of the reference genes was confirmed by the automatic M-value analysis performed by the Bio-Rad® CFX Manager 2.0 Software. For each gene, the relative gene expression values were obtained following calculation by the Bio-Rad® CFX Manager 2.0 Software. For each of the three biological replicates, after RNA extractions and cDNA synthesis, an independent qPCR analysis was performed with three internal technical replicates.

Table 2 – List of the primers used in this study (Conde et al., 2015). *VvACT1* accession number was obtained from GenBank.

Primer name	Primer sequence (5' - 3')	Amplicon length	Gene sequence accession number
*qVvPLT1*forward	AGCCGTCGGCATTCACTTCTTC	68 bp	KF319032
*qVvPLT1*reverse	TTCTTGGGCTGTAGAGGACGAC		
*qVvMTD1*forward	TACCCTATAGTTCCCGGACATGAG	118 bp	KF319033
*qVvMTD1*reverse	AGTGACAAGCTCCAACCATGC		
*qVvACT1*forward	GTGCCTGCCATGTATGTTGCCATTCAG	158 bp	AY680701.1
*qVvACT1*reverse	GCAAGGTCAAGACGAAGGATAGCATGG		

2.6. VvMTD and VvSDH enzyme activity

Grape berry tissue from both control and infected samples previously grounded to a fine powder in liquid nitrogen was used for protein extraction. The samples were mixed in a 1:3 ratio with the protein extraction buffer containing the following compounds: 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) at ph 7.5; 5 mM $MgCl_2$; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride (PMSF); 5 mM Dithiothreitol; 0,1% (v/v) Triton x-100 and 1% Polyvinylpolypyrrolidone (PVPP). Then, the obtained homogenate was centrifuged at 18000 x g for 20 minutes and the supernatant was kept on ice to be used on enzymatic assays.

For enzyme activity measurements, a reaction mixture was prepared with the following compounds: protein extract (300 mM BIS-TRIS propane (pH 9.0)), 1 mM NAD^+, 0.2 M D-mannitol, or 0.2 M D-sorbitol for measuring VvMTD and VvSDH enzymatic activities, respectively. Enzymatic assays were performed observing the reduction of NAD^+ in a UV-1700 Pharma Spec spectrophotometer (Shimadzu) at 340 nm. Enzymatic activities were performed at 37 °C with a final total reaction volume of 1 ml. Every reaction was initiated with the addition of the appropriate polyol. VvMTD activity in the direction of fructose reduction was also measured. In this case, instead of using mannitol to start the reaction, 0.2 M of fructose was used. Similarly, 0.1 mM NADH was used as a co-factor instead of NAD^+.

Total protein concentration was determined by the Bradford method (Bradford, 1976) using Bovine serum albumin as a standard. Sample absorbance was measured at OD = 595 nm in a UV-1700 Pharma Spec spectrophotometer (Shimadzu).

2.7. Statistical analysis

The results were statistically verified by Student's t-test using Prism v. 6 (GraphPad Software, Inc.). Post-hoc multiple comparisons were performed using the HSD Tukey test. Throughout the results, asterisks indicate statistically significance between columns (*$P \leq 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

3. RESULTS AND DISCUSSION

3.1. Phenotypic characterization of sampled grape berries

Control and infected grape berry bunches (figure 3) were harvested as detailed in the material and methods section. Infected berries with noble rot were assessed after observation of their characteristic phenotypic alterations which include a change in coloration to dark purple and purple-brown, dehydration, and shriveled aspect together with some mycelia development (Magyar, 2011; Blanco-Ulate *et al.*, 2015; Magyar and Soós, 2016; Jackson, 2020).

Figure 3 - Photographs taken on the sample harvesting date. On the left, a grapevine cluster with late-harvest control berries; on the right, several grape berries infected with *Botrytis cinerea.*

3.2. Berry water content

To determine if the infection by *Botrytis* affected the water content of the late-harvest grape berries (naturally dehydrated), infected and control ones were weighed before and after being totally dried in an incubator at 60 °C for 7 days. Results showed a slight difference (3.55%) between the infected berries and the control ones (figure 4). Importantly, our samples are from a cultivar that has extended ripening periods. Accordingly, with prolonged ripening periods, grapes tend to show weight loss due to xylem backflow in conjunction with loss of phloem functionality. As such, the initial water content on control

berries is expected to be slightly lower compared to grapes with regular harvest timings due to the extended maturation process (McCarthy, 1999; Tyerman *et al.*, 2004; Tonutti and Bonghi, 2013).

Differences between control and infected berries could be caused by the cracks in the cuticle and shriveling in infected grapes, symptoms of *B. cinerea* infection. Dehydration is a known symptom in noble rot leading to an increase in water loss compared to healthy berries (Ribéreau-gayon *et al.*, 2006; Magyar, 2011). Nevertheless, this negligible change does not suggest any influence on grape berry metabolism.

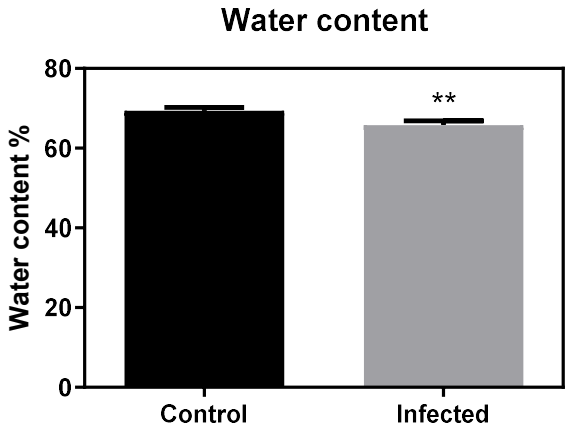

Figure 4 – Water content comparison of *V. vinifera* control grapes and grapes infected with *Botrytis cinerea*. Results indicate the mean and standard deviation of three independent experiments. Asterisks indicate statistically significant results (**P<0.01).

3.3. Sugar and polyol content in infected and control berries

Sugar content, particularly polyols, in infected and control grape berries, was determined by HPLC. The concentration of sucrose in control and infected berries was 24.03 $mg.g^{-1}$ DW and 19.74 $mg.g^{-1}$ DW, respectively. Glucose concentration was 393.59 $mg.g^{-1}$ DW in control berries and 354.28 $mg.g^{-1}$ DW in infected berries, while, the fructose concentration was, respectively, 368.78 $mg.g^{-1}$ DW and 340.22 $mg.g^{-1}$ DW (Figure 5). No statistically significant differences were found between control and infected berries in the concentrations of sucrose, glucose, and fructose, despite a slight decrease. Additionally, the glucose/fructose ratio was smaller in infected berries (Figure 6).

Sugar levels normally increase in noble rot berries since the concentrating effect of berry dehydration overcomes the opposing effect of *B. cinerea* sugar consumption. Additionally, in some cases, the glucose to fructose ratio diminishes (Ribéreau-gayon *et al.*, 2006; Hornsey, 2007; Magyar, 2011; Magyar and Soós, 2016; Thakur, 2018; Jackson, 2020). A comparison between these metabolites, in control and infected berries levels, is on display in Table 1 in the introduction chapter.

In previous works, the observed decrease of these sugars concentrations was suggested to be the result of the fungus metabolism (Ribéreau-gayon *et al.*, 2006; Magyar, 2011; Vannini and Chilosi, 2013; Jackson, 2020). Moreover, during infection, an increase in the need for carbohydrates by the plant for pathogen defense mechanisms could decrease the levels of the studied sugars as observed in *Arabidopsis thaliana* (Veillet *et al.*, 2017). Therefore, a similar phenomenon can be happening in the *Botrytis* infected grape berries.

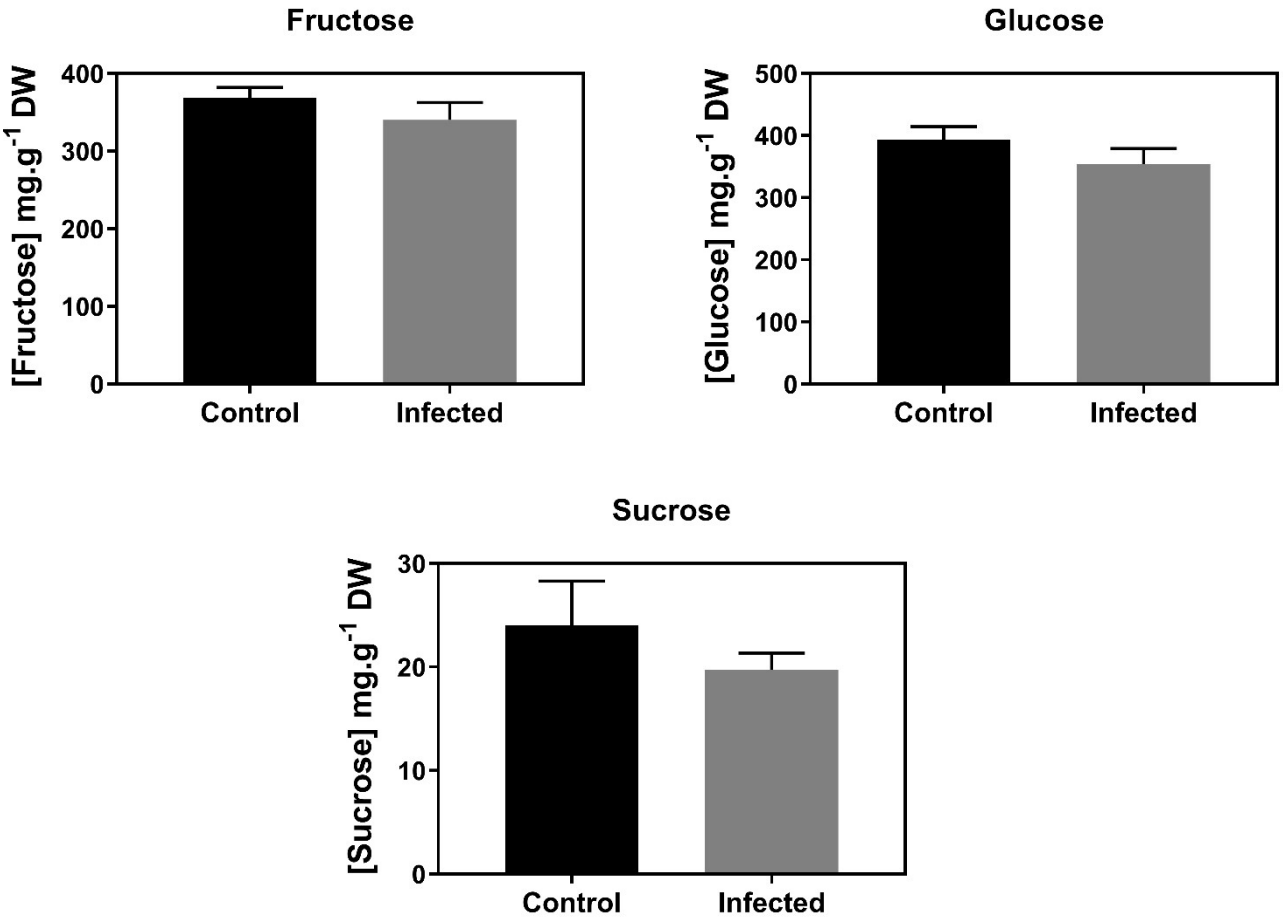

Figure 5 - Concentration of sucrose, glucose, and fructose in control grapes and grapes infected with *Botrytis cinerea* measured by HPLC. Results indicate the mean and standard deviation of three independent experiments.

A decrease in the glucose/fructose ratio has been previously reported in infected berries, given the preference for glucose metabolism compared to fructose by *Botrytis cinerea* which results in the production of gluconic acid (Magyar, 2011; Jackson, 2020). This ratio can reach lower levels, down to 0.80. Garcia-Jares and Médina, in 1997, considered the glucose to fructose ratio to be an indicator of noble rot infection progression. A decrease in the glucose/fructose ratio, similar to our results, was also observed during *Botrytis* infection of the grape berries (Blanco-Ulate *et al.* in 2015).

In a study which evaluated leaf lesion sizes of *Botrytis*-infected lettuce and respective plant metabolite composition, the authors suggested that the ratio of sucrose and fructose amongst plant soluble sugars (sucrose, glucose, and fructose) was more related to the induction of plant defense compared to individual sugar content suggesting that ratios rather than the absolute sugar levels were more relevant as indicators of disease progression. Nonetheless, a negative correlation was found for lesion size and total sucrose

content in *B. cinerea* infected plants (Lecompte, Abro and Nicot, 2013). Following this correlation and based on their results, the same authors hypothesized that sucrose was being used as a producer of antifungal signaling components more than as a carbon source for polyphenol synthesis. In a later study, in 2017, Lecompte *et al.* found that the ratio of fructose and main soluble sugars (sucrose, glucose, and fructose) was an indicator of tomato stem defense against *B. cinerea* infection pointing that the level of this ratio is key for maintaining plant defense. While the literature suggests that the decrease of the glucose/fructose ratio observed in our results is a result of the pathogen metabolism (Magyar, 2011; Jackson, 2020), the evidence found by Lecompte *et al.* points that this ratio's change in infected plants could be induced by the host plant as a means to defend itself from pathogen infection. The same authors proposed that invertases and sucrose synthases, which evenly catabolize sucrose into glucose and fructose, and UDP-glucose and fructose, respectively (Barratt *et al.*, 2009), could be a way for the host plant to adjust the relative ratio of fructose among main soluble sugars. Additionally, and with the same aim, Lecompte *et al.* suggested a role for hexokinase and fructokinase in infected tissues in the use of fructose and glucose in different proportions. Hexokinase is a catalytic enzyme which breaks down glucose and fructose, while fructokinase is only capable of catalyzing the transformation of fructose (Claeyssen and Rivoal, 2007; Stein and Granot, 2018).

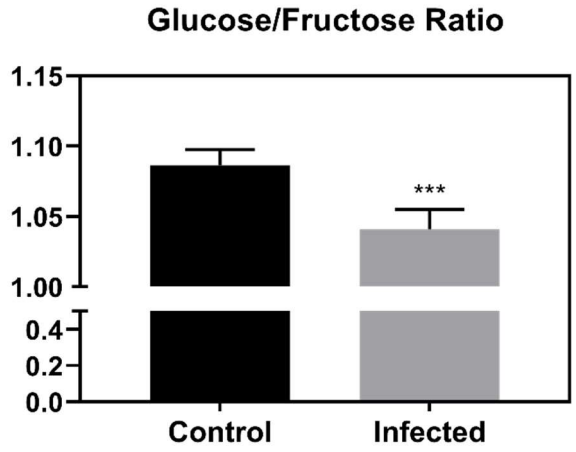

Figure 6 – Ratio of the concentration of glucose and fructose in *V. vinifera* control grapes and grapes infected with noble rot measured by HPLC. Asterisks indicate statistically significant results (***P<0.001).

Regarding mannitol and sorbitol concentrations, a statistically significant increase was found in infected berries compared to control ones (Figure 7). The results of the quantification of mannitol in control and infected berries were, respectively, 2,76 $mg.g^{-1}$ DW and 5,50 $mg.g^{-1}$ DW. The sorbitol measurements results were the following: 1,34 $mg.g^{-1}$ DW for control and 2,02 $mg.g^{-1}$ DW for infected.

As mentioned in the Introduction section, polyol levels tend to increase their concentration in wines from berries infected by *B. cinerea* as a result of the fungus sugar metabolism (Bertrand *et al.*, 1976; Ribéreau-gayon *et al.*, 2006; Magyar, 2011; Magyar and Soós, 2016; Thakur, 2018; Jackson, 2020). An increase in the mannitol and sorbitol levels was also observed in noble rotted berries of the Sauternes, german regions and the Sémillon, Merlot, Pinot Noir, Cinsaut and Auxerrois cultivars (Bertrand *et al.*, 1976; Ribéreau-gayon *et al.*, 2006; Magyar, 2011; Blanco-Ulate *et al.*, 2015; Jackson, 2020). It is plausible that some part of the mannitol was formed by the fungus metabolism of glucose and fructose. In sunflower infected with *B. cinerea,* Dulermo *et al.*, 2009 observed the conversion of plant host glucose and fructose to mannitol. The same conversion may be happening in infected berries.

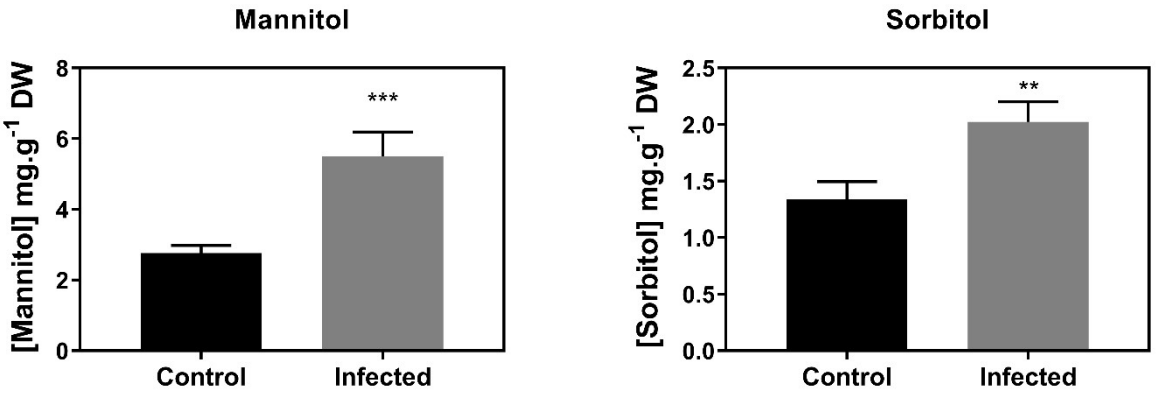

Figure 7 - Concentration of mannitol and sorbitol in control grapes and grapes infected with noble rot measured by HPLC. Results indicate the mean and standard deviation of three independent experiments. Asterisks indicate statistically significant results (**P<0.01; ***P<0.001).

3.4. *VvMTD1* and *VvPLT1* gene expression in infected and control berries

The gene expression of *VvMTD1* and *VvPLT1* was determined by qPCR in control and infected berries. As mentioned in the introduction section, VvPTL1 is a plasma membrane polyol transporter with high affinity to mannitol and sorbitol (Conde *et al.*, 2015). MTD is an oxidoreductase enzyme (EC: 1.1.1.255); in *Apium graveolens* (celery) this enzyme oxidizes mannitol to mannose in the cytoplasm by mannitol oxidation, with NAD^+ as a cofactor. In *Vitis vinifera,* this enzyme is responsible for the production of fructose instead of mannose but is also capable of the opposite reaction, i.e. fructose reduction into mannitol (Williamson *et al.*, 1995; Conde *et al.*, 2015).

Results showed an up-regulation of *VvMTD1* in infected berries compared to control ones (Figure 8). The upregulation was approximately 33%. Concerning *VvPLT1* transcripts, these were strongly reduced in infected berries compared to control ones, a 9-fold decrease. These results suggest, at a transcriptional level, an increase in the metabolism of mannitol and, simultaneously, a decrease in the uptake of mannitol to the cell in infected grape berries.

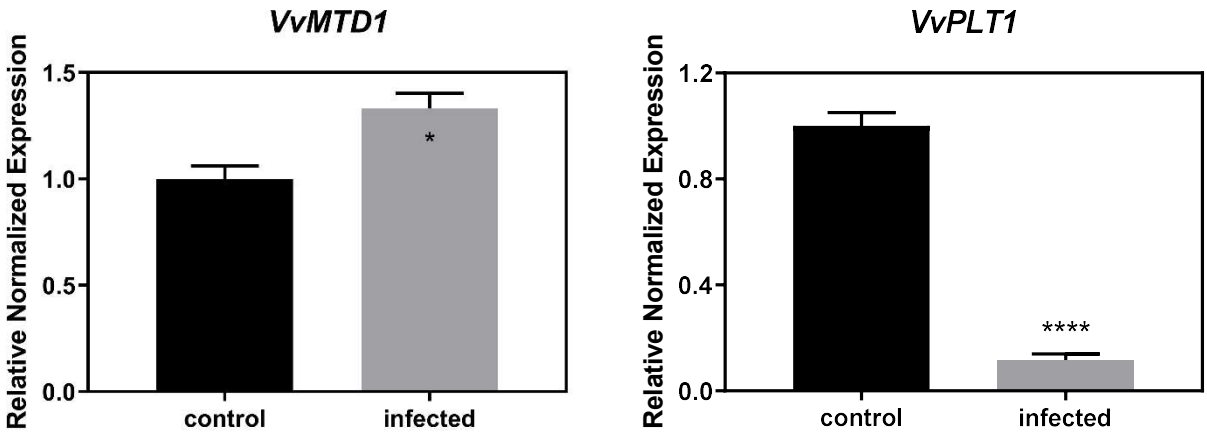

Figure 8 - Relative normalized expression of *VvMTD1* and *VvPLT1* genes of *Vitis vinifera* control grapes and *Vitis vinifera* grapes infected with noble rot measured by qPCR. Results indicate the mean and standard deviation of three independent experiments. Asterisks indicate statistically significant results (*P<0.05; ****P<0.0001).

3.5. Effect of *Botrytis* infection on the biochemical activity of mannitol dehydrogenase and sorbitol dehydrogenase

In grapevine, M6PR catalyzes the conversion of mannose-6-phosphate to mannitol-1-phosphate. Subsequently, mannitol is synthesized through mannitol-1-phosphate by dephosphorylation of this compound. Mannitol is then translocated to heterotrophic tissues where it can be stored as a reserve carbohydrate or oxidized to fructose in a reversible reaction (Stoop, Williamson and Masonpharr, 1996; N. Noiraud, Maurousset and Lemoine, 2001; Parvaiz and Satyawati, 2008; Conde *et al.*, 2015). The synthesis and catabolism of mannitol were studied in *Botrytis* infected and control grape berries. Regarding fructose production, our results indicate a strong, statistically significant, 3-fold increase in VvMTD activity in infected grape berries compared to control berries (Figure 9). At the same time, we did not detect any enzyme activity in the reaction direction of mannitol production. Overall, these results

coupled with the significant increase in expression of *VvMTD1* suggest that *Vitis vinifera* berries favor mannitol degradation when under attack by *Botrytis cinerea.*

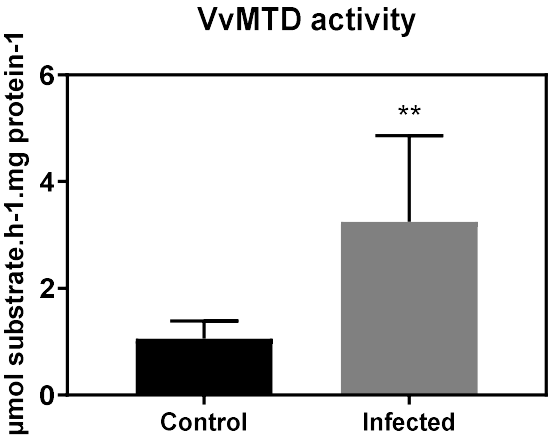

Figure 9 - Mannitol oxidation (V_{max}) by VvMTD in control grapes and noble-rot infected. Results indicate the mean and standard deviation of six independent experiments. Asterisks indicate statistically significant results (**P<0.01).

Using a similar method, enzyme assays were performed for VvSDH (EC 1.1.1.14) but no activity was detected in either control or infected berries. This enzyme catalyzes the oxidation of sorbitol to fructose, in a reversible reaction, in the grape berry (Shangguan *et al.*, 2014; Conde *et al.*, 2015, 2018).

The increase in MTD mannitol degradation activity in our results is in line with the existing evidence of MTD's role in plant defense in other plant species(Jennings *et al.*, 1998; Williamson *et al.*, 2002, 2013; Meena *et al.*, 2015; Patel and Williamson, 2016). The upregulation of *MTD* and *MTD* homologs has been observed in plants as a means to eliminate mannitol produced by pathogenic fungi, even in those that do not produce this polyol, like tobacco and *Arabidopsis thaliana* (Jennings *et al.*, 1998; Meena *et al.*, 2015; Patel and Williamson, 2016). It is suggested that, when MTD or/and its homologs are induced, the oxidative burst caused by ROS production has more efficiency in protecting the plant against the pathogen-produced mannitol (Meena et al., 2015; Patel and Williamson, 2016). Zonal geranium (*Pelargonium × hortorum*) showed increased effectiveness against *B. cinerea* infection when MTD was overexpressed, further solidifying the hypothesis that plants use MTD as a means of defense (Williamson et al., 2013). Additionally, salicylic acid (a molecule involved with biotic stress signaling) induction of MTD in celery and tobacco also points to MTD being a pathogenesis-related protein (Cheng *et al.*, 2009; Meena *et al.*, 2015; Patel and Williamson, 2016).

However, while MTD-driven catabolism of mannitol happens inside the cell in celery and grapevine (Loescher and Everard, 2000; Conde *et al.*, 2015), the plant oxidative burst occurs in the apoplast (Levine, Tenhaken and Lamb, 2003; Patel and Williamson, 2016). Interestingly, salicylic acid was found to induce the secretion of MTD to the apoplast in celery and tobacco by an unknown (non-Golgi complex related) mechanism, in response to *Alternaria alternata* infection (Cheng *et al.*, 2009; Meena *et al.*, 2015; Patel and Williamson, 2016). This suggests that MTD is localized in the apoplast to degrade infection-related mannitol (Cheng *et al.*, 2009; Cheng and Williamson, 2010; Patel and Williamson, 2016). While this possible localization was not yet studied in *Vitis vinifera*, *VvMTD1* has *cis*-acting elements related to salicylic acid present in its promoter (Conde *et al.*, 2015). Moreover, a synergy between MTD activity and the transport of mannitol is also suggested (N. Noiraud, Maurousset and Lemoine, 2001; Juchaux-Cachau *et al.*, 2007; Williamson *et al.*, 2013; Meena *et al.*, 2015; Patel and Williamson, 2016). Tobacco plants (non-mannitol producers) over-expressing a celery mannitol transporter (AgMaT2) showed higher resistance to infection by *Alternaria alternata*, a fungal pathogen that secretes mannitol. Afterward, Williamson *et al.*, (2013) hypothesized that the increased resistance to *A. alternata* was due to the fact that the tobacco plant would degrade apoplastic fungi-produced mannitol with the secreted MTD and also by increasing the uptake of mannitol to the cytosol to degrade.

The strong downregulation of *VvPLT1* in infected berries in our findings goes against the above hypothesis given that, in the case of noble rot infected berries, mannitol uptake to the cytosol is not increased. In fact, our results suggest that *Vitis vinifera* induces MTD to degrade fungi-produced mannitol, but, at the same time, does not to try to increase the uptake of mannitol for degradation (Figure 10). This last interaction could be explained by the fungus interference with plant defense responses as *B. cinerea* is suggested to interfere with grapevine hormone production and perception and more specifically with gene expression (Agudelo-Romero *et al.*, 2015; Blanco-Ulate *et al.*, 2015; Chanclud and Morel, 2016; Lovato *et al.*, 2019). Moreover, in an article covering the identification of the SWEET family of transporters, the authors suggested that fungal pathogens benefit from the induced expression of these transporters for their growth (Chen *et al.*, 2010). More precisely, Chen *et al.* suggested that *B. cinerea* infection caused the overexpression of several *A. thaliana* genes (*AtSWEET4I, AtSWEET15,* and *AtSWEET17*). In grapevine, *VvSWEET4*, a glucose uniporter, was up-regulated during *B. cinerea* infection (Chong *et al.*, 2014). In the referenced study, the authors suggested this increase in gene expression to be a manipulation by the fungal pathogen to export cytosol sugars more easily to the apoplast to help in its infection process. *VvSWEET7* and *VvSWEET*15 were also upregulated during *B. cinerea* infection in

Trincadeira, a cv. susceptible to this pathogen (Breia *et al.*, 2020). Therefore, the repression of *VvPLT1* is probably a direct effect of *B. cinerea* infection in grape berries.

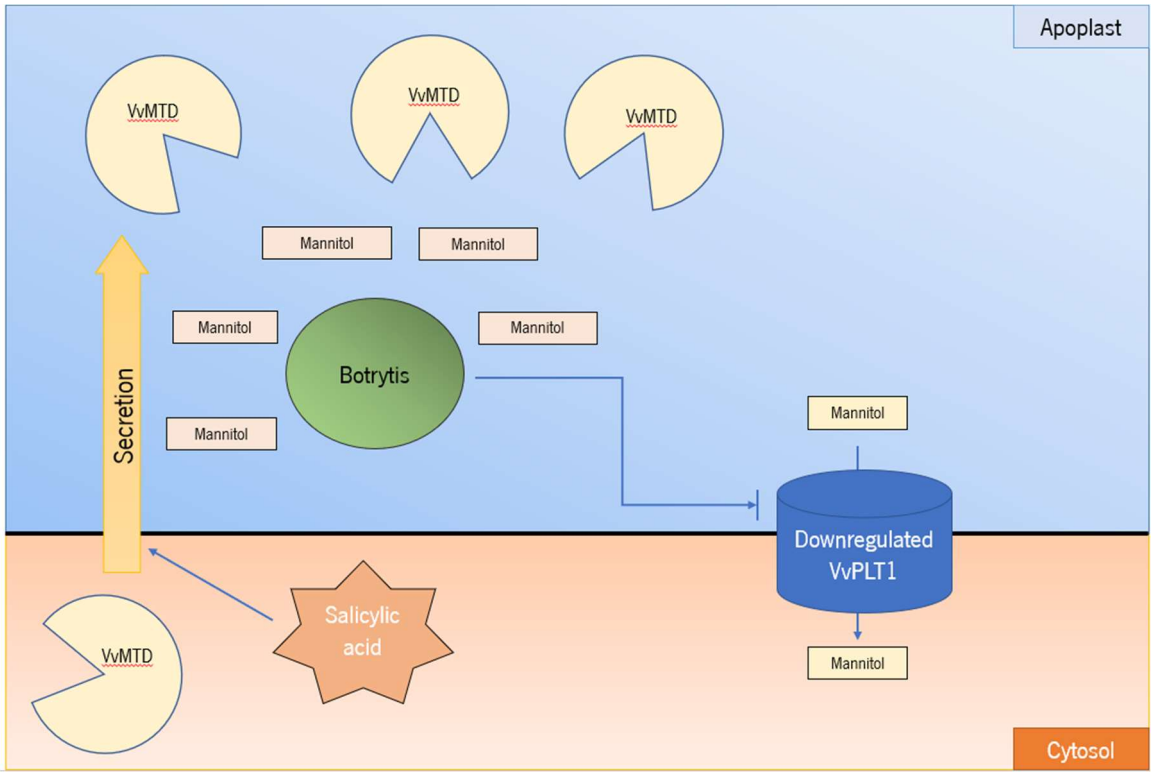

Figure 10 – Illustration of the hypothetic interaction between *B. cinerea* and the grape berry, per our results. In the infected berry, *Botrytis* mycelia produce mannitol for its protection against berry-derived ROS. The grape berry increases the expression of VvMTD and possibly secretes the enzyme to the apoplast region for the catabolization of fungal-derived mannitol. At the same time, *Botrytis* reduces the expression of the plasma membrane mannitol transporter VvPLT1, diminishing the possibility of apoplast-mannitol uptake by the berry.

3.6. Conclusion and future perspectives

Research has been made over the past decades regarding *Botrytis* interactions with its host plants in both the pathogen attack mechanisms and in plant defense systems. Hence, some breakthroughs have been achieved with bioengineering approaches, biocontrol strategies, and also with plant breeding programs to improve plant resistance, however, no complete tolerance was achieved to *Botrytis* diseases so far in any affected crop, therefore, fungicide application and field practices are still being the most prevalent form of disease control (AbuQamar, Moustafa and Tran, 2017).

By studying transcriptional and metabolic changes in the grape berry during *B. cinerea* infection we observed a decrease in the levels of several sugars (sucrose, glucose, and fructose) and an accumulation of mannitol and sorbitol, in infected berries. Also, we detected the upregulation of *VvMTD* together with an increase in its enzyme activity and the downregulation of *VvPLT1*. These data are in agreement with previous literature regarding the possible role of MTD in other plants' defense as a protein that degrades mannitol produced by pathogenic fungi and opens up a question related to the possible connection between MTD and mannitol transport.

The role of mannitol in the interactions between plants and pathogenic fungi has long been suggested (Chaturvedi *et al.*, 1996; Jennings *et al.*, 1998), but it was only in the last decade that mannitol has been implied to be part of these interactions. Nonetheless, the specific role of fungal-produced mannitol and its metabolism and transport in *Vitis* is a scarcely studied topic. Therefore, to further study this subject, additional molecular biology approaches, such as overexpressing *VvMTD* in *Vitis* suspension cells or in tomato plants to assess their resistance against *Botrytis* infection are important. It was previously mentioned that the overexpression of *MTD* has increased the resistance of several plants against this fungus, so it is plausible to expect similar results. Besides, the study of a mutant grapevine overexpressing *VvPLT1* could prove interesting to check it for decreased susceptibility to *Botrytis*. As mentioned previously, this gene was strongly downregulated in our results suggesting interference by *B. cinerea*. There is also a need for more investigation on the issue of MTD secretion to the apoplast. While MTD is secreted to the apoplast under salicylic acid treatment in celery and tobacco (Cheng *et al.*, 2009; Cheng and Williamson, 2010; Patel and Williamson, 2016), the mechanism by which MTD is secreted is unknown. Applying new findings on the unconventional secretion of proteins such as MTD that do not involve the Golgi complex in the process of said secretion can shed further knowledge on this question (Krause *et al.*, 2013; Pompa and Walker, 2016). Additionally, to complement our results, the observation of the sub-cellular localization of VvMTDs in *Botrytis* infected berries is of utmost importance.

Finally, the determination of *VvPMT1* gene expression, another polyol transporter expressed in grape berry would also be another informative study. Our studied transporter (*VvPLT1*/*VvPMT5*) is the most expressed polyol transporter in the grape berry, mainly in the fruit set stage. *VvPMT1* is another polyol transporter-coding gene that does not display the same levels of expression as *VvPLT1*/ *VvPMT5*. Nonetheless, this transporter is an interesting target gene for research in *Botrytis* infected berries due to its higher expression levels specifically at the berry ripe stage than *VvPLT1* (Afoufa-Bastien *et al.*, 2010).

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