

## AN OVERVIEW ON BOTRYTIZED WINES

### REVISÃO: VINHOS BOTRITIZADOS

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#### SUMMARY

Noble rot wine is a specific type of sweet wine that derives from the infection of grape berries by a fungus called *Botrytis cinerea*. These wines are produced in specific wine regions around the world, with Sauternes region of France and Tokay region of Hungary being the most famous ones. The purpose of the current article is to provide a systematic review on the different stages of botrytized wines production, including a detailed analysis of the technical aspects involved. Specifically, it describes the process and development of berry infection by *B. cinerea*, and special emphasis is given to the main stages and operations of winemaking, conservation, aging and stabilization. A complex combination of a number of parameters (e.g., very specific environmental conditions) explains the rarity of noble rot occurrence and highlights the uniqueness of botrytized wines.

#### RESUMO

Os vinhos botritizados representam uma categoria específica de vinhos doces, sendo obtidos a partir de bagos de uva infectados pelo fungo *Botrytis cinerea*, através de um processo designado por podridão nobre. Estes vinhos são produzidos em regiões específicas do mundo, sendo Sauternes e Tokay, originários de França e Hungria respectivamente, os exemplos mais conhecidos a nível mundial. No presente trabalho são revistos os principais aspectos relacionados com a produção de vinhos botritizados. É descrito o processo e desenvolvimento da infecção do bago por *B. cinerea*, e discutidas as principais etapas e operações de vinificação, conservação, envelhecimento e estabilização. A combinação complexa de muitos factores, desde logo a exigência de condições ambientais muito específicas, explica a raridade da ocorrência de podridão nobre e destaca a singularidade deste tipo de vinhos.

**Key words:** Botrytization, *Botrytis cinerea*, noble rot, sweet wines.

**Palavras-chave:** Botritização, *Botrytis cinerea*, podridão nobre, vinhos doces.

#### INTRODUCTION

According to the International Organisation of Vine and Wine (OIV), noble rot wines are included in the general category of “Special Wines”. “Special wines” are wines coming from fresh grapes, from musts or wines that have undergone certain treatments during or after their production and whose characteristics come not only from the grape itself, but also from the production technique used. This category includes flor or film wines, liqueur wines, sparkling wines, carbonated wines, sweet wines with residual sugar derived from grapes and icewine – eiswein (OIV, 2020).

The subcategory of “Sweet wines with residual sugar derived from grapes”, where noble rot wines are included, respects wines with a content of fermentation residual sugars, glucose plus fructose, above or equal to 45 g/L. These wines result exclusively from partial alcoholic fermentation (ALF) of grapes or grape must for which the sugar content was obtained naturally during the grape maturation, or may be obtained from overripe or slightly raisined or suffering from noble rot grapes, by means of raisining, selective sorting of grapes and cryoselection. The actual alcoholic strength by volume of the wine must not be less than 4.5% vol. The potential alcoholic strength of the grapes before

fermentation must not be less than 15% vol. (OIV, 2020).

Within the European (EU) legislation, noble rot wine integrates the category of “Wine of overripe grapes”, respecting the following requisites: a) is produced without enrichment; b) has a natural alcoholic strength of more than 15% vol.; and c) has a total alcoholic strength of not less than 15% vol. and an actual alcoholic strength of not less than 12% vol. (EEC, 2013).

### Noble rot wines

One of the main techniques to produce a sweet wine is by concentrating the grape juice during noble rot infection. The latter, refers to the development of the fungus *Botrytis cinerea* on mature grapes under very specific weather conditions (Jackson, 2008). Few rainy days after grapes ripening are vital for *B. cinerea* germination and colonization on berries. Sequential sunny days with foggy mornings and high humidity followed by afternoons with high temperatures are perfect for fungus development, water evaporation and berry’s volume concentration (Keller, 2015). The attack of this fungus via micro-fissures, generates a modification on berry’s chemical

composition and leads to grape epidermis rupture and excess water losses (Carrascosa *et al.*, 2011). The stage where the infection is visible to the naked eye is called *pourri plein*, while when the grape is at a stage known as *pourri rôti*, berry is shriveled and the sugar concentration is at maximum level (Figure 1). Due to asynchrony of fungus infection harvest takes place manually with parallel sorting on the vineyard, assuring that only well concentrated berries in the final stage of fungus development will end up in the winery (Thakur, 2018).

### History

Many are the theories for the region where accidental production of this type of sweet wine took place for the first time. One possibility is that it happened in Tokaj of Hungary, where the first reports dated back to 1560 (Thakur, 2018). Another possible region, is Schloss Johannisberg of Germany in 1775, in which the winemakers were allowed to harvest only after the permission of the owner who forgot it. As a result, the harvest was very late leading to development of *B. cinerea* on grapes (Thakur, 2018). Finally, in 1815 when the two Coalition armies invaded in Paris, late harvest and botrytization occurred (Keller, 2015).



**Figure 1.** Stages of botrytization in the ‘Semillon’ variety. Healthy stage (A), mild infection (B), *pourri plein* (C), and *pourri rôti* (D) (Blanco-Ulate *et al.*, 2015) (Photo courtesy of Château la Varière, Brissac-Quincé, France).

*Estádios do processo de botritização na variedade ‘Semillon’. Sem infecção aparente (A), infecção ligeira (B), pourri plein (C), e pourri rôti (D) (Blanco-Ulate et al., 2015) (Foto cedida por Château la Varière, Brissac-Quincé, França).*

### Types of noble rot wines

Only in some specific wine regions, mainly in Sauternes, Barsac, Loupiac, Anjou and Alsace in France, Moselle in Germany, and Tokaj in Hungary, *B. cinerea* can colonize on mature grapes resulting in concentration of compounds in berries that increase their qualitative characteristics (Magyar, 2011). The most well-known wines from these regions where botrytization process has positive effects are called ‘Sauternes’, ‘Barsac’ and ‘Côteaux du Layon’ (France), ‘Tokaji Aszú’ (Hungary), ‘Beerenauslese’ and ‘Trockenbeerenauslese’ (Germany and Austria) (Magyar, 2011).

### Main grape varieties used for noble rot wine production

*Vitis vinifera* L. cv. ‘Semillon’, ‘Sauvignon Blanc’, ‘Chenin Blanc’, ‘Furmint’, ‘Riesling’, ‘Picolit’, ‘Pinot Blanc’ and ‘Gewürztraminer’ are the main varieties used according to the region individually or as blend for botrytized wines (Jackson, 2008). Their specific characteristics, such as berry’s skin thickness

(> 150  $\mu\text{m}$ ) and oenological parameters, such as potential alcohol, sugar content and acidity, make them dominant varieties for the production of this category of wines (Ribéreau-Gayon *et al.*, 2006a). Table I displays typical analytical characteristics of noble rot wines produced with some of these grape varieties. Even though there is no direct correlation between the clone of each variety and specific qualitative markers that could indicate the quality of botrytization and so the quality of the wine, the clone of each variety seems to play a pivotal role in the relation between host and parasite (Ribéreau-Gayon *et al.*, 2006a). Clone sensitivity to *B. cinerea* is related to berry’s defense mechanism against infection (secreted substances), and clone’s morphological characteristics such as berry’s skin thickness and number of stomata present on berries that due their degeneration over the time will create peristomatic openings facilitating the infection (Thakur, 2018).

**Table I**

Analytical characteristics of noble rot wines from different French regions (Magyar, 2011)

*Características analíticas de vinos botritizados de diferentes regiões Francesas* (Magyar, 2011)

	Sauternes and Barsac	Coteaux du Layon / Coteaux de l’Aubance	Alsace
Grape variety	‘Semillon’, ‘Sauvignon Blanc’, ‘Muscat Blanc’	‘Chenin Blanc’	‘Gewürztraminer’, ‘Pinot Gris’ / ‘Riesling’, ‘Muscat Blanc’
Minimum must sugar content (g/L)	221	294	279 / 256
Typical must sugar content (g/L)	300-350	350-400	250-350
Typical wine sugar content (g/L)	50-150	50-150	50-150
Minimum alcoholic strength (% vol.)	12	11-12/11	
Typical alcohol content (% vol.)	13-14	11.5-13	12-13
Typical total acidity (g/L of tartaric acid)	6-8	7-9	7-10

## **BOTRYTIS CINEREA**

### Biology of *B. cinerea* and life cycle

*B. cinerea* Pers. Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] is an ascomycete necrotrophic pathogen from the Kingdom of Fungi, Phylum *Ascomycota*, Subphylum *Peizizomycotina*, Class *Leotiomycetes*, Order *Helotiales*, Family *Sclerotiniaceae*, and Genus *Botryotinia* (Williamson

*et al.*, 2007). Its reproductivity is mainly asexually and very rare sexually. Fungus overwinters on canes as a compact mass of hardened mycelium or sclerotia able to survive in extreme environmental conditions. It has a very short period of germination in optimal conditions (Carrascosa *et al.*, 2011). During spring or summer after the development of conidiophores, conidium can disperse and infect flower caps and pistils during flowering. After a quiescence phase, colonization on the berries just after veraison, with

the first visible signs in-between veraison and maturity, leads to early mold known as bunch rot. When the colonization takes place just before maturity and the first visible signs appear on grapes during maturity, the infection is then called noble rot (Carrascosa *et al.*, 2011). Several cycles of infection can happen during the season starting from the period in-between flowering and veraison until late maturation during middle of autumn (Thakur, 2018). Temperature between 15 °C and 25 °C and humidity above 90% for more than four hours are optimal conditions for conidium germination. Berries become more susceptible as they mature and varieties with compact thin-skin clusters are easily infected (Thakur, 2018).

#### **Difference between noble and gray rot**

In many cases, during the first stages of botrytization both gray and noble rot can be observed. An infection considered as beneficial in the beginning, is possible to turn to gray mold promoting grape spoilage. This is in accordance to water availability on berry at the first stages of *B. cinerea* colonization, in which high water activity ( $a_w$ ) close to 0.93 is beneficial for germination and mycelia development (Rousseau and Donèche, 2001). Extended humidity and heavy precipitations promote bunch rot occurrence and prolonged survival of *B. cinerea* in the berry under the presence of other microorganisms. When  $a_w$  is between 0.96 and 0.99, humidity on berries increases bacterial load, promoting thus the chemical alternation of infected grapes in an unfavorable way. This co-infection leads to sugar degradation below 230 g/L, tartaric and malic acids elimination and secretion of pathogens' metabolites, making impossible the production of wine without defects. On the contrary, limited humidity during morning dew with hot afternoons, cause fast berry's water evaporation, berry's shriveling, concentration of sugar and increase of berry's osmotic pressure that eventually kills the fungus (Rousseau and Donèche, 2001).

The fact that berries neighbor to infected ones can remain healthy when their skins are intact indicates that skin rupture is a necessary condition for botrytization (Pucheu-Planté and Seguin, 1978). The extent of berry's skin rupture is a decisive factor on whether the rot will turn to be gray or noble. In noble rot infection, berry's skin micro-fissures allow only *B. cinerea* to enter and germinate inside, leading to fungus growth in the interior of berry's skin where glycerol production in the anaerobic environment is supported (Pucheu-Planté and Seguin, 1978). According to Ribéreau-Gayon (1982), the origin of this very small ruptures that are invisible with naked

eye can be skin tensions during berry's volume growth from veraison to maturity or stomata present on berries. On the other hand, intensive ruptures produced by birds, insects, worms, hail, light detachment of the berry from the pedicel or berry's skin bursting due to heavy rainfalls, lead to bunch rot. Extreme ruptures, make berries very susceptible to gray rot and lead to fungus growth at the exterior part of the berry, where oxidation of sugars leads to gluconic acid accumulation (Pucheu-Planté and Seguin, 1978).

The dominant *Botrytis* species (sp.) causing grape mold and ruining harvests is *B. cinerea* (sensu stricto alias Group II) (Fournier *et al.*, 2013). This species along with *B. cinerea* Group I, also named as *B. pseudocinerea*, are living in sympatry on hosts such as grapevines (Walker *et al.*, 2011). The latter has an insignificant effect on bunch rot development (Walker *et al.*, 2011), while it is totally absent during noble rot infection (Fournier *et al.*, 2013). Studies conducted in three different regions in France (Loire Valley, Bordeaux and Alsace) on *B. cinerea* infection, showed that only in Alsace there was a small differentiation of *B. cinerea*'s genome compared to the other two regions. The fact that Alsace is geographically the most remoted region from the three, could explain this genetic diversity (Fournier *et al.*, 2013). Concerning a comparison between gray and noble rot cultures found in the same vine plots, no differentiation of the genome of *B. cinerea* was found for the two types of infection for each of the three regions separately. This indicated that in each region the same fungus with the same genotype and probably same strains, were responsible for both bunch and noble rot (Fournier *et al.*, 2013). What really changes between the two kinds of rots is the metabolic activity of the fungus in response to different environmental conditions. Microclimate is the most important factor promoting fungal development and secretion of metabolites beneficial or not for noble rot development (Fournier *et al.*, 2013). Another study on cv. 'Semillon' grapes infected by noble rot highlighted that only 12% of the expressed genes were similar with those expressed in bunch rot. In other words, different transcription factors were upregulated during noble infection in the berry leading to different enzymatic production and metabolic paths (Blanco-Ulate *et al.*, 2015).

It is well established for gray rot that the fungus uses more than 800 sRNAs during the infection, and 73 out of them are correlated with the defense mRNAs of the host cells (Weiberg *et al.*, 2014). These mRNAs are responsible for the production of proteins, in ribosomes, able to act as shield against the parasite (Weiberg *et al.*, 2014). Fungal sRNAs

after penetrating in berry cells silence the defense mRNA by binding to Argonaute 1 (AGO1), a plant protein normally found in cells playing a role of stopping genes' expression whenever is needed by ceasing mRNAs' activity. A better understanding of this pattern occurrence during noble rot infection justifies further investigation (Weiberg *et al.*, 2014).

#### ***Vitis vinifera* L. interaction with *B. cinerea***

Environmental conditions are crucial for triggering different metabolic pathways during noble and gray rot infection. The up- or down-regulation of specific genes that decrease the power of the virulence factors secreted during noble rot colonization by *B. cinerea*, and result in a "milder" infection, is responsible for these metabolic differences (Lovato *et al.*, 2019). *Vitis vinifera* L. also responds in a "gentler" way without activating all its defense mechanisms against the fungus as in gray mold case. The host decreases its defenses and produces lower number of phytoalexins responsible for the creation of hostile environment for the necrotrophic pathogen. Intense collision between host and parasite increases secreted compounds for both sides, leading to gray rot and decreasing grape quality (Lovato *et al.*, 2019). The disruption of grape hormones by *B. cinerea* could explain the up- and down-regulation of specific genes during noble rot infection (Blanco-Ulate *et al.*, 2015).

## **BOTRYTIZATION PROCESS**

### **Infection of the berry**

Wax presence around the skin (2  $\mu\text{m}$ ) is the first line of berry's defense against infections, while berry's skin is the second (Ribéreau-Gayon *et al.*, 2006a). The higher the berry's skin thickness, the higher the berry's resistance to microorganisms, and so the better the protection against *B. cinerea*. For example, 'Silvaner' (100  $\mu\text{m}$ ) is less susceptible to gray rot than 'Müller-Thurgau' (50  $\mu\text{m}$ ) (Ribéreau-Gayon *et al.*, 2006a). Varieties used for noble rot wines such as 'Sauvignon Blanc' and 'Riesling' with approximately 175  $\mu\text{m}$  of skin thickness and 'Gewürztraminer' with around 250  $\mu\text{m}$ , are rather resistant to gray mold before ripening. Their thick-skin berries make easier the sorting and picking during harvest (Ribéreau-Gayon *et al.*, 2006a).

Berry's skin is composed of cuticle, epidermis and hypodermis (König *et al.*, 2017). The infection starts, when conidia from external sources colonize on mature berries via pre-existing micro-ruptures on cuticle not visible on the naked eye (Carrascosa *et al.*, 2011). Under optimal microclimatic conditions their germination can start. *B. cinerea* uses cutinases

against cuticle but whether or not it is possible to degrade it in order to reach the epidermis is still unknown. Yet, this happens during mycelia growth towards the exterior at *pourri plein* stage (Kamoen, 1992; Carrascosa *et al.*, 2011; König *et al.*, 2017).

As a necrotrophic organism, *B. cinerea* penetrates directly in the berries intracellular. It enters through berry's epidermis by producing high range of enzymes that induce cell wall degradation and cell death (Jackson, 2008). One of these enzymes is pectinase, which causes degradation of epidermal cells' pectic structures, liberating pectins and galacturonic acid later in the must (Pucheu-Planté and Mercier, 1983). Mycelium growth continuous inside the berry until reaches hypodermis level by the expansion of its filaments. Due to chitinases and  $\beta$ -glucanases presence in berry's pulp cells, enzymes that can break fungus cell wall and are considered as pathogenesis-related (PR) proteins, fungus will remain inside the berry at the skin surface (Pucheu-Planté and Mercier, 1983).

At *pourri plein* stage of infection, *B. cinerea* starts its development towards the external part of grape skin. New conidiophores are dispersed by the wind over neighbor clusters in order to ensure new colonization. At this level of infection, oxidation phenomena are inevitable, so berries gain a characteristic brown color (Pucheu-Planté and Mercier, 1983). The results from the skin ruptures produced by the fungus are also related to high water evaporation and sugars concentration that lead to unbalanced change of osmotic pressure of the environment around the fungus that actually kills it. At the *pourri rôti* stage the withered grapes can be finally picked up (Pucheu-Planté and Mercier, 1983).

### **Induced botrytization**

The specific environmental conditions under which botrytization occurs, along with the effects of global warming in microclimates of wine regions, has aroused questions about a potential future incapacity of producing noble rot wines (Jackson, 2008). Studies were conducted in Italy, where noble rot occurrence is rather rare, regarding induced *B. cinerea* infection of red cv. 'Corvina' and 'Rondinella' and white cv. 'Garganega' (Fedrizzi *et al.*, 2011; Tosi *et al.*, 2012). Specifically, investigation on the effect of noble rot occurrence on traditionally produced 'Amarone' wines during a period of four months post-harvest grape drying, revealed that an artificial or not inoculation of *B. cinerea* could affect positively their organoleptic characteristics (Fedrizzi *et al.*, 2011; Tosi *et al.*, 2012).

The amelioration of the sensory characteristics of

straw wines known as ‘passito’, after man-made inoculation during grapes withering, has also been argued by Lorenzini *et al.* (2012). Moreover, Negri *et al.* (2017) have demonstrated that post-harvest raisining followed by grapes’ maintenance in humid chamber for natural *B. cinerea* infection have positively influenced the grapes’ character compared to the non-infected ones. A comparison between a ‘Protected Designation of Origin’ (PDO) noble rot wine and a wine produced by pre-harvest artificial botrytization of cv. ‘Chardonnay’ grapes, in terms of their aromatic profile, showed that typical aromas of noble rot wines were present in both wines (Wang *et al.*, 2017). Odorant compounds naturally present in noble rot wines such as volatile thiols, lactones and furans were also detected on the artificial botrytized wine (Wang *et al.*, 2017). Yet, whether or not a future pre- or post-harvest induced botrytization will be an acceptable technique to be implemented in PDO wine regions where traditionally noble rot wines are produced is still under discussion.

## ENVIRONMENTAL CONDITIONS

### Optimum weather conditions for noble rot infection

In general, production of very high-quality wines depends on many factors of equal importance such as microclimate, soil, viticulture and winemaking practices, vintage, among others (Pucheu-Planté and Seguin, 1981). For noble rot wine production, weather conditions are the key factor responsible for the quality of botrytization. Grapes should reach maturity healthy without skin ruptures to avoid bunch rot. The second step towards noble infection is *B. cinerea* presence on berries only under very specific sequential weather conditions (Pucheu-Planté and Seguin, 1981).

Taking Sauternes as an example, a region landmark for noble rot wine production, the average precipitation from January to July ranges from 500 to 600 mm. Ideally, from the end of August to the end of September, the weather should be as dry as possible with maximum average rainfall between 30 to 40 mm. Empirically, the first visual detection of *B. cinerea* is 15 to 20 days before berry’s full ripeness, where the unfavorable environmental conditions make fungus growth very sluggish (Pucheu-Planté and Seguin, 1981). During October, consecutive rainy days of 60 to 70 mm of precipitation increase the level of humidity on berries, promoting germination of conidia and fungus exponential growth in less than ten days, and leading to berry-skin rupture towards the exterior. In a faultless way, after berry-skin

rupture another ten sunny and dry days with humid nights and foggy mornings follow. Harvest start normally at the end of October when water losses and sugar concentration of berries achieve the desirable levels (Pucheu-Planté and Seguin, 1981).

In case that rain continues with insufficient number of sunny days, berries desiccation is impossible, while other undesirable fungus and bacteria develop. In case of insufficient amount of rain and humidity, *B. cinerea* development will be suppressed and the final wine will lose its distinctive character. The climate conditions of these three to five weeks after berry’s ripeness are decisive for the quality of the final product (Pucheu-Planté and Seguin, 1981).

### Climate change

Climate change, as one of the main topics discussed around the world, concerns every aspect of nature including human life. As a result, it arouses many queries and uncertainties also in wine industry (Bonnefoy *et al.*, 2010). First and above all, climate change is very important in the viticulture part of wine production. Changes in phenological cycle of plants, changes in microclimate of regions in which terroir plays pivotal role on wine’s organoleptic profile and increase of the possibility of annual severe weather phenomena, are only some of its effects (Bonnefoy *et al.*, 2010). In regions where specific climatic conditions are very important for noble rot development on berries, climate change could disturb their microclimate leading to vintages either drier or more humid, affecting botrytization process (Agenis-Nevers, 2006). This explains why the discussion about artificial induction of *B. cinerea*, even in regions with tradition in this type of wine production, proceeds (Agenis-Nevers, 2006).

## VITICULTURE PRACTICES

### Soil and rooting depth

Gray rot depends on the abrupt increase of berry’s volume due to intense water accumulation that leads to skin bursting. With extended ruptures, not only *B. cinerea* but any other microbial can colonize on berries, spoiling their qualitative characteristics (Pucheu-Planté and Seguin, 1978). In an area with same varieties, viticulture techniques and rootstocks, the problem of mold is always greater only in specific parcels where the vine roots are superficial and the soil is shallow. This observation, highlights the importance of soil depth and root system localization on potential gray mold infection (Pucheu-Planté and Seguin, 1978).

The soil is one of the most important parameters regarding the degree of vine water stress. Considering Sauternes, where irrigation is not allowed, two types of soil exist (Pucheu-Planté and Seguin, 1981). The first type is mainly gravel and sandy, located at the upper part of small hills with a maximum altitude of 50 m. This soil is rather homogenous and deep, with rooting depth up to five meters. The second type of soil consists mainly of clay beneath of a shallow sandy and silt layer of 50 to 60 cm depth that can cause water accumulation and roots asphyxia without a proper drainage system. In the first case, soil properties keep the vines during maturation under controlled water stress, ideal for grapes' ripening and aromatic enhancement (Pucheu-Planté and Seguin, 1981). Moreover, deep rooting leads to limited number of superficial roots, reducing water absorption and limiting berries' ruptures in case of intense precipitations during summer. Contradictory, vine's water uptake from shallow root system is easier due to increased water accumulation of the upper soil layers after heavy rainfalls (Pucheu-Planté and Seguin, 1978).

As the depth of the root system increases the percentage of gray mold decreases, starting from 100 cm with 70% occurrence of bunch rot up to 500 cm with only 5% (Pucheu-Planté and Seguin, 1978). When berries are in the stage of late maturity and botrytization is the objective, the more intense the precipitation the higher the possibility of berry bursting in case of superficial rooting system. Deep soils with good drainage and potential rootstocks able to create deep rooting system are crucial for the production of botrytized wines of high quality (Pucheu-Planté and Seguin, 1978).

### **Training system**

A study conducted over a period of four consecutive vintages on Sainte-Croix-du-Mont, a PDO region in Bordeaux, tried to identify differences between vines trained in two different trellis systems for the production of botrytized grapes and noble rot wines. Vertical shoot position (VSP) and Lyre were under examination, while the differences were based on sugar accumulation and aroma profile on wines (Carbonneau and Casteran, 1986). SP trellis system with 1.6 m vine distance between rows and trunk height at 40 cm, VSP trellis system with 3.2 m vine distance between rows and trunk height at 70 cm, and three types of Lyre training system (open, closed and truncated) with 3.2 m vine distance between rows and trunk height at 70 cm, were examined. Vines with VSP trellis system and narrow distance (1.6 m), due to their shorter trunks height compared to the other cases, were infected and reached *pourri plein* and

*pourri rôti* stages first. Vines with VSP trellis system and wide distance (3.2 m) were the last to be infected. The higher sugar content was detected in open Lyre samples and the lower was found in the sample from the VSP system with wide distance (Carbonneau and Casteran, 1986).

Tasting results on the aroma perception and intensity of the produced wines showed that the wines obtained from vines trained in open Lyre had the most complex and intense aromatic profile with the aromas of fruits, raisin and honey to be dominant (Carbonneau and Casteran, 1986). VSP samples with wide distances were ranked last in the tasting evaluation, having very a limited aromatic intensity and increased herbal aromas. The evaluation order from the best to the worst sample, according to the tasting panel, was: open Lyre, closed Lyre, truncated Lyre, narrow VSP and finally wide VSP (Carbonneau and Casteran, 1986). In total, Lyre system was the training system that produced the most qualitative results. It could be claimed that even if narrow plant density favored the infection at the beginning, at the end it was not an indispensable criterion for must and wine final quality. Although the VSP trellis system with narrow distance between rows and short trunk height produced qualitative wines, the Lyre training systems even with wide distances and long trunks were considered better. Manual harvest is the only option in respect to botrytized grapes both due to PDO regulations and sorting necessities. As a result, Lyre training system, in which mechanical pruning is also not possible, could replace training systems in VSP trellising, since their adaptability to mechanical pruning as one of their major advantages in this case is actually useless (Carbonneau and Casteran, 1986).

## **METABOLIC DERIVATIVES OF *BOTRYTIS CINEREA***

### **Polysaccharides**

The two main polysaccharides secreted by *B. cinerea* have different solubilities in alcoholic solution (König *et al.*, 2017). The first is a rather soluble heteropolysaccharide that consists of mannose 60%, galactose 30%, glucose 5%, rhamnose 5% and a protein part (König *et al.*, 2017). This heteropolysaccharide with molecular weight between  $2 \cdot 10^4$  and  $5 \cdot 10^4$  Da, known as botryticin, is a fungus antibiotic that acts toxically against plants (Dubourdieu *et al.*, 1981). Concerning winemaking, it constrains yeast development leading to sluggish fermentation, and increased glycerol and acetate production (Dubourdieu *et al.*, 1981). A homopolysaccharide called glucan or cinerean with

limited solubility in alcoholic solution is the second main polysaccharide (König *et al.*, 2017). Specifically,  $\beta$ -glucan with higher molecular weight between  $10^5$  and  $10^6$  Da consists of  $\beta$ -1,3 glucoses branched glycosidically ( $\beta$ -1,6) with another glucose molecule (Dubourdieu *et al.*, 1981).

*B. cinerea*'s cell wall consists of glucans and chitin, which explains glucans abundance in botrytized grapes. The exterior part of this cell wall's molecule not only protect the fungus from vine's responses but also plays a linkage role with berry's skin at the first stage of fungus colonization (Dubourdieu *et al.*, 1981). Harsh mechanical operations in the wine cellar, such as severe crushing and pumping, can increase the extraction of cinerean in the must, suppressing yeasts development and promoting glyceropyruvic fermentation. Later, in the wine, due to its limited solubility in alcohol, it is in suspension in the medium, producing clogging problems during filtration (Dubourdieu *et al.*, 1981). Botryticin and  $\beta$ -glucans excreted by the fungus are estimated up to 750 mg/L in a ratio of 1:6 (Dubourdieu *et al.*, 1981).

### Laccase

Laccase is an enzyme that belongs to the polyphenol oxidase (PPO) enzyme group (Dubernet *et al.*, 1977). Its enzymatic activity is optimal at temperatures around 30 to 40 °C and pH 4 (Dubernet *et al.*, 1977). This enzyme is responsible for oxidation of *o*-diphenols to semiquinones and subsequently to *o*-quinones. Caffeic and caftaric acids mainly, but also catechin and cyanidin, are the most important substrates of laccase (Dubernet *et al.*, 1977). Other PPOs present in berries, such as tyrosinase, oxidize also monophenols, such as *p*-coumaric acid, or other than caftaric hydroxycinnamoyl tartaric acids, by converting them to *o*-diphenols via their cresolase activity. In all the cases, the produced *o*-quinones are responsible for the brown-dark color of oxidized white musts (Ribéreau-Gayon *et al.*, 2006a).

Glutathione (GSH) is a tripeptide consisted of glutamic acid (Glu), cysteine (Cys) and glycine (Gly) that accumulates in berries during ripening (König *et al.*, 2017). It is one of berry's responses to oxidative stress as it participates in detoxification pathways and reacts against toxic metals and peroxides (König *et al.*, 2017). This compound is highly active with the produced *o*-quinones, forming 2-*S*-glutathionyl caftaric acid known as "Grape Reaction Product" (GRP) (Salgues *et al.*, 1986). The formed compound is actually colorless preventing the enzymatic

browning described above. Laccase is the only enzyme able to oxidize GRP by producing new *o*-quinones. These new compounds are combined in complex polymers contributing to the brown color of botrytized wines. GSH in surplus, is able to react with the produced *o*-quinones before their polymerization, producing another complex molecule with two GSH in its benzoic ring (alias GRP 2), not susceptible any more to laccase (Salgues *et al.*, 1986). Due to extended phenolic oxidation and existence of laccase especially in botrytized must, addition of GSH for preventing browning is not useful (Salgues *et al.*, 1986). *B. cinerea* secretes these enzymes as a response to berry's increased production of phenolic compound, that act as inhibitors of fungus growth (König *et al.*, 2017).

In general, white varieties are less sensitive to laccase activity compared to the red ones (Boulton *et al.*, 1999). Intensified coloring matter degradation, also known as "oxidasic casse", due to laccase severity, explains why noble rot wines are only produced from white varieties (Boulton *et al.*, 1999). In infected berries, enzymatic activity is increased until *pourri plein* stage of infection (Table II). At *pourri rôti* stage of infection, *B. cinerea*'s metabolic activity is gradually eliminated due to sugar concentration and increased osmotic pressure. Production of laccase is also decreased while its enzymatic activity falls below 40  $\mu$ U (Donèche, 1991). Sugar concentration of approximately 350 g/L reduces oxygen saturation level in musts from 8 mg/L to less than 6 mg/L, while oxygen consumption rate in botrytized musts is the same as in musts produced by healthy grapes (Donèche, 1991). This highlights the decrease of laccase activity in noble rot musts, yet the potential effects of this enzyme both in oxidation phenomena and color change should not be totally ignored during wine making. Therefore, hyperoxygenation technique in botrytized musts is not useful and thus not applicable (Donèche, 1991). Whether must heating treatment, with temperature at around 50 °C for laccase inactivation, is beneficial or not is still a debate. Unfavorable aroma modifications and losses are possible with this technique (König *et al.*, 2017). Induction of selected *B. cinerea*'s strains that produce lower amount of laccase could be another way to reduce their presence in grapes (König *et al.*, 2017). In must, 125 mg/L of total sulfur dioxide (SO<sub>2</sub>) is an adequate dose for laccase deactivation, while in wine total sulfur dioxide higher than 50 mg/L is sufficient (Jackson, 2008).

**Table II**

Laccase activity during botrytization in berries (Donèche, 1991)

*Atividade lacase em diferentes estádios da infecção do bago por Botrytis cinerea* (Donèche, 1991)

Stage of infection		Sugar concentration (g/L)	Laccase activity ( $\mu$ U/berry)
1	(healthy)	218	0
2		220	9.4
3		223	57.8
4	(pourri plein)	256	106.3
5		278	75.1
6	(pourri rôti)	357	39.1

### Proteases

Nitrogen availability is very important for *B. cinerea* development on berries, given that it is a vital element for the production of amino acids, peptides and proteins that play fundamental role in metabolic activities of every living cell (Thakur, 2018). *B. cinerea* degrades berry's proteins, assimilates the nitrogen and produces new ones beneficial for each own survival. This degradation takes place catalytically under the presence of proteolytic enzymes produced by the fungus, increasing finally the levels both of fungal proteins and fungal proteases in the berries (Thakur, 2018). An increase of proteins up to 25%, expressed in g/L of botrytized musts compared to musts from uninfected grapes, depends exclusively on fungal proteins that are concentrated after berry's dehydration (Ribéreau-Gayon *et al.*, 2006a).

PR proteins, such as thaumatin-like proteins (TLPs) and chitinases, even though are increased during infection are degraded by fungus proteases in berries and must (Girbau *et al.*, 2004). A study conducted on healthy and botrytized berries and the corresponding musts, argued that PR proteins were always reduced during botrytization. According to the study, proteases' activity rather than berry's down-regulation of genes for production of specific proteins is responsible for such a reduction (Girbau *et al.*, 2004). As polar molecules, fungus proteolytic enzymes are also present in must, in which, due to their resistance in pH, sulfur dioxide and heating, act against the remaining PR proteins (Marchal *et al.*, 2006). The degradation of these proteins, that are considered the most difficult proteins to eliminate from wine, is beneficial for protein stabilization (Reynolds, 2010).

### BERRY'S CHEMICAL COMPOSITION

#### Sugars and acids concentration on berry

During botrytization, berry's volume can decrease up to five times, intensifying concentration phenomena (Donèche, 1989). *B. cinerea* metabolizes sugars via Embden–Meyerhof pathway and hexose monophosphate shunt pathway (Donèche, 1989). Sugar decrease per berry, is up to 45% for 2.5-fold berry's volume concentration after desiccation. Yet, this lost is not noticeable as berry's shriveling results in an average sugar content of 350 g/L of must – Table III (Donèche, 1989).

Reduction of tartaric and malic acid per berry is up to 85% and 45%, respectively, again for 2.5-fold berry's volume concentration after desiccation (Ribéreau-Gayon, 1982). Tartaric acid decreases in g/L of botrytized must by 55% and malic acid increases by 45%. Thus, total acidity of around 8 g/L expressed as tartaric acid is almost unchangeable, compared to musts derived from healthy grapes - Table III (Ribéreau-Gayon, 1982). Lesser acidity would result in flabby wines with cloying perception. Citric and acetic acid of botrytized must, both increase in g/L by approximately 30% - Table III (Ribéreau-Gayon, 1982).

Finally, mucic acid, as another organic acid present naturally in grapes, can slightly increase after oxidation of galacturonic acid found in the must (Ribéreau-Gayon, 1982). The latter is abundant in botrytized musts after the extended enzymatic activity of pectinases during infection (Ribéreau-Gayon, 1982). Addition of commercial enzymes like polygalacturonases during pressing for better aromas extraction and more efficient settling during clarification is a treatment with limited effects, and therefore not recommended (Ribéreau-Gayon *et al.*,

2006a). The extended degradation of berry's pectic structure leads to the release of other sugars, such as arabinose, rhamnose, galactose, mannose and xylose, in the medium (Ribéreau-Gayon, 1982). As *B. cinerea* consumes mainly glucose, partial reduction of glucose concentration decreases the ratio between glucose and fructose below one, which can be used as indicator of infection (Garcia-Jares and Médina, 1997).

### Gluconic acid

Gluconic acid and 2-keto gluconic acid are produced enzymatically by oxidation of the aldehyde functional group of glucose and the hydroxy functional group of C<sub>1</sub> of fructose, respectively, under the presence of *B. cinerea* and oxygen (Donèche, 1989). In general, more than 0.5 g/L of gluconic acid in must can be

caused by bacteria or fungus contamination on berries (Moreno and Peinado, 2012). In berries infected by noble rot, gluconic acid concentration is above 3 g/L and it is not metabolized by yeasts during alcoholic fermentation (Moreno and Peinado, 2012). Finally, it is considered as potential parameter for monitoring the degree of *B. cinerea* infection on berries (Cuadrado *et al.*, 2005; Rolle *et al.*, 2012; Carbajal-Ida *et al.*, 2016).

### Polyalcohols

There are three main polyalcohols responsible for the increased viscosity and the "body" of botrytized wines. Butanediol, glycerol or glycerin and sorbitol are present in infected grapes while the first two are also produced during alcoholic fermentation (Moreno and Peinado, 2012).

**Table III**

Berry's chemical composition expressed in per liter of must and per 1000 berries (Ribéreau-Gayon *et al.*, 2006a)  
*Composição química do bago, por volume de mosto (L) e por número de bagos (1000) (Ribéreau-Gayon et al., 2006a)*

	Per liter		Per berries	
	Healthy must	Botrytized must	Healthy berry	Botrytized berry
Weight (g)			2020	980
Volume of must (mL)			1190	450
pH	3.33	3.62	3.33	3.62
Sugars (g)	247	317	294	143
Glycerol (g)	Not detected	7.4	Not detected	3.4
Total acidity (g of tartaric acid)	9.2	8.4	11	3.8
Tartaric acid (g)	5.3	2.5	6.4	1.1
Malic acid (g)	5.4	7.8	6.4	3.6
Citric acid (g)	0.17	0.22	0.20	0.10
Acetic acid (g)	0.32	0.41	0.38	0.14
Gluconic acid (g)	Not detected	2.1	Not detected	0.94
Ammonium (mg)	85	56	101	25
Proteins (mg)	2815	3795	3350	1708

Sorbitol concentration in berries does not exceed 600 mg/L, while 2,3-butanediol level is around 1 g/L (Moreno and Peinado, 2012). Glycerol accumulation on berry is maximized at *pourri plein* stage under the

lack of oxygen. Later it is oxidized to glyceraldehyde and decreases, reaching an average quantity of 5 g/L in berries (Ribéreau-Gayon *et al.*, 2006a). Moreover, it is the third most abundant component after water

and ethanol in wines. Given that glycerol is also produced during fermentation, its concentration in botrytized wines can be higher than 20 g/L (Moreno and Peinado, 2012). Glycerol along with gluconic acid are quality markers of *B. cinerea* infection (Ravji *et al.*, 1988; Rolle *et al.*, 2012; Tosi *et al.*, 2012).

## Aroma precursors

### Terpenes

Mevalonic acid (C<sub>5</sub>) is the key chemical compound for the production of terpenes (Blanco-Ulate *et al.*, 2015). Experiments conducted on botrytized berries showed that it is present in higher quantities compared to health ones (Blanco-Ulate *et al.*, 2015). This means that via the mevalonate pathway (MVP) and the methylerythritol-4-phosphate pathway (MEP), production of the main terpenoid precursors dimethylallyl pyrophosphate (DMAPP) and isopentyl pyrophosphate (IPP) take place. From these precursors a variety of aromatic compounds such as monoterpenoids (C<sub>10</sub>) and sesquiterpenoids (C<sub>15</sub>) can be present in the wine directly. While others, such as C<sub>13</sub>-norisoprenoids, can be present indirectly after enzymatic or oxidative cleavage of Carotenoids (C<sub>40</sub>) (Blanco-Ulate *et al.*, 2015).

These odorant compounds, which are considered as primary wine aromas and are found in berries in their glycosylated forms, seems to be oxidized after the aglycone part is being liberated catalytically under the presence of *B. cinerea*'s glycosidases (Ribéreau-Gayon *et al.*, 2006a). The process involves an  $\omega$ -hydroxylation reaction in which a hydrogen from the terminal methyl of a terpene such as geraniol, is substituted for a hydroxyl group ( $\omega$ -oxidation), converting geraniol to a less odorant diol while the wine loses its varietal character (Bock *et al.*, 1988). This explains the use of varieties such as 'Semillon' or 'Chenin Blanc' less depended on their aromatic character during botrytized wine production. 'Muscat' grapes, for example, diminished their aromatic profile rather than gain in complexity after botrytization (Jackson, 2008). Addition of glycosidases during or after alcoholic fermentation for enhancing the aromatic profile of botrytized wines is not recommended (Jackson, 2008).

### Thiol precursors

Degradation of fatty acids to C<sub>6</sub> compounds (alcohols and aldehydes with six carbons) normally occurs in healthy grapes (Blanco-Ulate *et al.*, 2015). The process during berry ripening starts from breakdown of unsaturated fatty acids within the lipoxygenase (LOX) cycle, which is catalyzed enzymatically and produces hydroperoxides (Blanco-Ulate *et al.*, 2015). These are also enzymatically degraded to C<sub>6</sub>

compounds such as (*E*)-2-hexenal known as leaf aldehyde and (*Z*)-3-hexen-1-ol known as leaf alcohol. GSH, whose the production increases during noble rot infection, is combined by glutathione *S*-transferases (GSTs) with C<sub>6</sub> compounds during detoxification pathways, creating *S*-glutathione conjugates that are actually thiol precursors. The expression of the genes responsible for GSTs formation are also upregulated during botrytization (Blanco-Ulate *et al.*, 2015). A sequential conversion of *S*-glutathione precursors leads to *S*-cysteinylated precursors by elimination of Glu and Gly from the molecule. A final cleavage from  $\beta$ -lyases during alcoholic fermentation liberates thiols in wine matrix, which is vital for the aroma enhancement (Blanco-Ulate *et al.*, 2015).

Quinones (oxidized polyphenols) are highly reactive with volatile thiols (Quideau *et al.*, 1995). In botrytized wines, polyphenols are almost eliminated by oxidization, polymerization and precipitation phenomena under the enzymatic activity of laccase (Quideau *et al.*, 1995). C<sub>6</sub> compounds are considered pre-fermentative aromas, derived from unsaturated fatty acids (such as linoleic and linolenic acids), being released in the must during maceration, destemming, crushing and pressing. The cleavage of linoleic and linolenic acids during pre-fermentation processes follows the same LOX pathway described above (Ferreira *et al.*, 1995).

Other higher alcohols present in these wines, such as 1-octen-3-ol (known for its mushroom distinctive aroma), derive from fatty acids degradation, during metabolic pathways of other microorganism such as bacteria present on berries during botrytization (Jackson, 2008).

A study conducted on cv. 'Semillon' and 'Sauvignon Blanc' grapes at every stage of botrytization process from healthy ripe to late *pourri rôti* berries, indicated that concentration of cysteinylated precursor of 3-mercapto-1-hexanol (3MH) for both varieties was around 100 times higher than the control until the stage of *pourri plein* (Thibon *et al.*, 2009). A later decrease of this compound in berries due to oxidation or combustion was not highly noticeable in mg/L due to parallel water evaporation. The same study tried to compare the differences between overripe sound grapes at late harvest and botrytized ones regarding the quantities of the Cys3MH in order to highlight the pivotal role of *B. cinerea* infection and how it affects directly or not berry's metabolic pathways (Thibon *et al.*, 2009). Indeed, an average ten times increase of this compound for both varieties during botrytization was ascribed to the previous argue. The diastereoisomeric ratio between R/S Cys3MH forms was actually the same between on and off-vine

botrytization and overripening and equal to 30:70. This expresses an indirect involvement of *B. cinerea* in 3MH production (Thibon *et al.*, 2009). Again, as above-mentioned *S*-glutathione precursors and the enzymes involved in berries seem to be two crucial steps for the release of these very aromatic thiols later in wine (Thibon *et al.*, 2009).

### **Benzaldehyde**

Benzyl alcohol is an aromatic higher alcohol normally absent from healthy grapes and dry wines (Swiegers *et al.*, 2005). It is produced by berries during *B. cinerea* infection as it is a limiting factor in fungus development (Goetghebeur *et al.*, 1993). *B. cinerea* acts against this compound enzymatically, by producing benzyl alcohol oxidase that converts benzyl alcohol to benzaldehyde (Goetghebeur *et al.*, 1993). The latter is only found in noble rot wines, in which its bitter almond aroma enchases their aromatic profile (Bailly *et al.*, 2006; Fedrizzi *et al.*, 2011; Negri *et al.*, 2017).

### **Phenylpropanoid pathway**

The phenylpropanoid pathway is one of the most important metabolic paths of red grape varieties (Chen *et al.*, 2006). Via this pathway, berry produces many secondary derivatives mainly located in the skin, such as phenolic acids, stilbenes, anthocyanins, flavanols and flavonols (Chen *et al.*, 2006). These compounds are the response of berries to the increased vine stress caused by biotic and abiotic factors, and play a vital role in the organoleptic characteristics of the final product (Chen *et al.*, 2006). Phenylpropanoid metabolic activity is marginal in white grape varieties except when they are infected by *B. cinerea* (Blanco-Ulate *et al.*, 2015). When infection takes place in white berries, their response derives from the upregulation of specific transcription factors followed by the expression of specific genes for the production of enzymes that will induce, among others, this metabolic path during berries' maturation (Blanco-Ulate *et al.*, 2015).

#### *Phenolic acids and hydroxycinnamic esters*

In the early stages of phenylpropanoid pathway during botrytization, increased production and accumulation of the three main hydroxycinnamic acids, *p*-coumaric, caffeic and ferulic in berries, is noticeable (Blanco-Ulate *et al.*, 2015). Yet, PPOs such as laccase and tyrosinase oxidize these acids along with their hydroxycinnamic esters such as caftaric and coumaric acids (Dubernet *et al.*, 1977). Accordingly, a study on cv. 'Chenin Blanc' from Loire Valley region during botrytization highlighted the oxidation of these esters during infection (Carbajal-Ida *et al.*, 2016).

### *Stilbenes*

Stilbenes are 1,2-diphenylethylene derivatives very important non-flavonoid compounds widespread in nature (Hart, 1981). They act as phytoalexins produced by plants in response to microbial or insect colonization (Hart, 1981). Resveratrol is one of the most well-known and studied stilbenes in wine industry due to its beneficial properties in human health (Frankel *et al.*, 1993). Specifically, *trans*-resveratrol seems to act as antioxidant, preventing the oxidation of low-density lipoprotein that is correlated with heart diseases (Frankel *et al.*, 1993). It is also considered as cancer-preventative substance that can deter or constrain tumor development (Jang *et al.*, 1997). Production of stilbenes is generally increased in botrytized grapes (Blanco-Ulate *et al.*, 2015).

*B. cinerea* seems to be very little affected by resveratrol's presence in berries. It dimerizes resveratrol oxidatively to inactivate products potentially under the enzymatic activity of laccase (Cichewicz *et al.*, 2000). A study carried out in Sauternes region concerning botrytized grapes highlighted that resveratrol increased as infection expanded in berries from cv. 'Semillon', while in 'Sauvignon Blanc' the opposite behavior was observed (Landrault *et al.*, 2002). In both varieties the quantity of *trans*-resveratrol was lower than 0.15 mg/kg of grapes. Other stilbenes found in these grapes, such as *trans*-astringin, *trans*-piceid and pallidol, were also present in very limited concentrations. Only  $\epsilon$ -viniferin, a resveratrol dimer, was an exception following an increasing trend in both varieties up to 2 mg/kg of grapes before infection reached *pourri plein* stage, while after was reduced to half (Landrault *et al.*, 2002).

#### *Other phenolic compounds*

Numerous phenolic compounds that are highly produced during infection, acting against *B. cinerea* and as antioxidants, are also relevant from the oenological point of view regarding the enhancement of the unique flavor of this kind of wines (Blanco-Ulate *et al.*, 2015). Flavonols such as quercetin and kaempferol in their glycosylated forms are eliminated from berries at the last stage of infection, while myricetin is identified only in *pourri roti* phase (Carbajal-Ida *et al.*, 2016). Flavanols such as catechin, epicatechin and galloyl epicatechin also increase during botrytization, reaching a maximum at the final stage of infection; according to Goetz *et al.* (1999) they act against fungus' enzymes, and more specifically against stilbene oxidases.

In total, for the cv. 'Chenin Blanc', the phenolic composition decreased up to 40% throughout noble

rot infection (Carbajal-Ida *et al.*, 2016). Even though berry activates every possible defense mechanism against *B. cinerea*, fungus finally prevails by degrading enzymatically most of the produced phenolic compounds (Ribéreau-Gayon *et al.*, 2006b). Phenolic composition, excluding tyrosol, is noticeably lower in botrytized wines compared to dry

wines – Table IV (Ribéreau-Gayon *et al.*, 2006b). Tyrosol as yeasts' by-product remains in the wine matrix after alcoholic fermentation, constituting 40% of the total phenolic compounds and having a strong honey wax reminiscence that contributes to the honey-like characteristic aromatic category of these wines (Jackson, 2008).

**Table IV**

Phenolic content of dry and botrytized wine (Ribéreau-Gayon *et al.*, 2006b)

*Composição fenólica de um vinho branco seco e de um vinho botritizado* (Ribéreau-Gayon *et al.*, 2006b)

	Dry white wine		Noble rot wine	
	Content (mg/L)	%	Content (mg/L)	%
Tyrosol	25	22.2	29	40.5
Gallic acid	1.4	1.2	1.0	1.4
Caffeic acid	13.5	12	0.2	0.3
<i>p</i> -Coumaric acid	2.6	2.3		
Quercetin	0.2	0.2		
Tannins (procyanidins)	70	62.1	41.3	57.7
Total phenolic compounds	112.7	100	71.5	100

### Quality markers

The exponential phase of *B. cinerea* development is carried out in the internal part of berry's skin, where deprivation of oxygen results to fungus incapacity of glucose assimilation (Donèche, 1989). Nicotinamide adenine dinucleotide (NAD) is a cofactor playing pivotal role in microorganisms' metabolic activities. Reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate is carried out enzymatically by glycerol-3-phosphate dehydrogenase (Donèche, 1989). This process guarantees fungus metabolic activity and survival, and leads to glycerol accumulation in berries (Donèche, 1989). As *B. cinerea* begins to grow at the external part of berry's skin at the stationary phase of its development, gluconic acid starts accumulating in berries due to glucose oxidation by glucose oxidase (GOx) (Ribéreau-Gayon *et al.*, 2006a). The duration of *B. cinerea* presence in internal and external part of grape skin, determines the concentration of glycerol and gluconic acid, respectively. Optimal environmental conditions lead to rapid berry shriveling, reducing the period of fungus occurrence externally and limiting gluconic acid accumulation on berries (Ribéreau-Gayon *et al.*, 2006a). The ratio between glycerol and gluconic acid is considered as noble rot quality index, the higher the ratio the higher the quality of the

potential wine (Ribéreau-Gayon *et al.*, 2006a).

## BERRY'S MICROBIOLOGY

### Other fungus

During noble rot infection, colonization of other molds on berry skin is always possible under the condition that *B. cinerea* is the dominant fungus (Pucheu-Planté and Seguin, 1978). Contrary to bunch rot, in noble rot infection populations of other microorganisms on grapes are 10.000 times less compared to *B. cinerea* (Pucheu-Planté and Seguin, 1978). The most common saprophytes on noble rot berries are *Penicillium expansum*, *Penicillium crustosum*, *Aspergillus niger* and *Alternaria alternata* (Lorenzini *et al.*, 2012). Experimentation on intended co-inoculation of different pairs of these molds with *B. cinerea* on grapes showed the different metabolic pathways of each one and how they affected berry's composition (Lorenzini *et al.*, 2012). *B. cinerea* along with *Penicillium* and *Aspergillus* spp. affected the level of shriveling due to increased water losses. These fungi promoted the oxidation of glucose to gluconic acid catalyzed by GOx. High amount of gluconic acid reduced the quality of infected grapes (Lorenzini *et al.*, 2012). In general, even if absolute

fungus monoculture infection is rather rare, the higher the purity of *B. cinerea* colonization on grapes the higher the quality of the final product (Lorenzini *et al.*, 2012).

### Bacteria

A study conducted in Sauternes revealed that *Gluconobacter* spp., and especially *Gluconobacter oxydans*, are present on berries during botrytization. Comparison of these bacteria's CFU with healthy musts highlighted the importance of the infection and its severity in berries' microbial load (Barbe *et al.*, 2001). Acetic acid bacteria are by far the most dominant bacteria found on berries during noble rot – Table V (Barbe *et al.*, 2001). The three main

compounds responsible for the high sulfur dioxide binding capacity of botrytized musts are gluconic acid, 5-oxofructose and dihydroxyacetone (DHA). According to Barbe *et al.* (2001), *Gluconobacter oxydans* present in botrytized grapes is directly correlated with the accumulation of these compounds produced by the oxidation of glucose, fructose and glycerol, respectively. *Acetobacter aceti* and *Acetobacter pasteurianus* dominated progressively noble rot musts during fermentation conducted at 21 °C in wood barrels. *Acetobacter aceti* is responsible for acetic acid production also during maturation in barrels, especially when pH and temperature are high (Joyeux *et al.*, 1984).

**Table V**

Presence of acetic acid bacteria in botrytized must for three consecutive vintages (Barbe *et al.*, 2001)

*Presença de bactérias acéticas em mostos de uvas botritizadas em três vindimas consecutivas (Barbe et al., 2001)*

Harvest	Samples	Acetic bacteria / Total bacteria		
		Minimum	Average	Maximum
1995	24	0%	69%	100%
1996	46	41%	95%	100%
1997	12	62%	94%	100%

## FROM GRAPE TO MUST

### Harvest

Due to high heterogeneity of berries' shriveling degree during infection, the presence of more than one variety for noble rot wine production is beneficial (Pucheu-Planté and Mercier, 1983). Sorting three to four times each harvest period, selectively hand picking of clusters or part of them at *pourri rôti* stage, is mandatory to ensure homogeneity and thus typicity of produced wines (Pucheu-Planté and Mercier, 1983). For this reason, the harvest for noble rot wines cannot be done mechanically (Thakur, 2018). The choice of the perfect harvest date is crucial for the production of this type of wines. In addition to berry's shriveling degree, harvest is based on botrytization process that promotes an intense enzymatic maceration of the grape skin, similar to red skin maceration in vats. The longer and deeper this process is in berries, the greater the release of varietal aromas such as thiols precursors in must (Ribéreau-Gayon *et al.*, 2006a). Compared to botrytized wines, the aromatic profile of straw wines is less complex. While berries are drying under the sun, their varietal

aromas undergo thermal cleavage, reducing the aromatic potentials of the final product (Ribéreau-Gayon *et al.*, 2006a).

### Markers for evaluating the infection and proposing harvest date

Standardization of the evaluation of *B. cinerea*'s infection on berries is crucial for noble rot wine production. The establishment of the optimum harvest date based on objective factors allows reducing human error (Lorenzini *et al.*, 2015; Carbajal-Ida *et al.*, 2016). Thus far, validation of fungus growth stage is based on the personal experience of winemakers while human eye is the main verification tool (Lorenzini *et al.*, 2015; Carbajal-Ida *et al.*, 2016). Puncture and compression tests are potential markers found in the literature for evaluating the infection (Carbajal-Ida *et al.*, 2016). Both are based on the principle that the higher the degree of infection the more extended the skin degradation by fungus's enzymes. In the puncture test, a sensor penetrates on the berry until the skin is ruptured, while in the compression test two parallel plates press the berry until its deformation reaches 70%. Both tests measure the required forces for achieving the targets, the lower

the applied force the more advanced the stage of infection (Carbajal-Ida *et al.*, 2016). Berry's myricetin levels is another proposal for evaluating botrytization as it is a phenolic compound normally absent from grapes (Carbajal-Ida *et al.*, 2016). It has been found only in berries at *pourri rôti* stage of infection probably produced by berries as response to parasite's presence (Carbajal-Ida *et al.*, 2016). Finally, the amount of proteins in berries is another evaluation test based on the principle that *B. cinerea* causes enzymatic degradation of proteins (Lorenzini *et al.*, 2015). The lower the amount of berries' proteins the higher the botrytization. Further evaluation of these procedures is required, namely in terms of time consuming, reliability of the results and applicability during the very stressful period of harvest (Lorenzini *et al.*, 2015).

### **Cryoextraction technique**

As harvest takes place manually and botrytization degree is firstly evaluated subjectively by the winemaker and then by the harvesters during hand picking, the method of cryoextraction seems very efficient (Reynolds, 2010). The principle of this technique derives from icewine production and it is based on the high sugar content of infected grapes by *B. cinerea* at *pourri rôti* stage (Reynolds, 2010). The higher the amount of sugars the lower the freezing temperature of berries. The harvested grapes are transferred in freezing chambers where temperature range from 0°C to -16 °C (Ribéreau-Gayon *et al.*, 2006a). The produced must derives only from grapes highly botrytized with high sugar content and more qualitative and full-bodied juice (Ribéreau-Gayon *et al.*, 2006a). The extractability of aromatic compounds from skin increase due to intense skin burns by frost (Ribéreau-Gayon *et al.*, 2006a). This technique can also be applied during harvests of below average vintages, when lack of sunny days do not allow sufficient berry desiccation leading to berry's water excess and dilution of sugar content (Ribéreau-Gayon *et al.*, 2006a).

### **Pressing**

A sorting table is recommended to be used after harvest for separating part of clusters that are less or not infected or co-infected by other fungus and bacteria (Thakur, 2018). In some cases, crushing without destemming, in which stems facilitate pressing and their extracted tannins facilitate protein stabilization, with subsequent maceration for sugar and aromas extraction in inert gas atmosphere are followed by pressings (Jackson, 2008). Direct pressing without crushing and maceration is another option that can be followed and depends on winemaker decision (Jackson, 2008). In general, the

main principle for this type of wine production is to avoid the release of particles such as glucans in the must (Jackson, 2008). These colloids will produce later clarification problems and will hamper the stabilization processes. Thus, minimization of frictions by using gravity during berries' transfer and decrease of harshness of every operation, such as pumpings, are vital (Jackson, 2008). Continuous presses increase liberation of undesirable particles in the must, while pneumatic ones provide with inadequate pressing, up to 2 bars maximum (Ribéreau-Gayon *et al.*, 2006a). Both types are not recommended for noble rot grapes. Yet, pneumatic pressing is a sustainable choice for cryoextraction technique (Ribéreau-Gayon *et al.*, 2006a). Moving-head presses are the best option as they can apply pressure on grapes up to 12 bars. The most qualitative juice with the higher sugar content is obtained by the last pressing cycles (Ribéreau-Gayon *et al.*, 2006a).

### **Settling before fermentation**

The high sugar concentration and the existence of colloidal particles in the must, make settling rather difficult (Ribéreau-Gayon *et al.*, 2006a). Yet, settling at temperatures lower than 10°C for 28 to 48 hours is beneficial. As lees is a source of fatty acids, sterols, among others, which are important for yeast development, excessive clarification increases the possibility of stuck or sluggish fermentation (Ribéreau-Gayon *et al.*, 2006a). The optimum turbidity, without facing problems of bitter, reductive and herbaceous notes is in-between 500 and 600 NTU (Ribéreau-Gayon *et al.*, 2006a). Addition of lees, saved from settling of previously produced dry white wine, in tank or barrel filled with botrytized must, is a boosting factor for yeasts development during fermentation (Ribéreau-Gayon *et al.*, 2006a).

## **IONS CONTENT OF BOTRYTIZED MUST**

### **Anions**

Phosphates and sulfates are the anions with the highest concentrations in must and wine (Moreno and Peinado, 2012). Phosphates are naturally present in grapes, while their normal levels in white wines are between 75 and 500 mg/L. This fluctuation depends on the use or not of diammonium phosphates as source of yeasts' assimilable nitrogen (Moreno and Peinado, 2012). Origins of sulfates are endogenous since their presence in grapes varies from 0.1 to 0.4 g/L expressed as potassium sulfate or exogenous deriving from viticulture treatments with copper sulfate against downy mildew (Moreno and Peinado, 2012). During winemaking, other possible sources are the addition of yeast nutrients like ammonium sulfate

and copper sulfate treatment against reductive notes (Moreno and Peinado, 2012). Yet, the most important origin of sulfates for botrytized wines is sulfur dioxide addition. Oxidation of sulfur dioxide, during barrel aging, leads to sulfate accumulation (Moreno and Peinado, 2012). Normal levels of sulfates found in other types of wines are between 0.15-0.7 g/L expressed as potassium sulfate, while for this specific category they can reach even 2 g/L (Moreno and Peinado, 2012).

### Cations

Potassium is the main cation present in wines and is responsible for tartaric instability (Moreno and Peinado, 2012). In botrytized and straw wines, it is highly increased up to 2 g/L, due to concentration effect of berry's shriveling (Moreno and Peinado, 2012). Calcium is also a source of instability mainly due to tartrate precipitation under the form of calcium bitartrate. It is considered the most difficult tartaric instability to control, as precipitation mainly occurs during storage (Jackson, 2008). Calcium concentration is increased up to 140 mg/L in noble rot wines, leading to formation of other salts except of tartaric, such as gluconic and mucic whose concentrations are also increased for this type of wines (Jackson, 2008).

## GLYCEROPYRUVIC AND ALCOHOLIC FERMENTATION

### Glyceropyruvic fermentation

Synthesis of glycerol in must from healthy grapes, with sugar content around 240 g/L, starts before alcoholic fermentation (Moreno and Peinado, 2012). Glyceropyruvic fermentation takes place providing the wine with an average concentration of 5 to 10 g/L of glycerol (Moreno and Peinado, 2012). In botrytized wines, concentration of glycerol is increased with an average of 16 g/L, partly due to the extended glyceropyruvic fermentation (Moreno-Arribas and Polo, 2009). More in detail, normally during alcoholic fermentation ethanol production by the reduction of ethanal is carried out since yeast needs to re-oxidize  $\text{NADH}_2$ , the reduced form of NAD, back to  $\text{NAD}^+$  in order to continue its metabolic activity. Limited amount of ethanal at the beginning of fermentation, parallel to its strong affinity with sulfur dioxide in sulfited musts like botrytized ones, block this step (Moreno-Arribas and Polo, 2009).

Alternative to ethanal, yeast uses dihydroxyacetone (DHA) that is reduced to glycerol, increasing consequently the concentration of the latter in the

must (Moreno-Arribas and Polo, 2009). Glycerol is very important not only for yeasts' high osmolarity and redox equilibrium but also for improvement of organoleptic characteristics of wine (Moreno-Arribas and Polo, 2009). Glycolysis still takes place during glyceropyruvic fermentation since breakdown of fermentative sugars up to 8%, leads to pyruvic acid accumulation and then to secondary products such as ethanal and acetate. This explains why, as it happens with glycerol, the major portion of volatile acidity (VA) is generated during yeasts' growth phase (Bely *et al.*, 2003). Glycerol content is independent of the maximum yeasts' population, the produced VA and the assimilable nitrogen present in must (Bely *et al.*, 2003). When sulfur dioxide decreases and ethanal is in sufficient amount, alcoholic fermentation and so ethanol production start (Bely *et al.*, 2003).

### Alcoholic fermentation kinetics

It is well known that "stuck" fermentation can be caused by many factors that affect and finally inhibit yeasts' metabolic activities (Bisson, 1999). During spontaneous fermentation one of these factors can be the problematic multiplication of *Saccharomyces cerevisiae* under hostile environment, as other yeasts species and microorganisms are present on must, making difficult its colonization and establishment in the medium (Bisson, 1999).

Following a spontaneous alcoholic fermentation of noble rot must with a sugar content of 320 g/L showed that after yeasts' growth phase, fermentation rate stayed stable and maximum during stationary phase, until the 11<sup>th</sup> day of fermentation when death phase started (Lafon-Lafourcade and Ribéreau-Gayon, 1979). At this moment the average daily degradation of sugars fell from 15 g/L to 5 g/L. At the 15<sup>th</sup> day from the beginning of fermentation, 170 g/L of sugar had been already metabolized. During death phase the number of living yeasts decreased rapidly and sugar consumption felt at 35 g/L in 24 days. Fermentation stopped after 40 days, with a level of residual sugars at 100 g/L (Lafon-Lafourcade and Ribéreau-Gayon, 1979). The extremely high sugar content of these wines affected yeasts' growth phase due to the hyperosmotic environment that eventually led to "stuck" fermentation. An experiment conducted in musts from sound grapes and different sugar contents, revealed this trend (Lafon-Lafourcade and Ribéreau-Gayon, 1979).

Different blends of two types of musts, one coming from botrytized grapes with sugar content of 420 g/L and one from healthy grapes with content of 220 g/L, all corrected at the same sugar level with sucrose addition, showed different fermentation rates (Lafon-Lafourcade and Ribéreau-Gayon, 1979). The lower

fermentation rate derived from the blend with the higher ratio of botrytized must indicated that specific compounds present on botrytized must act as inhibitors against yeasts' development (Lafon-Lafourcade and Ribéreau-Gayon, 1979). Isolation and addition of botryticin in noble rot musts showed increased production both of glycerol and acetic acid, highlighting its activity against yeasts' growth (Ribéreau-Gayon *et al.*, 2006a).

Fermentation kinetics of free-run and pressed botrytized must from different pressings, showed that the must from the last pressing caused the most sluggish fermentation (Lafon-Lafourcade and Ribéreau-Gayon, 1979). Compounds with *B. cinerea*'s origin, such as  $\beta$ -glucans, also act as yeasts' inhibitors. Their release in must is correlated with the harshness of wine making techniques (Lafon-Lafourcade and Ribéreau-Gayon, 1979). Finally, one other very important inhibitory factor for yeasts was also the lack of assimilable nitrogen, which was metabolized almost totally by the fungus (Lafon-Lafourcade and Ribéreau-Gayon, 1979). Addition of nutrients at the beginning of fermentation assured sufficient yeasts multiplication, increasing fermentation rate during stationary phase (Lafon-Lafourcade and Ribéreau-Gayon, 1979). In order to ensure that after stopping fermentation residual sugars would be less than 90 g/L, inoculation of specific yeast strains that can survive for longer time under all these unfavored conditions with parallel addition of nutrients are obligatory (Lafon-Lafourcade and Ribéreau-Gayon, 1979).

#### **Assimilable nitrogen correction in must**

After harvesting botrytized grapes and during alcoholic fermentation, high concentration of sugars up to 400 g/L along with limited assimilable nitrogen content can cause stuck fermentation and increase the VA from 1.1 to 1.6 g/L expressed as acetic acid (Bely *et al.*, 2003). In noble rot musts, VA produced by yeasts without bacteria participation can exceed the legal limits for white wines, 20 milliequivalents per liter or 1.20 g/L expressed as acetic acid (OIV, 2020), reaching a final content of more than 2.0 g/L (Bely *et al.*, 2003). In contrast to the average nitrogen content of 180 mg/L for bordelaise dry white musts, the average available nitrogen in noble rot grapes was estimated at around 84 mg/L with a worth mentioned minimum of even 25 mg/L (Bely *et al.*, 2003). A study conducted during the fermentation of the obtained musts with and without addition of ammonium salts, showed that increase of nitrogen content generated an increase on yeasts population that subsequently resulted to decreased VA production (Bely *et al.*, 2003).

Accumulation of 80% of the total VA in the samples took place during yeasts' growth phase. More than that, VA decreased up to 40% when must of 350 g/L of sugars reached 210 mg/L of assimilable nitrogen compared to the same must with content at around 100 mg/L (Bely *et al.*, 2003). Nevertheless, addition of nitrogen more than 210 mg/L resulted to increased yeasts bioactivity that led to acetate overproduction. The best time for nitrogen addition in the must was at the beginning of fermentation, at a density of around 1140 g/L and definitely before yeasts reached the stationary phase, two to three days after yeasts' inoculation (1100 g/L) (Bely *et al.*, 2003). Finally, a correction on must assimilable nitrogen up to 190 mg/L was proposed as the optimum concentration for a must with sugar content of 350 g/L (Bely *et al.*, 2003). All the above-mentioned highlight the importance of measuring the assimilable nitrogen in pre-fermented sweet musts and make the corrections without being based on general estimations, in order to avoid sluggish fermentation and high production of VA (Bely *et al.*, 2003).

#### **Thiamine addition**

Thiamine or Vitamin B<sub>1</sub>, as part of the enzyme carboxylase is very important for its production (Ribéreau-Gayon *et al.*, 2006a). It is an enzyme excreted by yeasts that removes the carboxylic groups from ketonic acids during fermentation. Pyruvic acid for example is converted to ethanal and later to ethanol. Thus, thiamine plays important role in yeasts metabolism by decreasing acetate production and VA (Ribéreau-Gayon *et al.*, 2006a). For botrytized musts, thiamine is vital for diminishing carbonyl compounds, like pyruvic and  $\alpha$ -ketoglutaric acid, which are counting for 20% and 16% of sulfur dioxide binding, respectively, by promoting their decarboxylation (Ribéreau-Gayon *et al.*, 2006a). In botrytized musts in which fermentation takes place under unfavorable conditions, production of these acids especially at the beginning is much higher than in white musts from sound grapes. Addition of thiamine in botrytized musts can decrease in average the bounded sulfur dioxide by 20 mg/L (Ribéreau-Gayon *et al.*, 2006a). The deprivation of this vitamin in berries and later in must is actually induced by *B. cinerea* (Thakur, 2018). Addition of sulfites in must inactivates even more the already feeble amount of this vitamin (Jackson, 2008). Addition of 50 mg/L of vitamin B<sub>1</sub>, along with other nutrients parallel to yeast inoculation in clarified must, is the best way to assure higher sulfur dioxide efficiency due to limited accumulation of pyruvic and  $\alpha$ -ketoglutaric acid and limited binding (Ribéreau-Gayon *et al.*, 2006a). The maximum amount of thiamine hydrochloride addition

in must is 60 mg/hL expressed as thiamine (EEC, 2009).

### Vat types for fermentation

Two types of containers can be used during fermentation, stainless-steel tank or wooden barrel (Ribéreau-Gayon *et al.*, 2006a). When fermentation takes place in barrel, wood pores allow oxygen dissolution in must that favors yeasts' activity, producing wines with less residual sugars and higher ethanol content. Temperature during fermentation in barrel ranges from 12 °C in Tokaj to 28 °C in Sauternes (Magyar, 2011). Temperature control at around 20 °C to 24 °C is much easier in stainless-steel tanks, favoring yeasts' fermentation rate (Ribéreau-Gayon *et al.*, 2006a). When fermentation takes place in stainless-steel tank, the contact of juice with oxygen is preferable after yeasts' growth phase, for beneficial ALF and reduced VA (Ribéreau-Gayon *et al.*, 2006a). It is advisable to conduct fermentation, regardless of the type of fermenter, in small volume containers for separating different batches of grapes that derive from sortings during harvest. In this way, these batches keep separately their unique character, gained during botrytization, facilitating blending at the end (Jackson, 2008).

### Yeast species during fermentation

#### *Candida* spp.

A study conducted on botrytized musts, tried to investigate the adaptation capacity and the evolution of yeast species during spontaneous fermentation at two different temperatures (20 °C and 30 °C) with pH 3.57 (Mills *et al.*, 2002). Only *Candida* and *Kluyveromyces* spp. persist during the 30 °C fermentation, while all the other species were actually eliminated in the early stages. An explanation could be the incapacity of non-*Saccharomyces* spp. adaptation under the very fast ethanol accumulation (Mills *et al.*, 2002). The durability of all the species at 20 °C was higher. During the 30 °C fermentation, the presence of *Candida* spp. was reduced sharply as soon as alcohol content reached 6% vol, while in the other case the population was not reduced until 12.5% vol of alcohol was achieved. *Candida* spp. were very important even in cases of dry yeast inoculated musts, with noticeable presence in the medium during fermentation (Mills *et al.*, 2002).

Investigation on benefits of *Candida stellata* on aroma profile of dry wines was carried out, revealing an amelioration on the analytical profile of wines after successive inoculation of *Candida stellata* and *Saccharomyces cerevisiae* during fermentation (Ciani and Ferraro, 1998), and an intensification of aromas of stone fruits and honey after monoculture

fermentation with this *Candida* sp. (Soden *et al.*, 2000). Bibliography also mentions an increase of glycerol production under the presence of *Candida stellata* parallel to a very low fermentation rate, while the trend of acetate production is quite controversial (Ciani and Ferraro, 1996; Ciani *et al.*, 2000; Soden *et al.*, 2000).

According to Sipiczki (2004), previously published articles concerning *Candida stellata* sp. most probably referred to *Candida zemplinina* sp. A research carried out on high sugar must fermentation after simultaneous inoculation of *Candida zemplinina* (syn *Starmerella bacillaris*) and *Saccharomyces cerevisiae* compared to monoculture fermentation of the latter, demonstrated 0.3 g/L lower VA expressed as acetic acid and sufficient ethanol and glycerol production (Rantsiou *et al.*, 2012). On the contrary, a fermentation after subsequent inoculation was sluggish in terms of alcohol accumulation while VA was 50% reduced, indicating the limited impact of high sugar content as yeasts' inhibitor during fermentation when other yeasts species are present (Rantsiou *et al.*, 2012). The fructophilic character of *Candida stellata* changes the ratio of glucose/fructose and helps *Saccharomyces cerevisiae* with sluggish problems. The role of *Candida stellata* or *Candida zemplinina* in co-inoculated, sequential inoculated or non-inoculated fermentation and their impact on wine's organoleptic characteristics, need further research before strong conclusions can be drawn (Magyar and Tóth, 2011).

#### *Torulaspora delbrueckii*

Finally, another study tried to investigate the potentials of *Torulaspora delbrueckii*, another yeast species known for limited levels of VA accumulation in fermentative musts (Bely *et al.*, 2008). Measurements after alcoholic fermentation of botrytized musts, with sugar content of 360 g/L, verified the above statement regarding VA, even though ethanol accumulation did not reach desired levels since the fermentation was actually sluggish. Co-inoculation with *Saccharomyces cerevisiae* provided wines with low VA and ethanal concentration meaning reduced binding capacity with sulfur dioxide. Subsequent inoculation again with *Saccharomyces cerevisiae* incorporation after five days was unsuccessful regarding the same two parameters (Bely *et al.*, 2008). Simultaneous inoculation of both the two species with ratio 20:1 in favor of *Torulaspora delbrueckii* provided the best results when compared to monoculture *Saccharomyces cerevisiae* fermentation, again in terms of VA and ethanal, with 53% and 60% decrease respectively (Bely *et al.*, 2008).

### “Mutage” operation

The antimicrobial properties of sulfur dioxide can be used for delaying the beginning of alcoholic fermentation or in higher doses even finishing it (Ribéreau-Gayon *et al.*, 2006a). Only molecular sulfur is active against *Saccharomyces cerevisiae* because it is the only form that can pass through the cell wall into the cytoplasm of the yeast where it dissociates due to the high pH 6-7 and kills the yeast from the interior by reacting with vital compounds and unbalancing the redox equilibrium (Ribéreau-Gayon *et al.*, 2006a). “Mutage” refers to an operation where addition of at least 100 mg/L of sulfur dioxide can cease fermentation (Sudraud and Chauvet, 1985). According to some studies, the safest dose for stopping fermentation is 1.50 mg/L of molecular or active sulfur. For assuring a good conservation of these wines without any refermentation problems after racking and aging, 1.20 mg/L of active sulfur is sufficient (Sudraud and Chauvet, 1985).

“Mutage” is carried out when sugar and alcohol levels are at a desired equilibrium, providing well balanced wines without excess alcohol or cloying perception (Ribéreau-Gayon *et al.*, 2006a). Empirically, the ratio of 13:3, 14:4 and 15:5 between the actual and potential alcohol strength is a potential guide to follow, but it is rather rare to achieve it when fermentation stops spontaneously. In most cases, “mutage” is applied when fermentation rate is very low, increasing VA without actually reducing sugars, or in the opposite case where sugar degradation is very extensive. The balance and the equilibrium in these wines is ensured during blending, in which different batches can be mixed (Ribéreau-Gayon *et al.*, 2006a). A common practice nowadays before adding sulfur dioxide is the decrease of wine temperature at -4 °C followed by centrifugation or racking in order to eliminate a high amount of yeasts. This will ensure a more effective treatment. The amount of total sulfur dioxide added to the must, in order to achieve around 50 mg/L of free, is between 200 and 300 mg/L (Ribéreau-Gayon *et al.*, 2006a).

## SULFUR DIOXIDE

### Binding capacity and antioxidant properties in botrytized musts and wines

Alcoholic fermentation of botrytized wines stops by “mutage”. As previously mentioned, the proper dose of sulfur dioxide for avoiding refermentation problems ranges between 200 and 300 mg/L. This dosage is higher than the sulfur dioxide addition in dry red or white wines, because botrytization

increases the number of sulfur dioxide binding compounds in must, and finally in wine (Barbe *et al.*, 2000). These compounds are partially produced by *B. cinerea* and bacteria present in infected grapes. Yet, most of the molecules with the strongest affinity with sulfur dioxide derive either from alcoholic fermentation or oxidation of sugars (Barbe *et al.*, 2000).

An experiment conducted on different musts and wines from Sauternes, tried to identify specific compounds that combine sulfur dioxide, thus increasing the amount of sulfur dioxide required to stabilize it. (Barbe *et al.*, 2000). According to the results, the three main substances with the stronger binding affinity identified in musts were carbonyl compounds. More specifically, these compounds were  $\gamma$ - and  $\delta$ -gluconolactone, both in chemical equilibrium with gluconic acid (only 10% was in acid's form), 5-oxo-fructose with an average content of 100 mg/L that was bound with 60% of the total sulfur dioxide and DHA. The latter, was in equilibrium with glyceraldehyde, explaining its very limited presence in must. DHA was metabolized by yeasts during alcoholic fermentation, while the other two were still present in wine accounting for 50% of the total binding capacity of sulfur dioxide. These compounds along with the following yeasts' derivatives (produced during ALF) - ethanal, pyruvic acid and 2-oxoglutaric acid - were considered responsible for binding with sulfur dioxide and increasing its addition in botrytized wines (Barbe *et al.*, 2000).

Different amounts of the three alcoholic fermentation by-products identified in different wines were correlated with different yeast strains (Masneuf and Dubourdieu, 2000). A study conducted in different musts, one fermented spontaneously by indigenous yeasts and three inoculated with different industrial dry yeast strains, intended to correlate the production of these compounds during fermentation with their binding capacity, for a aimed concentration of 50 mg/L of free sulfur dioxide after “mutage” (Masneuf and Dubourdieu, 2000). The wine from spontaneous fermentation had the second lower binding capacity with sulfur dioxide, while the wine from *Saccharomyces cerevisiae* BO213 fermentation, a strain highly appreciated in Sauternes region for the production of noble rot wines, needed the highest amount of total sulfur dioxide addition (336 mg/L). This strain produced in average 55 mg/L of ethanal, corresponding to approximately 82 mg of bound sulfur dioxide, 117 mg/L of pyruvic acid, and 130 mg/L of 2-oxoglutaric acid. Indigenous yeasts naturally present in botrytized must had the best adaptation in such a medium with so many

fermentation inhibitors (Masneuf and Dubourdieu, 2000).

Ethanal compared to other compounds has the strongest affinity with sulfur dioxide (Barbe *et al.*, 2000). In wines derived from already sulfited musts, a common practice applied in many wineries, ethanal's levels are higher than in wines from non-sulfited ones (Barbe *et al.*, 2000). Sulfur dioxide blocks yeast's metabolic pathways, leading to an increased ethanal accumulation as response (Barbe *et al.*, 2000). Empirically, it is known that approximately only half of sulfur dioxide added in the must is actually bound with compounds. For this reason, the usual winemaking strategy of adding 30 to 50 mg/L of sulfur dioxide in must, to avoid fermentation, microbial spoilages and laccase activity, is actually beneficial (Ribéreau-Gayon *et al.*, 2006a). Botrytized must composition and wine matrix varies from vintage to vintage according to the intensity of infection. Although an average of 200 to 300 mg/L of total sulfur dioxide can be added to reach the targeted 50 mg/L of free sulfur dioxide, there is no general rule on the ratio between combined and free sulfur dioxide. For all the above-mentioned reasons the maximum acceptable limits of total sulfur dioxide for these wines is 400 mg/L (EEC, 2009).

Sulfur dioxide not only suppresses the enzymatic activity of laccase but also reacts with the produced *o*-quinones, that are highly oxidative, by reducing them back to *o*-diphenols (Waterhouse *et al.*, 2016). Sulfur dioxide acts against hydrogen peroxide, a powerful oxidant produced by the oxidation of *o*-diphenols to semiquinones and then to *o*-quinones via the hypothetical mechanism: hydroperoxyl radical step (Waterhouse *et al.*, 2016). Without sulfur dioxide presence in must and wine, hydrogen peroxide is decomposed to a hydroxyl radical under the catalytic presence of iron, able to oxidize a large number of compounds, known as the "Fenton reaction". This explains the limited binding capacity of sulfur dioxide against oxygen. In fact, sulfur dioxide reaction with oxygen is rather slower in botrytized musts than in healthy musts (Waterhouse *et al.*, 2016).

## AROMATIC COMPOUNDS IN NOBLE ROT WINES

### Lactones

Lactones can be cyclic carboxylic esters of gamma ( $\gamma$ ) and delta ( $\delta$ ) hydroxy-acids easily formed under mildly acidic environment after the cleavage of fatty

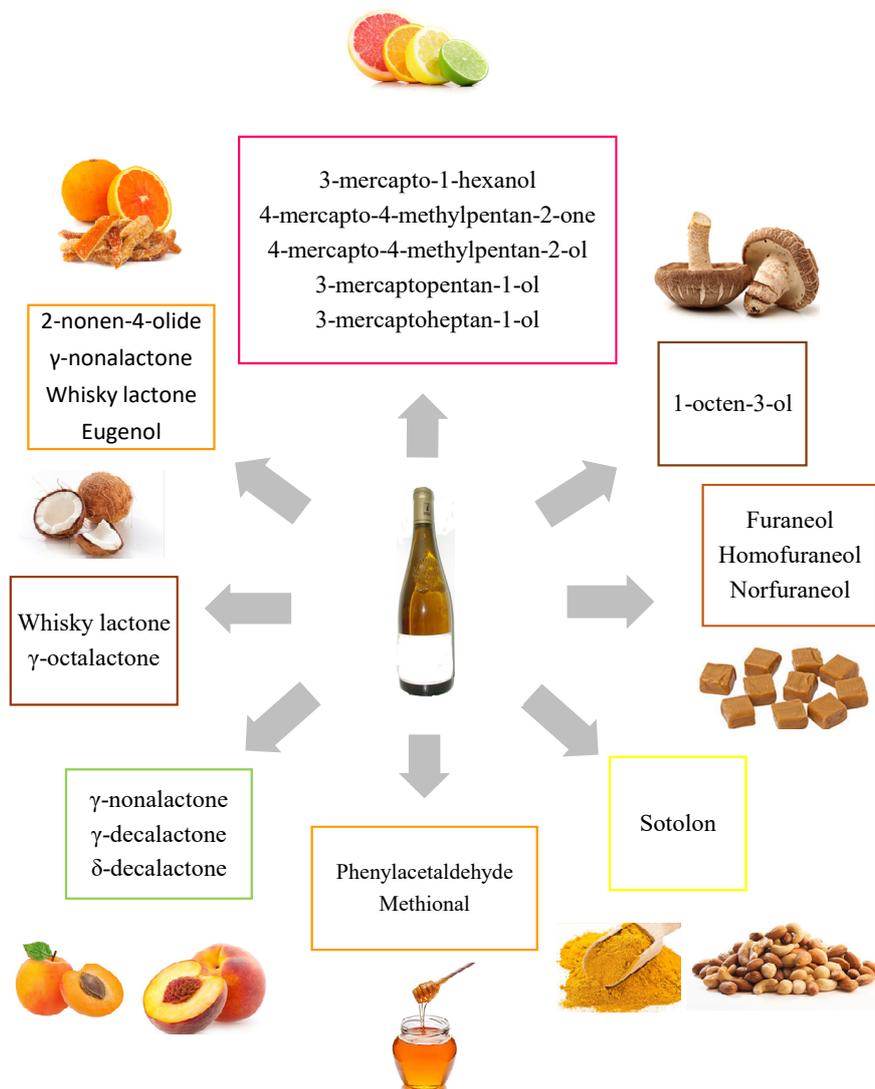
acids during berry's LOX pathway, a process known as "lactonization" (Moreno and Peinado, 2012). The produced  $\gamma$ -nonalactone,  $\gamma$ -decalactone and  $\delta$ -decalactone (Figure 2) with distinct aromas of apricot and peach have been identified in Sauternes wines (Sarrazin *et al.*, 2007a). In Australian botrytized wines,  $\gamma$ -nonalactone and  $\gamma$ -octalactone (coconut aroma) were identified in the highest concentrations 59 and 8.5  $\mu\text{g/L}$ , respectively, compared to a total of 58 white wines (Cooke *et al.*, 2009). Both of them were above their threshold limits, which are 25 and 7  $\mu\text{g/L}$ , respectively (Waterhouse *et al.*, 2016). Moreover,  $\gamma$ -3-methyloctalactone, known as whisky lactone, that is a tertiary aroma compound with coconut flavor extracted from wood during barrel aging, was also present in aged Sauternes wines (Sarrazin *et al.*, 2007a).

2-Furanone derivatives are other type of lactones, which are found both in botrytized berries and young Aszú wines from Tokaj region, enhancing their aromatic profile with stone fruit notes (Miklósý *et al.*, 2004; Miklósý and Kerényi, 2004). Even though 2-furanone or  $\gamma$ -crotonolactone is a lactone, its origin differs from the previously mentioned lactonization pathway. 2-Furanone, with caramel aroma, results from the oxidation of furfural, which is proposed to be previously formed by hydrolysis of C<sub>5</sub> sugar oligomers and subsequent dehydration of the produced monomer (Serrano-Ruiz *et al.*, 2012), or it is a Maillard reaction's product (Banerjee *et al.*, 1981).

HPLC analysis of botrytized wines revealed the presence of 2-nonen-4-olide, among other compounds characteristic of these wines (Stamatopoulos *et al.*, 2015). This compound is a 2-furanone type lactone, which has the second lowest perception threshold (10.8  $\mu\text{g/L}$ ) after whisky lactone, and is mainly associated with fruity and minty aromas only found in this type of wines. This compound along with whisky lactone, eugenol and  $\gamma$ -nonalactone seem to be responsible for the creation of the synthetic aroma of candied orange (Figure 2), highlighting the importance of lactones in wine matrix not only individually but also synergistically especially after barrel aging (Stamatopoulos *et al.*, 2015).

### Sotolon

3-Hydroxy-4,5-dimethylfuran-2(5H)-one, widely known as sotolon, is a compound landmark for Port aged wines with sensory character of curry, fenugreek, nuts and dried fruits, and a threshold of 19  $\mu\text{g/L}$  (Ferreira *et al.*, 2003).



**Figure 2.** Basic odorant compounds within the main four aromatic groups found in botrytized wines.

*Compostos odorantes dos principais grupos de compostos do aroma encontrados nos vinhos botritizados.*

Its accumulation was found to be induced under oxidative conditions and although its production pathway is not yet completely clarified, the involvement of glycolaldehyde and diacetyl, which are both by-products of Maillard reaction, was proposed (Ferreira *et al.*, 2003). Even though sotolon is considered as lactone and more precisely as 2-furanone derivative, it is preferable to be mentioned separately since it is identified in Sauternes wines - Figure 2 (Sarrazin *et al.*, 2007a).

The impacts of botrytization on grapes, such as oxidation and sugar concentration, seem to create the perfect substrate for sotolon production after aging (Sarrazin *et al.*, 2007a). According to Masuda *et al.* (1984), 5 to 20  $\mu\text{g/L}$  were present in these wines compared to a maximum of 1  $\mu\text{g/L}$  in the dry ones, while its threshold limit for these sweet wines was around 5  $\mu\text{g/L}$ . On the contrary, seminar studies from Hungary on Aszú wines from Tokaj, indicated a non-significant identification of sotolon on young wine

matrices (Tóth-Markus *et al.*, 2002; Miklósy *et al.*, 2004). However, before strong conclusions can be drawn further studies on aged Aszú wines are recommended.

### Thiols

3-Mercaptohexan-1-ol (3MH) is the most important thiol in botrytized wines (Table VI). It has been identified in concentrations of 5000 ng/L after years of storage (perception threshold of 50 ng/L), enhancing the aroma profile of wine with notes of

citrus peel, grapefruit and passion fruit (Bailly *et al.*, 2006; Tominaga *et al.*, 2006; Sarrazin *et al.*, 2007a; Thibon *et al.*, 2009; Blanco-Ulate *et al.*, 2015). Other thiols have been identified to act synergistically with each other improving the final aromatic profile of the wine (Sarrazin *et al.*, 2007b). Indicatively, 4-mercapto-4-methylpentan-2-one, 4-mercapto-4-methylpentan-2-ol, 3-mercaptopentan-1-ol and 3-mercaptoheptan-1-ol (Figure 2) are responsible for aromas of citrus fruits (Sarrazin *et al.*, 2007b).

**Table VI**

Concentration of 3MH for wines obtained by grapes in different stages of infection (Sarrazin *et al.*, 2007b)

*Concentração de 3MH em vinhos obtidos a partir de uvas em diferentes estádios de infecção (Sarrazin et al., 2007b)*

Variety	Botrytization stage	Mean grape volume (mL)	Variation of mean grape volume (%)	3MH (ng/L)
'Semillon'	Healthy	0.85	100	195
	<i>Pourri plein</i>	0.68	80	2326
	<i>Pourri rôti</i>	0.37	44	3678
	Late <i>pourri rôti</i>	0.38	45	6334
'Sauvignon Blanc'	Healthy	0.78	100	161
	<i>Pourri plein</i>	0.52	67	3003
	<i>Pourri rôti</i>	0.21	27	9648
	Late <i>pourri rôti</i>	0.29	37	9319

Multidimensional gas chromatography-mass spectrometry-olfactometry (MDGC-MS-O) analysis of young Sauternes wines indicated for the first time the existence of 3-propyl-1,2-oxathiolane, which is not a volatile thiol but has similar odor of citrus fruits (Sarrazin *et al.*, 2010). It has never been identified in white dry wines, and its origin is proposed to be 3,3'-dithiobis(1-hexanol), a 3MH disulfide, that is cleaved to 3-propyl-1,2-oxathiolane and 3MH after thermal degradation. The potential mechanism for the production of 3,3'-dithiobis(1-hexanol) at the first place involves *S-S* conjugation of two 3-sulfanylhexasan-1-ol molecules in oxidative environment without polyphenols presence in the medium (Sarrazin *et al.*, 2010).

Polyphenols in botrytized wines have almost been eliminated by oxidization, polymerization and precipitation phenomena under the enzymatic activity

of laccase, so they are actually inactive otherwise, after being oxidized to quinones would be highly reactive against volatile thiols including 3MH (Quideau *et al.*, 1995). Therefore, 3-propyl-1,2-oxathiolane cannot be present in dry white wines (Sarrazin *et al.*, 2010). Absence of it in noble rot wines during barrel aging under the presence of oxygen reinforced the hypothesis of 3MH dimer presence in wine (Sarrazin *et al.*, 2010). Identification of this compound will lend further support to this theory, and will allow a better understanding on thiols behavior during bottle aging and conservation in domestic conditions (Sarrazin *et al.*, 2010).

### Phenylacetaldehyde

Other very important odorant compounds in botrytized wines are the previously mentioned benzaldehyde and furfural, with almond-like aromas,

and phenylacetaldehyde, with aroma of honey - Figure 2 (Kikuchi *et al.*, 1983). In the past, these compounds were erroneously considered as *B. cinerea* metabolites (Kikuchi *et al.*, 1983). Phenylacetaldehyde was mentioned in recent studies as a compound identified in high concentration in botrytized wines, whose origin is strongly correlated with the fungus and the oxidative environment it creates on berries (Sarrazin *et al.*, 2007a). Its concentration increased during infection, while it was not found in sound grapes and musts.

A study showed that even though phenylacetaldehyde had a strong affinity with sulfur dioxide, its concentration was increased during fermentation (Sarrazin *et al.*, 2007a). A potential mechanism, where phenylpyruvic acid as precursor (produced enzymatically during botrytization) was converted by yeasts to phenylacetaldehyde during fermentation, was proposed. Finally, it was found that this aldehyde decreased during aging without being totally eliminated (Sarrazin *et al.*, 2007a).

#### **Furaneol and derivatives**

Furaneol, homofuraneol and norfuraneol contribute with their caramel-like notes to the aroma profile of noble rot wines since their concentration exceed the perception threshold - Figure 2 (Sarrazin *et al.*, 2007a). These three compounds have very limited affinity with sulfur dioxide due to their complex structure. Given that they were not identified in musts from both botrytized and healthy grapes but only in corresponding wines, they were considered to be present in increased concentrations in botrytized wines due to the shriveling effect of *B. cinerea* on the berry and not directly due to the infection (Sarrazin *et al.*, 2007a). In fact, water evaporation from berries during infection concentrates all the compounds, including the ones responsible for the aromas.

#### **Higher alcohols and esters**

Higher alcohols, which are alcohols with more than two carbon atoms on their molecule, are compounds produced by yeasts during ALF, mainly during amino acids degradation via a mechanism known as “Ehrlich reaction” (Reynolds, 2010). More specifically, 2-phenylethanol along with its ester, phenylethyl acetate, both with notes of rose, are very specific aromatic compounds identified in botrytized wines (Tóth-Markus *et al.*, 2002; Bailly *et al.*, 2009; Fedrizzi *et al.*, 2011; Tosi *et al.*, 2012). Another remarkable higher alcohol found in botrytized wines is methionol, with aroma of cooked potato (Sarrazin *et al.*, 2007a; Fedrizzi *et al.*, 2011; Tosi *et al.*, 2012). This higher alcohol is oxidized to methional during barrel aging of botrytized wines, which explains the increasing of methional over the time (Sarrazin *et al.*,

2007a). Although methional is not directly linked with honey-like aroma, it was identified in honey and it is considered that together with phenylacetaldehyde it contributes to wine’s aromatic profile (Sarrazin *et al.*, 2007a). Finally, ethyl hexanoate is an ester associated with the characteristic aroma of green apple, formed during fermentation by condensation of fatty acids and acetyl coenzyme A without participation of higher alcohols (Swiegers *et al.*, 2005). It is found in botrytized wines even after aging in bottle (Sarrazin *et al.*, 2007a; Bailly *et al.*, 2009).

#### **Sensory evaluation**

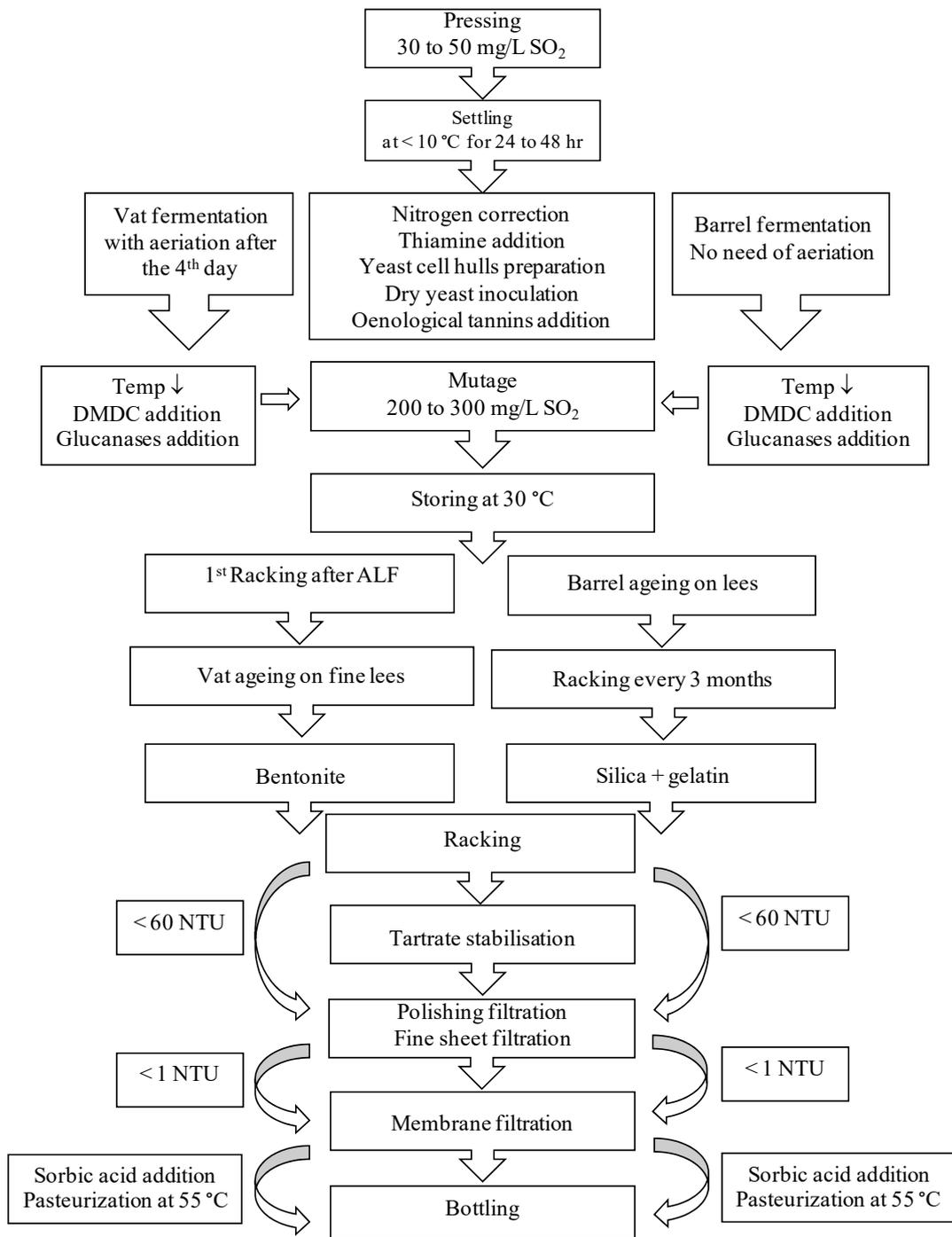
A comparative evaluation on the distinctive aroma profile of three types of sweet wines produced after botrytization, raisining, and fortification was conducted by experts in wine tasting under the method of citation frequency (González-Álvarez *et al.*, 2014). According to the results, citrus fruits, stone fruits, floral and animal aromas were dominant in botrytized wines. Between seven more specific characteristics, the differentiation of botrytized wines from the other two was much clearer. Aromas such as muscat, grapefruit, bay leaf and mushroom were much more intense than in the other two wine categories. Straw and fortified wines showed many similarities among them compared to noble rot wines (González-Álvarez *et al.*, 2014). Evaluation by professionals on the typicality of noble rot wines at a regional scale showed that the majority of bordelaise wines from Sauternes, Barsac and Loupiac were differentiated compared to other French sweet wines produced by botrytization or raisining (Sarrazin, 2008).

### **STABILIZATION AND AGING**

#### **Microbiological stabilization**

##### *Refermentation problems*

*Zygosaccharomyces bailii*, *Sacharomycodes ludwigii* and strains of *Saccharomyces cerevisiae* are very durable in the unfavored environment of botrytized wine matrix (Ribéreau-Gayon *et al.*, 2006a). These yeasts species are responsible for refermentation during barrel aging or bottling. A potential refermentation can easily start when temperature is higher than 15 °C and wine stays in contact with lees. (Ribéreau-Gayon *et al.*, 2006a). Cold treatment followed by “mutage” and clarification by racking, not only decrease the amount of sulfur dioxide that needs to be added to stop fermentation but also stabilize the wine in terms of potential future refermentations (Figure 3) (Ribéreau-Gayon *et al.*, 2006a).



**Figure 3.** Winemaking diagram for fermentation and aging of botrytized wines in barrel and vat.

*Diagrama de vinificação e envelhecimento de vinhos botritizados em barrica e em cuba.*

### *Pasteurization*

Due to the increased amount of both free and combined sulfur dioxide, the ratio in favor of free can change by increasing temporary the storage temperature from 15 °C to 30 °C right after “mutage” (Ribéreau-Gayon *et al.*, 2006a). Depending on the conditions, this can liberate up to 20 mg/L of combined sulfur dioxide, while the equilibrium between free and combined sulfur dioxide changes in favor of the first. The effect of mild heating not exceeding 50 - 55 °C for seven to ten minutes can be used to avoid potential refermentation during aging - Figure 3 (Ribéreau-Gayon *et al.*, 2006a). If this process is followed in regular intervals, when yeasts populations exceeds 1000 cells/mL, and just before bottling, the final product can be microbiologically stable. In this case, a significantly lower targeted amount of free sulfur dioxide, at around 30 mg/L, protects the wine. This contributes to a reduction in the total amount of sulfur dioxide of more than 60 mg/L (Ribéreau-Gayon *et al.*, 2006a).

### *Sorbic acid*

Sorbic acid is added to the wine just before bottling to avoid refermentation during storage - Figure 3 (Ribéreau-Gayon *et al.*, 2006a). It is an additive complementary to sulfur dioxide that acts against yeasts. It inhibits yeast growth without affecting bacteria or avoiding oxidation but it cannot be used for ceasing fermentation during “mutage” (Ribéreau-Gayon *et al.*, 2006a). The addition of sorbic acid during bottling can decrease the recommended dose of free sulfur dioxide only from 50 to 40 mg/L depending on the alcoholic strength and pH of wine (Ribéreau-Gayon *et al.*, 2006a). The maximum acceptable dose of sorbic acid is 200 mg/L, expressed as potassium sorbate, due to its high insolubility to water (EEC, 2019).

### *Dimethyl dicarbonate*

Dimethyl dicarbonate (DMDC) is an additive that acts against yeasts, and it is recommended to be used during bottling (Divol *et al.*, 2005). In the case of noble rot wines, it can be also used during “mutage” to decrease sulfur dioxide addition (Figure 3). DMDC cannot replace sulfur dioxide as antioxidant and antiseptic but it can cease more effectively ALF in healthy musts (Divol *et al.*, 2005). Besides, for the complex matrix of botrytized musts DMDC should always be used along with sulfiting. Methanol production when DMDC contacts with the wine is the main drawback of this operation; the maximum addition of 200 mg/L of DMDC releases by hydrolysis 96 mg/L of methanol (EEC, 2019).

Bearing in mind the maximum acceptable limit of methanol in white wines of 250 mg/L (OIV, 2020), the contribute of DMDC seems not be of special concern (Divol *et al.*, 2005).

### *Oenological tannins*

As aforementioned, even though laccase activity is reduced at the final stage of infection, the possibility of oxidizing phenolic compounds in the must is still high. In order to further suppress this enzyme along with sulfur dioxide, addition of oenological tannins before ALF, especially skin/seed tannins and gallotannins, is an efficient treatment - Figure 3 (Vignault *et al.*, 2020). Exogenous tannins when added in the medium, due to hydrogen bonds and van der Waals forces formed, combine and precipitate with proteins, including enzymes such as laccase, helping indirectly protein stabilization. They also act as antioxidants (Moreno and Peinado, 2012).

### **Protein stabilization**

#### *Bentonite*

Bentonite addition in noble rot must does not stabilize the medium from protein hazes during ALF and does not make easier the racking afterwards, since the amount of protective colloids such as glucans is at maximum levels at this time (Ribéreau-Gayon *et al.*, 2006a). In the case of fermenting must in barrels and age on lees, addition of bentonite after ALF is also not suitable. Contradictory to dry white wines, in botrytized wines addition of bentonite at this stage does not improve settling (Ribéreau-Gayon *et al.*, 2006a). Bentonite is used before bottling, when part of glucans has been eliminated by precipitation during aging, and only after conducting stability trials (Figure 3). As a fining agent, bentonite along with barrel aging and filtration reduce wine turbidity at desired levels (Ribéreau-Gayon *et al.*, 2006a).

#### *Stabilization with silica*

Another possible stabilization treatment to prevent protein turbidity in noble rot wines is the addition of siliceous earth, kieselsool or klebosol (Moreno and Peinado, 2012). It is an adsorbent treatment, like in bentonite case, where the dissociation of hydroxyl groups in an alkaline solution charges negatively the molecules, making possible their combination with the positively charged proteins, at wine pH, due to their high Isoelectric Point (IP) (Moreno and Peinado, 2012). This fining agent is active even in difficult mediums with high quantity of protective colloids, such as  $\beta$ -glucans, that make clarification very laborious. For this reason, silica is considered suitable for noble rot musts and wines (Moreno and Peinado, 2012). In the form of alkaline solution of 30%, the

recommended dose is between 25 and 100 mL/hL (Moreno and Peinado, 2012). For optimum results, better flocculation and settling, especially during barrel aging, it should be used in association with gelatin at a ratio 10:5 in favor of the silica - Figure 3 (Jackson, 2008). Silica should be added first to the medium, while both should stay in the wine for an optimum time of 14 days before racking (Moreno and Peinado, 2012).

### **Tartrate stabilization**

Stabilization by cooling is actually ineffective in botrytized wines, while stability trials with oxalic acid can indicate potential future problems due to its easy crystallization with calcium cations (Ribéreau-Gayon *et al.*, 2006b). Glucans as protective colloids can act sufficiently against tartaric crystallization. Effectiveness of the applied stabilization method should be estimated after six days at -8 °C for this complex wine matrix (Moreno and Peinado, 2012).

### **Clarification treatments**

#### *Filtration*

Wines produced from grapes affected by noble rot are difficult to clarify due to the high concentration of glucans that act as clogging colloids (Ribéreau-Gayon *et al.*, 2006b). Aging process along with glucanases addition reduce glucans and wine turbidity below 60 NTU. As a result, the use of diatomaceous earth with medium size kieselguhr for polishing filtration is possible. After filtration, turbidity below 1 NTU is guaranteed for no longer than one week (Ribéreau-Gayon *et al.*, 2006b). During this period and just before bottling, fine sheet filtration, where the sheet consists of cellulose matrix with kieselguhr and perlite, and membrane filtration for total sterilization of the medium is applicable (Figure 3). Sterilization of the equipment before and after every filtration reduces the possibility of refermentation during storage (Ribéreau-Gayon *et al.*, 2006b).

#### *Addition of beta-glucanases ( $\beta$ 1-3, $\beta$ 1-6)*

Beta-glucanases are very expensive enzymes able to hydrolyze  $\beta$ -glucans to glucose in order to avoid clogging problems during filtration. These enzymes are active up to 80% under an optimal temperature at around 40 °C, and at wine pH between 3.1 and 3.6. Total sulfur dioxide at 200 mg/L after “mutage” does not affect their enzymatic activity (Dubourdieu *et al.*, 1981). Taking into account that yeasts cell walls consist of glucans and mannoproteins, addition of the enzymes is advisable to be done after ALF to avoid fermentation problems - Figure 3 (Carrascosa *et al.*, 2011). In any case, mannoproteins liberation in the medium can be beneficial for both protein and

tartrate stabilization and improvement of organoleptic characteristics, explaining why these enzymes can also be used in other types of wines even without glucans presence (Waterhouse *et al.*, 2016). The maximum acceptable limit of glucanases addition is 3 g/hL (EEC, 2009).

### **Treatments to avoid for noble rot wines**

#### *Inoculation of lactic acid bacteria*

Lactic acid bacteria, such as *Oenococcus oeni*, are used for conducting malolactic fermentation (MLF) (Jackson, 2008). Inhibition of these bacteria can be achieved by the presence of free sulfur dioxide at quantities higher than 20 mg/L. Given that MLF is not desired for botrytized wines, because decrease of acidity will unbalance the wine by making it flabbier, their inoculation in wine is avoided (Jackson, 2008).

#### *Carboxymethylcellulose*

Carboxymethylcellulose (CMC) is a polar cellulose derivative, characterized by an electronegative charge, binding potassium tartrate crystals (electropositive charged), preventing their growth and precipitation (Moreno and Peinado, 2012; Pittari *et al.*, 2018). CMC efficiency in terms of time is limited as it cannot prevent the precipitation of calcium tartrate that generally occurs in wine after several years' aging. Given that CMC should only be used for wines without aging potentials destined to be consumed young, it is not recommended for the tartrate stabilization of botrytized wines (Moreno and Peinado, 2012).

#### *Ascorbic acid*

Ascorbic acid is an additive used before bottling to protect wines from oxidation. It is an antioxidant that acts against oxygen faster than sulfur dioxide, and it is mainly added in young white or rosé wines without aging potentials (Waterhouse *et al.*, 2016). The aromatic profile of these wines (fruity aromas) is based on the primary aromas of the grape varieties, the conservation of which is only possible under the absence of oxygen (Moreno-Arribas and Polo, 2009). The wine matrix of noble rot wines is totally different because it is already highly oxidized during botrytization, and in most of the cases these wines are passing through a period of barrel aging so the aromatic profile is mainly based on secondary and tertiary aromas. Given that the corresponding volatile compounds are stable in oxidative environment, the use of ascorbic acid is not necessary (Moreno-Arribas and Polo, 2009).

## **Wine aging**

### *Barrel aging*

The most iconic noble rot wines are aged from 16 to 24 months in oak barrels (Ribéreau-Gayon *et al.*, 2006a). The aging starts 15 days after “mutage” and each barrel is topped up very carefully once per week in the highest possible aseptic way. Given that possibility of refermentation is always high, it must always be taken under consideration by the winemaker. For wines fermented in vats, the first racking is carried out after the end of fermentation to eliminate yeasts still present in the wine. It is desirable that only fine lees remain in the wine to avoid production of reductive notes by sulfur reductase (Ribéreau-Gayon *et al.*, 2006a). For wines fermented in barrels, the first racking takes place during winter due to cold weather, while only fine lees remain in wine at that time. Finally, the last two rackings are done within the next six months - Figure 3 (Ribéreau-Gayon *et al.*, 2006a). During each racking practice barrels must be properly cleaned and treated with sulfur prior to reuse. Racking should always be a very delicate treatment in order to limit extended oxidation, preventing ethanal's formation that has very powerful affinity with sulfur dioxide. Decrease of free sulfur dioxide level always increases the instabilities of this type of wines (Ribéreau-Gayon *et al.*, 2006a).

Barrel aging is required for the evolution of the tertiary aromas. During this stage, flavors of dried or artificial fruits like apricot, quince, peach and grapefruit, honey, almonds and notes of wood are either intensified or developed (Ribéreau-Gayon *et al.*, 2006a). The exact physicochemical evolution of these wines during barrel aging is still not well clarified and studied.

### *Bottling aging*

A study on how aroma compounds were evolved during bottle aging of Sauternes wines revealed the total elimination of volatile thiols present in young botrytized wines except for 3MH (Bailly *et al.*, 2009). The concentration of this odorant compound can be even 50 times higher than its perception threshold even after six years of bottle aging (Bailly *et al.*, 2009). Furthermore, aromas from botrytization process, such as sotolon and others resulting from other 2-furanone derivatives, along with secondary aromas produced by fermentation like higher alcohols, and wood aged aromas deriving from barrel maturation, were still present in lower concentrations but still perceivable, in the samples. Finally, a furanone derivative known as abhexon, with honey and spicy flavor, was quantified at levels close to its

perception threshold of 7 µg/L after six years of bottle aging (Bailly *et al.*, 2009).

## **WINE SAFETY**

### **Mycotoxins**

Ochratoxin A (OTA) is a mycotoxin known for its toxic properties on living organisms (Purchase and Theron, 1968). In foods and fruits, including grapes and wines, it is present as a result of fungus infection, due to the metabolic pathways mainly of *Aspergillus* and *Penicillium* spp. (Purchase and Theron, 1968). Due to its toxic effect on human body, OTA's maximum acceptable level in wines is 2.0 µg/kg (EEC, 2006). Some types of wines are exempted from this regulation, but noble rot wines are not included in this group. Part of the botrytization process includes the simultaneous colonization of *B. cinerea* with other microbial on berries. Therefore, these wines are prone to potential contamination. In a study conducted on different noble rot wines derived from different regions across Europe, OTA was not identified in the samples analyzed (Valero *et al.*, 2008). What was proposed but it has not been proved yet, is that even if a small amount of OTA is produced by other microorganisms during infection, its degradation by *B. cinerea* is possible. The same trend of botrytized wines was followed in the case of icewines or late harvest sweet wines. On the contrary, toxin's content in sweet wines made of grapes left to dry in the sun was the highest (Mateo *et al.*, 2007; Valero *et al.*, 2008).

### **Biogenic amines**

Biogenic amines are toxic compounds that derive from decarboxylation of amino-acids under the enzymatic activity of decarboxylase (Bauza *et al.*, 1995). The origin of this enzyme is mainly correlated with microorganisms' presence in berries, such as *B. cinerea*, or in wines, such as lactic acid bacteria. Either way, a small amount is always produced by yeasts during alcoholic fermentation (Bauza *et al.*, 1995). The average values in wine vary from 1 to 2 mg/L, while when exceeds 10 mg/L, it can be harmful for allergic consumers. The most plentiful and important biogenic amines found in wines are histamine, tyramine, putrescine, cadaverine, phenylethylamine and isoamylamine (Bauza *et al.*, 1995). A study carried out on different types of wine showed that the levels of these amines were higher in noble rot wines than in the dry ones (Kiss and Sass-Kiss, 2005). Up to 13 biogenic amines were identified in botrytized wines; concentrations much higher than 1 mg/L were found for phenylethylamine, putrescine and tyramine. Phenylethylamine and agmatine were

connected with *B. cinerea*, while tyramine was correlated with fermentation and other winemaking techniques (Kiss and Sass-Kiss, 2005).

Concerning the amount of free amino-acids and biogenic amines in Tokaji Aszú wines, some studies indicated tyramine, putrescine and histamine as the main biogenic amines with a total content of 24 mg/L (Csomós and Simon-Sarkadi, 2002). The argue that botrytization changed the biogenic amines profile both on grapes and wine was supported by Hajós *et al.* (2000), who also highlighted that these amines' concentrations increased during aging. Finally, except for botrytization, other factors such as vintage, region, weather and winemaking practices need to be taken into consideration (Hajós *et al.*, 2000; Sass-Kiss *et al.*, 2000; Csomós and Simon-Sarkadi, 2002).

## CONCLUSIONS

Botrytized wines are very interesting in every aspect of winemaking, starting from grape to bottle. *B. cinerea* plays a critical role in their quality by changing all the chemical characteristics of berries and musts. The techniques used for the production of these wines show remarkable particularities. Even though these are wines produced for many years and well established in the market, systematic studying upon them, with the slight exception of Sauternes and Tokaji Aszú wines, is not as evolved as expected.

Earlier studies were focused on the direct effects of the fungus on berries and musts and tried to propose sustainable practices during winemaking. In contrast, current studies are searching for the activity of *B. cinerea*'s genome and how it affects berry's transcription factors that lead to specific metabolic pathways. Further research is needed to understand the behavior of sulfur dioxide in the complex matrix of noble rot wines in order to reduce its additions by proposing alternatives. It is vital for noble rot wine industry to adapt to the current marketing trends, since consumers are searching for more and more "healthier" wines with limited sulfur dioxide and ethanol content.

The aromatic profile of these wines is a subtopic studied in depth. On the contrary, wine's chemical evolution and oxygen participation during barrel and bottle aging need further investigation. The importance of climate change that might lead to problematic botrytization and whether or not artificial induction of *B. cinerea* is possible is a very controversial topic that also needs further discuss. The complex combination of many factors to produce noble rot wines, starting from the obligation of having very specific environmental conditions, explains the

rarity of noble rot occurrence and highlights the uniqueness of the final product.

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