Chapter 1

General Introduction

1 Fungal infections in the vineyard

Winemaking has a long history representing one of the oldest food processing techniques performed by humans since ancient civilizations. The earliest records of wine production were found between 5400 and 5000 BC in Iran at the Hajji Firuz Tepe site (Pérez-Torrado et al., 2018). Over the centuries, wine was part of the human culture concerning dietary and socio-religious aspects (Soleas et al., 1997), and it has always been seen as a beverage of the affluent community (Bisson et al., 2002). Since today, people expect a high quality standard of wine, being a product enjoyable in all sensory aspects, produced in an environmentally sustainable manner, and offering health-promoting properties (Bisson et al., 2002). To guarantee a good wine quality, winemakers need to compete with several challenges that concern horticulture as well as viticulture. A long-standing problem are fungal infections of the grapevine, which may impair wine quality in terms of sensory properties including aroma, flavor, and color.

The European grapevine *Vitis vinifera* L. is mainly used for commercial wine production. It is a perennial plant grown in temperate regions of the world with the capability to withstand environmental stress (Mullins et al., 1992). A number of plant diseases may affect the grapevine, among which fungal pathogens are the most important (Steel et al., 2013). Many parts of the grapevine are susceptible to fungal attacks including the roots, trunk, canes, leaves, and berries (Hocking et al., 2007). Fungal infections of the grapevine may result in large economic losses in wine production, mainly attributed to infections of the berries, which deteriorate grape and wine quality (Steel et al., 2013). The berries are commonly infected by the mildew pathogens *Erysiphe necator* (*Uncinula necator*) and

Plasmopara viticola, as well as *Alternaria* spp., *Aspergillus* spp., *Botrytis cinerea*, *Cladosporium* spp., *Penicillium* spp., and *Rhizopus* spp. (Hocking et al., 2007). Among these, *B. cinerea* is a pathogen frequently found on berries (Steel et al., 2013).

1.1 *Botrytis cinerea* infections

B. cinerea is a ubiquitous, filamentous, and necrotrophic fungus infecting a wide range of plants as a non-host specific organism (Leroux, 2007). It occurs in vineyards all over the world being responsible for the most serious diseases of the grapevine, the gray mold or *Botrytis* bunch rot (Steel et al., 2013). The infection of grapes can also be desired as the noble rot for the production of sweet wines when climatic conditions are favorable, whereas the infection in an early ripening stage with low sugar and high acid concentrations leads to spoilage of the crop (Vortkamp et al., 2013). The gray mold can have several negative consequences on qualities from economical and enological points of view (Ky et al., 2012). It leads to a drastically reduced yield at harvest, which in turn reduces the volume of the wine (Dubos, 1999). The enological quality impact affects the grapes' chemical composition, including major quality compounds such as sugars, organic acids as well as various aroma and phenolic compounds (Ribéreau-Gayon et al., 1980). Organoleptic deteriorations such as mushroom and earthy off-odors have been described (La Guerche et al., 2006). Further problems arise due to the production of a secretome by the fungus, mainly consisting of β-D-glucan, which causes clarification problems (Thakur et al., 2018). The glucan-rich secretome also contains extracellular enzymes as part of the fungal resistance mechanisms against host responses (Gil-ad et al., 2001). During the infection of the grape, *B. cinerea* utilizes several extracellular enzymes to penetrate the grape skin and epidermic layers, and to counteract antifungal agents produced in the grape skin, mainly phenolic compounds (Ky et al., 2012). The stilbene resveratrol is primarily synthesized by the grapevine after fungal infestation and is converted to derivatives like pterostilbene, and δ- and ε-viniferin (Hammerschmidt, 2004). Viniferins are known to exhibit antifungal effects against downy mildew (*P. viticola*) or gray mold (Ehrhardt et al., 2014). Concurrently to the defense of the plant, *B. cinerea* produces laccase to degrade and inactivate antifungal agents. Laccase together with grape tyrosinase represent polyphenoloxidases (PPOs), which are responsible for the quality deterioration of mainly white wine due to oxidative discoloration (Dewey et al.,

2008). The following sections describe important issues of wine quality which are connected to *Botrytis* infections of the grapes, but also to the disease management of fungal infections with copper-based fungicides.

2 Copper in winemaking

The application of copper-containing agents represents one of the oldest approaches to control fungal pathogens in viticulture. Intensive use of copper-based fungicides occurs in the European vineyard since the end of the 19th century to reduce major diseases like downy mildew (Komárek et al., 2010). The Bordeaux mixture was discovered in France in the 1880s. As a simple mixture of copper sulfate and lime, it was the first commercially successful fungicide (Ayres, 2004). Copper is also effective as bactericide, herbicide, and against several other insect pests and diseases (Provenzano et al., 2010). With a greater demand for organic wines, the use of copper- and sulfur-based biopesticides might still increase, since it is the most important pesticide in organic viticulture (Pedneault & Provost, 2016). The perennial application of copper-containing fungicides on the same land together with the low mobility of copper in the soil has led to copper accumulation in vineyard soils and groundwater, causing a risk for human health and the ecosystem (Besnard et al., 2001; Komárek et al., 2010). This problem occurs especially in regions where wet climate enhances the proliferation of fungal diseases (Mirlean et al., 2007). It further raises concerns for fermentation and wine quality, since processing of the grapes with soil copper which is transported over the roots through the transportation system to the grapes or copper attached on the grape surface may result in musts and wines with elevated copper concentrations (Sun et al., 2019). Copper levels in vineyard soils often exceed 200 mg·kg^{-1}, whereas the warning and critical legislative limits in the EU were set to copper concentrations in agricultural soils of 50 and 140 mg·kg^{-1}, respectively (Komárek et al., 2010). The determination of a threshold that causes risk is difficult because the mobility and availability of copper are always connected to soil properties such as organic carbon, texture, and pH (Ballabio et al., 2018). An educated use of copper is of great importance for the ecosystem and human health. The European Commission, thus, has limited the annual dose of copper compounds including copper hydroxide, copper oxychloride, copper oxide, Bordeaux mixture, and tribasic copper sulfate to 4 kg copper per ha in the Commission Implementing Regulation (EU) from December 2018.

2.1 Influence of copper on *Saccharomyces cerevisiae*

At cellular level, copper is an essential micronutrient (1–10 µM) required for metabolic processes as a cofactor of enzymes, and is involved in redox reactions and electron transport based on its ability to undergo Cu(I)-to-Cu(II) transitions (Cervantes & Gutierrez-Corona, 1994; Avery et al., 1996). Toxic concentrations, however, influence microbial metabolism and growth due to interactions with nucleic acids, enzyme active sides, and cellular or organellar membranes (Avery et al., 1996). Disruptive effects of copper are especially linked to the induction of oxidative stress within the cells. The copper-mediated Fenton reaction promotes the formation of an elevated cellular level of free hydroxyl radicals ($OH^{\bullet}$) (Shanmuganathan et al., 2004). Major targets of oxidation by $OH^{\bullet}$ and other reactive oxygen species (ROS) are the plasma membrane and proteins (Avery et al., 1996; Shanmuganathan et al., 2004). Free radicals and the redox-active nature of copper lead to a loss of membrane integrity and cell death, usually due to membrane lipid peroxidation. Several studies reported the copper-induced membrane permeabilization in *Saccharomyces cerevisiae*, which is manifested in an efflux of mobile cellular solutes (e.g., K^+) (Avery et al., 1996; Howlett & Avery, 1997). Proteins are affected by ROS due to oxidation of their amino acid side chains inducing hydroxyl or carbonyl derivatives or the cleavage of peptide bonds (Costa et al., 2002; Shanmuganathan et al., 2004). In *S. cerevisiae* high copper exposure predominantly affected catabolic enzymes by specific carbonylation. The majority of proteins susceptible to oxidation were enzymes participating directly in glycolysis or fermentation of the product of glycolysis, pyruvate, but the alcohol dehydrogenase (ADH) (isoform ADHI) has been described to be the most heavily oxidized enzyme (Shanmuganathan et al., 2004). In *S. cerevisiae*, seven genes have been found (ADH1–ADH7) which encode the isoenzymes ADHI to ADHVII (Smidt et al., 2008). Among these, the ADHI is responsible for reducing acetaldehyde to ethanol during fermentation. This was proven with ADHI-defective mutants which showed effects like acetaldehyde accumulation and reduced alcohol production (Ciriacy, 1997). The reversible reduction of acetaldehyde to ethanol generates NAD^+ or NADH depending on the direction (Mauricio et al., 1997). Another impact is the direct substitution of metals at the enzyme active site, which may reduce or inhibit enzyme activities of the yeast. Due to the tetrameric conformation of ADH (150 kDa) that contains structural and catalytic zinc

(Magonet et al., 1992), copper or other transition metals can replace zinc, leading to reduced specific enzyme activity (Cavaletto et al., 2000; Vanni et al., 2002). Copper has led to the highest inhibition efficiency of ADHI in *S. cerevisiae* compared to other transition metals (Cavaletto et al., 2000). All these mechanisms cause serious problems in winemaking when the yeast is exposed to copper stress during fermentation. **Chapter 2** shows the effects of different copper concentrations in the must on two commercially available wine yeasts strains, regarding their fermentation efficiency, vitality, specific ADH activity, and other important fermentation parameters.

2.2 Intracellular copper disposition in *Saccharomyces cerevisiae*

To prevent copper deficiency as well as toxicity, *S. cerevisiae* has developed a complex mechanism of copper trafficking inside the cell to maintain copper homeostasis. It is important to control the transport of this essential micronutrient to its targets, such as enzymes or mitochondria, to prevent spurious interactions. In case of toxic copper amounts, the regulation of the transport and copper sequestration is induced (Vest et al., 2019).

To overcome the plasma membrane as the major barrier of copper distribution in the cell, it contains high- and low-affinity transporters to import copper (Culotta et al., 1999). Copper is transported in its cuprous form, thus, an extracellular metalloreductase is involved in an efficient copper transport via the high-affinity copper transporter 1 (Ctr1) (Yamaguchi-Iwai et al., 1997). Once inside the cell, copper chaperones in the cytoplasm prevent inappropriate interactions by copper-binding and further induce the copper distribution to target enzymes (Vest et al., 2019). This leads to the estimation that the concentration of cytoplasmic free copper is equal to zero. Sequestration of copper is achieved by transport to organelles like the trans-Golgi network, vacuole, or mitochondria (Vest et al., 2019). Besides the chaperone-mediated binding, copper can also be complexed to metallothioneins to prevent redox cycling or uncontrolled binding reactions. In *S. cerevisiae*, two metallothioneins, the Cup1 (cuprum 1) and Crs5 (copper resistant suppressor 5) have been described (Winge et al., 1985; Culotta et al., 1994), but Cup1 is responsible for the majority of copper resistance in yeasts due to its high expression under copper exposure (Jensen et al., 1996). As a consequence of toxic intracellular copper

levels in the yeast cell, the transcription of metallothionein genes is, thus, induced and the degradation of the Ctr1 has been described to limit the toxic copper influx (Ooi et al., 1996; Vest et al., 2019). A further important role in heavy metal detoxification can be ascribed to the redox-active tripeptide glutathione (GSH), which is described in section 4.

2.3 Influence of copper on wine quality

Copper may deteriorate wine quality due to a stress-induced stuck and sluggish fermentation. This, however, strongly depends on the copper sensitivity of the yeast strain used (Ferreira et al., 2006). The delayed fermentation reduces alcohol production and increases residual sugar levels in the wine (Bisson, 1999). Furthermore, copper exposition of the yeast during fermentation might increase the volatile acidity of the wine, mainly due to an undesired acetic acid production (Ferreira et al., 2006). Since copper can inhibit important enzymes of *S. cerevisiae* including the ADH, the role of acetaldehyde as an important volatile aroma component in wine needs to be considered. At low levels, acetaldehyde gives a pleasant fruity flavor, but high amounts result in a pungent irritating flavor (Miyake & Shibamoto, 1993; Lachenmeier & Sohnius, 2008). The high affinity to the preservative sulfur dioxide (SO_2) reduces the aroma effect of acetaldehyde and the functionality of SO_2 (Jackowetz et al., 2011). A further influence on wine organoleptic properties arises due to the high reactivity of acetaldehyde with phenolic compounds. It mediates a fast polymerization of anthocyanins and tannins or flavanols, which increases color stability and intensity, but further polymerization with flavanols leads to precipitation, color instability, and color decrease (Es-Safi et al., 2002; Li et al., 2008).

Copper itself is well-known to cause oxidative spoilage of the wine since it catalyzes the first step of non-enzymatic browning in white wine. This follows the same pathways similar to the enzymatic browning described in section 3. Copper oxidizes phenols to reactive *ortho*-quinones, which further polymerize and enhance the browning degree of white wine (Li et al., 2008). Other influences of copper described are the pinking of wine as well as haze formation (Provenzano et al., 2010). This is mainly a problem during wine aging together with a loss of typical wine aromas. An example is the decrease of the strong aroma compound 3-mercaptohexanol, characteristic for a passionfruit odor, during

copper exposure of the wine (Ugliano et al., 2011). Copper reactivity with thiols is a known reaction in enology. It is also utilized to remove aromatic defects induced by thiol compounds by adding copper sulfate (Darriet et al., 2001). This is, however, an additional source of elevated copper concentrations in the wine, causing the risk of unwanted side reactions.

3 Influence of polyphenoloxidases on wine color

Wine color is an important quality attribute for the sensory properties of wine. It is usually used as the first clue for wine evaluation giving evidence, for example, of the age or degree of oxidation (Kennedy, 2010). Wine color depends on the chemical composition of the wine, primarily on phenols. Hydroxycinnamates like caffeic, ferulic, and *p*-coumaric acids, as well as their esters with tartaric acid such as caftaric acid, are the principal phenols in white must and wine, with small amounts of flavanols (Waterhouse, 2002). These phenols provide the characteristic yellow color of most white wines (Kennedy 2010). Anthocyanins are responsible for the color of red wines. In red grapes, six anthocyanidins have been identified, with malvidin as the most abundant in *V. vinifera* grapes (Garrido & Borges, 2013). Among the flavonoids, the predominant flavanol in wine is catechin. It is derived from the solid parts of grapes (skin and seeds) (Garrido & Borges, 2013). Seed extraction and extended maceration techniques obtain high levels of catechin (Waterhouse, 2002). The phenolic composition of must and wine strongly depends on winemaking techniques and vinification methods, but also on the grape variety, maturity, and environmental factors, e.g., climate and soil in the vineyards (Fang et al., 2008; Garrido & Borges, 2013). Despite the desired coloring of wine due to its phenolic composition, phenols also contribute to negative color changes throughout the whole winemaking process. The susceptibility of phenols to enzymatic oxidation especially in white must and wine is a well-known problem in winemaking.

3.1 Tyrosinase and laccase

PPOs are copper-containing enzymes responsible for the oxidation of phenols during the processing of grapes in the presence of oxygen. Tyrosinase (*o*-diphenol oxidoreductase, E.C. 1.10.3.1) is a PPO naturally produced by the grape berry, whereas the laccase (*p*-diphenol oxidoreductase, E.C. 1.10.3.2) is a PPO produced and secreted by *B. cinerea*

after the infestation of the grape (Dewey et al., 2008). In intact cells of the grape berry, tyrosinase located in the cytoplasm is not in contact with phenolic compounds, which are present in the vacuole, due to different cellular compartments (Wang, 1990). During grape crushing, membrane systems are disrupted and tyrosinase is released into the must, where it catalyzes the hydroxylation of monophenols to *ortho*-diphenols with consecutive oxidation of the catechol to *ortho*-quinones (Claus et al., 2014; Oliveira et al., 2011). These highly reactive species can undergo condensation with other phenolic compounds to form polymerized brown products (Singleton, 1987; Li et al., 2008). In contrast to tyrosinase, laccase causes a larger problem in winemaking regarding quality deterioration due to oxidative browning in white wine and color loss of red wine (Dewey et al., 2008). It is more active than tyrosinase, tolerant against SO_2 and heat treatment usually used in winemaking, stable against alcohol and low pH present in must and wine (Dewey et al., 2008; Ugliano, 2009). Furthermore, the laccase oxidizes a wider range of substrates including mono-, di-, and polyphenols, amino phenols, methoxy phenols, aromatic amines, and ascorbate in a reaction coupled to a four-electron reduction of oxygen to water (Madhavi & Lele, 2009). The first product of oxidation, an unstable free radical, may undergo a second enzyme-catalyzed reaction to form a *para*-quinone (Thurston, 1994; Oliveira et al., 2011). The free radicals as well as the quinones, may polymerize to form brown products (Thurston, 1994). This makes *Botrytis*-infected grapes commonly undesired in winemaking. However, there is strong evidence that different *B. cinerea* isolates do not secrete the same levels of laccases (Cotoras & Silva, 2005) and the degree of infection does not always correspond to laccase activities in the must (Macheix et al., 1991; Perino et al., 1994). **Chapter 3** demonstrates specific laccase activities and the varying catalytic activity of laccase-containing secretomes of different *B. cinerea* strains toward typical wine phenols.

Extracellular *B. cinerea* laccases show a large heterogeneity in their molecular properties. They may occur as different isoenzymes emerging from strain-specific variations, different culture conditions, and purification methods (Claus et al., 2014). Further variations were observed in their inducibility, substrate specificity, molecular weight, isoelectric point, and sugar content as well as temperature and pH optimum (Dubernet et al., 1977; Gigi et al., 1981; Marbach et al., 1984; Zouari et al., 1987; Bollag et al., 1988).

Inducibility of extracellular laccase production may be affected by the addition of grape juice or phenolic compounds, such as gallic and caffeic acids to liquid media (Gigi et al., 1980). Laccase activity *in vitro* is mostly quantified by photometric tests with the use of phenolic substrates by the formation of colored products (Johannes & Majcherczyk, 2000). Common tests utilize the phenolic substrates guaiacol, 2,6-dimethoxyphenol (Prillinger & Esser, 1977), and syringaldazine (Harkin & Obst, 1973), or the non-phenolic substrate 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Childs & Bardsley, 1975). The phenolic substrates form quinones, whereas ABTS results in a colored radical cation (Johannes & Majcherczyk, 2000).

3.2 Enological practices to reduce laccase activity

Some winemaking practices are available to mitigate laccase activity in must and wine. The first step is to minimize the use of infected grapes by selective hand harvesting and manual or automated grape sorting tables in the winery (Steel et al., 2013). Cool conditions at harvest (Wilker, 2010a) followed by gentle and fast transport and immediate processing of the grapes also reduce the effects of laccase (Steel et al., 2013). Common SO_2 levels used in winemaking do not inhibit laccase activity (Dewey et al., 2008), however, high concentrations of SO_2 (100–200 $mg \cdot L^{-1}$) added to infected fruits or musts (Steel et al., 2013) with concurrent cooling to 10–12 °C might impede oxidation due to the reduction of laccase activity (Wilker, 2010b). Depending on the substrate, *B. cinerea* laccase activity decreases at temperatures above 60 °C (Marbach et al., 1984). Inactivation of laccase in the must, thus, requires pasteurization temperatures exceeding 60 °C, while 80 °C with a holding time of 5 s has been recommended (Steel et al., 2013). Since the laccase requires oxygen to catalyze substrate transformation, a limited exposure of infected grapes to normal atmospheric conditions can control oxidative browning reactions. This can be achieved by the use of inert gas covers during procedures such as pressing, transfers, and for in-tank ullage space (Wilker, 2010a). Further practices such as whole bunch pressing reduce oxygen exposure during white wine production, in contrast to maceration steps of crushing and destemming (Steel et al., 2013). Total oxygen exclusion, however, is not appropriate since yeast starter cultures require small amounts of oxygen during fermentation (Wilker, 2010b). Copper chelating-agents such as EDTA, citric acid, and oxalic acid, as well as the heavy metals copper and

cadmium, have been described to serve as laccase inhibitors *in vitro* (Lorenzo et al., 2005), however, their use in winemaking is inappropriate. To avoid the necessity of all these laccase-inhibiting procedures during winemaking, a good disease management is required to inhibit the fungal attack of the grapevine and, thus, reduce the occurrence of laccase in must and wine.

4 Glutathione in winemaking

The lack of suitable alternatives to copper-based fungicides in organic viticulture requires a tool to reduce copper stress for the yeast as well as oxidation in wine. GSH provides a potent natural antioxidative additive in winemaking and simultaneously offers a defense mechanism of the yeast against oxidative stress. Its natural occurrence in the grapes and yeasts presents an ideal requirement for its increased use as an additive to must and wine.

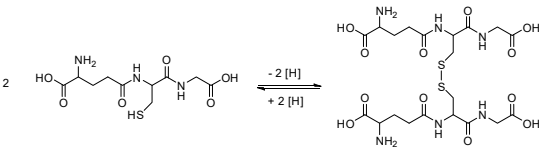

Figure 1.1: Reduced glutathione (GSH, left) and the oxidized glutathione disulfide (GSSG, right).

4.1 The role of glutathione at cellular level

GSH is the major non-protein thiol present in high amounts up to 10 mM in most living cells from prokaryotes to eukaryotes (Penninckx, 2000; Penninckx, 2002). The pseudo-tripeptide of L-glutamate, L-cysteine, and glycine is synthesized intracellularly in two ATP-dependent steps by γ-glutamylcysteine synthetase (GSH1) and GSH synthetase (GSH2) (Grant et al., 1996). GSH is generally present with >90% in the reduced form in the cell while the rest occurs in its oxidized form as glutathione disulfide (GSSG) (see **Figure 1.1**) and mixed disulfides, GS-S-Cys and GS-S-CoA (Kritzinger et al., 2013). The NADPH-dependent glutathione reductase can reduce the GSSG back to GSH being again available for the cell (Grant & Dawes, 1996). GSH acts as a strong cellular redox buffer (Penninckx, 2002) and has a strong nucleophilic property related to its thiol moiety of the cysteine residue that protects cells by scavenging free radicals (Grant & Dawes, 1996)