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ABSTRACT

Enzymatic parameters (K_M and V_{max}), residual activity, effect of bentonite and electrophoresis characterization of laccase in the presence of different oenological tannins (OT) were investigated in relation to *B. cinerea* negative effects in grapes and musts. Five OT were tested (gallotannin, ellagitannin, quebracho, grape-skin and grape-seed) in comparison with ascorbic acid (AA), sulfur dioxide (SO_2) and bentonite. We added OT, AA, SO_2 and bentonite to botrytized must obtained by inoculation of grapes with B. *cinerea* strain (strain 213). Laccase activity was measured by the syringaldazine method at different concentrations of substrate. Enzymatic parameters were determined using Michaelis-Menten and Lineweaver-Burk plots. The B. *cinerea* strain was also grown in a liquid medium for laccase production. Molecular weight of laccases and effect of OT upon these laccases were studied by SDS-PAGE. Results confirm that bentonite, contrary to OT, did not permit to reduce laccase activity. Regardless the tannin considered, V_{max}, K_M and laccase activity were reduced and gallotannin, grape-skin and grape-seed tannin presented the greatest ability. Efficiency of grape-seed tannin addition in order to reduce the laccase activity, was comparable to that of AA or SO_2 at the typical doses employed in oenology for each one. Oenological tannins appear to be excellent processing aids to prevent laccase effects and contribute to reduce the use of SO_2 in grapes and musts.

Keywords: oenological tannins, grapes, musts, Botrytis bunch rot, laccases, kinetics parameters, electrophoresis.

1. Introduction

Botrytis cinerea, is a ubiquitous, filamentous and necrotrophic fungus excreting metabolites (glycerol, gluconic acid, β-glucans) and enzymes (pectinases, proteases, tyrosinases and laccases) in the host cells (Steel, Blackman, & Schmidtke, 2013). Laccases (EC 1.10.3.2) are o-diphenol and p-diphenol: dioxygen oxidoreductases. These multi-copper glycoproteins use molecular oxygen to oxidize various aromatic and

non-aromatic compounds by a radical-catalyzed reaction mechanism (Claus, 2004). In this pathogen, two genes have been found encoding laccases with molecular weights of about 60 kDa (Claus, Sabel, & König, 2014) and the molecular mass of the monomer ranges from about 50 to 100 kDa (Claus, 2004). An important feature of laccases is to be very stable under wine conditions and they have serious impacts on the phenolic composition and quality of musts and wines (More et al., 2011).

Under particular environmental and grape growing conditions, *B. cinerea* can affect positively the grapes by causing "noble rot" leading to high-priced, natural sweet white wines, such as Tokaji Aszú, Sauternes or Passito wines for example (Magyar, 2011). This particular infection pathway, associated with noble rot, promotes favorable biochemical changes in grape berries, notably by the accumulation of secondary metabolites enhancing the grape composition (Blanco-Ulate et al., 2015). Nevertheless, *B. cinerea*, can also cause grey mould or botrytis bunch rot (BBR), responsible for huge economic losses each year for the wine industry worldwide. Infection of bunch by the pathogen provokes serious biological and chemical changes impacting negatively organoleptic qualities of wines (J. Ribéreau-Gayon, Ribéreau-Gayon, & Seguin, 1980). For a qualitative red wine, a vintage contaminated by the pathogen at harvest at the rate of 5% in severity show irreversible consequences on the wine organoleptic features (Ky et al., 2012).

As recently reviewed and thoroughly investigated, when climatic conditions are wet under mild weather conditions, *B. cinerea* can infect directly grape berries, in particular from veraison onwards (Ciliberti, Fermaud, Roudet, & Rossi, 2015; Hill, Beresford, & Evans, 2018). The infection by the fungus begins in the grape berry and continues in the grape juice, leading to the oxidation of polyphenols and then the alteration of the color. The phenomenon of polyphenol oxidation is induced by the production of laccases from *B. cinerea*. The laccases produced, oxidize the polyphenols into quinones which themselves polymerized forming brown compounds. This reaction is called oxidase breakage and corresponds to a color degradation and an increase in instability (Pourcel, Routaboul, Cheynier, Lepiniec, & Debeaujon, 2007). The oxidase breakage can take place in red grape juice as well as in white inducing brick tints or brown tints, respectively. Moreover, changes in color due to the laccases produced by the pathogen are accompanied by others changes in the organoleptic wine qualities which are impacted by changing their equilibrium, body and/or mouthfeel (Claus et al., 2014). Several studies have shown that grey mold leads to the development of organoleptic deviations in grapes and wines and depreciation of botrytized wines have been attributed to off-flavors such as ‘damp earth’, ‘vegetal/herbal like’ and ‘mushroom’ (Ky et al., 2012). Various authors have reported organoleptic defects, in musts as in wines made from rotten grapes, such as mushroom, mouldy, camphoric odors (Lopez Pinar, Rauhut, Ruehl, & Buettner, 2016). Geosmin and 1-octen-3-one have been also identified in musts and wines made from botrytized grapes (La Guerche, Chamont, Blancard, Dubourdieu, & Darriet, 2005; La Guerche, De Senneville, Blancard, & Darriet, 2007). Regarding mouthfeel, botrytized red wines were described as less astringent, according with a decrease of their mDP (Ky et al., 2012).

Oenological tannins are usually classified in two families which are hydrolysable and condensed tannins. Hydrolysable tannins include gallotannins and ellagitannins whereas condensed tannins include procyanidins/prodelphinidins and profisetinidins/prorobitenidins. Each group of tannins present different composition, nature, and chemical structure associated with different properties. During the winemaking process, oenological tannins can be used at different doses, i.e. 5 to 50 g/hL of grape must, as a common

practice (Obradovic, Schulz, & Oatey, 2005). The oenological tannins are commonly used by winemakers to improve and stabilize the color of red wines since they are efficient copigments and/or can form new pigments (Neves, Spranger, Zhao, Leandro, & Sun, 2010; Versari, du Toit, & Parpinello, 2013). Winemakers, also used them as antioxidant or antioxidasic compounds in order to protect wine against oxidation (González-Centeno et al., 2012; Pascual et al., 2017; Vignault et al., 2018). They can also be used in order to i) help protein fining and prevent protein haze (P. Ribéreau-Gayon, Dubourdieu, & Donèche, 2006), ii) improve wine structure/mouthfeel (Preys et al., 2006) and iii) eliminate reduction odors (Vivas, 2001). Nevertheless, until very recently , addition of oenological tannins was only accepted as an aid practice to facilitate the fining of musts and wines, by the International Organization of Vine and Wine (OIV, 2015). Very recently, OIV has also authorized its use for two new purposes. The first is to contribute to the antioxidant protection of components of the must and wine, and their second is to promote the expression, stabilization and preservation of color of wines (OENO-TECHNO 17-612 and OENO-TECHNO 17-613).

Even though properties of oenological tannins have been widely described in the literature, antioxidasic properties (anti-laccase) are not yet well documented. Until now, it is only possible to inactivate or inhibit the laccase enzyme by thermovinification or by sulfur dioxide addition (P. Ribéreau-Gayon et al., 2006). Inert gas or ascorbic acid in presence of sulfur dioxide may also be used to protect the grape juice against oxidation (Li, Guo, & Wang, 2008). Nevertheless, in our previous study (Vignault et al., 2019), oenological tannins have been shown as good candidates to prevent the damage induced by laccases produced by *B. cinerea*. Oenological tannins allowed us to protect color of wines infected by *B. cinerea* and to reduce the laccase activity.

The aim of our present research was to complete our previous study by understanding the mechanisms of action of oenological tannins against laccases produced by *B. cinerea* (strain 213) in grapes and musts. Our objective was also to compare the effects of oenological tannins against laccase activity in grapes/musts with the two usual oenological additives used in the wine industry nowadays: ascorbic acid and sulfur dioxide.

2. Materials and methods

2.1. Chemicals and equipment

All samples and standards were handled without any exposure to light. L-(+)-tartaric acid, sodium hydroxide, sodium acetate, polyvinylpolypyrrolidone (PVPP), Tween 80, glycerol, gallic acid, L-histidine, $CuSO_4$, $NaNO_3$, NaCl, KCl, $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, KH_2PO_4, $MgSO_4.7H_2O$, Bradford reagent, bovine serum albumin (BSA) and syringaldazine were purchased from Sigma-Aldrich (Madrid, Spain). D-(+)-glucose, peptone, agar and yeast extract were purchased from Panreac (Barcelona, Spain). Ethanol (96 % vol.) and hydrochloric acid were supplied by Fisher Scientific (Madrid, Spain). Yeast (Zymaflore® Spark), nutrients (Nutristart®) and bentonite (MICROCOL® ALPHA) were provided by Laffort (Floirac, France).

The equipment used was as follows: a spectrophotometer UV-Vis Helios Alpha™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); an incubator IPP 260 (DD Biolab, Barcelona, Spain); a centrifuge

Heraeus™ Primo™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); and a CB Standard Balance (Cobos, Barcelona, Spain). All the materials for the micro-vinification were provided by the cellar "Mas dels Frares" of the Enology Faculty of the Rovira i Virgili University (Constanti, AOC Tarragona, Spain).

2.2. Commercial tannins

Five commercial tannins, representing the main botanical origins, were used including three condensed tannins: one procyanidin from grape seeds, one procyanidin/prodelphinidin from grape skin and one profisetinidin from quebracho. Furthermore, two hydrolysable tannins were studied: one gallotannin from nut gall and one ellagitannin from oak. All these tannins were provided by Laffort (Floirac, France).

2.3. Fruit sampling, inoculation with B. cinerea and botrytized-must elaboration

During the 2017 vintage, healthy grapes (*V. vinifera*, cv. Muscat d'Alexandrie; Variety number VIVC 8241) were collected on September 18th (around 50 kg) from the experimental vineyard, planted in 1992, in the Enology Faculty of the Rovira i Virgili, University in Constanti (AOC Tarragona; 41°8′54.17″ N and 1°11′53.89″ E). The vineyard is at 87 m height above sea level, and groundwater is located at a depth of around 4 m. The vines were trained on a vertical trellis system and arranged in rows 2.80 m apart, with 1.20 m spacing between vines. They were pruned using a double "Cordon de Royat" system, with 16 buds, 8 on each cane.

The *B. cinerea* single-spore isolate 213, originally isolated from grapevine leaf in 1998, was selected from the collection of UMR SAVE, Bordeaux (Martinez et al., 2003). It was selected because of its virulence on grapevine leaves and berries and because it is a *transposa* type strain (Ky et al., 2012; Martinez et al., 2003; Martinez, Dubos, & Fermaud, 2005). The pathogen was cultured on Yeast Peptone Dextrose Petri plates (YPD: 20 g/L of peptone and glucose; 10 g/L of yeast extract and 17 g/L of agar in distillated water) and incubated about 1 week at 20°C before use. The harvested grapes were placed in five plastic boxes (600 x 400 x 200 mm) and were inoculated by spraying a spore suspension (1.10^6 conidia/mL; 1 drop of Tween 80; 50 g/L of glucose; sterilized water) until the complete fruit surface was covered by the spore suspension. The plastic boxes containing the grapes were then incubated, for around three weeks, at 20°C surrounded by two plastic boxes containing sterile water to maintain the relative humidity, i.e. RH ranging from 90% to 100%.

The botrytized grapes were beforehand sorted visually to remove undesirable rotten berries due to other fungal development, such as notably *Penicillium spp* (blue-green color) and/or other fungal species (*Alternaria spp*. or *Clostridium spp*.), or acetic bacteria (red-pink color). Then, these selected botrytized grapes were crushed and pressed to obtain the botrytized grape juice using a small pneumatic press (Venmhidrprei-040, Invia, Vilafranca del Penedes, Spain). The juice was recovered under dry ice in order to keep it protected from oxidation, but without any addition of sulfur dioxide for not inhibiting laccase activity. The botrytized grape juice was centrifuged at 8,500 rpm for 5 minutes and immediately stocked in glass bottles at - 4°C.

2.4. Laccase activity assays

Tannins solutions were prepared in model wine solution (12% of ethanol, 4 g/L of tartaric acid and pH 3.5) at different doses: 10, 20, 30 and 40 g/hL. Then, five different solutions were prepared by adding 4 ml of botrytized must (obtained in 2.3) to 1 ml of each tannins solutions (samples) or 1 ml of model wine solution (control). The mix was left four minutes in contact before being added by 0.8 g of PVPP, stirred and centrifuged for 10 minutes at 8,500 rpm. Laccase activity was determined using the syringaldazine test method as previously described (Vignault et al., 2019). All analyses were performed in triplicate. A laccase unit (UL) corresponds to the amount of enzyme catalyzing the oxidation of a micromole of syringaldazine per minute. The following equation was used for the calculation of laccase activity by using the slope from a calibrating linear regression (ΔA) expressed in absorbance units/minute:

$$Laccase\ activity = 46.15\ x\ \Delta A\ \mu mol.L^{-1}.min^{-1} = 46.15\ x\ \Delta A\ UL$$

Moreover, the residual laccase activity was determined for each sample using the following equation:

$$\%\ of\ residual\ activity = (laccase\ activity\ sample/laccase\ activity\ control)\ x\ 100$$

2.5. Determination of kinetics parameters (Km and Vmax)

Solutions were prepared (see previous paragraph 2.4) and the enzyme kinetic of the *B. cinerea* laccase was studied using different concentrations of syringaldazine as the substrate. The Michaelis-Menten plot was represented for each sample allowing us to represent the Lineweaver-Burk curve (double reciprocal plots). Then, the values of K_M and V_{max} were determined from the linear regression of double reciprocal plots obtained by varying the final concentration of syringaldazine in the reaction medium from 0 to 33 µM.

2.6. Influence of bentonite treatment

Increasing volumes of a 200 g/hL bentonite suspension were added to 3 mL of botrytized must (as obtained in 2.3) to reach final bentonite concentration between 0 and 100 g/hL. The samples were left 20 minutes in contact before being added by 0.5 g of PVPP, stirred and centrifuged for 5 min at 8,500 rpm. Laccase activity was then determined as previously described (paragraph 2.4).

2.7. Extracellular laccase production

Extracellular laccases were produced from the strain 213 of *B. cinerea* as previously described (paragraph 2.3). The strain was kept as a stock suspension of spores in 20% glycerol and stored at -80°C. *B. cinerea* laccases were produced as previously described by (Quijada-Morin et al., 2018) with some modifications. Briefly, cultures on YPD medium were kept for 1 week at 20°C in incubator to induce growth and sporulation. The spores were then gently scraped from one 55 mm diameter Petri dish to inoculate 125 mL of sterilized culture medium in an Erlenmeyer flask of 500 mL. Sterilized culture medium was composed as followed (in g/L): glucose, 40; glycerol, 7; L-histidine, 0.5; $CuSO_4$, 0.1; $NaNO_3$, 1.8; NaCl, 1.8; KCl, 0.5; $CaCl_2{\cdot}2H_2O$, 0.5; $FeSO_4{\cdot}7H_2O$, 0.05; KH_2PO_4, 1.0; and $MgSO_4{\cdot}7H_2O$, 0.5. After 3 days of incubation (20°C, dark, 140 rpm), 100 mL of these pre-cultures were transferred into an Erlenmeyer flask of 5 L containing

1.4 L of the culture medium. After 2 days of growth (20°C, dark, 140 rpm), gallic acid was added at 2 g/L and cultures were maintained 5 more days under the same conditions. Then, the liquid medium was filtered through a filter paper and kept until use at -80°C. Fresh fungal biomass was lyophilized and dried biomass weighed. Protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as standard (Okutucu, Dınçer, Habib, & Zıhnıoglu, 2007). Initial laccase activity of the liquid medium was also determined.

2.8. Enzyme electrophoresis

B. cinerea laccase was analyzed by SDS-PAGE as previously described (Cilindre, Castro, Clément, Jeandet, & Marchal, 2007) with some modifications. Briefly, 10 µL of the enzyme solution was mixed with Laemmli buffer: 62.5 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol and 0.01% bromophenol blue. The gel was a 10% Mini-PROTEAN® TGX™ Gel (Bio-Rad Laboratories, Hercules, CA, USA) and the electrophoresis was carried out at 120 V, using the Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA, USA). A Spectra™ Multicolor Broad Range Protein Ladder (10-260 kDa, Thermo Fisher Scientific Inc., Waltham, MA, USA) was included in each electrophoresis run as standards for molecular weight estimation. The gel was stained with a Pierce™ Silver Stain Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). A digitized image of the gel was obtained by photography. The molecular weight, intensity and relative quantity in proteins of the bands were determined with GeneTools analysis software (Syngene, Cambridge, UK).

2.9. Statistical analysis

All the chemical and physical data were expressed as mean values ± standard deviation. The statistical analyses were carried out using the XLSTAT 2017 statistical package. The normality and homoscedasticity of the data were tested for all parameters by using the Shapiro-Wilk test and Levene's test, respectively. When populations were distributed normally and presented homogeneity in variance, parametric tests were used, i.e. ANOVA and Tukey. In contrast, when data were not distributed normally and/or presented heterogeneity in variance, non-parametric tests were used (Kruskal-Wallis and Pairwise-Wilcox). Differences were statistically significant at p-value < 0.05.

3. Results and discussion

Enzyme kinetics

As expected, laccase showed a classical Michaelis-Menten kinetics since the reaction rate shows a first-order kinetics behavior when the substrate concentration is low and becomes a zero-order kinetics when the substrate concentration is saturating. Supplementation with increasing concentration of oenological tannins, sulfur dioxide and ascorbic acid in botrytized must induced a diminution of the maximal reaction rate (V_{max}) (Figure 1) which confirm that all these additives exert an inhibitory effect on laccase activity. However, this inhibitory effect does not seem to be similar for all these products. Oenological tannins and sulfur dioxide seems to exert uncompetitive inhibition since the Lineweaver-Burk plots of laccase kinetics

in the presence of these additives were parallel with that obtained without any addition (Figure 2). An uncompetitive inhibition takes place when an enzyme inhibitor (I) binds only to the complex "ES" formed by the enzyme (E) and the substrate (S). This phenomenon induced the reduction of the concentration of ES complex which can be explained by the fact that ES complex may be essentially converted into ESI complex ("enzyme-substrate-inhibitor"), thus considered a separate complex altogether. This reduction in ES complex concentration decreased V_{max}, since it takes longer for the substrate or product to leave the active site. The reduction of the binding affinity (K_M) can also be linked back to the decrease in ES complex concentration.

In contrast, ascorbic acid seems to exert a noncompetive inhibition since Lineweaver-Burk plots of laccase kinetics in the presence of this additive the x intercept stays constant in comparison with the control plot. In non-competitive inhibition, the inhibitor will bind to an enzyme at its allosteric site; therefore, the K_M of the substrate with the enzyme will remain the same. On the other hand, the V_{max} will decrease relative to an uninhibited enzyme.

In this way, Michaelis-Menten (Figure 1) and Lineweaver-Burk plots (Figure 2) allowed to qualify oenological tannins and sulfur dioxide as uncompetitive inhibitors, whereas ascorbic acid can be rather qualified as non-competitive inhibitor. To confirm this graphical statement, kinetics parameters were calculated as follows.

Kinetic parameters (Determination of K_M and V_{max})

The values of V_{max} and K_M were calculated by the extrapolation of the curve obtained from the Lineweaver-Burk plot. The enzyme obtained for the *B. cinerea* strain 213 had a V_{max} of 0.364 ± 0.026 µmol/L · min and a K_M of 0.697 ± 0.133 µmol/L.

According to the previous statement, oenological tannins and sulfur dioxide presented, as excepted, a significant decrease in their V_{max} and K_M compared to the control (without any inhibitor) (**Table 1**). Likewise, except for quebracho tannin, the significant differences of V_{max} were already shown at the lowest dose of inhibitor compared to the control (**Table 1**). Nonetheless, at the highest dose, sulfur dioxide presented the greatest ability to reduce the V_{max} (0.003 ± 0.002 µmol/L · min), while ellagitannin (0.274 ± 0.002 µmol/L · min) had the lowest ability. The other oenological tannins presented similar effects (0.201 ± 0.007 µmol/L · min to 0.220 ± 0.006 µmol/L · min), since no significant differences were observed between them (**Table 1**). All the inhibitors tested, i.e. oenological tannins and sulfur dioxide, at the highest dose showed the same ability to decrease K_M without any significant difference. The initial K_M was diminished from 0.697 ± 0.133 µmol/L (control) at 0.417 ± 0.048 µmol/L (gallotannin) to 0.506 ± 0.019 µmol/L (ellagitannin). Nevertheless, for both ellagitannin and quebracho tannin, the lowest dose was not enough to diminish significantly the enzyme's K_M compared to the control. Indeed, 40 g/hL and 20 g/hL were necessary, respectively. However, 10 mg/L and 25 g/hL were enough for sulfur dioxide and the other oenological tannins, respectively.

In the case of ascorbic acid, as it was a non-competitive inhibitor, the enzyme's V_{max} decreased significantly with addition of the lowest dose (50 mg/L) compared to the control (0 mg/L). Moreover, at the highest dose (200 mg/L), V_{max} reached values three fold time lower than control, ranking from 0.364 ± 0.026 µmol/L · min to 0.156 ± 0.004 µmol/L · min. However, K_M values remained constant, since no significant differences

were observed with control, even if values were ranked between 0.667 ± 0.121 µmol/L (100 mg/L) and 0.996 ± 0.137 µmol/L (200 mg/L).

Based only on the determination of V_{max} and K_M parameters, it was then not possible to compare the effect of enological tannins with ascorbic acid, since they are different type of inhibitors. It must also be considered that laccase enzyme, in our reaction conditions, has two subtracts, syringaldazine and oxygen. Oenological tannins, sulfur dioxide and ascorbic acid can react with oxygen, decreasing therefore its concentration in the medium (Pascual et al., 2017; Vignault et al., 2018). Consequently, this reduced the V_{max} of the reaction. However, considering the oxygen consumption rate of oenological tannins and sulfur dioxide, as reported by (Pascual et al., 2017), the oxygen consumption by these compounds can be considered as negligible. Indeed, the oxygen consumption of oenological tannins and sulfur dioxide at normal concentrations (400 mg/L for oenological tannins and 50 mg/L for sulfur dioxide) represent less than 0.2 % compared with the consumption capacity of laccase in our botrytized grape juice, for 30 UL (Figure 3). In contrast, ascorbic acid consumes oxygen much faster and can really compete with laccase (Pascual et al., 2017). Specifically, the oxygen consumption rate of 100 mg/L of ascorbic acid is around the 50 % of the oxygen consumption rate of the botrytized must use with a laccase activity of around 30 UL. Thus, the inhibitory effect of ascorbic acid on laccase activity seems to result from a competition with laccase for the oxygen present in the medium.

In this way, the residual activity was additionally determined, for each sample, to make possible the comparison between the different oenological tannins.

Residual laccase activity

Initial activity of the control was 27.3 ± 0.6 UL and was considered as the 100 ± 6.40% of residual activity (**Table 1**). All the samples tested, including oenological tannin, sulfur dioxide and ascorbic acid, diminished significantly the residual activity at the lowest dose tested. Sulfur dioxide clearly appeared as the most efficient to reduce laccase activity, since with 100 mg/L, no residual laccase activity was detected (0.9 ± 0.4%). Ascorbic acid and grape-seed tannin appeared also as great candidates to reduce laccase activity, since at the highest dose (200 mg/L and 40 g/hL respectively), only 11.5 ± 3.2% and 12.8 ± 6.4% of the laccase activity remained present, respectively (**Table 1**). Similarly, gallotannin and grape-skin tannin at the highest dose presented an equivalent effect, since they reduced by half the laccase activity: 44.6 ± 7.8% and 50.7 ± 5.5%, respectively (**Table 1**). Nevertheless, gallotannins reached this value at the lowest dose used (10 g/hL), while grape-skin tannin only reduced around by 35% the laccase activity at the lowest dose (65.6 ± 3.2%). Concerning ellagitannin and quebracho tannin, their ability to reduce laccase activity was clearly lower than in the case of the other oenological tannin. Indeed, including at the highest dose (40 g/hL), they were able to reduce the activity only between 20 and 30%, respectively (**Table 1**).

Influence of bentonite treatment

Bentonite is an inorganic clay finning agent, negatively charged, which is responsible for the binding of proteins who have a net positive charge at wine pH, resulting in their removal from the wine (Vincenzi, Panighel, Gazzola, Flamini, & Curioni, 2015). Bentonite is universally used in the wine industry to remove

wine proteins by electrostatic adsorption, because it is efficiency , low cost, and because its use needs a simple batch process that does not require any specialized equipment or knowledge (Lira et al., 2015). Laccases produced by *B. cinerea* are proteins which have a isoelectric point lower than usual wine pH (Baldrian, 2006) and, consequently, they are charged negatively at wine pH. Theoretically they cannot be removed with bentonite. The influence of a bentonite treatment on botrytized white must is showed in **Figure 3**. No significant differences were observed regarding laccase activity, by comparing the botrytized white must not treated and the botrytized must treated at different doses of bentonite. The initial laccase activity was around 12 $\mu mol.L^{-1}.min^{-1}$ and remained stable when the botrytized must was previously treated with bentonite. According to these results, it was decided to use bentonite treatment for the enzyme electrophoresis when it was necessary to remove other proteins without affecting laccase activity .

Enzyme production

The strain 213 produced extracellular enzymes when cultured under the conditions described in material and methods (section 2.7.). The amount of the dry fungal biomass was 0.0429 g/mL and the content of protein found in the culture medium was 0.15 mg/mL. The yield of protein per gram of dry fungal biomass was 3.49 mg/g for the strain 213. These results were in accordance with previous published ones (Quijada-Morin et al., 2018) in similar conditions for three other different *B. cinerea* strains: yields values ranging between 2 and 5 mg/g.

Enzyme electrophoresis

The SDS-PAGE of laccase sample showed the presence of only one band corresponding to the laccase proteins (**Figure 4** – well 1). The strain produced laccase enzymes with a molecular weight of 97 kDa, which is in accordance with previous results published (Quijada-Morin et al., 2018; Slomczynski, Nakas, & Tanenbaum, 1995). Nevertheless, values ranging from 36 kDa to 97 kDa have been also reported, suggesting a great variability between the enzymes with laccase activity produced by different strains of the pathogen. When bentonite (100 g/hL) was added to laccase sample (**Figure 4** – well 2), a part of proteins was removed, since the band at 97 kDa appeared weaker suggesting that bentonite has eliminated other proteins of similar molecular weight but not laccase since its enzymatic activity remains stable. The SDS-PAGE of the white wine (**Figure 4** – well 3) showed the presence of two bands with a molecular weight of 57 kDa and 20 kDa, as previously reported (Esteruelas et al., 2009). When bentonite was added to the wine (**Figure 4** – well 4), these two proteins bands were not detectable. It has been previously shown (Sauvage, Bach, Moutounet, & Vernhet, 2010) that more than 50% of the whole protein content was removed with only 50 g/hL of bentonite, but around 15% of this protein content remained non-adsorbed even for a bentonite concentration as high as 150 g/hL. This means that the dose of 100 g/hL could be the most appropriate in order to remove all the protein content, nevertheless such a high addition of bentonite can decrease the quality of the wines (Sauvage et al., 2010). After bentonite treatment of laccase samples and white wine, 70% and 100% of the proteins were removed respectively (**Table 2**). Therefore, at this dose, bentonite was able to remove all the proteins from wine. However, bentonite was not able to remove all proteins of laccase sample since 30% of them remained after the treatment. These results are not in

accordance with those from (Riebel et al., 2017) in which laccase was not removed by bentonite fining.. Nevertheless, as it has been commented above, laccase activity is not impacted by bentonite treatment (**Figure 3**), meaning that, probably, bentonite have removed some other proteins of similar molecular weight but not laccase. Additionally, the SDS-PAGE of white wine supplemented by laccase sample (**Figure 4** – well 5) presented three band at 97 kDa, 57 kDa and 20 kDa corresponding to the bands obtained for laccase sample and white wine proteins in wells 1 and 3, respectively. When bentonite was added to the mixture made of the white wine and laccase (**Figure 4** – well 6), only the band corresponding to laccases remained visible. This result confirms once again the ability of bentonite to precipitate white wines proteins, but not laccase.

Regarding the ability of oenological tannins to precipitate laccase proteins in white wine treated with bentonite, grape tannins (grape-seed and grape-skin) followed by gallotannin were the most efficient ones (**Figure 4** – well 7, 10 and 11). Grape-seed and grape-skin led to precipitate 77.8% and 74.5% of laccase proteins, respectively (**Table 2**). Gallotannin allowed to precipitate almost 50% of laccase proteins (**Table 2**). In contrast, ellagitannin and quebracho tannin did not present any ability to precipitate laccase proteins (**Figure 4** – well 9 and 12), since 100% of laccase proteins remained present (**Table 2**). These results are in accordance with the previous ones obtained in **Table 1**, in which grape tannins and gallotannins were the most efficient products tested to reduce laccase activity. Nevertheless, gallotannin showed a lower ability to precipitate laccase proteins, but presented similar kinetics parameters and ability to reduce laccase activity than grape-skin tannin. This could be explained by the fact that, on one hand, oenological tannins can precipitate laccase proteins, thus reducing laccase activity, or on the other hand, by inhibition of laccase activity without protein precipitation. Indeed, the binding of an inhibitor and its effect on the enzymatic activity are two distinct and quite different mechanisms. According to this hypothesis, grape tannins and gallotannin presented the ability to inhibit laccase activity and to precipitate (bounds) laccase proteins. Specifically, gallotannin was the most efficient to inhibit laccases produced by *B. cinerea*, whereas grape-skin tannin was most efficient regarding laccase proteins precipitation. Additionally, grape-seed tannin was the most efficient in the oenological tannins to inhibit laccase activity and precipitate laccase proteins. Concerning quebracho tannin, it should be noted its ability to inhibit laccase activity, but its ability to precipitate laccase proteins was almost inexistent. Ellagitannin presented the lowest ability to inhibit and to precipitate laccase from *B. cinerea*. Nevertheless, even though ellagitannin presented low ability to inhibit laccase, it can reduce the activity by 20%.

4. Conclusions

As expected, bentonite did not permit to reduce laccase activity in white must, even if it was responsible for precipitation of other proteins similar in molecular weight to laccase from *B. cinerea* strain 213. Oenological tannins were characterized by different abilities regarding prevention of *B. cinerea* damage, since they showed different ability to precipitate laccases produced and/or reduce their activity. They were all uncompetitive inhibitors, but gallotannin, grape-skin and even more so grape-seed tannin presented the greatest ability to reduce V_{max}, K_M and residual laccase activity. Addition of grape-seed tannin was efficient to reduce laccase activity with a similar level effect than that of ascorbic acid and that of sulfur dioxide at traditional doses employed in oenology during winemaking for each one. Our results

confirmed our previous study (Vignault et al., 2019), and demonstrated that oenological tannins and more specifically, grape tannins and gallotannin, are excellent processing aids to prevent *B. cinerea* damage in grape musts. Oenological tannins used on botrytized grapes should permit also to decrease sulfur dioxide use during winemaking. Further studies are required to further study the laccase inhibitory mechanisms of oenological tannins, but the actual findings justify why the OIV has included this functionality in the OIV International Oenological Codex.

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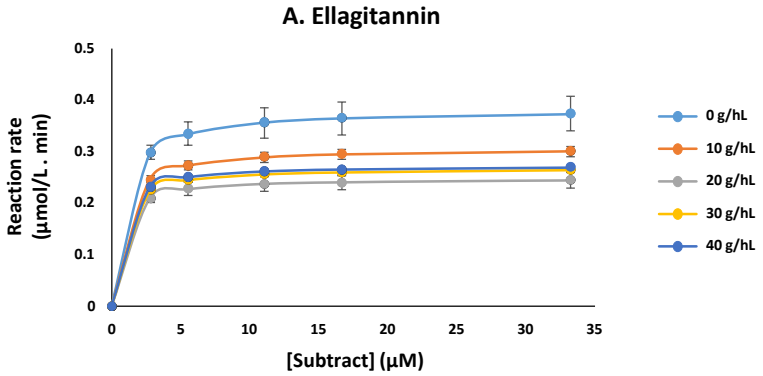
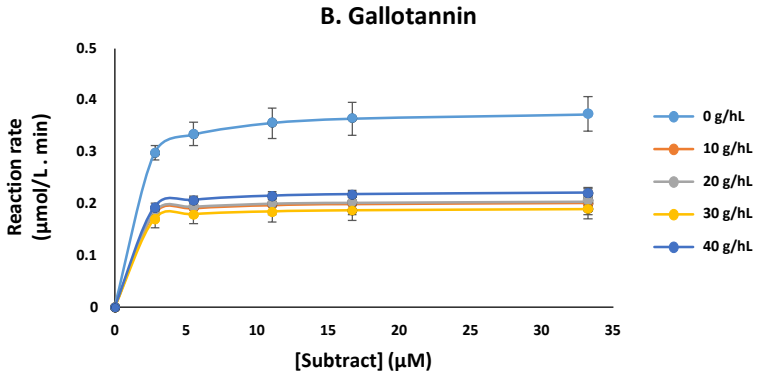
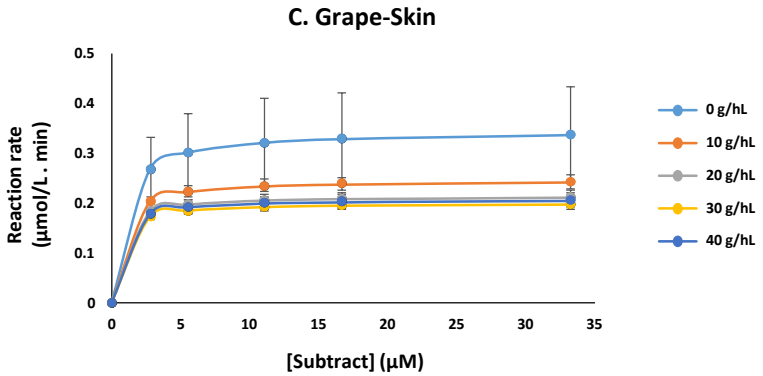
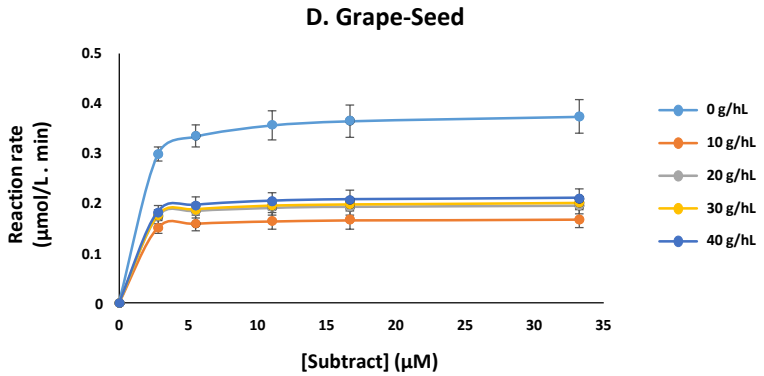
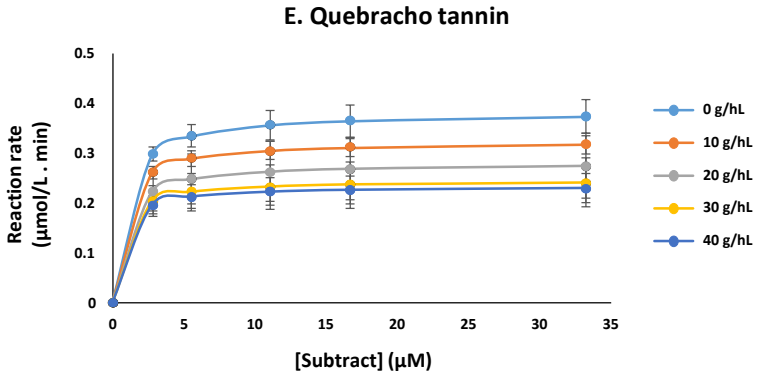
Figure 1. Michaëlis-Menten plots to visualize the kinetics of laccase and the inhibitory effect of oenological tannins (A, B, C, D, E), SO_2 (F) or ascorbic acid (G)

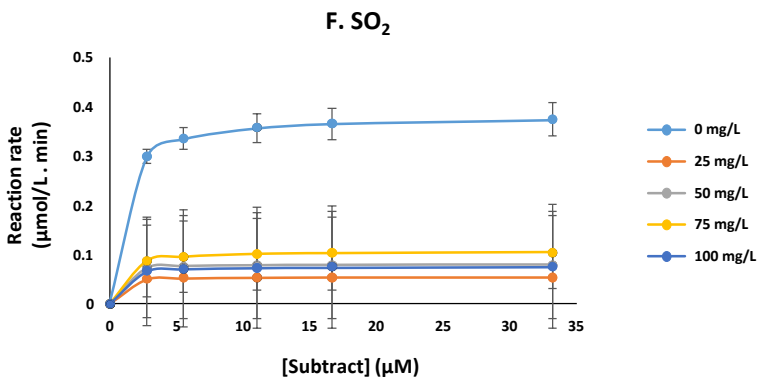


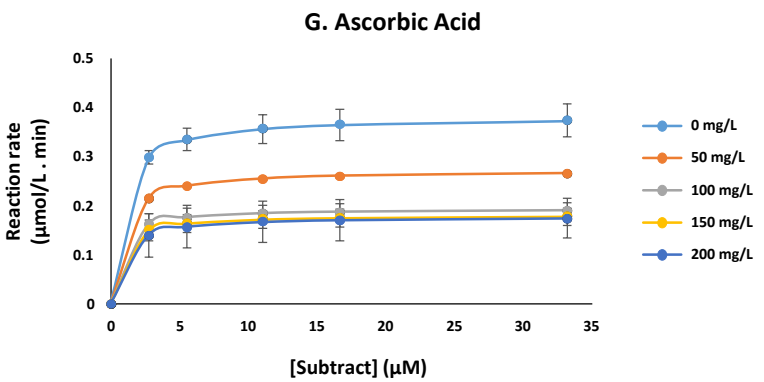






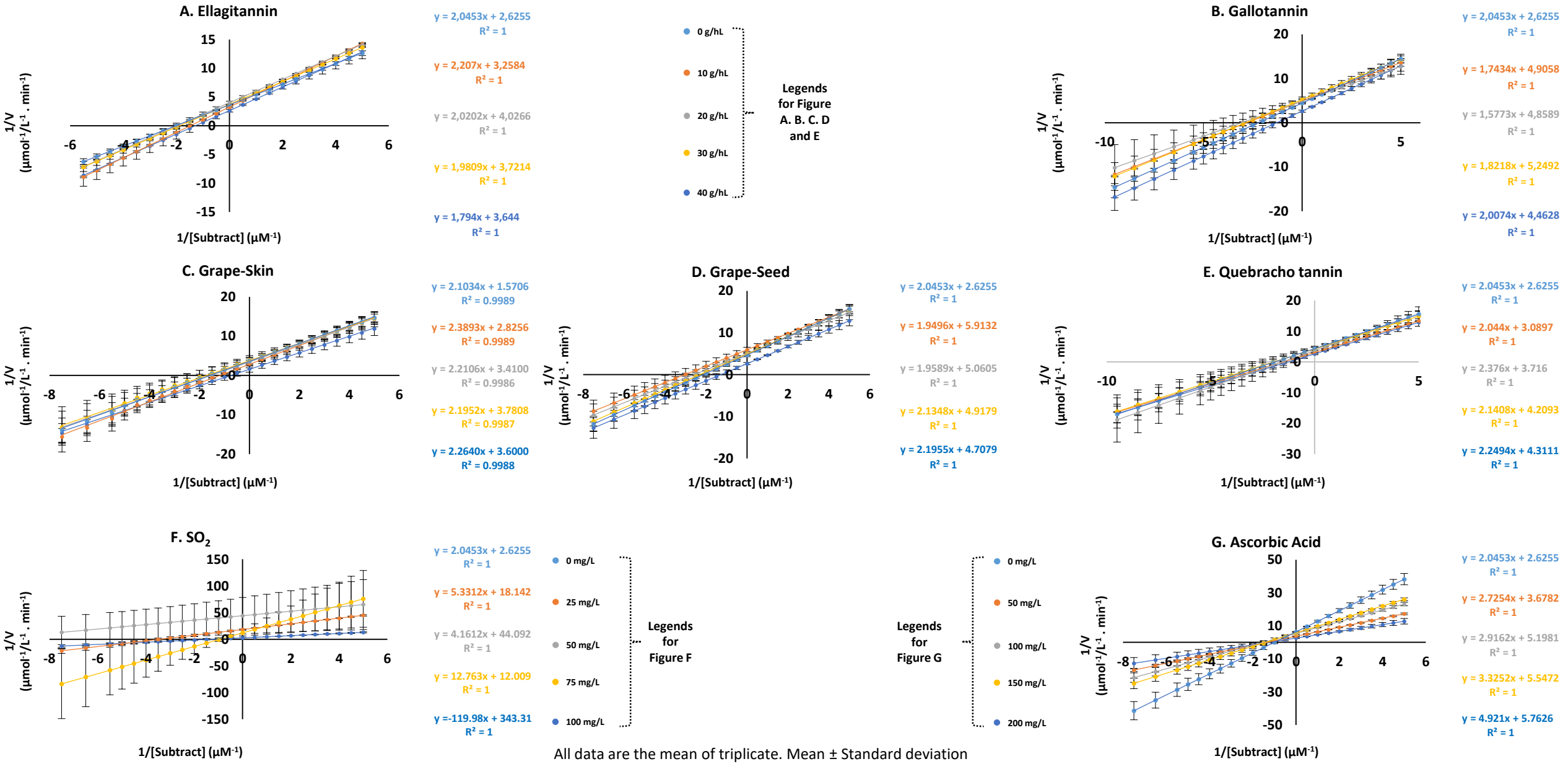






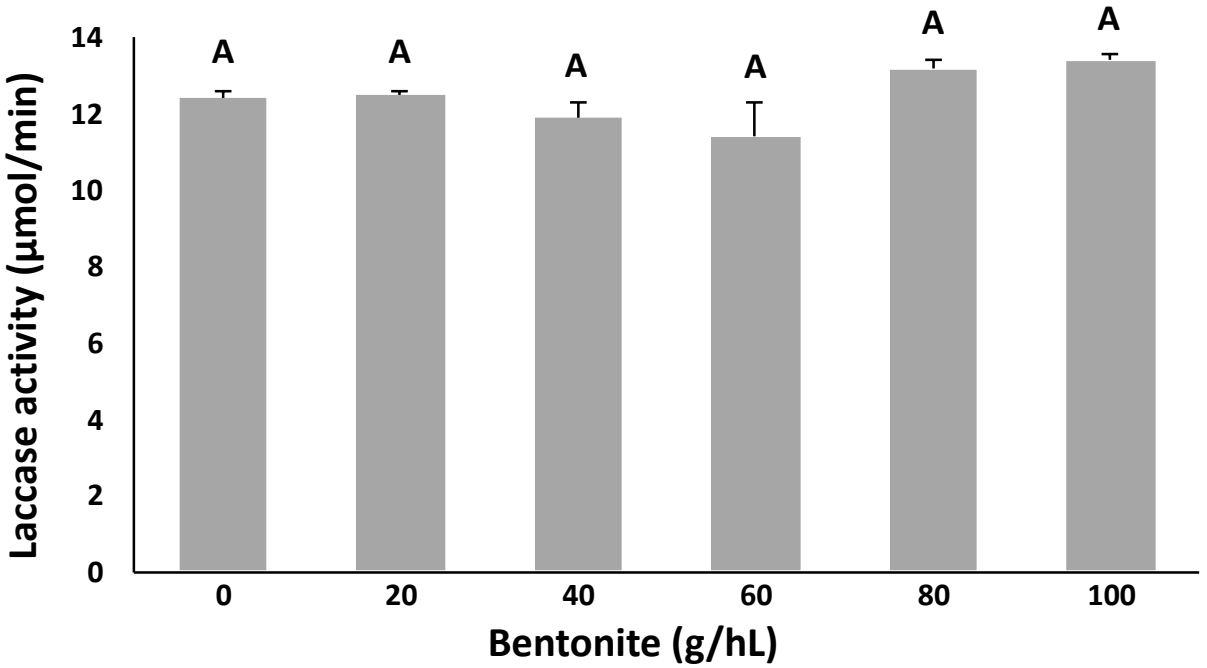
All data are the mean of triplicate. Mean ± Standard deviation

Figure 2. Lineweaver-Burk plot for determining the kinetic constants of laccase and the inhibitory effect of oenological tannins (A, B, C, D, E), SO_2 (F) or ascorbic acid (G)



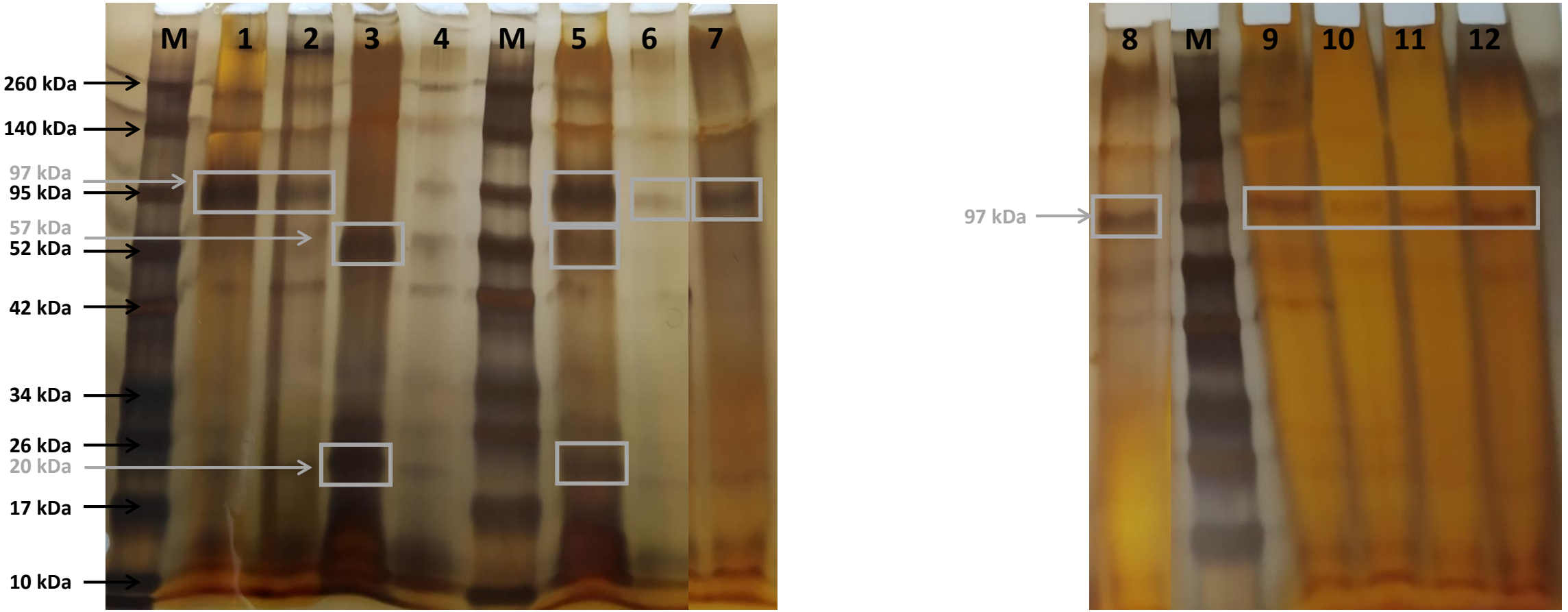
All data are the mean of triplicate. Mean ± Standard deviation

Figure 3. Influence of bentonite treatment at different doses on laccase activity of botrytized white must



All data are the mean of triplicate. Mean ± Standard deviation

Figure 4. Electrophoresis gels of laccases produced by *Botrytis cinerea* and supplemented by oenological tannins (40 g/hL) with a bentonite treatment (200 g/hL)



M: Molecular Weight marker
1: Laccase (L)
2: Laccase + Bentonite (B)
3: White wine (Ww)
4: Ww+ B
5: Ww+ L
6: Ww+ L + B
7: Ww+ L + B + Gallotannin 40 g/hL
8: Ww+ L + B
9: Ww+ L + B + Ellagitannin 40 g/hL
10: Ww+ L + B + Grape-seed tannin 40 g/hL
11: Ww+ L + B + Grape-skin tannin 40 g/hL
12: Ww+ L + B + Quebracho tannin 40 g/hL

The arrows indicate the different molecular weight of the corresponding bands. The brackets correspond to the different bands of interest.

Table 1. Kinetics parameters (Vmax and Km) and residual laccase activity of botrytized white must added by oenological tannins or SO_2 or ascorbic acid at different concentration

Samples	Doses	V_{max} (µmol/L . min)	K_M (µmol/L)	Residual activity (%)
Ellagitannin (g/hL)	0	**0.364 ± 0.026 A α**	**0.697 ± 0.133 A α**	**100.0 ± 6.40 A α**
	10	0.307 ± 0.010 **B α**	0.678 ± 0.024 **A α**	78.4 ± 6.4 **B α**
	20	0.257 ± 0.011 **C β**	0.534 ± 0.042 **AB αβ**	80.4 ± 3.2 **B α**
	30	0.269 ± 0.006 **C α**	0.525 ± 0.011 **AB β**	76.0 ± 3.9 **B α**
	40	0.274 ± 0.002 **C α**	0.506 ± 0.019 **B β**	81.8 ± 3.2 **B α**
Gallotannin (g/hL)	0	**0.364 ± 0.026 A α**	**0.697 ± 0.133 A α**	**100.0 ± 6.4 A α**
	10	0.212 ± 0.010 **B γ**	0.396 ± 0.053 **B β**	41.5 ± 3.9 **B γ**
	20	0.221 ± 0.019 **B βγ**	0.407 ± 0.100 **B αβ**	39.9 ± 6.4 **B γ**
	30	0.202 ± 0.015 **B β**	0.364 ± 0.020 **B β**	45.6 ± 3.9 **B γ**
	40	0.220 ± 0.006 **B β**	0.417 ± 0.048 **B β**	44.6 ± 7.8 **B γ**
Quebracho (g/hL)	0	**0.364 ± 0.026 A α**	**0.697 ± 0.133 A α**	**100.0 ± 6.4 A α**
	10	0.334 ± 0.013 **A α**	0.663 ± 0.037 **A α**	73.6 ± 3.2 **B αβ**
	20	0.318 ± 0.043 **AB α**	0.571 ± 0.032 **B αβ**	80.4 ± 8.5 **B α**
	30	0.245 ± 0.051 **BC αβ**	0.411 ± 0.088 **B β**	73.6 ± 17.8 **B αβ**
	40	0.217 ± 0.010 **C β**	0.462 ± 0.015 **B β**	67.9 ± 11.7 **B αβ**
Grape-skin (g/hL)	0	**0.364 ± 0.026 A α**	**0.697 ± 0.133 A α**	**100.0 ± 6.4 A α**
	10	0.248 ± 0.015 **B β**	0.620 ± 0.061 **AB α**	65.6 ± 3.2 **B β**
	20	0.215 ± 0.015 **BC βγ**	0.386 ± 0.070 **C αβ**	61.5 ± 3.2 **BC β**
	30	0.200 ± 0.010 **C β**	0.386 ± 0.052 **C β**	53.7 ± 3.9 **BC βγ**
	40	0.201 ± 0.007 **C β**	0.426 ± 0.036 **BC β**	50.7 ± 5.5 **C βγ**
Grape-seed (g/hL)	0	**0.364 ± 0.026 A α**	**0.697 ± 0.133 A α**	**100.0 ± 6.4 A α**
	10	0.180 ± 0.013 **B δ**	0.295 ± 0.057 **B β**	13.5 ± 3.2 **B δε**
	20	0.209 ± 0.001 **B βγ**	0.343 ± 0.009 **B αβ**	16.2 ± 0.0 **B δ**
	30	0.210 ± 0.005 **B β**	0.481 ± 0.025 **B β**	15.6 ± 3.2 **B δ**
	40	0.214 ± 0.019 **B β**	0.470 ± 0.072 **B β**	12.8 ± 6.4 **B δ**
SO_2 (mg/L)	0	**0.364 ± 0.026 A α**	**0.697 ± 0.133 A α**	**100.0 ± 6.4 A α**
	25	0.055 ± 0.003 **B ε**	0.274 ± 0.031 **B β**	4.1 ± 0.0 **B δε**
	50	0.016 ± 0.001 **C δ**	0.237 ± 0.141 **B β**	2.0 ± 0.0 **B ε**
	75	0.065 ± 0.000 **B γ**	0.349 ± 0.000 **B β**	1.4 ± 1.0 **B δ**
	100	0.003 ± 0.002 **C δ**	0.451 ± 0.000 **B β**	0.9 ± 0.4 **B δ**
Ascorbic acid (mg/L)	0	**0.364 ± 0.026 A α**	**0.697 ± 0.133 A α**	**100.0 ± 6.4 A α**
	50	0.272 ± 0.003 **B β**	0.741 ± 0.030 **A α**	21.3 ± 3.9 **B δ**
	100	0.203 ± 0.007 **C γ**	0.667 ± 0.121 **A α**	12.8 ± 0.0 **B δε**
	150	0.193 ± 0.001 **C β**	0.724 ± 0.153 **A α**	19.6 ± 3.2 **B δ**
	200	0.156 ± 0.004 **D γ**	0.996 ± 0.137 **A α**	11.5 ± 3.2 **B δ**

All data are the mean of triplicate. Mean ± Standard deviation. Capital letters indicate significant differences between the different doses for the same sample ($p<0.05$). Greek letters indicate significant differences between samples for the same doses ($p<0.05$)

Table 2. Molecular weight and intensity of the bands of laccases produced by Botrytis cinerea; Influence of treatment with oenological tannins (40 g/hL) and with bentonite (200 g/hL)

		1	2	3	4	6	7	9	10	11	12
Band 1 (laccase)	**MW (kDa)**	97	97			97	97	97	97	97	97
	Quantity (%)	100.0	31.6			100.0	48.3	94.4	22.8	25.5	105.3
Band 2 (wine)	**MW (kDa)**			57	57	57					
	Quantity (%)			100.0	n.d	n.d					
Band 3 (wine)	**MW (kDa)**			20	20	20					
	Quantity (%)			100.0	n.d	n.d					

1: Laccase (L)
2: Laccase + Bentonite (B)
3: White wine (Ww)
4: Ww+ B
6: Ww+ L + B
7: Ww+ L + B + Gallotannin 40 g/hL
9: Ww+ L + B + Ellagitannin 40 g/hL
10: Ww+ L + B + Grape-seed tannin 40 g/hL
11: Ww+ L + B + Grape-skin tannin 40 g/hL
12: Ww+ L + B + Quebracho tannin 40 g/hL