Strain effect on extracellular laccase activities from *Botrytis cinerea*

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Abstract

Background and Aims: Laccase enzymes produced by *Botrytis cinerea* are involved in the oxidation of phenolic substances during the development of grey mould, which causes significant economic losses. The aim of this work was to study the structural and activity characteristics of the laccase enzymes secreted by three *B. cinerea* strains that are involved in the development of grey mould.

Methods and Results: Laccase enzymes obtained from three *B. cinerea* strains [one reference strain (B05.10) and two strains obtained from two French vineyards (VA612 and RM344)] were characterised. Analysis by LC-QTOF-MS revealed that the three strains contained a mixture of Laccase-2-BcLCC2 and Laccase-3-BcLCC7. The structural characteristics of the laccases from the three strains, such as molecular weight and glycosylation degree, were identical. Nevertheless, their catalytic activities were significantly different.

Conclusions: Differences in catalytic activities could be due either to possible differences in the relative amount of Laccase-2-BcLCC2 and Laccase-3-BcLCC7 produced by each strain or to differences in the glycosidic fraction of the enzymes.

Significance of the Study: The severity of the infection caused by *B. cinerea* may be not only related to the infection level but also to the strain involved.

Keywords: Botrytis cinerea, laccase, Laccase-2-BcLCC2, Laccase-3-BcLCC7

Introduction

Botrytis cinerea is a ubiquitous, filamentous and necrotrophic fungus attacking over 1400 plant species including horticultural crop plants such as rose and also many fruits, such as cucumber, tomato, strawberries, kiwifruits and grapes (Elad et al. 2016). It is one of the principal causes of quantitative and qualitative degradation in many crops.

Botrytis cinerea is responsible for one of the most serious diseases affecting grapes (*Vitis vinifera*), namely grey mould or Botrytis bunch rot (Ky et al. 2012). This fungus, which is the most frequently encountered bunch rot pathogen of mature grape berries, occurs worldwide, particularly in vineyards exposed to cool and wet conditions during the ripening period (Steel et al. 2013). It can cause considerable economic losses linked to grape desiccation, rot and biochemical changes that reduce wine quality (Steel et al. 2013). For instance, a decrease in the quality of red wine has been reported when it is made from grapes with an infection rate as low as 5% for *B. cinerea* (Ky et al. 2012), mainly due to the appearance of off-flavours such as geosmin or 1-octen-3-one. The colour of red wines is damaged as a consequence of the decrease in the concentration of anthocyanidins and other phenolic substances, which can be quickly oxidised by laccase enzymes (Steel et al. 2013). The enzymatic oxidation of gallic acid induced by laccase enzymes has been reported to be up to three orders of magnitude higher than chemical oxidation (Zinnai et al. 2013). Moreover, grey mould is the most important cause of postharvest decay of tablegrapes during storage, transit to markets and commercialisation (Latorre et al. 2015).

Botrytis cinerea can also cause the infection known as noble rot, which promotes favourable changes in grape berries and the accumulation of secondary metabolites that enhance grape composition for the production of sweet white wines (Blanco-Ulate et al. 2015, Magyar and Soós 2016). The processes at molecular and biochemical level that contribute to the development of either noble rot or bunch rot during *Botrytis* infection of grape berries have not been elucidated yet. A combination of environmental effects, cultural practices, intrinsic characteristics of the grape cultivar and the berry microbiome would influence the type of rot developed (Ribéreau-Gayon et al. 2000, Fournier et al. 2013). Genomic studies of strains causing noble rot or grey rot did not reveal genetic differences between the strains, thus it is thought that the development of one or the other kind of infection is more related to metabolic regulation due to microclimatic conditions (Fournier et al. 2013).

Several problems are related to the presence of *B. cinerea* in grapes for the vinification process. Their presence in grapes may lead to the de novo synthesis of compounds not present in healthy grapes or wine or to the modification of existing grape substrates (Steel et al. 2013), such as phenolic

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substances that are highly related to wine quality. It also alters the composition of wine polysaccharides due to β-glucan secretion, and causes the formation of several polyols, such as mannitol, sorbitol and sugar oxidation products such as gluconic acid (Ribéreau-Gayon et al. 2000). The presence of a high molecular weight β-glucan leads to the appearance of aggregates and colloidal particles which make wines made from grapes affected by grey mould difficult to clarify (Ribéreau-Gayon et al. 2000). The clarity of the wines is thus diminished and the appearance of oxidised compounds will contribute to the alteration of the appearance, taste, mouthfeel, fragrance and antimicrobial properties of the wine (Jacometti et al. 2010).

In addition to this, during winemaking, the presence of *B. cinerea* can hinder the alcoholic fermentation process. It is responsible for the oxidasic haze in red wines derived from rotten grapes, and the grapes and wines obtained are often marked by characteristic mould or undergrowth odours (Ribéreau-Gayon et al. 2000). The degradation of phenolic substances, such as proanthocyanidins or anthocyanidins, caused by laccase activity leads to changes in wine colour and colour stability (Steel et al. 2013). The loss of general quality and the alteration of colour, smell and taste in white wines elaborated from grapes affected by bunch rot has also been reported (Meneguzzo et al. 2008).

Botrytis cinerea can produce extracellular laccase enzymes (EC 1.10.3.2, *p*-diphenol : dioxygen oxidoreductases). These enzymes are multi-copper proteins that use molecular oxygen to oxidise various aromatic and non-aromatic substrates by a radical-catalysed reaction mechanism (Claus 2004). They oxidise phenolic substances to form quinones, which readily condense to form compounds associated with accelerated browning in wines (Ribéreau-Gayon et al. 2000). The production of laccase facilitates the infection process (Thurston 1994), and the amount of laccase activity in must has been taken as indicative of the degree of Botrytis rot. The genome of the reference strain B05.10 has been fully sequenced (Amselem et al. 2011, Van Kan et al. 2017) and 13 genes that encode putative laccase proteins have been identified (*BcLCC1* to *BcLCC13*).

Laccase enzymes from different sources have been widely studied (Mayer et al. 1977, Bollag and Leonowicz 1984, Tinoco et al. 2001, Shleev et al. 2004, Zouari-Mechichi et al. 2006, Abou-Mansour et al. 2009, Frasconi et al. 2010) and an increasing interest has been reported due to their industrial or technological applications, such as their use as oxidisers in green catalysis (Jeon et al. 2012), for the decolouration of textile dyes (Zouari-Mechichi et al. 2006, Si et al. 2013), for lignolysis (Niku Paavola et al. 1988), for hair colouring (Saito et al. 2012), as biosensors (Di Fusco et al. 2010, Tortolini et al. 2010), and for waste water reparation (Le et al. 2016), among other applications. Among the fungal laccases, a great variability is observed in physicochemical and kinetic properties, as well as their degree of polymorphism (Bollag and Leonowicz 1984, Frasconi et al. 2010). They present some variability for their optimum activity in response to pH and temperature and they also have different substrate specific affinities. The heterogeneity of the activity of laccase enzymes has been reported not only between different fungal species (Frasconi et al. 2010) but also among the enzymes produced by the same species under different environment conditions (Gigi et al. 1980, Marbach et al. 1983, 1984) or even by different strains of *B. cinerea* (Zouari et al. 1987). The study of this heterogeneity is of great interest in order to minimise the impact of the infection in the case of rot of vegetable crops or to choose the correct enzyme for the desired industrial application. The polymorphism and the variability of the physicochemical and kinetic properties of laccase enzymes need to be investigated in order to determine the relevance of the strain effect involved in the damage caused by laccases for crops or beverages.

The aim of this work was to study the structural and activity characteristics of the laccase enzymes secreted by three *B. cinerea* strains, B05.10, VA612 and RM344, that are involved in the development of grey mould.

Materials and methods

Chemicals

All aqueous solutions were prepared with high-purity deionised water obtained from a Milli Q water purification system (Millipore, Bedford, MA, USA). All reagents were of analytical grade and included: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Na_2HPO_4, Na_2CO_3, $NaHCO_3$, Bradford reagent, bovine serum albumin (BSA), caffeic acid, catechin, epicatechin, quercetin, *trans*-ferulic acid, *p*-coumaric acid, phloroglucinol, and gallic acid acetonitrile (ACN), trifluroroacetic acid (TFA) and dithiothreitol (DTT) purchased from Sigma (Saint Quentin Fallavier, France); 2-mercaptoethanol and NaH_2PO_4 from Fluka (Illkirch-Graffenstaden, France); NaOH from Riedel de Haen (Illkirch-Graffenstaden, France); and tartaric acid from Carlo Erba (Peypin, France). The reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Sigma.

Strains and microbiological procedures

Extracellular laccases were produced from three strains of *B. cinerea*. The B05.10 strain is the Botrytis research community reference, whose genome is fully sequenced (Van Kan et al. 2017). Strain RM344 was collected in 1998 in a vineyard in St Julien (Médoc, Bordeaux, France) from grapes of the Merlot cultivar (Martinez et al. 2003). Strain VA612 was collected in 2005 in a vineyard in Hautvillers (Champagne, France) from the Pinot Noir cultivar (Walker et al. 2015). The three strains exhibited distinct single sequence repeat (SSR) genotypes [not shown (Fournier et al. 2002)]. Strains were kept as a stock suspension of spores in 20% glycerol at −80°C.

Botrytis cinerea laccases were produced as previously described by Slomczynski et al. (1995) but modified with the addition of gallic acid as suggested by Fortina et al. (1996). Briefly, cultures on solid malt yeast (MY) medium were kept for 1 week at 24°C under blue light to induce sporulation. For each strain, the spores were then gently scraped from one 55 mm diameter Petri dish to inoculate a 500 mL Erlenmeyer flask containing 125 mL of culture medium with the following composition (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; $CaCl_2{\cdot}H_2O$, 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 (24°C, dark, 150 rpm agitation), 100 mL of these pre-cultures were transferred into 5 L Erlenmeyer flasks containing 1.4 L of the same medium. Gallic acid (2 g/L) was added after 2 days of growth and the cultures were maintained for a further 5 days under the same conditions. The liquid medium was then filtered through a 100 μm filter and kept at 4°C until use. Fresh fungal biomass was lyophilised and dried biomass was weighed.

Enzyme purification

The filtered liquid medium was centrifuged for 30 min at 8600 × *g*, 4°C. The supernatant was carefully removed and filtered through a 0.45 μm filter before being submitted to tangential filtration in a Quixstand filtration system (GE Healthcare UK, Little Chalfont, England) equipped with a 30 kDa molecular weight cut off membrane. Following separation, the concentrate was subjected to a diafiltration against distilled water. Only the fractions that presented oxidant activity against ABTS, determined by the method described in the following section, were kept at −80°C for the remaining analysis. The amount of protein present in each sample was determined with the Bradford method (Bradford 1976) with BSA as the standard for calibration. These measurements were in triplicate.

Activity assays

Laccase activity was measured using ABTS as the substrate for the enzyme (Johannes and Majcherczyk 2000). In order to mimic the conditions for the enzyme activity in wine, a reaction buffer was prepared containing 12.5% ethanol and 4 g/L tartaric acid, and pH was adjusted to 3.6 with 0.1 mol/L NaOH solution. The reaction was monitored by the change of absorbance at 415 nm (A_{415}) at 25°C for 3 min with an Agilent Cary 60 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Under these conditions, initial reaction rate (V_0) was estimated as a measure of the enzyme activity. All the measurements were in triplicate.

Enzyme electrophoresis

Botrytis cinerea laccase was analysed by SDS-PAGE as previously described (Hue et al. 2016). In brief, 50 μL of the enzyme solution was mixed with Laemmli buffer (62.5 mmol/L Tris–HCl pH 6.8, SDS 2%, glycerol 10% and bromophenol blue 0.002%). Electrophoresis was carried out at 40 mA, using the Novex Gel Cassettes (Invitrogen, Life Technologies, Carlsbad, CA, USA) with a 3.75% (v/v) acrylamide/bis-acrylamide (29:1) stacking gel and a 10% (v/v) acrylamide/bis-acrylamide (29:1) resolving gel in the presence of 0.1% (v/v) SDS (gel length, 60 mm). The gel was stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA) (50% EtOH, 10% acetic acid, R250 blue 0.1%) for 2 h and destained by acetic acid 10% (v/v). A low molecular weight calibration kit (14.4–97 kDa, Pharmacia, Biotech, Piscataway, NJ, USA) was included in each electrophoretic run as standards for molecular weight estimation. A digitised image of the gel was obtained with a transmission scanner (GS710, Bio-Rad Laboratories).

The polymeric nature of the enzyme was determined by treating 60 μL of each sample with 1 μL of 2-mercaptoethanol at 100°C for 5 min prior to SDS-PAGE in order to achieve separation of the subunits that could be present in the enzyme structure. The electrophoretic conditions were as previously described.

The extent of glycosylation of the enzyme was studied by treating 60 μL of each sample with 12 μL of Endo-H recombinant glycosidase (500 U/mL) (New England Biolabs, Ipswich, MA, USA) at 37°C for 15 h prior to SDS-PAGE, which was conducted as previously described.

Enzyme identification

In-gel digestion. Bands of interest corresponding to B05.10, VA612 and RM344 *Botrytis* strains at 94 kDa (non-deglycosylated) and at 30 kDa (deglycosylated) were excised from preparative 10% SDS-PAGE gels, washed sequentially with 150 μL 50% ethanol, then 150 μL 50 mmol/L ammonium bicarbonate (pH 8.4) and repeated once before drying on Speedvac (Thermo Fisher Scientific, Villebon sur Yvette, France). The bands were reduced (10 mmol/L DTT, 56°C, 1 h) and alkylated (55 mmol/L iodoacetic acid, 37°C, 45 min), before dehydration [50% ethanol, 50 mmol/L ammonium bicarbonate (pH 8.4)] and drying on a Speedvac. Bands were covered with the trypsin digestion solution (12.5 mg/mL in 25 mmol/L ammonium carbonate) and digested overnight at 37°C. The digestion was stopped by adding 15 μL of pure formic acid (pH < 4). The generated tryptic peptides were desalted on BRAVO AssayMap (Agilent Technologies) with C18 Tips primed with 50 μL of 70% ACN/0.1% TFA, equilibrated with 50 μL of 0.1% TFA, loaded with sample, washed two times with 50 μL of 0.1% TFA and eluted with 50 μL of 70% ACN/0.1% TFA. Samples were dried with a vacuum concentrator (Labconco, Kansas City, MO, USA) and resuspended in 10 μL of phase A (A, 0.1% formic acid, 2% ACN in water) for LC-MS/MS injection.

Mass spectrometric analysis. Samples (7 μL) were injected into an Ultimate 3000 RSLCnano system (Thermo Fisher Scientific). This LC system was operated in nanoflow mode (<1 μL/min) NanoFlow LC and coupled to a QTOF MS instrument (Impact II, Bruker Daltonics, Champs sur Marne, France) through captive spray ion source operating with a nanobooster. In the LC part, samples were desalted and pre-concentrated online on a PepMap u-precolumn (300 μm × 5 mm, C18 PepMap 100, 5 μm, 100 Å) (Thermo Fisher Scientific). The capillary pump was set at 20 μL/min and the mobile phase was 0.05% TFA, 2% ACN in water. Peptides were separated on an analytical column (75 μm × 500 mm; Acclaim Pepmap RSLC, C18, 2 μm, 100 Å). The peptides were eluted from the reverse-phase column with a gradient consisting of 7–30% B for 45 min and 90% B for 4 min (A, 0.1% formic acid, 2% ACN in water; B, 0.1% formic acid in ACN) at 400 μL/min. Temperature was set at 50°C.

Peptides were identified by data dependent acquisition (DDA) and a lock-mass [*m/z* 1222, Hexakis (1H, 1H, 4H-hexafluorobutyloxy) phosphazine] was used as the internal calibrator. Instant Expertise software (Bruker Daltonics) selected as many as possible of the most intense ions per cycle of 3 s and active exclusion was performed after one spectrum during 2 min only if the precursor ion exhibited an intensity three times greater than the previous scan.

Peptide identification. All MS/MS spectra were searched against a homemade database based on annotated expressed sequence tags (EST) for *B. cinerea* (B05.10 assembly, Database version 87.3asm83294v1). The number of sequences was 12 104 gene transcripts for the *B. cinerea* database. Mascot v 2.4.1 algorithm (Matrix Science, http://www.matrixscience.com/) was used with the following settings: enzyme: trypsin; variable modifications: oxidation (M) and deamidated (N,Q); fixed modifications: carbamidomethyl (C); missed cleavages: 2; instrument type CID: ESI-QUAD-TOF; peptide tolerance: 10.0 ppm; MS/MS tolerance: 0.05 Da; peptide charge: 1+, 2+ and 3+; mass: monoisotopic; C13: 1; minimum peptide length: 5; peptide decoy: ON; adjust FDR (%): 1; percolator: on; ions score cut-off: 12; ions score threshold for significant peptide IDs: 12.

Gene sequencing. The sequences of *BcLcc2* (Bcin14g02510) and *BcLcc7* (Bcin02g07640) from B05.10 were extracted from the genome sequence (Van Kan et al. 2017). For *BcLcc2*, PCR primers MV18 (5′CCTCATCT CATCCTCACCACA3′) and MV19 (5′GCGAGAGGAGGTTGG AAGAT3′) and the high fidelity polymerase Accuzyme (Bioline, London, England) were used to produce a 2226 bp amplicon, covering the totality of the coding sequences and introns. Sanger sequencing was achieved by Eurofins (Hamburg, Germany) by using the primers MV18, MV19, MV22 (5′ACGCTGATGGACTCTTTGGA3′), MV23 (5′GTTGG ATGAGCAGGTGGTTC3′) and MV26 (5′TCCAAAGAGTCCA TCAGCGT3′). The gene *BcLcc7* was amplified with the primers MV32 (5′TCTCGTGTGCATTGACATGG3′) and MV3 3 (5′AAGCGAACATAAGAAGGCCG3′), and the additional primers MV34 (5′TACTGGTTCCGTGCTGAAGT3′) and MV 35 (5′AGCGGAAGATGATGGAGTGG3′) were designed for sequencing. Consensus sequences and alignments were produced using the MUSCLE procedure implemented in CodonCode Corporation software (Centerville, MA, USA).

Enzyme characterisation

pH. Citrate-phosphate buffer, phosphate buffer and carbonate-bicarbonate buffer were used to reach a pH value ranging from 2.8 to 10.9. Spectrophotometric measurements were in triplicate at a constant temperature of 25°C using ABTS as the substrate for the enzyme as described previously.

Temperature. The assays at a range of temperature were undertaken with a Peltier (Agilent Technologies) thermostated multicell holder coupled to an Agilent Cary 60 spectrophotometer (Agilent Technologies). Spectrophotometric measurements were recorded in triplicate at 20, 30, 40, 50 and 60°C at 415 nm as described above.

Relative activity test with several phenolic substrates

The rate of oxygen consumption of a solution containing a phenolic substrate and the enzyme was measured using a micro cathode oxygen electrode (Clark-type polarographic electrode) calibrated as previously described (Lambert et al. 2003). The system was coupled to a dual channel oxygen meter and equipped with two RC350 respiration cells. Oxygen consumption was monitored by Strathkelvin 928 System Data Analysis Module software (version 2.3.1.4) (Strathkelvin Instruments, North Lanarkshire, Scotland). Oxygen consumption was specified as the amount of oxygen disappearing from the respiration chamber over time per microgram of protein [(μmol O_2/min)/μg protein].

Electrochemical measurements

The electrochemical measurements were made with an Autolab Potentiostat/Galvanostat (PGSTAT302N) equipped with the GPES software (Metrohm-Autolab, Zofingen, Switzerland), which were carried out at 20 ± 2°C in deaerated (with argon) citrate-phosphate buffer (pH 3.5). Screen-printed electrodes (4 mm diameter, SPGE 220 BT, Dropsens, Parque tecnológico de Asturias, Llanera, Asturias, Spain) were used in a three-electrode configuration comprising a gold working electrode with a silver reference electrode and a platinum counter electrode. An aliquot of 100 μL of laccase solution (0.5 mg/mL) was cast onto the surface of the electrode [covered by a polyamide meshed membrane (Dropsens) in order to minimise the deposited volume of solution] and the electrochemical measurements were made immediately. All the measurements were done in triplicate. Electrode reference values were provided by Dropsens.

Statistical analysis

ANOVA and post-hoc Tukey HSD test were performed using the software package for Windows XLSTAT Version 2016.04.32525 (Addinsoft, Paris, France). The confidence level for the post-hoc Tukey HSD was set at 95%.

Results

Purification of extracellular laccases

Laccase activity was tested in the fractions obtained during the purification process as previously described. For the three strains, only the fractions with a molecular weight higher than 30 kDa showed laccase activity and were retained for further analysis.

Enzyme production

The three strains produced extracellular laccase enzymes when cultured under the described conditions. The mass of the dry fungal biomass was 5.56, 7.49 and 4.63 g for B05.10, VA612 and RM344 strains, respectively.

The content of protein found in the active fraction of each culture media was 24.3 mg for B05.10 strain, 18.1 mg for VA612 and 17.1 mg for RM344 strain. The yield of protein per gram of dry fungal biomass was 4.35 mg/g for B05.10 strain, 2.42 mg/g for VA612 strain and 3.69 mg/g for RM344 strain. The strains collected from grapes presented a lower protein production per gram of biomass than that of the reference strain B05.10.

Molecular weight subunit composition

The SDS-PAGE of the active fractions showed the presence of only one band corresponding to the laccase proteins. The three strains produced laccase enzymes with a molecular weight of 94 kDa according to their migration in the 10% SDS-PAGE gel (Figure 1a). This value is in good agreement with the value previously reported by Slomczynski et al. (1995). Nevertheless, values ranging from 36 kDa (Marbach et al. 1984) to 97 kDa (Slomczynski et al. 1995) have been reported, suggesting that there is a great variability in the enzymes with laccase activity produced by *B. cinerea* strains. The monomeric nature of the enzymes is confirmed after the treatment with 2-mercaptoethanol previous to the electrophoresis of the enzymes (Figure 1a).

Carbohydrate content

The molecular weight of the enzymes, after removal of the glycosidic residues, is 30 kDa for the three strains (Figure 1b). This indicates that approximately 68% of the enzyme weight is due to glycosidic residues.

Mass spectrometry identification

Laccase-2-BcLCC2 (*BcLCC2* gene) and Laccase-3-BcLCC7 (*BcLCC7* gene) protein were identified with high confidence in the bands excised from the SDS-PAGE gel (Figure 1b) (Sequences shown in Appendix S1). According to the abundance of MS fragments, the quantity of Laccase-2-BcLCC2 appeared to be higher than the quantity of Laccase-3-BcLCC7 in all the excised bands.

Sequence of genes encoding laccase 2 and laccase 3

The *BcLcc2* gene of the two strains isolated from grape were sequenced and compared to the sequence of the gene of the

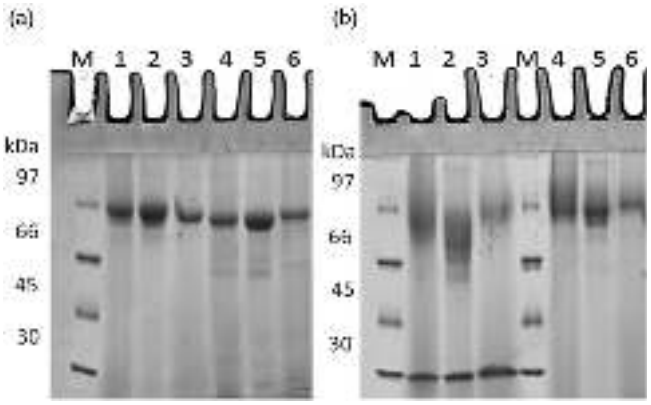

Figure 1. (a) Scan of SDS-PAGE gel showing: (M) molecular weight marker; (1), B05.10 laccase; (2), VA612 laccase; (3), RM344 laccase; (4), B05.10 laccase treated with mercaptoethanol; (5), VA612 laccase treated with mercaptoethanol; and (6), RM344 laccase treated with mercaptoethanol. (b) Scan of SDS-PAGE gel showing: (M), molecular weight marker; (1), B05.10 laccase treated with Endo-H recombinant glycosidase; (2), VA612 laccase treated with Endo-H recombinant glycosidase; (3), RM344 laccase treated with Endo-H recombinant glycosidase; (M), molecular weight marker; (4), B05.10 laccase; (5), VA612 laccase; and (6), RM344 laccase.

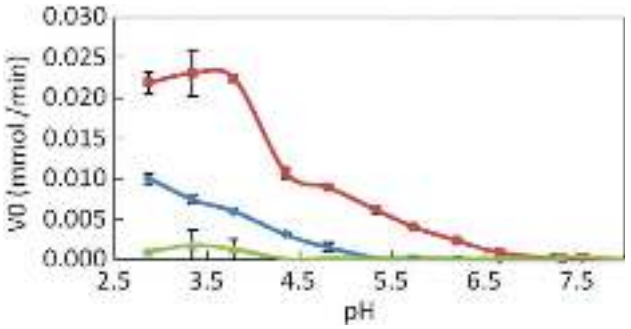

Figure 2. Effect of pH of the medium on the initial reaction rate (V_0) for laccase B05.10 (●), VA612 (●) and RM344 (●). Error bars show SD.

reference strain B05.10, that is Bcin14g02510.1. The sequence of *BcLcc2* was identical in the three strains (Appendix S2).

The *BcLcc7* gene was also sequenced. Slight modifications between the strains isolated from grapes and the reference strain (Bcin02g07640) were observed, nevertheless, most of them were silent mutations or affected intron regions. Only two of the observed modifications led to changes in the amino acid sequence of the laccase enzyme: one of them is a change from serine in B05.10 strain to threonine in RM344 and VA612 strains and the second one is a change from serine in B05.10 to asparagine in RM344 and VA612 (Appendix S3).

In conclusion, the *BcLcc2* and *BcLcc7* amino acid sequences are identical in the RM344 and VA612 strains, and only *BcLcc7* differs slightly (i.e. two amino acid changes) from the sequence of the reference strain B05.10.

Effect of pH and temperature on enzyme activity

The assays conducted in ABTS solutions ranging from pH 2.8 to 10.9 pointed out slight differences between the enzymes obtained from the three strains (Figure 2). All showed high activity at acidic pH while they were not active at basic pH. The maximal activity was reached at pH 2.8 for the laccases obtained from the B05.10 strain, while the maximum activity of the enzymes obtained from VA612 and RM344 strains was at pH 3.3. It is also noteworthy that the laccases from the RM344 strain lost their activity in a medium with a pH greater than 3.8; the enzymes from B05.10 strain were inactive at pH greater than 5.8 while the laccases from VA612 strain remained active to pH 6.7.

Temperature affected the activity of the enzymes obtained from the three strains (Figure 3). The laccases obtained from VA612 were quite stable between 20 and 50°C and a decrease in activity level (30.2% of the activity determined at 20°C) was noticeable only when the temperature reached 60°C (Figure 3c). The increase in the reaction rate was proportional to the temperature: at 20°C 2 min were needed to achieve the maximum activity level, while at 50°C it was reached in 69 s. The enzymes isolated from B05.10 (Figure 3a) presented their highest activity at 20°C. A slight decrease (11.3%) in the activity was observed when the reaction was carried out at 30 and 40°C, but with no difference between them. For the highest temperature assayed (50 and 60°C) a considerable decrease in the activity was observed (reduction of 40.8 and 60.9%, respectively) compared to the maximum activity level measured at 20°C.

The enzymes isolated from RM344 were sensitive to temperature increase (Figure 3b). The maximal activity decreased by 17.9, 40.7, 49.2 and 52.8%, respectively, at 30, 40, 50 and 60°C compared to the activity level at 20°C.

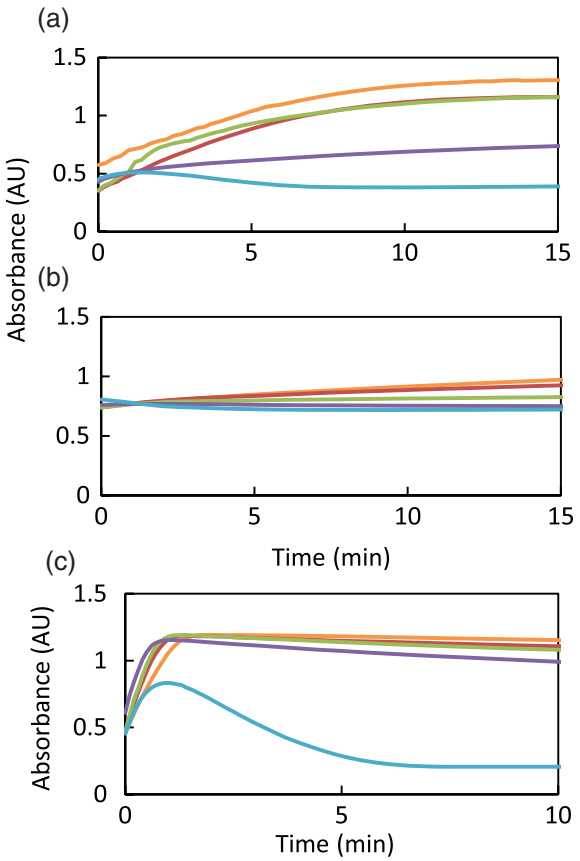

Figure 3. Effect of temperature on enzymatic activity of (a) enzyme isolated from B05.10 strain; (b) enzyme isolated from RM344 strain; and (c) enzyme isolated from VA612 strain. 20°C (—); 30°C (—); 40°C (—); 50°C (—); 60°C (—).

Table 1. Oxygen consumption of the laccase enzymes in the presence of phenolic substrates.

Phenolic substrate	Oxygen consumption [(μmol O_2/min)/μg protein]		
	B05.10	**VA612**	**RM344**
Caffeic acid	$1.34\ (\pm0.2) \times 10^{-04}$ a	$2.44\ (\pm0.6) \times 10^{-03}$ bcd	$6.11\ (\pm2.9) \times 10^{-05}$ a
Catechin	$1.63\ (\pm0.7) \times 10^{-04}$ a	$1.39\ (\pm0.5) \times 10^{-03}$ ab	$1.90\ (\pm2.6) \times 10^{-05}$ a
Epicatechin	$1.60\ (\pm0.5) \times 10^{-04}$ a	$1.87\ (\pm0.4) \times 10^{-03}$ abc	$4.59\ (\pm3.5) \times 10^{-05}$ a
Gallic acid	$8.02\ (\pm4.0) \times 10^{-05}$ a	$4.09\ (\pm1.4) \times 10^{-03}$ cd	$3.44\ (\pm3.3) \times 10^{-05}$ a
p-Coumaric acid	$5.33\ (\pm3.2) \times 10^{-05}$ a	$2.17\ (\pm0.9) \times 10^{-04}$ ab	$4.74\ (\pm3.4) \times 10^{-05}$ a
Phloroglucinol	$4.44\ (\pm2.9) \times 10^{-05}$ a	$6.40\ (\pm3.5) \times 10^{-05}$ a	$2.77\ (\pm2.2) \times 10^{-05}$ a
Quercetin	$5.29\ (\pm2.0) \times 10^{-04}$ b	$4.70\ (\pm0.2) \times 10^{-03}$ d	$8.90\ (\pm4.8) \times 10^{-05}$ a
trans-Ferulic acid	$2.32\ (\pm0.7) \times 10^{-04}$ a	$1.48\ (\pm0.5) \times 10^{-03}$ ab	$6.29\ (\pm2.4) \times 10^{-05}$ a

Values are (mean ± SD). Different letters indicate statistical differences using Tukey HSD test (α = 0.05) among samples.

Enzyme kinetics (K_M and V_{max} determination)

The activity of the mixture of the enzymes (Laccase-2-BcLCC2 and Laccase-3-BcLCC7) obtained from the three strains was investigated using ABTS as a substrate at a concentration ranging from 10 to 500 mg/L. The values of K_M and V_{max} were calculated by the extrapolation of the curve obtained for the Lineweaver–Burk plot. The enzymes obtained from the B05.10 strain had a V_{max} of 0.025 ± 0.005 mmol/L · min and a K_M of 0.12 ± 0.01 mmol/L; the enzymes from VA612 strain had a V_{max} of 0.15 ± 0.006 mmol/L · min and a K_M of 0.20 ± 0.05 mmol/L; and the laccases from RM344 had values of 0.002 ± 0.001 mmol/L · min for V_{max} and 2.19 ± 0.83 mmol/L for K_M. The catalytic constant K_{cat}, which can be considered as a measure of the enzyme efficiency, took the values of 71.20/s for B05.10, 222.05/s for VA612 and 1.20/s for RM344.

Relative activity test with several phenolic substrates

The ability of the enzymes to oxidise different substrates was tested with eight phenolic substances. The consumption rate of oxygen in the cell chamber is related to the ability of the enzyme to oxidise each phenolic substrate. Table 1 shows the results obtained for the three strains. Different letters indicate significant differences between samples, taking into account both the phenolic substrates and the strain that produced the laccase enzymes. Quercetin was the best substrate for the enzymes obtained from the three strains, despite that with the RM344 laccases the difference between quercetin and the other substrates tested was not significant. The lack of significance was probably due to its low activity rate, which provoked an increase in the standard deviation of the sample since its activity is quite near to the detection limit of the instrument. The VA612 laccases showed the highest activity for all substrates tested, with a significant difference between the eight compounds, which allows the establishment of four groups with a significant difference between them. The B05.10 laccases showed low oxygen consumption; nevertheless, their activity is from two- to fivefold higher against all the substrates than that exhibited by the RM344 laccases. With the B05.10 laccases, a significant difference was observed only for quercetin when compared to the other phenolic substrates tested.

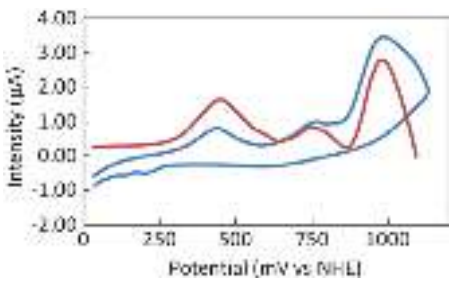

Figure 4. Cyclic voltammogram (——) of RM344 laccase in citrate-phosphate buffer (pH 3.5) at a scan rate of 100 mV/s (current background subtracted) and square wave voltammogram (——) of RM344 laccase in citrate-phosphate buffer (pH 3.5) at frequency 25 Hz (current background subtracted).

Electrochemical measurements

The cyclic voltammogram of the RM344 laccases is presented in Figure 4. Three distinct anodic peaks corresponding to an oxidation reaction at the working electrode can be observed. The cathodic peaks corresponding to the anodic waves for the reduction reaction during the return scan are not present. The square wave voltammogram obtained for these enzymes shows also three well-separated peaks at around the same potentials value as for the cyclic voltammetry experiment. This correspondence between the potentials value was also obtained for the high redox laccase POXA 1b from *Pleurotus ostreatus* (Macellaro et al. 2014). Similar cyclic and square wave voltammograms were obtained for the laccases produced by the other two strains (B05.10 and VA612) (data not shown) with the presence of three well-separated anodic peaks.

An estimate of the redox potential when an oxido-reduction reaction occurs is given from the cyclic voltammogram by the potential (E_{mid}) mid-way between E_{peak}, anodic and E_{peak}, cathodic and by the potential (E_{hw}) halfway between E_{peak}, anodic/2 and E_{peak}, anodic, which can be used when no return cathodic peak is produced (Kilmartin et al. 2001). The values (E'_0) obtained relative to the three waves on the cyclic voltammograms are given in Table 2. For all laccases, the first peak value is around 410 mV

Table 2. Redox potential for the three laccases isolated from *Botrytis cinerea* related to the three anodic waves on cyclic voltammograms.

Strain	E'_0 (Peak 1, mV) versus NHE	E'_0 (Peak 2, mV) versus NHE	E'_0 (Peak 3, mV) versus NHE
RM344	410 b	740 a	960 a
B05.10	410 b	740 a	970 a
VA612	430 a	740 a	970 a

Redox potential (E'_0) was determined from the halfway potential (E_{hw}) = ($E_{peak}/2 + E_{peak}$)/2 for the three anodic waves on cyclic voltammogram [citrate-phosphate buffer (pH, 3.5)]. Different letters indicate a statistical difference among samples using Tukey HSD test (α = 0.05).

(430 mV for the VA612 enzymes), the second one at 740 mV and the third one at 970 mV (960 mV for RM344 laccases), all values versus NHE (normal hydrogen electrode).

Discussion

Our study compared the structural and activity characteristics of laccases produced by three strains of *B. cinerea*. The choice of strains was B05.10, which is the reference strain for *B. cinerea* and has been fully sequenced, and two strains (VA612 and RM344) which were collected in two French vineyards.

The enzymes were isolated from the culture media with a simple procedure, with a good recovery and avoiding the presence of other proteins. For the strains VA612 and RM344, a similar amount of protein was isolated, while for the reference B05.10 strain, the amount isolated was slightly higher. This could be due to a higher production or release of the enzymes from this last strain. Differences in the laccase activity in musts obtained from grapes with a similar degree of contamination are often observed and could be related either to a higher enzyme production or to the presence of enzymes with higher catalytic activity. The conditions of the culture medium, however, used in this study are different to those that could occur under natural conditions, and the production and release of the laccases could be induced by interactions with the host plant or by environmental stress conditions.

It has been previously reported that the production of Laccase-2-BcLCC2 is induced by the presence of resveratrol (Schouten et al. 2002), tannic acid (Schouten et al. 2008) or non-phenolic substances such as a furyl derivative (Caruso et al. 2011) in the culture medium. According to our results, the production of Laccase-2-BcLCC2 and Laccase-3-BcLCC7 could be also induced by the presence of gallic acid in the culture medium. Gallic acid has been previously used as an inducer of the production of laccase enzymes in liquid medium (Gigi et al. 1980), but the identity of the laccase enzymes produced in that study is unknown. To our knowledge, there are no reports concerning the production of Laccase-3-BcLCC7 by *B. cinerea*. The enzymes isolated from the three strains presented the same apparent molecular mass as determined by SDS-PAGE, and the use of mercaptoethanol prior to SDS-PAGE confirmed the monomeric nature of the enzymes. The glycosylation degree of the laccases was also calculated to be 68.1% of the protein total weight. The enzymes isolated from the three strains presented the same degree of glycosylation as determined by SDS-PAGE. This is an unusual and high content for glycosylated proteins. Nevertheless, a similar proportion has been previously reported for *B. cinerea* laccases (Marbach et al. 1984, Thurston 1994). Previous studies have reported high variability in the glycosylation level of laccases of different origins, describing values ranging from 11% in *Neurospora crassa* laccase (Thurston 1994) to 84% reported by Marbach et al. (1984) for *B. cinerea* laccase. In this last study, values ranging from 55 to 71.8% for the glycosidic fraction and from 75 to 84% for the protein fraction of the laccase were found depending on the method employed to determine the glycosylation level and the culture conditions of the fungus. Mass spectrometry revealed the coexistence of Laccase-2-BcLCC2 and Laccase-3-BcLCC7 in the three samples. The molecular weight calculated for the enzymes using MS is 63.4 and 49.3 kDa, which is much lower than that estimated by SDS-PAGE. This discrepancy can be explained by the high carbohydrate content of the laccase enzymes, which would lead to an altered elution on SDS-PAGE, as previously noted by other authors (Slomczynski et al. 1995).

Gene sequencing did not reveal any difference between the genes *BcLcc2* that encode Laccase-2-BcLCC2 enzymes secreted by each strain (sequences provided in Appendix S2). The modifications detected in the sequence of *BcLcc7* genes would provoke the substitution of two amino acids in the protein sequence of Laccase-3-BcLCC7 of RM344 and VA612 strains when compared to the sequence of the enzyme produced by the reference strain B05.10. The VA612 laccases presented a higher activity level when compared to that of RM344 and B05.10. The lack of difference in the protein sequence of RM344 and VA612 for the two enzymes and the minor differences detected with the sequences of B05.10 enzymes allow us to conclude that the activity differences are not related to the protein fraction of the enzymes.

An aliquot of the isolated enzymes was freeze-dried in order to evaluate their stability during this process. A considerable decrease in the activity of the enzymes was observed after this process. The reduction of the enzyme activity during the freeze-drying process has been previously reported by other authors (Shleev et al. 2007), and this result forced us to store the isolated enzymes in 1 mL aliquots at −80 °C until use to avoid stability problems.

Activity assays revealed dissimilarities between the three enzymes. Their calculated K_M, V_{max} and K_{cat} were quite different. The laccase enzymes obtained from VA612 strain were the most active while the enzymes from RM344 had a low activity level when compared to that of the others. The K_M values determined for the enzymes isolated from the three strains denote the greater affinity of B05.10 enzymes for the substrate than VA612 and RM344 enzymes. Nevertheless, the enzymes isolated from VA612 strain presented higher V_{max} and K_{cat}, which are related to the higher activity level exhibited by the enzymes isolated from VA612 strain. Different levels of laccase activity have already been reported for different producing strains and could be related to their pathogeny (Thurston 1994) and in the case of grapes, these differences may have critical consequences for wine quality. The low activity level of the RM344 strain suggests that the infection caused by this strain of *B. cinerea* would have a minor impact on wine quality compared to the infection caused by the other strains studied. From the values of K_M and K_{cat} it is possible to calculate the value of the specificity constant (K_{cat}/K_M) which represents a measure of the catalytic efficiency of an enzyme. The calculated values for this constant were 591.24/(mmol · s/L) for B05.10, 1100.56/(mmol · s/L) for VA612 enzymes and 0.55/(mmol · s/L) for RM344 enzymes. The values determined for the specificity constant also confirm that VA612 enzymes were the most active ones, followed by B05.10 enzymes, while RM344 showed a low activity. These results reflect the great difference regarding the activity level for the enzymes isolated from the three strains and also confirm the variability of the negative effects of the infection in fruits and other vegetables. Mass spectrometry analysis identified Laccase-2-BcLCC2 and Laccase-3-BcLCC7 (Appendix S1) with high confidence in the three strains. To our knowledge, it is the first time that the production of a mixture of Laccase-2-BcLCC2 and Laccase-3-BcLCC7 by *B. cinerea* strains has been reported.

The different activity levels observed in the three strains could be due to the different proportion of these enzymes secreted by each strain or to modifications in their glycosidic

fractions that could lead to differences in substrate affinity. The ability of Laccase2-BcLCC2 to oxidise phenolic substances has been studied by Schouten et al. (2002). To our knowledge, there are no reports concerning the activity characteristics of Laccase-3-BcLCC7.

Under variable medium conditions, the enzymes obtained from the VA612 strain were more resistant to changes in pH and temperature than the laccases obtained from the other two strains. The laccases from B05.10 showed an intermediate behaviour between the enzymes from the other two strains, being more resistant to changes in pH and temperature than RM344 but less than VA612. The activity of RM344 enzymes was sensitive to changes in both parameters. The laccases from the three strains showed their maximal activity at acidic pH, which is in good agreement with the results provided by other authors for laccase enzymes from different origins (Dubernet et al. 1977, Bollag and Leonowicz 1984). Nevertheless, there are some reports for laccase enzymes that are active at basic pH (Saito et al. 2012, Chairin et al. 2014).

The effect of temperature was different for the enzymes from the three strains but they were all more active at 20°C than at higher temperature. Compared to the other two strains, the enzymes obtained from VA612 showed little loss of activity when the temperature was increased. The thermic treatment of wines at 50°C has been used for several years in order to inactivate laccase enzymes, among other enzymes present in wines, and it is reported as the only treatment that can eliminate the risk of oxidation of phenolic substances (Ugliano 2009). These results suggest that the treatment efficiency could be closely related to the strain of *B. cinerea* that causes the infection, since the ability of temperature to inactivate them is not the same for the three strains. Moreover, it is possible that other strains may produce laccase enzymes with even greater resistance to high temperature, and as a result the heat treatment would not be effective. The high resistance of VA612 laccases to changes in pH and temperature could be of interest for industrial applications of *B. cinerea* laccase.

The oxygen consumption in the presence of phenolic substances revealed the differences in the relative activity between the three enzymes (Table 1). The VA612 laccases have higher oxygen consumption than that of the laccases from B05.10 and RM 344, which indicates that this strain would be more efficient for the oxidation of phenolic substances than the other two strains. It is noteworthy that quercetin is the best substrate for the three enzymes while phloroglucinol was the poorest one for two of the laccases.

For the VA612 laccases, there were three phenolic substrates that showed a significant higher activity: quercetin, gallic acid and caffeic acid. It is worth noting that those three compounds contain at least two hydroxyl groups in the *para* and *meta* positions in the phenolic ring while the other compounds tested do not present this structure in their respective molecules. This structure may be involved in the recognition or in the assembly to the active site of the enzymes, as previously reported by Mayer and Staples (2002). Those diphenolic compounds are oxidised by laccase enzyme in musts, producing their corresponding quinones, which are unstable and react with other phenolic and non-phenolic substances, leading to brown products that are involved in wine spoilage (Steel et al. 2013).

Phloroglucinol and *p*-coumaric acid were in general the poorest substrates for the laccase enzymes. Of the phenolic substances tested, they are the only two that do not present two hydroxyl or methoxy groups in adjacent carbons of a phenolic ring, which confirms that this type of substitution is important for the enzymatic interaction.

The different pattern of oxidation of the laccases from the three strains could be critical for the consequences of the infection by *B. cinerea* in fruits and vegetables.

A focus on the determination of the redox properties of laccases has occurred during the past few decades. Indeed, laccases catalyse oxidation of various organic substances, including phenolic substances, coupled with a direct reduction at the same time of dioxygen to water without the production of hydrogen peroxide as an intermediate (Morozova et al. 2007). The laccase enzymes are multicopper oxidases with a catalytic site composed of four copper ions per molecule and these ions can be classified into three types: one type 1 (T1), one type 2 (T2) and two type 3 (T3). The function of the T1 site in this type of enzyme involves an electron capture from the reducing substrate (electron donor) followed by an electron transfer to the T2–T3 copper cluster. The key characteristic of laccase is the standard redox potentials of its redox centres: the T1, T2 and T3 sites.

Among these redox potentials, the redox potential of the T1 site is a key parameter for substrate specificity of the enzyme, since the higher the laccase redox potential is, the wider the range of oxidised substrates (Macellaro et al. 2014). In other words, the catalytic efficiency of laccases for some reducing substrates depends linearly on the redox potential of the T1 copper, meaning that the higher the potential of the T1 site the higher the catalytic efficiency. Thus, laccases with a high redox potential of the T1 site are of special interest in biotechnology, for example producing water as the only by-product. From an electrochemical point of view, all laccases can be divided in three categories depending on the copper T1: high-, medium- and low-potential enzymes, depending on plant or fungal source of laccases (Morozova et al. 2007). The low potential category includes laccases from trees, *Rhus vernicifera* laccase for example, with a T1 site potential of about 430 mV (vs NHE). The middle category is composed of laccases from basidiomycetes, for example *Myceliophtora thermophila*, basidiomycete C30, *Rhizoctonia solany*, *Coprinus cinereus*, with potentials in the range 470–710 mV. At last, the high-potential laccases are from fungi: *Trametes hirsuta*, *Trametes versicolor* and *Trametes villosa*, for example, have a potential around 780 mV (Shleev et al. 2005b).

Shleev et al. (2005a) have suggested that the T2 copper center might have the same formal potential value close to 400 mV vs NHE for many multi-copper oxidases. Indeed, the formal redox potentials of the T2 site under anaerobic conditions for totally different sources are close to each other. For example, for the low-potential laccase from *R. vernicifera* it is known that $E_0' \approx 390$ mV vs NHE and a value of 405 mV vs NHE was found for the high-potential laccase from *T. hirsuta*. The conservation of the redox potential of the T2 site to around 400 mV versus NHE would require that the structure of the T2 redox centres in laccases was conserved in terms of ligands and copper-ligand distances (Macellaro et al. 2014). Thus, from crystallographic data for some laccases and other copper oxidases, Shleev et al. (2005a) showed that such conservation is really retained. All oxidases presented have no significant difference in their structure of the T2 site (e.g. ligand environment and distances between ligands and copper ions or copper–copper distances in the T2–T3 cluster between the T2 copper and the T3 copper), whereas the structures

(ligands) and the redox potentials of the T1 sites for these enzymes are different.

At last, a redox potential value for the T3 site higher than that for the two other sites around 900 mV versus NHE has been observed for *T. hirsuta* laccase under anaerobic condition (Shleev et al. 2006).

The voltammograms obtained by both cyclic and square wave voltammetry were characterised by three anodic peaks independent of the laccase. One can expect that each peak corresponds to three different types of copper ion. During an electrochemical experiment, a random orientation of the laccase versus the electrode can occur. First, some molecules can be oriented on the electrode via the T1 site, that corresponds to the natural transfer from the T1 site to the T2/T3 cluster and thus to the reduction of water. This corresponds to the second peak on the cyclic voltammogram with a determined formal potential at 740 mV. Among the three peaks, the anodic peak intensity is the weakest, showing that only a small fraction of the bound laccase is oriented to the electrode through the T1 site of the enzyme as already observed by Shleev et al. (2006) for *T. hirsuta* laccase and also by Ramirez et al. (2008) for *Trachyderma tsunodae* bilirubin oxidase (also a multicopper oxidase). Table 2 shows that there is no difference between the redox potential of the three enzymes for the T1 site.

Moreover, other laccase molecules can be oriented by their T2/T3 cluster during electrochemical experiment, and in this case both a low and a high potential process, corresponding to the redox transformation of the T2 and T3 sites, are respectively observed (Shleev et al. 2006). As expected, the same values were obtained for the redox potential of the T2 site around a little more than 400 mV whatever the laccase studied, this one being considered to be invariant in many copper oxidases (conservation of the structure) whereas for the highest redox potential of T3 site, no significant difference in potential value was obtained, taking the value around 960–970 mV (Table 2). It can be also observed on the voltammograms that the intensity of the third peak (T3 site) is the most important as it was also obtained for *T. hirsuta* laccase (Shleev et al. 2006). Moreover, the absence of a cathodic peak in relation to the corresponding three anodic waves illustrates the irreversible electrochemistry of these laccases. The absence of cathodic peak shows that the oxidation process of the different copper ions in the active sites of these laccases is significantly faster than that of the reduction process. This behaviour has also been previously obtained for other laccase enzymes (Pita et al. 2006). As previously discussed, the catalytic efficiency of the laccase enzymes is related to their redox potential. The laccases from the three strains presented the same redox potential, but their catalytic activity against the phenolic substances tested was quite different, as well as their catalytic parameters such as V_{max} or K_M. It is possible that the differences among the laccases from the three strains are more related to enzyme-substrate assembly and release than to the catalytic capacity of their active centres.

Conclusions

Two types of laccase, Laccase-2-BcLCC2 and Laccase-3-BcLCC7, are present for each strain of *B. cinerea* as shown by MS. Mass spectrometry did not show any difference between the enzymes produced by each strain, which also presented the same structural characteristics, including molecular weight and glycosylation proportion. Genetic sequencing showed only slight differences in the sequence of Laccase 3-BcLCC7 from the three strains while the sequence of Laccase 2-BcLcc2 remained unchanged across the three strains. Nevertheless, the activity of the enzymes measured against ABTS and other phenolic substrates presented large differences, which could be due to differences in the glycosidic fraction of the enzymes. A second hypothesis to explain the activity differences would be possible differences in the relative amounts of Laccase-2-BcLCC2 and Laccase-3-BcLCC7 produced by each strain. Further research is needed to explain the relative activity observed when the three strains of *B. cinerea* were cultivated under similar standardised conditions. The observed differences in the activity level of the enzymes could have important consequences in the infection process and also in the quality alterations caused by the presence of *B. cinerea* in grapes.

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Supporting information

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Appendix S1. Amino acid sequence of the two identified laccases. The specific sequences used for the identification from MS analysis are shown in bold letters.

Appendix S2. Alignment of the laccase *BcLcc2* encoding gene for the three strains.

Appendix S3. Alignment of the laccase *BcLcc7* encoding gene for the three strains.