Fungi from industrial tannins: potential application in biotransformation and bioremediation of tannery wastewaters

TITLE: FUNGI FROM INDUSTRIAL TANNINS: POTENTIAL APPLICATION IN BIOTRANSFORMATION AND BIOREMEDIATION OF TANNERY WASTEWATERS

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Abstract

Tannins are a complex family of polyphenolic compounds, widely distributed in the plant kingdom where they act as growth inhibitors towards many microorganisms including bacteria, yeasts, and fungi. Tannins are one of the major components of tannery wastewaters and may cause serious environmental pollution. In the present study, four different tannins (the hydrolysable chestnut ellagitannin and tara gallotannin and the condensed quebracho and wattle tannins) were characterized from a mycological point of view with the aim of selecting fungal strains capable of growing in the presence of high tannin concentration and thus potentially useful in industrial biotransformations of these compounds or in the bioremediation of tannery wastewaters. A total of 125 isolates of filamentous fungi belonging to 10 species and four genera (*Aspergillus*, *Paecilomyces*, *Penicillium* and *Talaromyces*) were isolated from the tannin industrial preparations. Miniaturized biotransformation tests were set up with 10 fungal strains and the HPLC (High Performance Liquid Chromatography) analysis pointed out a strong activity of all the tested fungi on both chestnut and tara tannins. Two strains (*Aspergillus tubingensis* MUT 990 and *Paecilomyces variotii* MUT 1125), tested against a real tannery wastewater, were particularly efficient in COD (chemical oxygen demand) and tannin removal (>60%), with a detoxification above 74%. These results indicate that these fungi are potentially exploitable in the treatment of tannery wastewaters.

Keywords: Tannin, fungi, tannery wastewater, biotransformation, bioremediation

Introduction

Tannins are secondary metabolites widely distributed in the plant kingdom. Plants generally contain 5-20% tannin by weight, but those ones used for tannin production may contain even up to 40% (van Diepeningen et al. 2004). Most used raw materials for industrial production are chestnut wood (18% of tannin in wood on dry matter, DM), quebracho hardwood and mimosa barks (24% in both), tara pods and Chinese or Turkish gallnut (50% in both). Tannins are located in vacuoles of intact plant cells, but upon microbial attack (i.e. viruses, bacteria, and fungi), they are released helping to fight the infection of plant tissues (Silva et al. 1997). In addition, tannins offer protection against ruminants due to the formation of complexes between plant tannins and animal proteins such as hydroxyproline-rich proteins. Formation of such complexes results in a bitter and disagreeable sensation, which deters potential predators (Edelmann and Lendi 2002).

From a chemical point of view, tannins are a complex family of water-soluble polyphenolic compounds with molecular weight between 500 and 3,000 Da (Aguilera-Carbo et al. 2008). They can be classified into hydrolysable and condensed tannins. Hydrolysable tannins could be gallotannins or ellagitannins depending on the substances that are produced by hydrolysis (by acids, bases or certain enzymes): glucose and gallic acid or glucose and ellacig acid. Tara tannin has been shown to be mainly composed by a mixture of esters of gallic acids on quinic acid, where the predominant structure is a pentagalloyl quinic acid (Giovando et al. 2013). The tannin contained in Tara pods and in Chinese or Turkish gallnuts, after extraction and purification, is known as tannic acid, a molecule with ester groups connecting until 10 gallic acid moieties on a glucose core (Fig. 1A).

In ellagitannins, one or more hydroxydiphenoyl residues are linked to glucose as diester but can be further hydrolysed to ellagic acid. Chestnut tannin is part of this class of products. It contains castalagin (Fig. 1B), which represents, with the isomer vescalagin, around 30% of the product. It has been shown that these substances and their higher oligomers are present in this tannin and are quite stable because are coming from rearrangement of polypentagalloylglucose naturally occurring in the chestnut wood (Pash and Pizzi 2002). The higher oligomers contain repeating units of polygalloylglucose chain where galloyl groups can be linked differently to each other (Pizzi et al. 2009). The chestnut tannin has been found to be composed also of digalloyl glucose, glucose and gallic acid (Radebe et al. 2013a).

Condensed tannins are polymers of a mixture of flavan-3-ols or flavan-3,4-diols. They are often referred to as “flavolans”, recently becoming known as proanthocyanidins. Quebracho and wattle extracts are the tannins the most produced industrially and have clearly shown to be respectively predominantly composed by oligomers of profisetinidins and prorobinetinidins. Moreover, wattle tannin is heavily branched due to the presence of lot of angular units in its structure, while quebracho tannin is mostly a linear oligomeric structure (Pash et al. 2001). The angular molecular structure of wattle tannin in theory makes it more difficult to depolymerize compared to quebracho tannin (Radebe et al. 2013b). Catechin, gallocatechin and epi-gallocatechin are all precursors of condensed tannins (Chowdhury et al. 2010). In particular quebracho tannin contains 95% of proathocyanidins being a mixture of oligomers of epicatechin and fisetinidin that could be represented by a trimer (Fig. 1C) (Venter et al. 2012).

Tannins act as growth inhibitors towards many microorganisms including bacteria, yeasts, and fungi. Their antimicrobial properties are due to their irreversible interaction with microbial proteins or polysaccharides. Besides, tannins are recalcitrant to enzymatic degradation (Silva et al. 1997). In particular, condensed tannins are more resistant to microbial attack than hydrolysable tannins, being toxic for foodborne pathogens (Aguilera-Carbo 2008).

The capability of microorganisms to tolerate the presence of tannins and/or use these compounds as carbon source is associated to the presence of the enzyme tannin acyl hydrolase, also known as tannase (EC 3.1.1.20) (Deschamps et al. 1983; Govindarajan et al. 2016; Knudson 1913). Tannin derivatives can directly act as inducers of tannase production in filamentous fungi of the genera *Aspergillus* and *Penicillium*, but also in yeasts and bacteria (Aguilera-Carbo et al. 2008; Bhoite and Murthy 2015; Ma et al. 2015; Silva de Lima et al. 2014). Tannase catalyses the hydrolysis of ester bonds in hydrolysable tannins such as tannic acid that releases glucose and gallic acid (Lekha and Lonsane 1997). Tannase-producer fungi are an undoubted biotechnological attraction, since tannase is a commercially valuable enzyme, extensively used in a variety of fields including food, beverage, chemical, and pharmaceutical industries (Govindarajan et al. 2016; Ma et al. 2015). Fungi could perform very well biotransformations of polyphenolic substances contained in tannins. Moreover, filamentous fungi capable of degrading tannins could have a strong environmental impact as bioremediation agents. Tannins are indeed one of the major components of tannery, which may ultimately cause serious environmental pollution (Cassano et al. 2003; Romero-Dondiz et al. 2015), having for instance cytotoxic effects in Australian parakeets (de Souza et al. 2017). However, biodegradation of tannins is in an incipient stage and further studies have to be carried out to exploit the potential for large-scale application in food, fodder, medicine, and tannery effluent treatment (Aguilera-Carbo et al. 2008; Govindarajan et al. 2016).

In the present study, the air of a tannin facility warehouse and the tannins' industrial preparations themselves were characterized from a mycological point of view using four tannins (two hydrolysable and two condensed) as sole carbon source. The aim was to select fungal strains capable of growing in the presence of high tannin concentration and thus potentially useful in industrial biotransformations of these compounds or in the bioremediation of tannery wastewaters that, notoriously, contain high concentrations of tannins, usually not degraded by activated sludge. Therefore, two fungi previously isolated from the studied tannin substrates were tested against a real tannery wastewater, collected from a tannery wastewater treatment plant.

Materials and Methods

Tannins

Four different tannins, mainly used for vegetable tanning, were kindly provided by Silvateam S.p.A. (Italy, San Michele di Mondovì): two hydrolysable tannins, chestnut tannin (from wood of *Castanea sativa*) and tara tannin (from pods of *Cesalpinia spinosa*), and two condensed tannins, quebracho tannin (from wood of *Schinopsis lorentzii*) and wattle tannin (from barks of *Acacia mearnsii*). Wattle tannin (trade name Mimosa OP) and quebracho tannin (trade name Tan'Activ BPQ) were extracted with hot water at around 130 °C from barks and hardwood, respectively. The raw

extracts were concentrated at 50% of dry material and spray-dried to obtain the respective tannin in powder form. Chestnut tannin (trade name Tan'activ C) was extracted with hot water at around 110 °C. The first extract, removed from hemicelluloses and gums by purification steps, was concentrated at 50% of dry material and spray-dried to obtain a powder. The tara tannin used in this study (trade name Ormotan T) is a grounded powder obtained milling the pods of the plant. The chemical characteristics of the four tannins are reported in Table 1.

Isolation and identification of tannins mycobiota

In order to select fungi capable of growing in the presence of high concentrations of tannins as sole carbon source, culture media were prepared solubilizing tannins in sterile water at the concentrations of 30% and 50% w/v. Tara tannin, due to its low solubility in water, was solubilized at a maximum concentration of 44% w/v.

For each culture medium, 10 plates (15 cm diameter) were set up, each containing 50 ml of medium; 5 plates were exposed for 1 h to the air of the warehouse of the Silvateam facility where all these tannins pass through; the other 5 plates were kept closed (not-exposed plates) to directly isolate fungi from the tannins' industrial preparations.

In addition, in order to make a comparison with the airborne mycoflora of the same environment, 10 plates containing malt extract agar-MEA (glucose 20 g/l, malt extract 20 g/l, peptone 2 g/l, agar 18 g/l) and 10 plates containing dichloran glycerol agar-DG18 (Oxoid, Rodano, Italy) where exposed for 1 h in the same environment.

The plates were incubated at 25 °C in the dark. At regular time intervals, the colony forming units (CFUs) were counted and the different fungal morphotypes were isolated in pure culture.

Fungi were identified with a polyphasic approach, which combines morpho-physiological studies with molecular ones. After determination of genera according to macroscopic and microscopic features (Domsch et al. 1980; Kiffer and Morelet 1997; von Arx 1981), fungal isolates were transferred to the media recommended by the authors of selected genus monographs for species identification. Molecular identification was performed by amplification and sequencing of the appropriate DNA region, according to the genus (Gnavi et al. 2017; Poli et al. 2016). PCR products were purified and sequenced at Macrogen Europe Laboratory (Amsterdam, The Netherlands).

The resulting sequences were compared with reference sequences in online databases provided by the CBS-KNAW Collection (Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands) and the NCBI National Center for Biotechnology Information (Bethesda MD, USA).

Isolates of the same species were subjected to minisatellite screening using the universal M13 primer (Poli et al., 2016) in order to recognize identical strains and to exclude duplicates from further analyses. Representative strains of each species isolated in pure culture during this work are preserved at Mycotheca Universitatis Taurinensis (MUT, www.mut.unito.it) of the Department of Life Sciences and Systems Biology, University of Turin (Italy). The accession numbers of the sequences deposited in GenBank are: MF150872 – MF150903.

Statistic analysis

For each species, the mean number of colonies per plate and the relative abundance (%) per substrate of isolation were calculated.

All statistical analyses were performed using the statistical package PRIMER 7 (Plymouth Routines In Multivariate Ecological Research, Albany Auckland, New Zealand) (Clarke and Warwick, 2014). Significant differences among mycobiota of different substrates were evaluated by applying a Permutational Multivariate Analysis of Variance (PERMANOVA; pseufo-F index; $p < 0.05$) and visualized by The Non-Metric Multi Dimensional Scaling (NMDS). The contribution of single species (in percentage) to the diversity observed within and between groups was assessed by SIMilarity PERcentage (SIMPER) analysis.

Tannin biotransformation tests

Tannin biotransformation tests by submerged fermentation were performed using 10 fungal strains selected for their ability to grow at the highest tannin concentrations of and/or developed in plates not exposed to air.

Fungi were inoculated as conidial suspension (final concentration of $2.5 \cdot 10^5$ conidia/ml) in 100 ml Erlenmeyer flasks containing 50 ml of malt extract medium and incubated at 25 °C for 7 days in agitated condition at 80 rpm with a Minitron Infors orbital shaker (Bottmingen, CH) in order to obtain biomasses for the subsequent steps of the test. After incubation, the cultural medium was completely removed and replaced with 50 ml tannin solution (30% w/v chestnut, quebracho or tara tannin in water). Incubation with tannin solutions was performed under the conditions described above. Samples of tannin solutions without biomass were used as abiotic control. Each trial was performed in triplicate.

At the beginning of the experiment (t_0) and after 3 (t_3), 7 (t_7), 10 (t_{10}) and 14 (t_{14}) days, 2 ml of tannin solutions were sampled for HPLC analysis. At the end of the experiment, the fungal growth (dry weight) in presence of tannins as sole carbon source was evaluated.

Tannery wastewater treatment

Aspergillus tubingensis MUT 990 and *Paecilomyces variotii* MUT 1125 were used to treat a tannery wastewater collected from a tannery wastewater treatment plant (Consorzio Cuoio Depur s.p.a., Italy). The wastewater had an initial chemical oxygen demand (COD) (Spectroquant, COD Cell Test, Merck, Darmstadt, Germany) and total tannins (Folin-Ciocalteu, phenol equivalent) of 2375 mg/l and 19.98 mg/l, respectively. The pH was adjusted at pH 7.

Fungi were pre-grown in 250 ml Erlenmeyer flasks containing 150 ml of Malt Extract medium; the inoculum was made from a mycelium homogenate, prepared by homogenizing agar squares (1 cm^2) derived from the margins of an overgrown colony using Ultraturrax (IKA, Staufen, Germany). After 7 days, the medium was replaced with 150 ml of the wastewater supplemented with low amount of glucose (1 g/l). Flasks were incubated at 25°C in the dark at 120 rpm for 10 days. Three biological replicates were set up.

At the beginning and at the end of the experiment, the liquid was sampled for color, COD and total tannins assessment, HPLC and ecotoxicological test evaluating the effects of the tannery wastewater on the algal growth. Color removal was determined as previously described (Anastasi et al.

2010).

The ecotoxicological test followed the ISO 8692:2004 protocol, using the microalga *Raphidocelis subcapitata* (Korshikov) Hindak. The test was performed with the non-treated and the fungal-treated wastewater, evaluating the inhibition percentage (I%) of the algal growth. As the method procedure, for each sample dilutions were prepared: algal growth was evaluated in the presence of the wastewater from 100% v/v (no dilution) to 3% v/v (1:33 diluted). Data of the untreated wastewater at 25% v/v dilution were not reliable due to the high toxicity, close to the maximal sensitivity of the test: the comparison between untreated and treated effluent evaluated data at 12.5% (1:8 diluted).

Significance of differences ($p \leq 0.05$) was calculated by the Mann-Whitney test (IBM SPSS Statistics for Windows, Version 24.0, IBM Corp., Armonk, NY, USA).

HPLC analysis

After filtration on 0.45 μm membranes and after appropriate dilution, sample solutions were injected on a Waters Alliance HPLC system. The column was LiChroCART 125-4 LiChrospher® 100 RP-18 (5 µm, Merck KGaA). A diode array detector (DAD) was set to take signal at 280 nm. After fluxing at 1 ml/min for at least 10 minutes with an aqueous solution prepared with 95% of diammonium hydrogen phosphate 5 mM and phosphoric acid 5 mM and 5% acetonitrile (all the chemicals HPLC grade, from VWR International Srl, Milano, Italy), the sample solutions were injected. The elution took place within 30 min with a linear gradient up to 65% of acetonitrile. Since all components comes out from the column before 25 min, that time was set as the end of the chromatograms.

Results

Mycological analysis

A total of 125 isolates of filamentous fungi belonging to 10 species and 4 genera (*Aspergillus*, *Paecilomyces*, *Penicillium* and *Talaromyces*) were isolated from plates (not exposed and exposed to the air) containing tannins as sole carbon source. The mean number of colonies per plate for each species and culture medium is shown in Table 2. The highest number of species (10) was isolated from tara tannin, followed by quebracho (6 species), chestnut and mimosa tannins (4 species). The plates exposed to air allowed isolating a higher number of colonies and species, with the only exception of tara tannin, in this case no significant difference between exposed and non-exposed plates were recorded.

Among the species grown on chestnut tannin only *P. variotii* was isolated from plates not exposed to the air. This fungus was also the only one able to grow at the highest concentration of chestnut tannin (50%). Four species (*Aspergillus niger*, *A. tubingensis*, *Penicillium minioluteum* and *Aspergillus acidus*) were isolated from not exposed plates containing the highest tara concentration (44%). *A. acidus* and *A. niger* developed also on quebracho not exposed plates. *A. niger* was also able to grow in the presence of the maximum concentration of tannin (50%), though in plates exposed to the air. As regards wattle, only *Penicillium spinulosum* was isolated from not exposed plates, even if at the lowest

tannin concentration (30%), whereas *A. niger* and *A. tubingensis* were able to develop at the maximum tannin concentration (50%).

The relative abundance of each species on different tannins (Fig. 2) highlights that the culturable mycoflora composition changes according to the substrate: on chestnut there was a high prevalence of *P. spinulosum* (41.9%) and *P. variotii* (39.5%), whereas on the other three substrates the most represented species were *A. niger* and other species belonging to the "black *Aspergilli*" group (*A. acidus*, *A. awamorii*, *A. costaricaensis*, *A. tubingensis*, *A. vadensis*). Three species (namely *A. niger*, *A. tubingensis* and *P. spinulosum*) were constantly present on all the tannin substrates, although with different values of relative abundance, whereas *A. awamori* and *A. costaricaensis* were exclusive of tara tannin.

From the plates containing the generic culture media MEA and DG18, 120 fungal isolates, belonging to 8 genera and 12 species were isolated (Table 2). Four species were isolated from MEA, of which the predominant were *Trichoderma harzianum* and *Mucor plumbeus* (38.9% and 16.3%, respectively). The DG18 culture medium allowed the isolation of 12 species, of which the most abundant was *Penicillium brevicompactum* (42.4% relative abundance). *A. niger* was the only species isolated from all the culture media (both generic and containing tannins).

The reciprocal relationship between the different mycobiota can be visualized by Non-Metric Multi Dimensional Scaling (NMDS) analysis (Fig. 3): the fungal communities isolated from chestnut, quebracho and wattle nest together and are separated from those isolated from tara, MEA and DG18 (which are also very distant from each other). Focusing exclusively on tannins mycobiota, quebracho and wattle are very close, while chestnut and tara are spaced from one another. Actually, the PERMANOVA test shows that quebracho and wattle mycobiota are similar ($p = 0.3353$), whereas among the other mycobiota there are significant differences ($p < 0.05$). These differences are ascribable to the isolation substrate since all the groups of samples have proven to be very homogeneous (SIMPER test).

Tannin biotransformation tests

Fungal growth rate

The 10 fungal strains selected for the tannins biotransformation tests are listed in Table 3. The biomass increase for each fungus/substrate pairwise after 14 days of incubation is shown in Fig. 4. All fungi were able to grow on chestnut, quebracho and tara and, in most cases, there was a biomass increase greater than 300% respect to t_0. For most fungal strains, the excessive development of biomass on tara tannin did not allow a reliable assessment. In fact, tara tannin is a very dense substrate and at the orbital shaking speed used during the experiment (80 rpm) many fungi gave rise to a thick mycelial felt, which prevented the separation between fungus and substrate at the end of the test and, therefore, the HPLC analysis at t_{14}.

HPLC analysis

The chromatograms analysis pointed out a strong activity of the fungi, on both chestnut and tara tannins, whereas, no significant change was observed in the chromatogram profile of quebracho tannin.

Chromatograms do not show any changes from t_0 to t_{14} in the non-inoculated samples, confirming the chemical stability of the tannin solutions; hence, any modification of the chromatogram profiles were due to fungi biotransformation.

In the chestnut tannin chromatograms (Fig. 5), in most cases, the peaks of vescalin and castalin (indicated as peak 1 and 2) greatly increase while the peaks of vescalagin and castalagin (peaks 4 and 5) decrease already after 3-7 days of incubation, as a product of the fungus biotransformation activity. The technique used, however, did not allow monitoring the ellagic acid, only present in the water insoluble fraction.

In general, the analysis of chromatograms shows that fungi can be divided into three groups according to their biotransformation activity: the first group including those fungi (*A. tubingensis* MUT 943, *A. niger* MUT 1014, and *T. subinflatus* MUT 1136) for which there was a constant increase of the peak 1 and 2 until the end of the test (Fig. 5A). The second group (*A. niger* MUT 918, *A. acidus* MUT 971, *A. tubingensis* MUT 990, *P. minioluteum* MUT 1123 and *P. spinulosum* MUT 1124) comprising those cases in which the same two peaks reached a maximum at t_7, and then gradually decreased (Fig. 5B). The third group comprising the two strains of *P. variotii* MUT 1083 and MUT 1125, with a peculiar chromatogram profile characterized by two unidentified substances giving peak 6 and 7 increasing from t_7 to t_{14} (Fig. 5C).

Tara chromatograms can be classified with three different conversion rates in gallic acid as shown in Fig. 6. Simultaneously with the growth of the correspondent peak of gallic acid, a total flattening of the peaks eluted from 10 to 16 minutes occurred meaning total hydrolysis of larger oligomeric esters of gallic acid and quinic acid, typically present in this tannin. This was particularly evident for *A. tubingensis* MUT 943 and MUT 990, *A. niger* MUT 918, *A. acidus* MUT 971, *P. spinulosum* MUT 1124 and *T. subinflatus* MUT 1136 where the test had to be stopped at t_7 because of the excess of fungal biomass (Fig. 6A).

As regards *P. variotii* MUT 1083, *A. niger* MUT 918, *A acidus* MUT 971 and *P. minioluteum* MUT 1123, they had an intermediate conversion rate in gallic acid (Fig. 6B), while *P. variotii* MUT 1125 displayed a moderate activity toward hydrolysis and gallic acid production (Fig. 6C).

The analysis of the chromatograms allowed identifying other peaks that progressively increased during the biotransformation process coming from a progressive hydrolysis made by fungi, particularly peak 4 that is an isomer of galloylquinic acid.

With very few exceptions, the profile of quebracho tannin chromatograms after incubation with fungi did not show significant differences compared to the control. Only *P. minioluteum* MUT 1123 (Fig. 7) and *P. spinulosum* MUT 1124 caused a slight increase of peak 1 of the gallic acid and decrease of the peaks eluted from 14 and 18 minutes.

Tannery wastewater treatment

Due to their capability to convert tannins, *A. tubingensis* MUT 990 and *P. variotii* MUT 1125 were chosen to treat real wastewaters. Both fungi were capable of growing in the presence of a tannery wastewater representing almost the sole source of nourishment. The initial addition of glucose was indeed completely consumed within the first 48 h, but fungi grew and remained active untill the end of

the experiment despite the high toxicity of the wastewater. Actually, according to the ecotoxicological assay based of the algal growth inhibition, the tannery wastewater was very toxic, causing the almost complete inhibition of algal growth even when diluted at 25% w/w (1:4 diluted).

Fungi displayed an active metabolism, being active against color, COD and tannins (Table 4). A moderate decolorization (around 30%) was associated to a high COD and tannins removal (above 60%). No significant differences could be observed between the two fungi. Noteworthy, after the fungal treatment the wastewater was less toxic than the beginning: the algal growth inhibition percentage (I%) of the untreated effluent was 30% but decreased up to 6-8% after the fungal treatment (> 70% detoxification).

Discussion

Mycological analysis

Tannery industry is a global concern, producing a turnover of 48 billion euros only in Europe. Following Directive 2010/75/EU on industrial emissions, tannery wastewaters have to be treated before their discharge. The strong environmental pressure of these effluents is associated also to tannins, which represent a major fraction of the organic matter of the effluents. Tannins act as growth inhibitors towards many microorganisms ultimately affecting the receiving ecosystem. Even though vegetable tanning agents are natural materials, they are known for low biodegradability and the presence of phenol content and colour in the effluents (Kanth, 2009). An efficient tannery wastewater treatment plant is the final barrier to contain the problem, but they often work below their potential due to the inhibition of the oxidising bacteria of the biological treatment caused by tannins (Munz et al. 2009). The process clearly needs to be improved, and increasing attention has been giving to those microorganisms capable of surviving in the extreme conditions of tannery wastewaters and degrading tannins. Fungi are known to populate usual and unusual ecological niches with even a strong anthropization pressure (Chambergo and Valencia 2016). Therefore, particular attention should be given to strains isolated from matrixes rich in tannins since they may have developed a unique adapted metabolism in order to exploit tannins as source of nourishment. Lewis and Starkey (1969) reported that pure cultures of some soil fungi grew on media containing tannins as sole carbon source. Certain Aspergilli and Penicillia have been observed to grow on the surface of liquids of tannery pits and tannery wastes, on the surface of tannin-rich woods such as quebracho, European or American chestnut (Bhat et al. 1998). *Penicillium verrucosum* grew on coffee pulp as sole nutrient degrading up to 65% of tannins with up to 3.93 fold higher tannase concentration (Bhoite and Murthy 2015).

Even though there are numerous references to the isolation of fungi from soils, plant material or extreme environments capable of degrading tannins (Lara-Victoriano et al. 2017; van Diepeningen et al. 2004), studies of fungi growing on vegetable tannin raw materials and extracts are missing. To the best of our knowledge, this is the first study on the culturable mycoflora of industrial formulates of tannins belonging to different chemical classes.

The first aim of this study was to investigate from the mycological point of view four different tannins with the aim of isolating fungi adapted to grow on these substrates: the hydrolysable chestnut ellagitannin and tara gallotannin and the condensed quebracho and wattle tannins. Comparing non-

selective (MEA, DG18) and tannins-based media, two distinct mycofloras were observed: isolated fungal species never overlapped, with the only exception of one species (*A. niger*), which was commonly found in all the cultural conditions. Exposing plates to the air of the production line, cosmopolitan airborne fungi grew on MEA (a generic culture medium that allows the growth of the majority of fungal species) and DG18 (allows the preferential isolation of xerophilous and xerotolerant species), but due to the lack of a specific secondary metabolism, they could not grow in the presence of tannins. The fungal strains isolated on MEA and DG18 indeed belong to species commonly present in the air of indoor and outdoor environments and, presumably, they have no ability to grow in the presence of tannins as the sole carbon source or even tannins exert on them an antimicrobial action. More in detail *Cladosporium* is a ubiquitous and widespread genus, often very abundant in indoor and outdoor air samples (Bardei et al. 2017; Demirel et al. 2017) but it is also sensitive to tannins: in a recent study Messini et al. (2017) demonstrated the inhibiting effect of chestnut tannin extract on the proliferation of *C. cladosporioides* on sheep cheese rind during the ripening. In the present study, three species of *Cladosporium* (*C. cladosporioides*, *C. herbarum* and *C. sphaerospermum*) were isolated on DG18 only. This result suggests that fungi isolated from tannins belong to the autochthonous microflora of the industrial non-sterile tannins that actively competed with the airborne propagules.

A prevalence of black *Aspergillus* species was recorded on tannins (exposed and non-exposed plates). Our data are in agreement with van Diepeningen et al. (2004), who used a medium containing 20% tannin to select Aspergilli from soil samples: each of the 642 isolates belonged to the black *Aspergillus* group, *A. niger* and *A. tubingensis* being the most abundant species. They also hypothesized that the ability to grow on 20% tannin could be an exclusive trait for black *Aspergillus* species since 12 different non-black *Aspergillus* species could not grow on media containing more than 5% tannin. The unique ability of black Aspergilli to utilize high concentrations of tannic acid sets them apart from all related fungi. The worldwide occurrence of black *Aspergillus* species may be attributed to their unique role in the degradation of tannin-rich plant compounds and related nitrogen release. Similar results about the capacity of black *Aspergillus* strains to degrade tannic acid were obtained by Lara-Victoriano et al. (2017) studying fungi isolated from soil and plants in extreme environments.

The comparison among the cultivable mycobiota isolated from tannins did not reveal significant differences between quebracho and wattle mycobiota only: being both condensed tannins with very similar chemical composition they could have favoured the establishment of a similar mycobiota.

Regarding chestnut and tara, instead, the most abundant species were *P. variotii* and *A. tubingensis*, respectively. Although they both are hydrolysable tannins, chestnut and tara belong to different categories, as ellagitannin and gallotannin, respectively. Among tannins, tara allowed to isolate the highest number of species (10), four of which (*A. acidus*, *A. awamori*, *A. subinflatus* and *T. costaricaensis*) exclusive of this substrate. This greater biodiversity and fungal abundance (mean number of colonies per plate) could be explained by the fact that this tara tannin was not an extract but milled vegetable material: by grinding the pods, fungal propagules that presumably were on the plant at harvest time can be better preserved. On the contrary, the extreme physical extraction treatments commonly foreseen for chestnut, quebracho and wattle tannins could deeply affect the mycoflora.

Five out of ten species isolated by tannins (*A. awamori*, *A. niger*, *A. tubingensis*, *P. variotii* and *P.*

spinulosum) have long been known to be tannase producers (Aguilar et al. 2007; Silva de Lima et al. 2014; Govindarajan et al. 2016; van Diepeningen et al. 2004). As regards the other five species (*A. acidus*, *A. costaricaensis*, *A. vadensis*, *P. minioluteum*, *T. subinflatus*), this is the first report about their isolation from tannins and the demonstration of their ability to activate a secondary metabolism aimed at converting tannins into carbon source. In addition, all the fungi isolated from tannins have the common physiological feature of being xerophilous or xerotolerant species (Samson et al. 2010).

Noteworthy in the present study, fungi were isolated on substrates with a higher tannin concentration than what generally reported in the literature (30-50% vs. 5-20%). These strains are certainly adapted to live in environments with high tannin content since the environment exerts a selective pressure on the fungal community. These adaptation skills should not be underestimated and could present a tool for a real in field application. Despite the great theoretical potential, bioremediation processes are still fighting for the industrial exploitation, being the discovery of strong and robust microorganisms a critical issue. Therefore, the choice of isolating autochthonous fungi is generally an appropriate practice to select strains with high applicative potential (Bhoite and Murthy 2015; Prigione et al. 2009; Tigini et al. 2014).

Tannin biotransformation tests

Particular attention was given to those strains isolated from tannins and their actual capability to transform them was evaluated: biotransformation tests of three tannins were set up with 10 fungi. The criteria for the selection of fungi were: i) the growth at the highest tannin concentrations; ii) the isolation from plates non-exposed to the air (in order to prefer fungal strains already present the in polyphenolic matrices); iii) the possibility to represent all the isolated genera (in order to explore deeply the fungal biodiversity); iv) on the basis of the minisatellite screening results, the choice of different strains even if belonging to the same species.

Tannase production experiments usually are based on liquid-surface fermentation or solid-state fermentation, but most of the paper work with a single strain (Ascacio-Valdes et al. 2016; Cruz-Hernandez et al. 2006). This study evaluated and innovative and original solution to follow tannins degradation by a screening approach allowing to investigate the tannase production capability of 10 strains in a limited time. All fungi showed a high capacity to grow and produce biomass on all the substrates. In particular, on tara tannin the mycelial growth was so abundant and the substrate so dense that it was not possible to separate them preventing a proper evaluation of the fungal growth. On quebracho (condensed tannin) very high biomass growth rates were recorded, even higher than those obtained on the hydrolysable chestnut tannin. This surprising result is in contrast to what reported by Govindarajan et al. (2016), who indicated that condensed tannins were more recalcitrant for the growth of *Penicillium*, *Aspergillus*, *Fomes*, *Polyporus* and *Trametes* strains.

These data are of great importance as they unequivocally indicate that the 10 selected fungal strains are able to use polyphenolic matrices not only for an explorative growth, but also for a mass growth, by making important transformations of the substrates.

The HPLC analysis allowed to evaluate from a qualitative point of view the biotransformation on tannins and to follow the trend over a period of 14 days.

Among the different hydrolysable tannins, ellagitannins represent the less studied group mainly due to their diversity and chemical complexity. Actually, ellagitannins have the typical C-C bound, which makes them more difficult to degrade than gallotannins (Aguilar et al. 2007). In the present study, chestnut tannin has proved to be an easily degradable substrate from all the tested fungi that, however, have shown different biotransformation activities, especially as regards the time trend. It has been observed a flattening of the peaks eluted between 11 and 16 minutes and the simultaneous appearance of the peak of the gallic acid, which is one of the products of ellagitannins biodegradation by tannase (Aguilera-Carbo et al. 2008). This means that fungi are able to hydrolyse the higher oligomeric structures present in this type of tannin, even if it is composed also by ellagic acid and higher galloyl condensates. In the specific case of the two strains of *P. variotii* a different profile of the chromatogram was observed, with the appearance of two characteristic peaks over 6 minutes and at 9 minutes, never detected for the other fungi.

The fungal biotransformation of tara gallotannin was even more clear: as regard the peak of gallic acid, an increase was recorded already within the first 7 days of incubation, followed by the complete flattening of the peaks eluted from 11 to 16 minutes. Each fungal treatment made in this study on tara tannin shows a production of a big amount of gallic acid due to the hydrolysis of the esters groups linking together gallic acid with the core of quinic acid. This result demonstrates that it is really easy to decompose these groups by the action of fungi. As described before for chestnut, also for tara tannin the two strains of *P. variotii* showed characteristic chromatogram profiles with unique peaks, suggesting that this species could have a different degradation mechanism. In addition, as indicated by the substantial differences between the chromatogram profiles of two strains of *P. variotii*, a marked intraspecific variability was observed. This is in agreement with other findings. For instance, tannase production by *Aspergillus* depended on the strain, showing very different production patterns (Aguilar et al. 2007). The tests made on tara and chestnut tannins show that the activity of fungi is not easily depressed by the increasing presence of gallic acid or ellagic acid.

The evaluation of the chromatograms after the action of fungi on the condensed tannin quebracho shows almost no modification in the polyphenolic molecular structures, probably due to its angular molecular form. Probably these fungi are not able to break the carbon-carbon bonds between monomeric structures or to open the heteroatomic ring. The results obtained from the analysis of the chromatograms of quebracho are in contrast with what emerged from the growth tests: all the fungi were capable of growing on this substrate, but the chromatograms profile was slightly modified; a striking case was *P. variotii* MUT 1125 which displayed a biomass growth rate above 1300% with no changes in the peaks profile. A possible explanation of this phenomenon may lie in the limits of the technique used to monitor the biotransformation; for example, fungi could degrade part of the substrate present in the insoluble fraction and therefore not detectable. This could also indicate that fungal biomass development may be attributable to some sugars, mainly pentoses that are known to be present in this tannin.

The higher recalcitrance of condensed tannins than hydrolysable ones was not unexpected (Bhat et al. 1998). The transformation pathway is mostly unclear: “classical tannases” seem not to be involved in the hydrolization and initial degradation steps are carried out by mono- or di-oxygenases

but further studies are needed to characterize the process (Aguilar et al. 2007). Noteworthy, *P. minioluteum* MUT 1123 and *P. spinulosum* MUT 1124 caused a slight but evident decrease of the peak eluted around 16 minutes. Therefore, these two strains can be considered particularly interesting from an applicative point of view.

Tannery wastewater treatment

Having the actual interest to exploit fungi at industrial level, it was mandatory to assess selected fungi in not controlled conditions, facing real problems. Few reports in literature discussed the fungal treatment of tannery wastewaters but most of them are focused on chromium removal by mycelium absorption (Sathvika et al. 2015, Prigione et al., 2009). The removal of total organic content and the tannins transformation have been rarely investigated. *A. tubingensis* MUT 990 and *P. variotii* MUT 1125 confirmed their robustness, surviving in this extreme environment without any particular modification to the tannery wastewater. On the contrary, *Botryosphaeria rhodina* led to the maximal COD reduction only when the effluent was diluted at 1:10 (Hasegawa et al. 2011). Confirming what observed with other fungi (Hasegawa et al. 2011; Okoduwa et al. 2017), *A. tubingensis* MUT 990 and *P. variotii* MUT 1125 were particularly efficient in COD and tannin removal (> 60%). Moreover, the environmental risk assessment was not limited to the tannins removal but was considered also the ecotoxicological impact of the treated wastewaters. Actually, the transformation of tannins has no value whether the treatment leads to an increase of the toxicity. Noteworthy, in the present study, the fungal treatment strongly reduced the toxicity.

In conclusion, the mycological characterization of four industrial tannins belonging to different chemical categories allowed the isolation of several fungal strains that can grow in the presence of high concentrations of tannins, which, furthermore, represented the only available carbon source. These fungi are undoubtedly endowed with high application potential in the biotransformation field, as demonstrated by the tests carried out, particularly with regard to chestnut and tara tannins. Even more interesting from an applicative point of view are the two strains able to drastically reduce the COD and toxicity of a real industrial effluent from a tanning industry. Obviously, this preliminary result must be confirmed by further tests in conditions similar to the real ones.

Compliance with ethical standards: This article does not contain any studies with human participants performed by any of the authors.

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Table 1 Chemical characteristics of chestnut, quebracho, tara and wattle tannin

	Chestnut tannin	Quebracho tannin	Tara tannin	Wattle tannin
Moisture content	3-5%	6%	7-9%	6%
Tannins on DM (ISO14088 method)	80%	87%	58-65	80%
Non-tannins on DM (ISO14088 method)	~ 14	~ 4	~ 5	~ 14
Insolubles on DM (ISO14088 method)	1-2%	2.5-3%	25-28%	0%
pH at10% in water	3.3-3.5	4.6-4.8	3.6	4-5
Ash content at 550°C	1.5%	2-3%	4.3%	4%

Table 2 Number of colonies per plate (mean of 5 or 10 replicates) for each species and culture medium

	Chestnut				**Quebracho**				**Tara**				**Wattle**				**MEA**	**DG18**
	Not exposed		**Exposed**		**Not exposed**		**Exposed**		**Not exposed**		**Exposed**		**Not exposed**		**Exposed**		**Exposed**	
Species	**30%**	**50%**	**30%**	**50%**	**30%**	**50%**	**30%**	**50%**	**30%**	**44%**	**30%**	**44%**	**30%**	**50%**	**30%**	**50%**		
Aspergillus acidus Kozakiewicz	-	-	-	-	0.2	-	-	-	-	0.2	-	-	-	-	-	-	-	-
Aspergillus awamori Nakazawa	-	-	-	-	-	-	-	-	0.4	-	-	-	-	-	-	-	-	-
Aspergillus costaricaensis Samson & Frisvad	-	-	-	-	-	-	-	-	0.2	-	0.4	-	-	-	-	-	-	-
Aspergillus niger Tieghem	-	-	0.6	-	0.4	-	1.6	0.8	8.0	2.0	4.2	0.8	-	-	1.2	0.2	0.1	7.5
Aspergillus tubingensis Mosseray	-	-	2.6	-	-	-	0.2	-	1.0	4.8	3.2	21.2	-	-	-	0.2	-	-
Aspergillus vadensis de Vries	-	-	-	-	-	-	-	-	0.8	-	-	-	-	-	0.2	-	-	-
Cladosporium cladosporioides (Fresen.) G.A. de Vries	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16.6
Cladosporium herbarum (Pers.) Link	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.4
Cladosporium sphaerospermum Penzig	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	17.7
Epicoccum nigrum Link	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2
Eurotium rubrum Jos. König	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5
Mortierella sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-
Mucor plumbeus Bonorden	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16.3	1.1
Paecilomyces variotii Bainier	0.4	-	0.2	6.2	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-
Penicillium aurantiogriseum Dierckx	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	22.6
Penicillium brevicompactum Dierckx	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	42.4
Penicillium minioluteum Dierckx	-	-	-	-	-	-	0.2	-	1.0	2.2	-	2.0	-	-	-	-	-	-
Penicillium solitum Westling	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.1
Penicillium spinulosum Thom	-	-	7.2	-	-	-	0.8	-	-	-	0.2	-	0.2	-	0.8	-	-	-
Talaromyces subinflatus Yaguchi & Udagawa	-	-	-	-	-	-	-	-	0.2	-	0.8	-	-	-	-	-	-	-
Trichoderma harzianum Rifai	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	38.9	4.2
Unidentified fungus	-	-	-	-	-	-	-	-	0.2	-	0.2	-	-	-	-	-	-	-
Mean number of colonies	**0.4**	**-**	**10.6**	**6.2**	**0.6**	**-**	**3.0**	**0.8**	**11.8**	**9.2**	**9.0**	**24.0**	**0.2**	**-**	**2.2**	**0.4**	**55.4**	**123.3**
Number of species isolated for each of the conditions	**1**	**-**	**4**	**1**	**2**	**-**	**5**	**1**	**8**	**4**	**6**	**4**	**1**	**-**	**3**	**2**		
Total number of species isolated on each substrate	**4**				**6**				**10**				**4**				**4**	**11**

Table 3 Fungal strains selected for the tannin biotransformation tests and their isolation substrates

Species	**MUT number**	**Isolation substrate**		
		Tannin	**Concentration**	**Exposition to air**
Aspergillus tubingensis	MUT 943	chestnut	30%	exposed
Paecilomyces variotii	MUT 1083	chestnut	50%	exposed
Paecilomyces variotii	MUT 1125	chestnut	30%	not exposed
Penicillium spinulosum	MUT 1124	chestnut	30%	exposed
Aspergillus niger	MUT 918	quebracho	50%	exposed
Aspergillus acidus	MUT 971	tara	44%	not exposed
Aspergillus niger	MUT 1014	tara	44%	not exposed
Aspergillus tubingensis	MUT 990	tara	44%	not exposed
Penicillium minioluteum	MUT 1123	tara	30%	not exposed
Talaromyces subinflatus	MUT 1136	tara	30%	exposed

Table 4 Percentage of decolorization, COD and tannins removal (mean ± standard deviation) after the tannery wastewater treatment with *A. tubingensis* MUT 990 and *P. variotii* MUT 1125. The ecotoxicological data are expressed as decrease percentage of the I% after the fungal treatment compared to the untreated wastewater at a sample diluition of 12.5%.

	Decolorization (%)	COD removal (%)	Tannins removal (%)	Decrease of I% (%)
A. tubingensis	23.9 ± 0.9	68.4 ± 5.8	60.3 ± 8.5	73.8 ± 3.1
P. variotii	32.0 ± 4.5	64.2 ± 1.2	62.1 ± 3.3	81.1 ± 2.2

Fig. 1 Molecular structure of tannic acid (A), castalagin (B) and proanthocyanidin trimer isolated from *Schinopsis balansae* tannin [ent-fisetinidol-(4 β →8)-catechin-(6→4 β)-ent-fisetinidol] (C)

Fig. 2 Relative abundance (%) of each species on different culture media containing tannins as sole carbon source

Fig. 3 NMDS analysis with all the samples and repeated excluding MEA and DG18 (in the box)

Fig. 4 Biomass growth rate (%) for each strain and substrate tested

Fig. 5 Chestnut tannin chromatograms at t_0, t_7 and t_{14} of the experiment (dashed line=t_0; grey line in B=t_7; solid line=t_{14}). *Talaromyces subinflatus* MUT 1136 (A); *Aspergillus niger* MUT 918 (B); *Paecilomyces variotii* MUT 1083 (C). The majority of the original substances disappear during the treatment. Peak 6 and 7 are two unidentified new substances that appear during the treatment. Peak 1: vescalin; peak 2: castalin; peak 3: gallic acid; peak 4: vescalagin; peak 5 castalagin; peaks from 10 to 20 minutes: larger oligomers. AU: arbitrary units

Fig. 6 Tara tannin chromatograms at t_0, t_7 and t_{14} of the experiment (dashed line=t_0; solid line=t_7 in A and t_{14} in B and C): *Aspergillus tubingensis* MUT 943 (A), *Paecilomyces variotii* MUT 1083 (B) and *Paecilomyces variotii* MUT 1125 (C). Peak 1, 2 and 4: isomers of galloylquinic acid; peak 3: gallic acid; peak 5 and 6: isomers of digallic acid; peaks from 10 to 16 minutes: larger oligomers. AU: arbitrary units

Fig. 7 Quebracho tannin chromatograms at t_0, t_7 and t_{14} of the experiment (dashed line=t_0; solid line= t_{14}) with *Penicillium minioluteum* MUT 1123. Peak 1: gallic acid. AU: arbitrary units

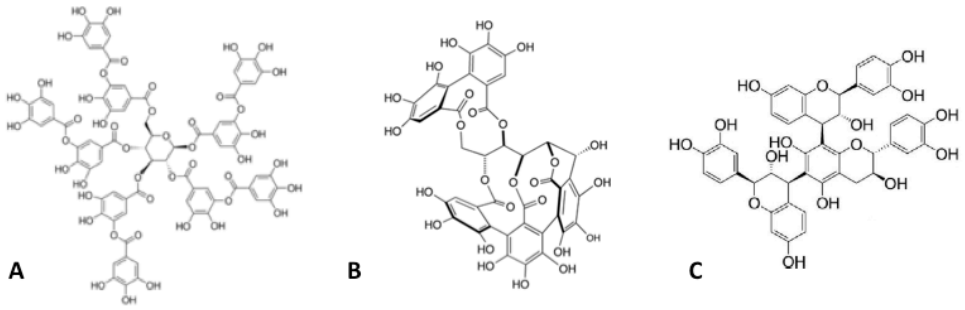
A
B
C

Fig. 1

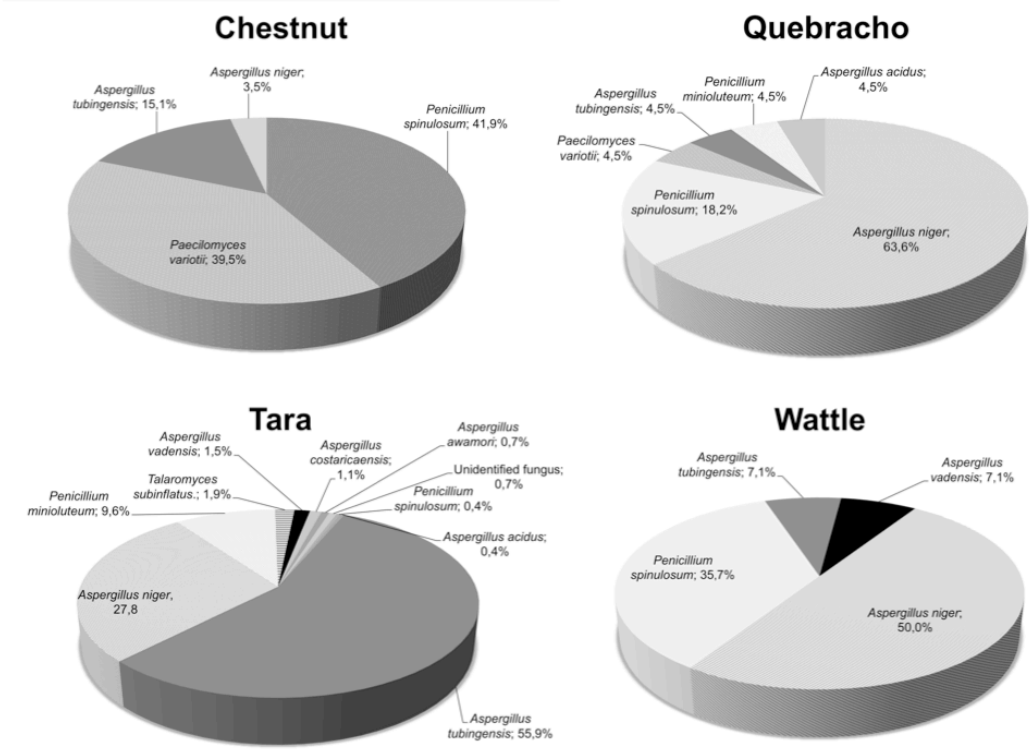
Chestnut
Aspergillus niger; 3,5%
Aspergillus tubingensis; 15,1%
Penicillium spinulosum; 41,9%
Paecilomyces variotii; 39,5%
Quebracho
Aspergillus acidus; 4,5%
Penicillium minioluteum; 4,5%
Aspergillus tubingensis; 4,5%
Paecilomyces variotii; 4,5%
Penicillium spinulosum; 18,2%
Aspergillus niger; 63,6%
Tara
Aspergillus vadensis; 1,5%
Aspergillus costaricaensis; 1,1%
Aspergillus awamori; 0,7%
Unidentified fungus; 0,7%
Talaromyces subinflatus.; 1,9%
Penicillium spinulosum; 0,4%
Penicillium minioluteum; 9,6%
Aspergillus acidus; 0,4%
Aspergillus niger, 27,8
Aspergillus tubingensis; 55,9%
Wattle
Aspergillus tubingensis; 7,1%
Aspergillus vadensis; 7,1%
Penicillium spinulosum; 35,7%
Aspergillus niger; 50,0%

Fig. 2

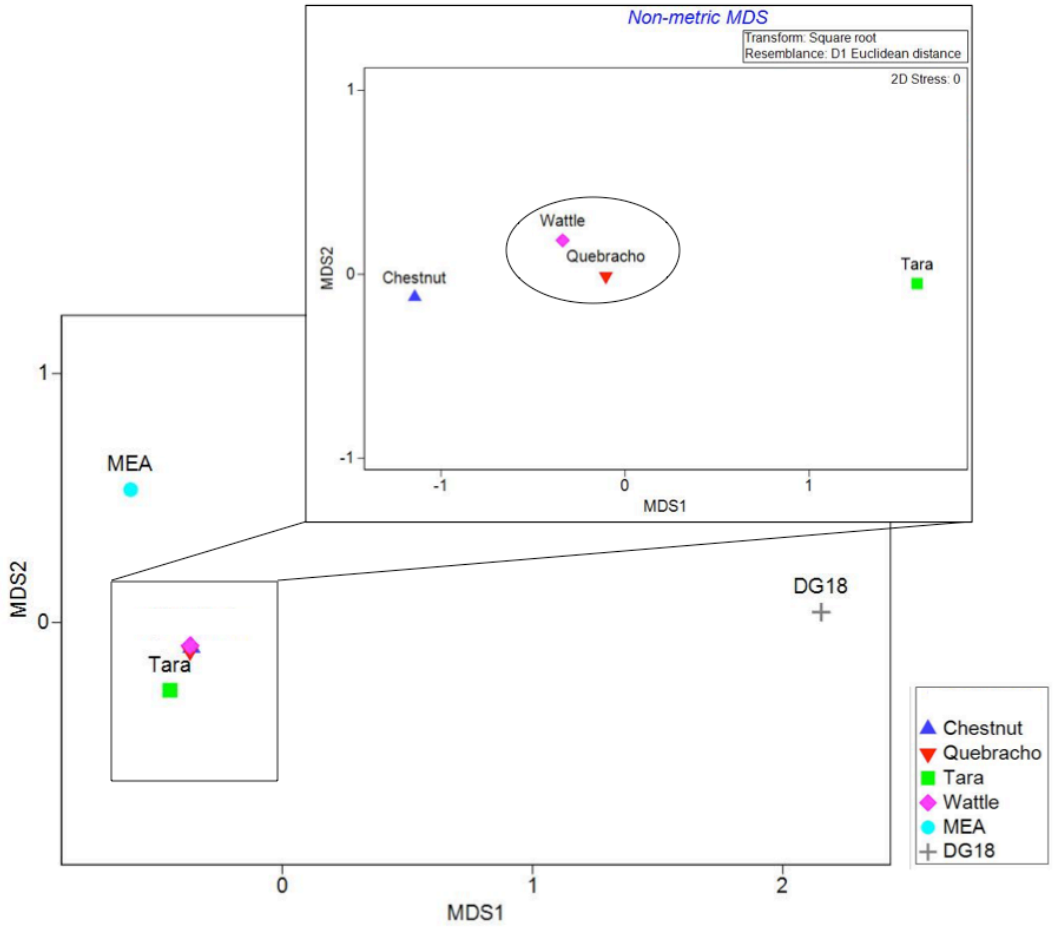
Non-metric MDS
Transform: Square root
Resemblance: D1 Euclidean distance
2D Stress: 0
Wattle
Quebracho
Chestnut
Tara
MDS1
MDS2
MEA
DG18
Chestnut
Quebracho
Tara
Wattle
MEA
DG18

Fig. 3

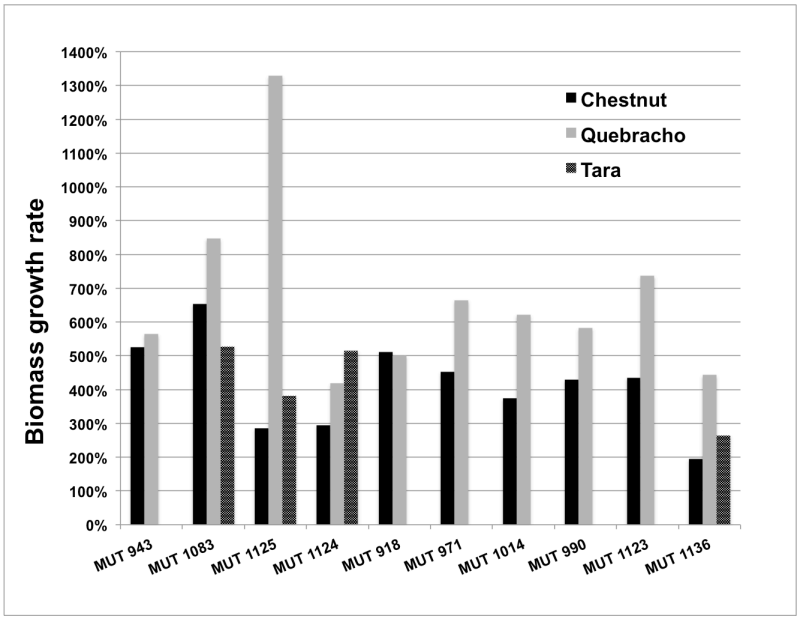
Biomass growth rate
1400%
1300%
1200%
1100%
1000%
900%
800%
700%
600%
500%
400%
300%
200%
100%
0%
Chestnut
Quebracho
Tara
MUT 943
MUT 1083
MUT 1125
MUT 1124
MUT 918
MUT 971
MUT 1014
MUT 990
MUT 1123
MUT 1136

Fig. 4

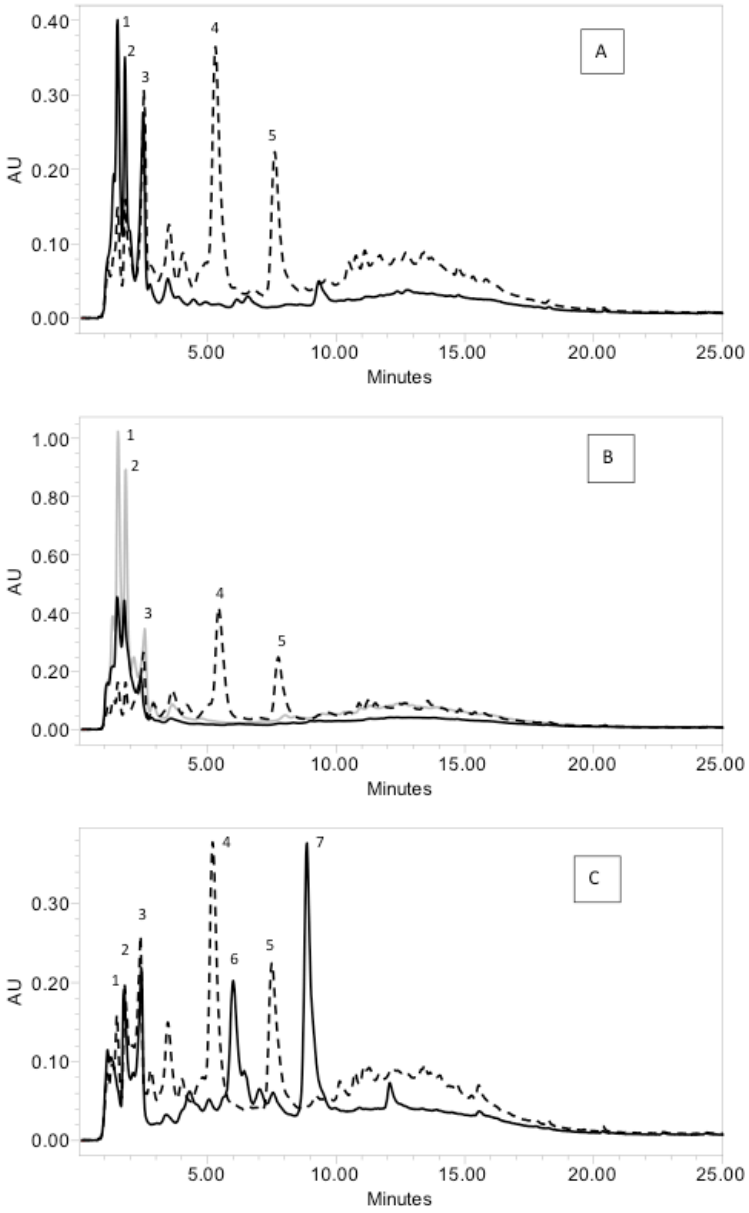
A
B
C
AU
Minutes
0.00
0.10
0.20
0.30
0.40
0.60
0.80
1.00
5.00
10.00
15.00
20.00
25.00
1
2
3
4
5
6
7

Fig. 5

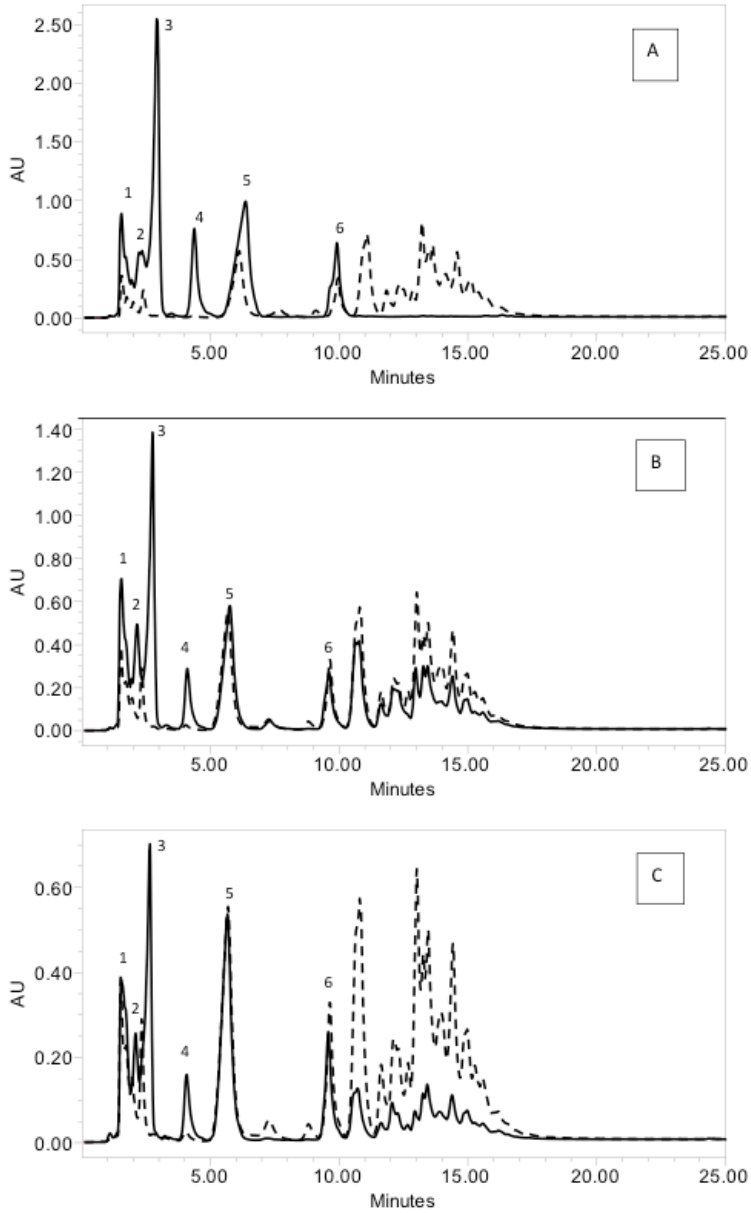
A
B
C
AU
Minutes
1
2
3
4
5
6
0.00
0.20
0.40
0.50
0.60
0.80
1.00
1.20
1.40
1.50
2.00
2.50
5.00
10.00
15.00
20.00
25.00

Fig. 6

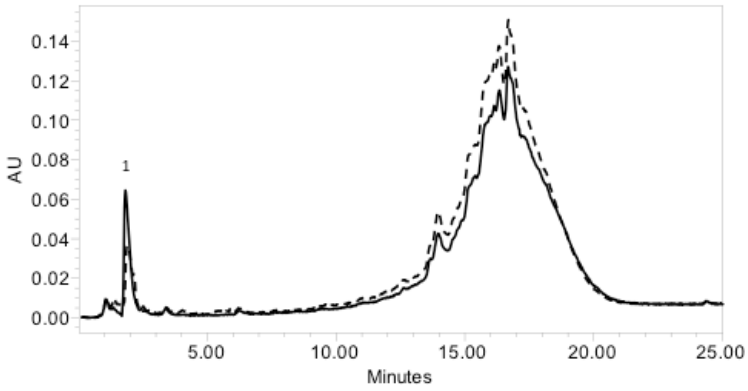
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
AU
1
5.00
10.00
15.00
20.00
25.00
Minutes

Fig. 7