Dynamics of cork mycobiota throughout stopper manufacturing process: from diversity to metabolite

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Knowledge Creation

"Time is life itself, and life resides in the human heart."
Michael Ende

To João, Sofia and my parents

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Summary

Cork, the continuous layer of outer bark of the Quercus suber L. tree, has physical and chemical properties that are unique. Portugal possesses 33 % of the world's cork oak forests and accounts for approximately half of total global cork production.

The manufacture of cork discs (or stoppers) comprises several stages, including two boiling stages, during which slabs of cork are steeped in boiling water. In days following the boiling the humidity of the slabs decreases and they become completely covered in a white mycelium of *Chrysonilia sitophila* until the cork achieves a certain water activity level (ca 0.9 aw). Below this level other fungal species (e.g. *Penicillium*, *Aspergillus* or *Trichoderma*) can germinate and shift the fungal colonization of the cork slabs.

The two main objectives of the research described in the presented PhD thesis are (1) a taxonomic identification of the mycobiota present in cork slabs throughout the manufacture of cork discs, and (2) an investigation into the chemical compounds, which can give unfavourable properties to the cork, produced by these fungi.

To perform the identification of the fungi present in the cork samples, one culture-dependent (isolation) and two culture-independent methods (denaturing gradient gel electrophoresis and cloning technique) were employed. Results show that most of the isolated fungi belong to the *Penicillium*, *Eurotium*, *Chrysonilia*, *Cladosporium* and *Mucor* genera with the most commonly encountered isolated fungal species being *Penicillium glabrum* which was detected in 70 % of the samples.

Consequently, a detailed taxonomic study of *Penicillium glabrum* complex was carried out. One isolate with unique phenotypical and molecular characteristics has been classified as a new species (*Penicillium subericola*).

All employed methods indicate that the mycobiota occurring in the samples taken prior to the first boiling stage appear to be distinct from the population present in subsequent manufacturing stages. Furthermore, the cloning technique confirmed the presence of uncultivable fungi, Ascomycota and endophytes in the raw cork and uncultivable fungi in the samples taken after the first boiling. In the remaining stages the samples were mostly composed of *Penicillium glabrum*, *Penicillium* sp. and *Chrysonilia sitophila* phylotypes.

The possible production of exo-metabolites by some fungal isolates that colonize cork slabs in the resting stage after the first boiling was assessed in one cork-based and two semi-synthetic media cultures. The studied fungi in the cork-based medium culture produced few metabolites with some isolates not producing any metabolite. However, the addition of *Chrysonilia sitophila* remains to the cork-based medium enhanced the exo-metabolome profiles of almost all studied fungi. Deleterious exo-metabolites or mycotoxins were not produced by the studied fungal species in either cork media culture employed.

The study of the chemical compounds produced by the fungi focused on the volatile compounds released by microbial communities during the cork manufacturing process. Results show that the majority of volatiles was produced during two stages: resting stage after the first boiling and cork discs (nontreated) The volatile profiles produced during both stages are similar.

The releasable volatile compounds produced by five isolated fungi either in pure or mixed cultures were analysed using gas chromatography coupled with mass-spectroscopy. Results show that 1-octen-3-ol and esters of fatty acids (medium chain length C8–C20) were the main volatile compounds produced either in pure or mixed culture. *Penicillium glabrum* seems to be the fungal species that contributed most to the global volatile composition obtained by the fungal mixture.

Preliminary results in the analysis of releasable 2,4,6-trichloroanisole (TCA) and eventually produced by these fungi in cork-based media were studied. Results show that the production of releasable TCA cannot be attributed to any of the assayed fungal isolates.

Results show the necessity to control the humidity levels of the cork slabs after the boiling stage to avoid the colonization by fungal species that could impart any unpleasant sensory properties to the final cork product.

Sumário

A cortiça é a camada externa e contínua do tronco da árvore de *Quercus suber* L. e tem propriedades físicas e químicas únicas. Portugal possui 33% das florestas de cortiça mundiais e contribui com aproximadamente metade da produção de cortiça global.

A manufactura de discos de cortiça (ou rolhas) compreende várias etapas, incluindo duas cozeduras, durante as quais as pranchas estão mergulhadas em água em ebulição. Nos dias seguintes à cozedura, o nível de humidade das pranchas diminuiu e estas ficaram completamente cobertas pelo micélio branco de *Chrysonilia sitophila* até a cortiça atinjir um determinado nível de actividade de água (ca 0.9 a_w). Abaixo desse nível outras espécies de fungos (exemplo: *Penicillium*, *Aspergillus* ou *Trichoderma*) podem germinar mudando assim as colonizações fúngicas existentes nas pranchas.

Os dois principais objectivos da investigação efectuada durante este Doutoramento foram: (1) identificação taxonómica do mycobiota presente nas pranchas de cortiça durante toda a manufactura dos discos de cortiça (2) investigar os compostos químicos produzidos por esses fungos, que podem transmitir à cortiça propriedades desfavoráveis.

Para identificar os fungos presentes nas amostras de cortiça, usou-se um método dependente de cultura (isolamento) e dois métodos independentes (electroforese em gel de gradiente e a técnica de clonagem). Os resultados mostraram que a maioria dos fungos isolados pertenciam aos géneros *Penicillium*, *Eurotium*, *Chrysonilia*, *Cladosporium* e *Mucor*, sendo *Penicillium glabrum* a espécie predominante detectada em 70% das amostras. Como consequência, foi efectuado um estudo taxonómico detalhado no grupo ao qual o *Penicillium glabrum* pertence. Um dos isolados apresentou características fenotípicas e moleculares únicas, sendo por isso classificado como espécie nova (*Penicillium subericola*).

Todos os métodos utilizados indicaram que o mycobiota presente em amostras colhidas após a primeira cozedura aparentou ser distinto da população fúngica presente nos subsequentes estádios de manufactura. Para além disso, a técnica de clonagem confirmou a presença de fungos não cultiváveis, Ascomycota e endófitos, na cortiça crua, e de fungos não cultiváveis em amostras colhidas após a primeira cozedura. As amostras colhidas nos restantes estádios continham filótipos pertencentes maioritariamente a *Penicillium glabrum*, *Penicillium* sp. e *Chrysonilia sitophila*.

Foi estudada a possível produção de exo-metabolitos por alguns dos isolados fúngicos, que colonizam as pranchas na fase de descanso após a primeira cozedura, nos seguintes meios de cultura: um de cortiça e dois meios semi-sintéticos. Os fungos estudados produziram poucos metabolitos no meio de cortiça, havendo mesmo alguns isolados que sem produção detectável quando crescidos nesse meio de cultura. No entanto, a adição de restos de micélio de *Chrysonilia sitophila* ao mesmo meio de cortiça aumentou os perfis exo-metabolómicos da maioria dos fungos estudados. As espécies fúngicas analisadas, quando crescidas em qualquer um dos meios de cortiça, não produziu qualquer exo-metabolito prejudicial ou micotoxina.

O estudo de compostos químicos gerados pelos fungos focou-se nos compostos voláteis libertados pelas comunidades microbianas presentes em amostras de cortiça colhidas durante o processo de manufactura. Os resultados mostram que a maioria dos compostos voláteis foi detectada durante dois estádios de manufactura: fase de repouso após a primeira cozedura e discos de cortiça (não tratados). Os perfis dos voláteis produzidos nestas duas fases é semelhante.

Foram analisados os compostos voláteis produzidos por cinco fungos isolados, tanto em cultura pura como mista, usando

cromatografia gasosa acoplada com espectrofotometria de massa. Os resultados mostraram que os principais compostos voláteis detectados, em ambas as culturas, foram o octen-3-ol e ésteres de ácidos gordos (de cadeia média C8-C20). A espécie fúngica que contribuiu mais para a composição volátil global obtida pela mistura fúngica foi, segundo os resultados obtidos, o *Penicillium glabrum.*

Estudou-se também a eventual produção de 2,4,6-tricloroanisole (TCA) por estes fungos em meio de cortiça. Os resultados indicam que a produção de TCA não pode ser atribuída a qualquer uma das espécies fúngicas estudadas.

Os resultados mostram ainda a necessidade de controlar os níveis de humidade das pranchas de cortiça após a fase de cozedura, para evitar a colonização destas por espécies fúngicas que podem produzir alterações sensoriais desagradáveis no produto final da cortiça (rolha).

Chapter 1

Introduction

1. Cork oak forests – Montado

The *Quercus suber* L. forests are spread along the western Mediterranian basin occupying 2 million hectares across Portugal, Spain, Algeria, Italy, Morocco, Tunisia and France (Pereira, 2007b). Portugal owns the world largest area of cork oak forest with 730 thousands hectares (WWF data; http://www.wwf.org.uk). These forests (*montado* - Portugal) are located in the South part of the country and are well adapted to dry summers (Gourlay, 1998). This unique ecosystem reduces the soil erosion and prevents soil desertification since these trees possess deep root systems that capture water from deep soil depths. These forests are biodiversity hotspots that serve as habitat for a number of animals, like the endangered Iberian lynx and the Spanish Imperial eagle and also for certain plants used in culinary (e.g. aromatic) and medicine (WWF, 2006).

Quercus suber trees are typical national species and constitute the basis of several economic activities with national interests; law protects it since 1927. In 1988 a new decree (Decree-law nº 172/88) was written to assure an efficient protection of this national species. It is a very strict law that forbids the cutting of cork oak trees, dead or alive (Oliveira, 2000). *Quercus suber* forests are also protected by the European Union (Habitats Directive 92/43/EEC).

1.2 Cork structure

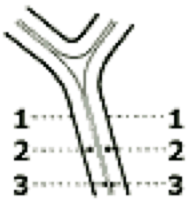
Cork is the continuous layer of bark produced on the outer layer of the *Quercus suber* L. tree. It is the suberized parenchyma

originated by the suber-felodermic meristem and constitutes the outer layer of trunk and branches. The meristem grows continuously to the exterior that is an important characteristic of cork.

The cork structure can be seen spatially according to three axes in relation to its original position in the tree, radial (horizontal), axial (vertical) and tangential (horizontal angle) (fig.1).

Fig. 1 Cork cell A. Longitudinal section B. Transversal section (Adapted from Quimar Sugheri: the cork)

Structurally, cork is constituted by several cells in tangential section having a polygonal shape, disposed in a regular and compact manner without any empty spaces. Images from scanning electron microscope show that cork cells have a structure similar to a honeycomb. During its growth their cellular content disappears and latter a suberization process (impermeability) of its cellular membranes occurs (Gil, 1998). These micro-cells are filled with a gas similar to air around 60 – 85% of the total volume (Maga, 2005). Their cell walls have five layers: two formed by cellulose that surrounds the cellular cavities; followed by two middle layers suberized (with suberin and waxes) and lately one more internal constituted by lignin (that confers the rigidity and structure) (fig 2).



1- Cellulose
2- Suberine
3- Lignin

Fig. 2- Cell membrane composition of a cork cell
(Adapted from Quimar Sugheri: the cork)

1.2.1 Chemical composition of cork and their mechanical properties

The chemical and structural composition of the cork oak cells is responsible for their unique mechanical properties. Cork cell walls are constituted by structural and non-structural components. The structural components are macromolecules, of polymeric nature, insoluble that confers most of their physical and chemical properties to the cork cell. The cork structural components are:

-Suberin (45%) that confers compressibility and elasticity to the cork

-Lignin (27%) that contributes to the cell wall structure

-Polysaccharides (12%) mainly cellulose and hemicelluloses polysaccharides that are linked to the cork structure.

The non-structural components are divided in:

-Extractable compounds including organic low molecular mass components (*e.g.* waxes) that repel the water and contribute to the cork impermeability and tanins (6%) that give colour and protection to the cork against the attack of biological organisms.

-Non-extractable components - ashes (4%)(Gil, 1998 & 2000).

Cork is the lightest solid material since 50% of its cell volume consists of air, so the cells can resist to great pressure without

breaking and retain 90% of its original form after the pressure is released, so maintaining their dimensions in one direction even when the pressure is applied in another one (Fortes, 2004; Gil, 2007).

1.3 Cork forest sustainability

The cork oaks trees can live up to 350 years and produce cork continuously during its life (Fortes, 2004).

The first harvest (virgin cork) is made when the tree is approximately 25 years old. The first two harvests produce poor quality cork, which are not used to produce stoppers. The following harvests are done every 9-12 years, when the cork is able to produce a layer of cork with the adequate thickness to produce stoppers.

Cork harvesting is done entirely by hand, and it is not a harmful operation. It is done at the end of the spring and during summer, so the cork cells can regenerate and continue to divide (Fortes, 2004).

Cork is a biodegradable product and has a positive impact in carbon fixing thus reduction the greenhouse gas emissions that cause the climate change (htttp://www.corkcomposites.amorim.com/client/skins/english/samples.asp?produto=23).

The needs to provide an adequate management service of the cork oak forests thus assuring its sustainability lead to the certification of the montados. In Portugal the Forest Stewardship Council (FSG) attributed around 28 thousand hectares of certified cork forest.

1.4 Cork applications

The unique physical properties of cork make it a material suitable for many applications. Traditionally, cork has been used since the early antiquity as floating device, sealant and insulator. Several cork devices were found (e.g. fishing items, cork lids, sandals soles) in China, Egypt and in the Mediterranean area. However, the most important application of cork is to seal wines, drinks and sparkling wines as stoppers, which started in the 17th century (http://www.corkfacts.com/nchoice1.htm).

The manufacturing of cork stoppers originates many remains however all of them used. The by-products from the cork stoppers manufacturing are used in other purposes.

Some of these products can be used in vibration, thermal and acoustic insulators in roofs, walls and floors (Gil, 1996). The lipophilic extractives of cork and cork by-products are promising sources of bioactive chemicals or chemical intermediates for the synthesis of value added compounds (Sousa, 2006).

Some of these wastes can be have several uses like hockey and baseball balls, golf cubs and rackets. They can also be used in computer printers, handbags, fishing rods, floats, carpets and helmets (APCOR) and in electrical and automobile Industries (Silva, 2005)

In recent years, the interest in cork increased and it is a material used by architects, designers and decorators due to the fact that it as natural resource extremely easy to use, renewable and environmental friendly. This noble material also serves to make clothes (http://www.trendhunter.com/trends/cork-clothing-gets-

fashionable), little pieces of furniture (e.g. cushions, sofas and pillows) (http://www.squidoo.com/decorate-with-cork) and even an entire house made from cork (http://www.tvleak.com/overflow/house-made-of-cork/).

Recently, the cosmetic investigations discovered the anti-aging effect of the cork extract. This fact is mainly due to the suberin content present in the cork cell wall, specially its hydroxycarbolic acids that provides special mechanical and chemical properties originating the possible smoothing effect on the surface skin (Coquet, 2005).

1.5 Manufacturing of cork stoppers

On the early times of cork stopper industry, all the procedures were quite rudimentary and mainly based on empiric knowledge. Although, the cork stopper is still punched with its axis synchronized to the plank axial position and the noblest part of the cork plank is used.

The Industry introduced some new technological innovations to guarantee the consumers the manufacture of high quality products. Moreover, their production processes have been checked and objectively analysed so the European cork federation (CELIEGE) elaborated the International Code of Cork Stopper Manufacturing Practice (SYSTECODE), which consists of several rules to assure the quality system and accreditation of the cork Industry (Celiège, 2006).

According to the SYSTECODE, the manufacture of cork stoppers includes several manufacturing stages starting with the stripping of the cork trees in the forest. After stripping, the cork is pilled

and stacked in the forest or in the factory outdoors, exposed to rainfall, for 6 months to one year, in order to become flattened and to enable the elimination of most of their dirt, also to oxidize most of their polyphenols and to partially stabilize their structure (Gourlay, 1998). In a second stage, cork slabs are prepared for the industrial processing. The cork slabs showing visible defects are immediately separated and the other ones are arranged in pallets with 3 levels.

The next stage in the Industrial processing is the boiling of cork slabs in water (95 – 100ºC) for about 1 hour. This is a very important stage as humidity allows the raw slabs to flat, and expand the cork tissue and to stabilize it dimensionally, mainly in the radial direction. Also cork becomes softer and more elastic (Fortes, 2004; Silva, 2005; Pereira, 2007b). Some tannins and minerals salts of the cork are partially extracted during this stage. To prevent the cork contamination by chlorinated compounds, no chlorine treated water is used and the boiling water is periodically renewed as some cork phenolic compounds, soluble solids or volatile compounds can be extracted from it. Their accumulation can be a contamination source for cork slabs during subsequent boiling since cork can absorb many volatiles and phenolic compounds (Pollnitz, 1996).

The boiling stage does not promote the microbial sterilization of cork. Although the water temperature reaches near 100º C, the specific physical-mechanical and chemical properties of cork namely its waterproof properties do not allow the water to reach some deeper layers of cork slabs. Moreover, the particular structure of cork with its lenticels filled with air leads to a specific

niche where water does not normally enter so the fungal spores are not destroyed.

After boiling, the cork slabs are left to dry in the factory atmosphere for some days (3-4 days). During this period the slabs will become more flat and straight. The humidity levels starts to decrease immediately after boiling and after two days attain a humidity level ranging from 14–18% (0.9 of water activity (a_w) (Pires, 2007), which is the adequate level for cork transformation (slabs slicing and stoppers or discs punching) (Pereira, 2007b). After this period the cork planks are selected once again, and separated according to their thickness and quality. Then they are sliced and punched according to its thickness in stoppers or for cork discs.

Regarding the manufacturing of cork discs, the slabs are first cut into strips, where the inner and outer-backs are removed. Then the slabs are punched into discs using an automated process. Furthermore, the discs and stoppers are washed and bleached with hydrogen peroxide aqueous solution; dried until they reach a humidity level that ranges between 5–8%, and dimensionally rectified. Both, cork stoppers and discs are then carefully chosen using optometric and manual choice. Therefore they are classified according to different quality classes mainly based on their visual appearance.

Lately, the cork stoppers are washed, dried and their surface is treated with a lubricant film to reduce friction and to allow a better introduction and extraction into the bottleneck. Additionally, regarding the technical stoppers there is the assembly of the different parts of their constituents to form the stopper (e.g.

champagne cork stopper an agglomerated body that is glued to two natural cork discs in one side).

2. Fungi on cork

Several different types of fungi co-exist in the same habitat as *Quercus suber* trees. Some of them are important to the tree adaptation to the habitat, establishing a mutual relation with the tree roots, as mycorriza. Others colonize the tree itself in the trunk, leaves or branches. When the cork is harvested several fungi colonizing it constitute a specific mycobiota as seen in non-boiled cork planks.

Fungi are eukaryotic organisms possessing absorptive heterotrophic nutrition. Their vegetative body is constituted by million of threads like structures, with or without septa, called hyphae constituting a multinucleate structure that exhibit apical growth. They form spores, as reproductive and/or resistance structures (Bennet, 2001). Their walls are constituted mainly by chitin, a polymer of N-acetylglucosamine.

The number of known fungi is estimated to be around 1.5 million members (Hawksworth, 1991). Most of their members colonize the terrestrial habitat, although some of them can be marine or aquatic. Fungi possess a remarkable ability to utilize almost any carbon source as food. Moreover, they colonize most habitats, surviving some extreme physiological conditions (e.g. temperature, pH, water activity and oxygen) (Alexopoulos, 1996)

Fungi own an important role in the decomposition and recycling of the ecosystem (Pitt, 1997) because they produce a number of

extracellular enzymes, able to decompose recalcitrant substrates into more easily metabolised ones.

Fungi are members of the kingdom *Fungi* constituted by five phyla: *Chytridiomycota*, *Gloemeromycota*, *Zygomycota*, *Ascomycota* and *Basidiomycota* (Carlile, 2001). The latter three phyla belong to the *Eumycota* Division. *Eumycota* members reproduce by spores and can have an asexual stage (anamorph) or sexual stage (teleomorph) or both (holomorph) phases. When the teleomorph of some fungi are not known, they are named mitosporic fungi (Hawksworth, 1995). The most important fungi involved in food and airborne contamination are included in this group. Recently the holomorph of more fungal species was known using heat shock treatment of the anamorph species (Houbraken, 2008).

The fungal genera *Aspergillus* and *Penicillium* are the most well known to be common air and foodborne habitants and may also be involved in the spoilage of food products. They are taxonomic placed in the Eurotiales, within the phylum *Ascomycota* (Frisvad, 2004).

2.1 Fungi present in cork and cork environment

The presence of fungi colonizing the cork was described in earlier works by Mathieu (1900), Bordas (1904) and Sharf & Lyon (1958) according to Riboulet (Riboulet, 1982).

Several studies concerning the identification and characterization of the cork mycoflora along several stages of cork stoppers manufacturing process were made along the time.

The quality of the air environment of a cork factory was also assessed to characterize the fungal spores present in there (Lacey, 1973; Ávila, 1974). Additionally, since cork is produced mainly in a country and then exported elsewhere, the cork planks that were transported in the ships containers and the cork bales were investigated to see if any fungi were present in them (Lacey, 1973; Ávila, 1974). Additionally, cork stoppers exported to Australia were studied to detect the eventual presence of natural mycoflora and to see if the ship conditions could originate the fungal contamination (Davis, 1981; Lee, 1993).

The whole cork manufacturing process involves the shift of humidity levels along the time. This factor is of the outmost importance as it allows the germination and development of fungal mycelia in cork. All the cultivable fungi that colonize cork grow at 0.97 a_w, some of them continue to grow at 0.85 a_w, however very few are able to grow at 0.80 a_w (Simpson, 1990). On the other had, the critical point for *C. sitophila* development is 0.9 w_a (Pires, 2007), which is the fungal species that covers the cork slabs after boiling with its white to pink abundant mycelium. In the beginnings of cork stoppers industry it was assumed that there presence indicate that the slabs had the adequate humidity to be sliced and punched. When the humidity levels decrease below 0.9 other fungi mycelium develop on the cork slabs, like *Penicillium*, *Aspergillus* and *Trichoderma*. So it is important that the maturating stage should last a maximum of 3 to 4 days. It was recommended that the cork Industry respected that period of time. In the case that they could not continue the process during that time, a new boiling step should be done to maintain the humidity levels to stop the growing of other fungi.

The cork mycoflora has been characterized over the past decades. The predominant fungal genera found were: *Chrysonilia*, *Penicillium*, *Aspergillus*, *Trichoderma*, *Mucor* (Davis, 1981; Daly, 1984; Simpson, 1990; Lee, 1993; Danesh, 1997; Silva Pereira, 2000b; Alvarez-Rodriguez, 2002; Oliveira, 2003). However, some studies refer genera like *Rhizoctonia* (Daly, 1984), *Aphanocladium* (Simpson, 1990), *Fusarium*, *Acremonium*, *Paecylomyces* (Alvarez-Rodriguez, 2002).

The yeast community was also studied and the predominant genera are *Rhodotorula, Candida* and Streptomyces (Lefebvre, 1983). Although, *Candida famata*, *Sporodiobolus johnsonii* and *Rhodotorula glutinis* were species also isolated from cork (Danesh, 1997). Recently, a polyphasic approach used on the identification of some yeasts isolated from cork resulted in the characterization of new genera like *Rhodosporium*, *Debaryomyces* and *Trichosporon* (Villa-Carvajal, 2004) and the characterization of *Rhodotorula subericula*, a new species isolated from cork oak (Belloch, 2007).

2.2 Fungal communities

Fungal communities have been traditionally studied using culture-based methods, where the fungal species were isolated through the use of several media cultures. These methods are not only time consuming but can only detect a limited number of microorganisms. Some previous studies have calculated that only 1 – 10% of the soil microflora is known by these methods. The low biodiversity encountered when using this approach is maybe due to the interdependence of the different

microorganisms upon each other. Some species depend on other ones and are not able to grow on artificial media cultures, also the culture conditions that are normally used are not suited to isolate most of the species that exist in a certain habitat (Muyzer, 1998).

To overcome this fact, some culture independent methods, which are DNA or RNA based, have been developed and their use has increased our knowledge of the biodiversity and functioning of more mycobiota present in some habitats (Simon, 1993; Vainio, 2000; Vandenkoornhuyse, 2002; Anderson, 2003b; Vandenkoornhuyse, 2003).

Most of these techniques have an initial step that consists in the extraction of nucleic acids, directly or indirectly from the sample, followed by amplification using polymerase chain reaction (PCR) of a specific part of the genomic DNA/RNA. The method used to extract DNA or RNA is vital for the success of the subsequent steps, since the quality and the purity of the extracted nucleic acids is important for the subsequent PCR amplification.

Another aspect to take into account is the primers choice for doing the PCR reaction. Some primer pairs amplify preferentially some fungal species, usually the ones that are present in higher concentrations at the expenses of the ones that are in lower concentrations in the template (Dickie, 2002). The excessive specificity of the primers can create this bias in the fungal biodiversity of the sample.

Another factor to consider when dealing with the fungal genome is the multicopy nature of some target region in the fungal genome. The rRNA operon, which is widely used in these studies, is assumed to have equal number of copies for each fungal

species. However, the number of copies in the genome of different fungal species is different and the exact number of the copies it is unknown for most of the species (Hibbet, 1992).

The techniques used to investigate the fungal communities are able to identify different fungal species, genera, families or higher taxonomic groups depending on their sensitivity (Nannipieri, 2003). Most of the molecular methods have intermediate resolution and have been used to detect mycota groups instead of mycota species such as denaturing gradient gel electrophoresis (DGGE).

DGGE technique have been based in the electrophoretic mobility of DNA fragments of the same size, previously amplified by PCR, in polyacrylamide gels that possess a linear gradient denaturant made with urea and formamide. The DNA double-stranded that is subjected to increasing concentrations of denaturant starts to partially melts in the so-called “melting domains”. The DNA mobility depends on its base composition and this technique allows the differentiation of two DNA molecules differing in only one single base (Muyzer, 1999). Moreover, an optimal resolution is obtained when the molecules do not completely denature this is assured by the addition of a 30 to 40 GC clamp to one of the PCR primers. This technique is simple and rapid to perform and allows the identification of shifts or changes that occur in the fungal community composition through a period of time (van Elsas, 2000; Vainio, 2000; Anderson, 2003b). Furthermore, the bands obtained after DGGE can be excised, re-amplified and sequenced. The obtained DNA sequences can be compared with known sequences present in databases. The objective is to identify the fungal species corresponding to each amplicon

(Nielsen, 2005). The primers choice is crucial for the technique sucess. Most of the used primers correspond to some parts of the rDNA region, which is a multicopy gene present in each fungal cell. However, the use of one copy gene primer is starting to be taken into consideration to be used.

The rapid analysis and comparisons between different samples in one gel, and also amongst several gels, is quickly made. However, that comparison depends on the use of suitable internal standards that allows an accurate analysis amongst all gels. The reproducibility between gels has been mentioned as one of the main pitfalls of the technique that can be overcome using standardized equipment to prepare each gel.

One disadvantages of this technique are the use of shorter DNA amplicons (<500 bp), thus limiting the taxonomic information obtained from excised band gels, although some larger PCR products have been used successfully (Ranjard, 2000; Landeweert, 2004). However, the obtained biodiversity profiles could not correspond to the real variety of the sample.

Another unfavourable condition is the staining method that often is not too sensitivity to detect the less dominant members of the fungal community. Besides, in some cases, a single band on the gel does not correspond to a fungal isolate, because some DNA amplicons with different base compositions co-migrate together. To overcome this problem the excised band is cloned and then sequenced.

Another technique use to study fungal communities is the cloning of the amplified PCR amplicons from specific samples to be studied (Chen, 2002; Anderson, 2003a). The obtained clones can be screened using restriction fragment length polymorphism

technique. (RFLP). This reduces the number of clones to be sequenced since the use of restriction enzymes groups the clones into different OTUs. So the choice of how many and which restriction enzymes are going to be used is an important step to have in consideration, since previous studies using *Pisolithus* isolates and using two restriction enzymes showed that the same species of *Pisolithus* can have different RFLP profiles (Hitchcock, 2003). The advantages of this technique are that it provides a diversity profile of the fungal community existing in a specific habitat. The disadvantages are that it takes a lot of time to obtain clones with different operational taxonomic units (OTUs) and to sequence them being a laborious and potentially costly technique. The presence of chimeras (DNA sequence originated from two different organisms) is another factor to take into account.
Moreover, the taxonomic identification of the clones depends on the correct identification of the sequences deposited in the known public sequence databases.

3. Secondary metabolites produced by fungi

Secondary metabolites were defined in opposition to primary metabolites (or central metabolites), which are common to all fungi and serves for its survival in the producing of carbon flux and energy.
Exo-metabolites (or secondary metabolites) are natural chemical compounds that are synthesized by fungi in response to an interaction with the surrounding environment (Thrane, 2007). They are energetic costly chemical products usually produced late in the cell differentiation or development processes and

normally produced with sporulation (Calvo, 2002). The production of exo-metabolites is not consistent for all fungi; instead it is specific to certain genera and species (Frisvad, 1998). Although, some researchers claim that it can be strain specific (Engel, 1982).

Some of the exo-metabolites produced by fungi are considered to be mycotoxins. According to the definition proposed by Frisvad, Thrane and Samson (2007), mycotoxins are chemical compounds that when present in low concentrations, are toxic to any vertebrate animal. Some of them can be neurotoxins, or carcinogenic, while others can lead to the kidney or liver deterioration. Some of them can also interfere with the protein synthesis producing several effects like, skin sensitivity to extreme immunodeficiency (Sweeney, 1998). The fungal genera that produce most of the fungal exo-metabolites and mycotoxins are, *Penicillium*, *Aspergillus* and *Fusarium* genera and their teleomorphs.

In the last decades, some exo-metabolites showed potential as source of new pharmaceutical compounds like, antibiotics, immunosuppressant's and antiviral compounds (Larsen, 2005). These data added interest in the demand to search new molecular active molecules.

The exo-metabolism specificity encouraged the mycologists to use the exo-metabolite profile as a taxonomic tool (Karlovsky, 2008). The *Penicillium*, *Aspergillus* and *Fusarium* genera have had good results. The first two are known to be present in food and also in environment and the former genus is constituted mostly by plant panthogens (Sweeney, 1998).

The production of exo-metabolites by fungi is studied in the laboratory using a solid culture media, since it is quite similar to fungal natural substrata (*e.g.* food, plants, decaying wood). The exo-metabolites production depends on several factors. The substrate ingredients of the culture media are carefully chosen to obtain the production of metabolites. Some of them are produced under certain environmental conditions and only when certain trace metals are present (*e.g.* iron or cupper).

The physiological conditions of the strains are a very important factor and several transfers can deteriorate them, as well as the accumulation of carbon dioxide can inhibit the metabolite production. The investigation done by some research groups (Filtenborg, 1990), (Frisvad, 1989) showed that the substrates that have easily assimilable nutrients originates the production of most exo-metabolites (Thrane, 2007). Most of the synthetic media cultures, like yeast extract sucrose agar (YES), malt extract agar (MEA), potato dextrose agar (PDA) and oatmeal agar (OA) are the most used and give the best results.

Furthermore, the study of the mycobiota composition of food or products indirectly used in the food chain is necessary to assess the possible existence of mycotoxins to assure the quality and safety of those products, for instance in wheat and white wheat (Weindenboerner, 2000) and in maize (Soriano, 2004).

3.2 Volatile compounds produced by fungi

Volatile organic compounds (VOCs) produced by the fungi normally contribute its intense and characteristic odours. Their production has been shown to be consistent and related to

cultural conditions and abiotic environment (Larsen, 1998). This characteristic can be used taxonomically to distinct different species at least in some fungi groups, *e.g. Trichophyton* (Sahgal, 2006) or *Penicillium* (Larsen, 1995a), (Larsen, 1995b), as dissimilarity among them is a difficult task.

Moreover, volatile metabolites can have some allergic effects on humans (Fischer *et al*, 2000). Their presence in foodstuff (Schnurer, Olsson & Borjesson, 1999) and inside buildings has been widely studied (Girman *et al*, 1999; Hodgson et al, 2003).

VOC-mediated positive, negative or neutral interactions can occur between a very wide range of soil bacteria and fungi. These effects include both stimulation and inhibition of growth, the enzyme production being only an example of the different surviving strategies used by the biological communities. Many organisms are known to modify the environment in order to construct an adequate niche where natural selection can take place (Brown, 2009).

3.3 Cork taint and off-flavours

Taint is by definition a foreign taste or odor imparted to a product (ISO, 1992), which gives disagreeable taste and most often results in consumer rejection of the product.

Cork is referred in most studies as the main source for the cork taint in wines. The main reason lies in the fact that several microorganisms colonize the cork tree and most of them are lodged in their cork lenticels and structural fissures (Macku, 2009). Latter the microorganisms can also colonize cork along the stopper manufacturing process. These microorganisms can

metabolize chemical compounds causing the cork taint in the presence of some humidity from: a) some compounds belonging to the cork lignin or suberin b) some chemical compounds that does not exist in the nature but were used for many decades as pesticides, wood preservatives, fungicides. However, some investigations also showed that the taint can be originated from a) contaminated oaks barrels b) contaminated winery machinery used for bottling the wines c) airborne fungus eventually present in the winery environment and d) molds present in wooden barrels and wine making devices (Haas, 2010).

The first presence of cork taint in bottled wine was described in 1904 (Lefèbvre et al, 1983). The report of several types of cork taint was well described by Duncan (1995) although, the real definition of “cork taint” refers to a very unpleasant odour still of unknown origin extremely rare to occur (Ribéreau-Gayon et al., 1998). The term taint can include several odors like earthy, musty, medicinal, moldy, mushroom-like, earthy (Margalit, 1997), depending on what compound originated the taint.

The proportion of wines contaminated with cork taint has been the subject of some divergence depending on the informative source. According to the Industry, the problem affects 0.7% of the bottled wine (Hall, 2002). On the other hand, the studies done so far indicate that the real value should be around 1-5% (Soleas, 2002). These values will depend which chemical compounds originated the taint as well as the origin of the taint, since the compounds detection threshold can vary.

Several chemical compounds have been identified as responsible for the taint in wines. Some of them possess different flavors (Pena-Neira et al, 2000; Alvarez-Rodriguez et al, 2003).

However, in most cases not only one but also a mixture of compounds can be detected in tainted wines (Silva Pereira, 2000). These compounds can act alone or synergistically; a phenomenon by which the effect is produced by all the components that is greater than the sum of their individual contribution. This fact makes it difficult to assess the contribution that each components gives to the taint.

The main chemical contaminants identified as responsible for taints are haloanisoles.

3.3.1 Haloanisoles

Haloanisoles are the most common volatile compounds detected in tainted wines, mainly chloro- and bromoanisoles (Amon, 1989; Pollnitz, 1996; Chatonnet, 2004; Coque, 2006; AFGC, 2007). Chloro- and bromoanisoles are derivatives of the anisole (or methoxybenzene) that possesses at least one substituted chlorine (or bromide) in the phenolic ring. These chemical compounds are volatiles produced by fungal methylation, when environmental humidity level and the corresponding halophenols precursors are present (Alvarez-Rodriguez, 2002). This chemical reaction occurs as a detoxification way, to transform the toxic halophenols in non-toxic haloanisoles. S-adenosyl-L-methyonine (SAM)-dependent methyltransferase is the enzyme that catalyses this chemical reaction resulting that the halophenol is converted into the respective anisole (Coque, 2003).

Chloro and bromoanisoles have been detected in most cases of tainted wine (Amon, 1989; Chatonnet, 2004; Coque, 2006; AFGC, 2007) as being responsible for that defect. According to Coque *et*

al. (2006) the most important chloroanisoles implicated in the contamination of wine are: 2,4,6-trichloroanisole (TCA), 2,3,4,6-tetrachloroanisole (TeCA), pentachloroanisole (PCA) and 2,4,6-tribromoanisole (TBA). However, 2,4- and 2,6- dichloroanisole were also found in some tainted wines by some researchers (Pollnitz, 1996). Moreover, in most cases not one but several compounds were detected in tainted wines (Pollnitz, 1996).

Apart from the wine (Pollnitz, 1996; Chatonnet, 2004) several cases of food tainted with haloanisoles have been reported, *e.g.* eggs (Curtis, 1974), sake ({MIki, 2005 #1265) and coffee (Spadone, 1990). Some studies indicate that these chemical compounds are probably the main contamination source of tainted food (AFGC, 2007). Some studies indicated that the main source contamination comes from the use of chlorophenols mainly in the last decades as fungicides for wood protection and preservatives (AFGC, 2007). However, now their use is forbidden specially in Europe, United States as it has been classified as "highly hazardous pesticides" by the International pesticide action network (PAN International) and is listed in the highly hazardous pesticides (International, 2010). Around the 1980s, other compounds were detected in tainted products, namely tribromoanisoles. Bromophenols are now used instead of the banned chlorophenols. They are produced by the industry and used as antifungal agents and flame-retardants, on wood, plastic and paintings (Chatonnet, 2004). Moreover, the TBA can be naturally found in the marine environment since it is synthesized by the red algae *Polysiphonia sphaerocarpato* to remove excessive bromine (Flodin, 2000). Although these compounds are less toxic to the environment, they are present in many cases

of food taint, like contaminated oysters (Watanabe, 1983) and in several Australian Food products (AFGC, 2007), especially in wine (Coque, 2006).

The main problem concerning the chloro- and bromoanisoles contamination is that these compounds possess a low threshold detection levels, in ng/L range. This results in the product rejection when these chemical contaminants are present in low amounts (Saxby, 1996). The necessity to detect their presence in very low quantities, at least sensitive to the human nose, as a standard quality, has lead to the improvement of methods used to detect the chemical compounds responsible for the off-flavours. Such methods are: gas chromatography-mass spectrometry (GC/MS) (Soleas, 2002; Chatonnet, 2004; Dias, 2008) or stir bar sorptive extraction (SBSE) and thermal desorption GC/MS (Hoffman, 2000) or even gas chromatography-olfactometry (GC-O) (Plutowska, 2008).

3.3.1.1 2,4,6 -Trichloroanisole (TCA)

Tanner (1981) published the first work relating the cork taint with the presence of 2,4,6-trichloroanisole (TCA) but Amon (1989) determined its content by gas chromatography-m ass spectrometry (GC-MS) and found its major contribution on cases of cork taint.

The most studied compound by the Cork Industry involved in the cork taint is TCA (Riboulet, 1982; Daly, 1984; Rigaud, 1984; Simpson, 1990, Lee, 1993; Pollnitz, 1996; Duncan, 1997; Sefton, 2005; Coque, 2006; Prak, 2007). This compound was detected in 80-90% of the taints (Maga, 2005) and its concern resides on the

low detection threshold, which lies between 1.4 ng/L and 10 ng/L (Buser, 1982; Silva Pereira, 2000a; Alvarez-Rodriguez, 2002). The detection of low quantities of TCA is difficult in a more complex matrix than water (Teixeira, 2006). When this compound is present in wines the rejection odor threshold is 10 ng/L (white wine) and 16 ng/L (red wines) according to Teixeira *et al*. (2006). Most of the studies performed until now focused on the ability of the cork isolated fungus to methylate TCP into TCA (Lee, 1993; Pollnitz, 1996; Silva Pereira, 2000a; Howland, 2008; Maggi, 2008). Other studies refer the mycobiota diversity present in tainted corks and related them to the TCA presence (Daly, 1984; Caldentey, 1998; Alvarez-Rodriguez, 2002; Prak, 2007). Although the fungal species content in cork is different for each research studies, some fungal genera isolated are common to most of the studies, like: *Penicillium* sp., *Chrysonilia* sp., *Cladosporium* sp., *Trichoderma* sp., *Aspergillus* sp. (Davis, 1981; Lee, 1993; Hill, 1995; Silva Pereira, 2000b; Alvarez-Rodriguez, 2002).

Silva-Pereira (2000) incubated some cork fungi in a medium culture containing TCP and analysed their capacity to convert the chlorophenol into TCA. The work showed that the isolated fungi have low potentially to convert TCP into TCA. *C. sitophila* was able to metabolize TCP but had a conversion rate of 0.03 %, appearing to be a non-TCA producer (Silva Pereira, 2000b).

Alvarez-Rodriguez (2002) also studied the capacity of 14 cork isolates belonging to several fungal genera to convert TCP into TCA. The results showed that *Fusarium* sp. and *Trichoderma* sp. were strong producers transforming 25% of TCP into TCA; the moderate producers were two *Penicillium* strains, *Acremonium*

strictum, *C. sitophila* and *Cladosporium oxysporium* that converted between 10-25% of the TCA into TCA, *P. viridis*, *P. chrysogenum* and *V. psalliotae* were low producers and *M. alpine*, *M. plumbeus* and *P. decumbens* non-producers (Alvarez-Rodriguez, 2002).

Other researchers showed that *Mucor* sp., *Paecylomyces* sp., *Penicillium* sp., *Trichoderma* sp. were able to convert TCP into TCA. However, the best yields were obtained by *Paecylomyces* sp. and *P. chrysogenum*. Additionally, in this study *C. sitophila* and *Penicillium* sp. did not produce TCA (Prak, 2007).

Another research work reported the capacity of the fungi *Cryptococcus* sp., *Rhodotorula* sp., *P. glabrum* and *P. variable* to produced TCA to a great extent (Prat., 2009).

The results obtained concerning the capacity of some fungal isolates to produce TCA from TCP leads to the hypothesis that this feature could be a strain dependent effect, thus explaining in different works by the different methylation capacities obtained for different isolates of the same species, *e.g. C. sitophila* and *P. glabrum*. Moreover, no direct relation between the methylation of TCP to TCA and the amount of TCP on the cork could be established, except for the work of Simpson and Lee that reported that the tested fungus had 74-100% of conversion to TCA, however, only 0-23% of them was converted to TCA (Simpson, 2007). All the other works reported lower conversion rates suggesting that TCP metabolites can be incorporated in the cellular material (Silva Pereira, 2000b; Alvarez-Rodriguez, 2002).

The quantity and diversity of moulds present in cork stoppers, some of them with TCA while others with none were studied. The results showed that the number of moulds is identical in both

cases, although the biodiversity is higher in TCA-containing stoppers (Prak, 2007).

It was observed that the number of corks that possess TCA or TCP is higher compared to the percentage of the wines that became tainted (Juanola, 2005). This fact was the starting point to to look for the factors that affect the TCA transference into the wine (Capone, 2002; Soleas, 2002; Sefton, 2005; Juanola, 2005; Alvarez-Rodriguez, 2009).

Additionally, in case of contamination of tainted wines present in the winery environment and absorbed by the cork, the main question is the TCA capacity to migrate through the cork to contaminate the wine.

Research showed that TCA has more affinity to the cork than to the hydro-alcoholic solution or wine (Juanola, 2005; Sefton, 2005; Alvarez-Rodriguez, 2009), so if this compound is present in the wine the cork will absorb at least most of it. However, if the cork is the source of contamination depending on the cork's surface of contact with the wine the percentage of the TCA that passes into the wine depends on the time, temperature as well as the compound amount that is present. In cases of bottled wine could range between 0.7 to 2.7% (Soleas, 2002) or less than 0.1% (Juanola, 2005).

The amount of TCA is not uniformly distributed in the cork stopper. Research done so far indicates that TCA migrates poorly inside the cork and stays mainly at its surface (Capone, 2002), (Sefton, 2005; Alvarez-Rodriguez, 2009). Moreover, after 24h of cork exposure to d_5-TCA the compound was restricted to the 2 mm of the outer layer, although 15 to 25% penetrated beyond this layer, possibly *via* lenticels (Capone, 2002).

However, in long term experiments that tries to mimic the long storage of wines, the studies done so far indicate that the surface equilibrium attained by the TCA-cork stopper is disturbed and some level of TCA can be released into the wine (Capone, 2002), (Juanola, 2005). It seems that TCA located in the cork matrix reaches more accessible layers and is slowly released into the wine. This could happen after 12 months storage and migration ranges from 0.7 to 2.7% (Soleas, 2002). Additionally, the releasable pollutants analysed (TCA included) could not be completely extracted by soaking into a hydro-alcoholic solution (Alvarez-Rodriguez, 2009). The study concluded that the TCA does not present a problem to wine closed with natural stoppers.

3.4 Other chemical compounds capable of producing taint

Some other chemical compounds were detected in tainted wines, which are capable to contribute or cause the off-flavor. Several compounds can impart a musty earthy off-flavor to the wines *e.g.* geosmin, 2-methylisoborneol (Karahadian, 1985; Darriet, 2000), 2-methoxy-3-isopropylpyrazine (IPMP), 2-methoxy-3,5-dimethylpyrazine (MDMP), guaiacol (Álvarez-Rodríguez, 2003), 1-octen-3-ol and 1-octen-3-one (Karahadian, 1985; Darriet, 2000). The contribution that each compound gives to the taint is not equal; it depends on the perception threshold and the chemical stability of each one of them in the wine, as well as the wine composition. The detection thresholds of these compounds are in the ngL^{-1} range (μgL^{-1} for guaiacol and 1-octen-3-ol) (Prat, 2008).

Among these compounds geosmim appears to cause less problems since it is chemically unstable in wine (Sefton, 2005).

4 Economic consequences of wine cork taint

Portugal is the world leader of the production and manufacturing of cork stoppers, responsible for 70 % of the global exporting (WWF, 2006). According to the Portuguese National Institute of Statistics (INE) cork exportation registered an increase of 0.6 %, export value in 2007 compared with 2006. In 2007, natural cork stoppers are the major Portuguese exported product ranging values of 415 million euros followed by champagne cork stoppers with 88 million euros and agglomerates with 86 million euros. The main destination countries of cork exportation in 2007 were: France (20.6 %), USA (15.7 %), Spain (13 %), Germany (8 %) and Italy (7,6 %).

It is reported that cork taints occur in 1 % to 8 % of the commercialised wines (Pereira, 2006) and its occurrence is responsible for economic losses suffered by either the cork and wine industry (Álvarez-Rodríguez, 2009). Even if the responsible for the wines contamination cannot always be imputed to the cork, the Industry is ensuring a high standard product to decrease its trustworthy.

However, the cork taint occurrence even with a low incidence, can origin high losses for both the cork stopper and wine Industries. As a consequence, in the nineties the synthetic wine stoppers to seal wine bottles developed and acquired an increased importance mainly due to economic interests of non-cork producing countries. The main drawback of these devices is

their negative environmental impact. Furthermore, to our knowledge, they possess low quality, at least, to seal high quality wines that usually require long storage periods to ensure the proper aging of the wine bouquet. Several studies were done to investigate the impact of the closure in wine aroma and aging, using wines sealed with both synthetic closures and natural cork stoppers (Karbowiak, 2010; Skouroumounis, 2005). The results showed that wines with synthetic closures exhibit a relatively oxidised aroma, a brown colour and low levels of sulphur dioxide. Natural cork stoppers, on the other hand, showed negligible reduced aroma characters. The closures act primarily as a barrier to oxygen, since if this gas is present in low or high levels can impart an oxidized or reduced flavour, respectively. Moreover, micro-oxygenation enabled by the cork porosity is a very important process to allow wines age correctly inside the bottles (Mills, 2006; Toit, 2006).

Currently, new cork products manufactured according the Industry technological research knowledge are available. These new products were developed to eradicate all TCA precursors and to be well adapted to the demands of the wine market.

i) natural cork stoppers are 100% natural product and can be made with a) single piece of cork and it is recommended for seal reserve wines or wines that need to age in the bottle or b) a multipiece natural stoppers which comprises more than one piece of cork glued together by FDA approved contact glues.

When the wines do not need to age in bottles the stoppers can be made of:

ii) colmated cork stoppers which are natural cork stoppers that contain pores or lenticels preserved with glued cork dust. They are used for the same wines as the multipiece stopper

iii) champagne and sparkling wines stoppers that are constituted by an agglomerated body with two cork discs glued to one side

iv) Technical cork stoppers which are constituted by an agglomerated body possessing a) one cork disc in each side or b) two cork discs in one side. These closures are used to seal wines that need to be consumed within shorter periods.

v) agglomerated cork stoppers formed from granulated corks which are constituted by little pieces of corks glued together. They are perfect for wines that require storage for no more than 12 months.

vi) capsulated cork closures. These stoppers derives from a) natural cork or b) colmated cork stoppers which possess the upper portion tied with glass, metal, porcelain, wooden or PVC. They are used to seal fortified wines and spirits, and can be reused (http://www.cork.pt/cork-stoppers.html).

Some studies done with “technical” cork stoppers (Neutrocork), natural cork stoppers, and synthetic closures (Nomacorc) showed that for natural and technical closures the oxygen diffuses into the bottle during the first 12 and 24 months of storage, respectively. Equally, under the study conditions, “Nomacorc” closures showed to be permeable to the atmospheric oxygen (Lopes, 2007). In conclusion, mimicking the heterogenic cellular structure of the cork even if the material had the same impermeability and mechanical properties can be a very difficult task to achieve. Throughout recent years, the cork stopper continues to be the noblest product used to seal wines.

5 Fight against cork taint

Several measures were implemented in the cork manufacturing process in order to eliminate or reduce the TCA from the cork, as a way to decrease the cork responsibility in the wine spoilage. Research has been developed either in some industries or in straight cooperation with public research institutions.

Since the nineties, several cork stoppers producers in conjunction with several investigation groups developed some processes that aim to eliminate TCA. However the presence of this compound remains to be not completely solved. Some of the actually most important available strategies can be summarized:

- Amorim & Irmãos Company developed the ROSA (http://www.amorim.com/cor_ied_rolhas.php) method based on a steam cleaning process, which efficiency in the TCA removal was reported by several authors (Sefton, 2005).

-The OENEO group, formerly known as Sabaté developed a method called “Diamont” based on the use of supercritical carbon dioxide extraction. The haloanisoles are selectively removed from the raw cork material. Several independent studies showed the good results obtained by this technique. (http://www.beveragedaily.com/Financial/Oeneo-and-the-Diamond-route-to-recovery).

-A group of researchers and cork companies developed a method called DELFIN (direct environmental load focused inactivation) that use a technology based on microwaves to remove TCA from the stoppers. They claim that reduces TCA levels at least 90% and it kills microbes throughout the stopper.

(http://www.winespectator.com/webfeature/show/id/Research-Group-Claims-to-Have-Eliminated-Wine-Cork-Taint_20322).

- A technique based on the use of gamma radiation was developed to remove TCA from cork. This technique transforms the TCA existing on the cork in molecular residues that do not possess the same organoleptic characteristics (Pereira, 2007a) (Portuguese patent application PT 103006).

- A CTCOR developed a technique called SYMBIOS, which consists in the addition of an addictive to the boiling water. This compound reduces the manufacturing process to one boiling that takes one hour and a half. This technique reduces the microbiological population on the post-boiling stage (*e.g.* development of mainly *C. sitophila* and *Mucor* mycelium in the cork surface) increasing also the extraction of polyphenolic compounds from the cork (http://www.ctcor.com/fotos/gca/SymbiosE.pdf).

II– Aims and layout

The main objective of this thesis was to isolate and identify fungal species present along the main stages of the manufacturing of cork discs, including raw cork, using either molecular or phenotypical techniques. The uncultivable mycobiota present at each stage of the manufacturing was also assessed by DGGE (denaturing gradient gel electrophoresis), cloning and subsequent sequencing. The elucidation of the relationship between cork fungal community and the different cork niches created along cork stoppers manufacturing process was also envisaged.

The most predominant fungal isolated fungal species or the most susceptible of producing harmful exo-metabolites were inoculated in several culture media: semi-synthetic and cork based culture media. Their exo-metabolite profiles were analysed and compared among them and the possible production of mycotoxins was evaluated especially in cork-based culture medium.

Additionally, the production of volatiles and TCA by some isolated fungal species inoculated in cork-based culture medium was investigated.

The main results obtained along this work were published or submitted to publication and they constitute the following chapters of this thesis:

- Taxonomic studies of the fungal mycobiota presented in cork samples collected throughout cork manufacturing discs
- Exo-metabolites produced by some fungal isolates in several media cultures
- Volatile compounds produced by cork mycobiota

Chapter 2

Taxonomic studies of the fungal mycobiota present in samples collected throughout cork manufacture discs

This chapter focus the identification of cork mycobiota present in some cork samples taken along the manufacturing of cork discs. Two different approaches were used: one culture-dependent (isolation) and two culture independent (DGGE and cloning technique). In the course of this work it was found that about half of the isolated species belong to *Penicillium* genus, mostly to *Glabra* series and taxonomic studies of the *Penicilllium glabrum* complex were done. Additionally, one isolate showed to have phenotypical and molecular characteristics to be included in a new species (*Penicillium subericola*).

This chapter consists of two scientific articles:

- Unveiling the fungal mycobiota throughout cork stopper manufacturing process (submitted to *FEMS Microbiology journal*).
- Taxonomic studies of the *Penicillium glabrum* complex and the description of a new species *P. subericola* (*Fungal Diversity*, 2011, 49:23-33).

The experimental work presented in this chapter was done by the author except the taxonomic studies that were made in collaboration with the Applied and Industrial Mycology Laboratory at Fungal Biodiversity Centre, CBS-KNAW, Utrecht, Netherlands. Cork was ground with the help of Mário Gil Dias, Susana Marcelino and Liliana Pinto. The cloning technique was done by Biopremier Inovação e Serviços em biotecnologia, S. A., Lisbon. Both manuscripts were written by the author and revised by the other co-authors of the articles.

Unveiling the fungal mycobiota present throughout cork stopper manufacturing process

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Abstract

The knowledge of the fungal population present in the main stages of the manufacturing process of cork discs allow us to evaluate which species could produce any chemical compounds that could spoil cork final product.

The fungal mycobiota using both culture dependent (isolation) and independent-methods (denaturing gel gradient electrophoresis and cloning of the ITS1-5.8S-ITS2 region) was studied. The mycobiota present in the samples taken in the stages before and after the first boiling seems to be distinct from the population present in the subsequent manufacturing stages. Most isolated fungi belong to the genera *Penicillium*, *Eurotium* and *Cladosporium*. The presence of uncultivable fungi, Ascomycota and endophytes in raw cork was confirmed by sequencing technique. The samples taken after the first boiling possessed uncultivable fungi, still in few samples some isolated fungi were also detected. The main detected taxa present in the following stages were *Chrysonilia sitophila*, *Penicillium glabrum* and *Penicillium* spp. All applied techniques had complementary outcomes.

The main driven factors of shift in cork fungal colonization seems to be the high levels of humidity and temperature, which the slabs are subjected during the boiling process.

Keywords: Mycobiota diversity, uncultivable fungal species, cloning, DGGE, cork

Introduction

Cork constitutes the outer layer of *Quercus suber* L. tree. It is a natural resource that is continuously produced during the tree life span. Its unique chemical composition and physical properties (compressibility, impermeability, resilience and chemical inertness) make it a unique material suitable to be used in cork stoppers. Cork appears to be a specific habitat due to its special chemical and physical constitution that harbours a unique mycobiota population.

The European cork federation (CELIEGE) elaborated an International Code of Cork Stopper Manufacturing Practice (SYSTECODE) that defines the necessary steps to acquire the accreditation in the cork Industry. To assure the high standard of the final product the cork industry follows this code.

The manufacturing of the cork discs (stoppers) comprises several stages and the most important ones are the boiling of the cork during 1 hour and their subsequent resting stage. These two stages allow the cork to become flat, softer and more elastic and thus being workable. The high humidity levels of the boiling make cork a good habitat for the development of the cork mycobiota. Few days after boiling the cork slabs become completely covered by white/salmon mycelium of *Chrysonilia sitophila*. When the moisture levels decrease below 0.9 a_w, mycelia from other fungi (*Penicillium*, *Trichoderma*, *Cladosporium*, *Mucor* and *Aspergillus*) start to germinate and replace *C. sitophila* mycelium (Danesh et al. 1997; Alvarez-Rodriguez et al. 2003; Oliveira et al. 2003; Basilio et al. 2006; Prat et al. 2009).

Fungi start to colonize the cork in the forest and different types of fungi can colonize the root, trunk, leaves and even the surrounding soil. Some fungi will establish saprobyotic relations with the tree, while others can be parasytic or mycorhizic. Most of these fungal communities will also colonize the cork layer and could be actively growing on them. The effect that the manufacturing process and mostly the boiling stage do in the original fungal

communities it is not known. However, several studies indicate that the manufacturing process does not kill all the mycobiota already present in it (Alvarez-Rodriguez et al. 2002; Basilio et al. 2006). However, the origin of the mycobiota present in each stage (tree forest or the factory environment), as well as the contribution that each one can have to the mycobiota that exists in the cork slabs at each stage is unknown. Some fungal species described in several works, like *C. sitophila* and *P. glabrum*, appear to constitute the predominant mycobiota and were also detected and mentioned in other works. However, some fungal species are described in some works but are not constantly detected in all cork and seems to be dependent on the geographic location of the cork tree. Some authors even describe the existence of new fungal species in cork, either filamentous or yeasts, although most of them were isolated in raw cork (Belloch, et al. 2007; Barreto et al. 2011b).

Most of the studies in the mycobiota of cork are made using cultivation-dependent methods and show that cork is colonized by a particular mycobiota. However, our knowledge of the uncultivable fungal species present is scarce. Prat et al (2009) studied the microbial community structure of cork stoppers and discs with and without marked musty-earthy aromas. Denaturing gradient gel electrophoresis (DGGE) was the culture-independent method employed. The predominant fungi that were present in all samples were: *P. glabrum* and *Neurospora* spp. However, the sole use of the final product (cork stoppers and discs) does not provide the knowledge about the mycobiota constitution in each manufacturing stage and also the microbial shifts occurring in each stage. A study covering more stages of the manufacturing cork discs is necessary to understand the mycobiota composition and their variance along the manufacturing process. Moreover, the presence of uncultivable species to our knowledge was never studied, although their presence is known from the studies done in the diversity of fungal communities present in other substrates and habitats (Vandenkoornhuyse et al. 2002; Schat et al. 2003; Nagano et al. 2010). Furthermore, the

endophytes presence was also studied in cork oak seedlings (Linaldeddu et al. 2009), however it was not known if they were present in the cork slabs.

In this work, both the cultivable and uncultivable mycobiota of several cork samples taken along the manufacturing of cork discs were studied. The cultivable methods were used to study cork sample originated from Portugal and Spain. From each geographic location two different sampling were made. The cork samples were taken from five stages of the manufacturing of cork discs. Two culture-independent methods were used: denaturing gel gradient electrophoresis (DGGE) and cloning methods. DGGE was employed to investigate the mycobiota shifts occurring in the cork samples taken along the manufacturing process. Fungal diversity was accessed through the cloning technique and their respective relative clone frequencies were calculated.

The objective was to study the mycobiota diversity that exists in the cork using both cultivable dependent and independent methods, relating them with the respective manufacturing stages.

Materials and Methods

Sample collection

The cork samples used in this work were originated from Portugal and Spain. All the samples were taken along the main stages of the processing of cork discs of the same batch of cork slabs, to assure that traceability is maintained. Two samplings of cork from each origin were: Portuguese (20/11/2006–batch 4 (autumn) and 23/07/2006–batch 38 (summer)) and Spanish (20/2/2006 –batch 59 (winter) and 20/11/2006 –batch17 (autumn)). All cork was assembled and processed at a factory, localised in the centre of Portugal. The samples were collected in the following manufacturing steps: before cork slabs boiling (BB), immediately after the first boiling (1B), during the resting stage after the first boiling (PB), immediately after the second boiling (2B) and non-treated cork discs (D). An alphanumeric code was used to designate the cork samples. The criteria followed to designate the

116 samples name included the first two letters of the sampling stage added the respective batch
117 number (e.g. BB4 or 2B38).
118 The slabs are normally disposed in stacks inside the factory; each stack has three levels
119 being separated by a metal structure. A piece of cork with about 20 cm side was taken in
120 three points on the upper part of each level, in the slab's diagonal.
121 The same amount of cork was collected for the non-boiled samples, which are stored in one
122 stack in the factory yard.
123 Samples of cork discs consisted of approximately 300 gr, a weight very similar to the cork
124 taken in the other stages.
125 After the samplings, all the cork samples were ground to a fine powder (60 mesh) using a
126 centrifuge mill (Retsch, Haan, Germany).
127 **Fungal isolation and characterization**
128 The dilution Plating Method was used to isolate the fungi. One g of ground cork was
129 weighed and added to 9 mL of 0.1% (w/v) peptone in water. Serial dilutions (10mL of final
130 volume) were used and 1 mL of each dilution was plated in three culture media (in
131 duplicate). In the case of the batch 59 Malt extract medium (MEA, Oxoid, Basingstoke,
132 Hampshire, United Kingdom) with chloramphenicol, Dichloran and Rose Bengal agar with
133 chloramphenicol (DRBC, Oxoid, Basingstoke, Hampshire, United Kingdom) and Dichloran
134 with 18% glycerol and chloramphenicol (DGC18, Oxoid, Basingstoke, Hampshire, United
135 Kingdom) were used. For the remaining samplings the culture medium used was DG18.
136 The plates were incubated for 5 days at 25 ° C and 99% humidity. After the first 24 h and
137 every subsequent 24 h, the presence of fungal colonies was registered. After 5 days of
138 incubation the total numbers of fungal colonies was counted and, for each sample all the
139 different morphological types of colonies were isolated. The isolated specimens were
140 transferred to MEA without antibiotic and purified by serial inoculations to obtain pure

141 cultures. As soon as the fungal isolates constitute single cultures, spore suspensions were
142 made for each isolate and maintained in 30% glycerol at -80 ° C (Samson et al, 2010).

143 **Sequencing and data analysis of the fungal isolates**

144 The fungal isolates obtained from all samples were grouped according to their morphological
145 characteristics on MEA. Among the groups that possessed similar morphological
146 characteristics up to two or three isolates were chosen. These isolates were grown for 2-3
147 days at 25ºC on malt peptone medium (Samson *et al.*, 2010). Total DNA of the selected
148 strains was extracted using Ultraclean ^{TM} Microbial DNA Isolation Kit (Mobio, Solana Beach,
149 U.S.A.) according to the manufacturer's instructions. A fragment of the β-tubulin gene was
150 amplified and later sequenced according to the procedure previously described by
151 Houbraken *et al*. (2007). Regarding the Penicillium *glabra* group, the partial sequence of the
152 calmodulin gene was also amplified and sequenced according to Houbraken et al. (2008).
153 The obtained DNA sequences were added to the bioloMICS database (CBS, Utrecht, the
154 Netherlands) and their identity was obtained by comparison with DNA sequences of the type
155 strains that exist in the database.
156 The fungal DNA sequences previously obtained were deposited on the Genbank nucleotide
157 sequence database under the following numbers (or designation) JN858112 to JN858147.

158 **Phenotypic identification of the fungal isolates**

159 The isolates that gave a homology equal or greater than 99% with a particular type strain
160 were only studied in MEA culture medium to confirm its pureness and identification. The
161 isolates that gave homologies smaller than 98% with the type strain were studied in more
162 detail. One, two or several isolates, depending on the number of isolates in each cluster that
163 were positioned in different clades on the dendrograms obtained from the alignment of the
164 partial sequences of β-tubulin gene were morphological characterized. The selected strains
165 were grown on MEA, Czapek Yeast autolysate agar (CYA), creatine agar (CREA) and Yeast
166 Extract Sucrose agar (YES) (Samson *et al.*, 2010). The inoculation was done in three -

points and the plates incubated at 25ºC for 7 days. Moreover, the CYA plates were incubated at 30ºC and 37ºC. Following incubation, the culture characteristics were recorded. Microscopic features were obtained in MEA and CYA.

The obtained data was used to construct a qualitative table (presence/absence) of the fungal biodiversity isolated in all samples. Unweight pair-group method using arithmetic averages (UPGMA) was applied to analyze the results. A principal component analysis (PCA) was computed and the minimum spanning tree was superimposed on the projections.

NTSYS-pc software (Rohlf, 2000) was used in the data analysis.

Fungal genomic DNA extraction and PCR amplification

The fungal DNA was extracted from the cork samples and amplified to be used in two experiments: DGGE and cloning. The samples used in the DGGE experiment were originated from batches 4 and 38. Contrary, to the ones used in the cloning process that came only from batch 4. Both experiments included samples from all five sampling stages. The same methods were used to extract DNA and amplify it in both experiments.

Around 5 g of the powdered cork samples was used to extract total fungal DNA according to a protocol previously described (Gadanho & Sampaio, 2004). After the extraction, the DNA was kept at -20ºC.

Amplification of the internal transcribed spacer (ITS) was used in both cloning and DGGE techniques. A nested-PCR was used in the DGGE technique. In the first PCR round the primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al, 1990) were used. On the second step the following primers were used: ITS 5 plus a 40bp GC clamp (Muyzer et al, 1993) and ITS4. The PCR conditions follow those previously described by Pereira et al. (2010).

Regarding the cloning technique ITS1F and ITS4 were the used primers and the PCR reactions and conditions were the same as previously described by Gadanho and Sampaio (2006).

DGGE technique

The PCR amplicons were analysed using the Dcode™ Universal Mutation Detection System (Bio-Rad Laboratories Inc., CA, USA) according to the manufacturer's instructions. The denaturing gradient made with formamide-urea (Bio-Rad Laboratories Inc., CA, USA) was 35%-42% in a gel of 6% Acrylamide/Bis 40% After 5h at 200V, each gel was embedded in a solution of SYBR® Gold (1:10,000, Invitrogen™ Molecular Probes, OR, USA) and revealed when exposed to the UV light and photographed.

Data analysis of DGGE

The photographed gels were analysed by the software BioNumerics (version 5.0; applied Maths, Sint-Martens-Latem, Belgium). Lanes were manually established and normalization of the gels was done using standards to assure a comparison between samples from different DGGE gels. A binary matrix was constructed using DICE product- moment correlations, which provide similarity values for the presence or absence of bands existing in all fingerprint profiles. Dendrograms showing DGGE profile similarity were calculated by the unweight pair group method with arithmetic mean (UPGMA) algorithm.

Cloning technique

Amplicons obtained with the primers ITS1F and ITS4 were cloned into the pGEM-T vector (Promega, Madison, WI, USA) according to the manufacturing instructions. Competent cells of *Escherichia coli* were transformed and the target gene amplified by PCR following the protocol described by Gadanho & Sampaio (2006). Clone screening was performed using restriction fragment length polymorphism (RFLP) using the following restriction enzymes: AluI, DdeI, MseI and RsaI (New England Biolabs). All the restriction profiles were compared and grouped according to their identity. For identification, one clone of each group was selected and sequenced using primer ITS4 as previously described (Gadanho and Sampaio, 2006). The clones were sequentially named according to Table 2.

ITS rDNA sequencing

DNA sequences originated from the cloning technique were obtained using standard protocols. The nucleic sequences were compared with those previously deposited at the GenBank database and identified using the Basic Local Alignment Search Tool (BLAST) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi (Altschul et al., 1990). The sequences obtained by cloning were checked for chimeric regions by dividing each sequence in three parts and performing a BLAST search for each part to confirm the identification of the entire sequence.

Phylogenetic analysis

Sequences of the ITS region obtained were aligned with reference sequences retrieved from the GenBank online database. Alignments were made using ClustalX 1.83 (Thompson *et al.*, 1997) and visually corrected. Phylogenetic trees were computed with the PAUP software (Swofford, 2001), using the Neighbour-Joining method (Saitou & Nei, 1987). Distances between sequences were calculated using Kimura's two parameter model (Kimura, 1980) and bootstrap analysis (Felsenstein, 1985) was based on 1000 random re-samplings.

Results

Identification and characterization of the cultivable fungal species

Fungi lodged in the cork samples taken along the main stages of cork manufacturing discs were isolated. Regarding the cork sample originated from batch 59 three culture media were employed: DRBC, MEA and DG18. In DG18, the number of fungal spores per g of cork and the diversity of the species isolated in those samples were similar or even higher than the ones present in the other two culture media (data not shown). However, cork is a dry substrate and the culture medium recommended to enumerate fungal species in foods with reduced water activity (i.e. less than 0.95 a_w) is DG18, according to Pitt and Hocking (2009). In the remaining samplings only DG18 culture medium was used. This procedure agrees

244 with previous studies using different dry substrates like dried fruits, dried fish, meat products
245 and cereals (Hocking and Pitt, 1980; Samson et al, 2010).
246 Filamentous fungi and yeasts were isolated from the cork slabs sampled along the main
247 stages of the manufacturing of cork discs using the dilution plate method. Total fungi isolated
248 in each sample onto DG18 culture medium were counted and their species diversity was
249 also assessed. The results are presented in Table 1.
250 All the isolates were studied by sequencing the partial region of β-tubulin gene and most of
251 the isolates were identified to species level, except three isolates from the Portuguese cork,
252 two isolates collected in the before boiling cork (BB4 and BB38) and another from the non-
253 treated cork discs stage (D38) that shared no homology with any type strains deposited at
254 the CBS database (data not shown).
255 Most of the isolates belonged to *Penicillium* (52.5%), followed by *Eurotium* (18.9%),
256 *Chrysonilia* (6.7%), *Cladosporium* (5.0%) and *Mucor* (5.0%), respectively. Furthermore, most
257 of the *Penicillium* isolates belonged to the *Glabra* series (Pitt, 1979), being *P. glabrum* the
258 main isolated species. In fact, sixty-nine strains belonging to *Glabra* series were
259 taxonomically studied and their partial β-tubulin and calmodulin gene sequences were
260 amplified. The results showed that all the isolates accommodate in three groups: most of the
261 isolates presented high homology with *P. glabrum* type strain, a second group of isolates
262 share similarity with *P. spinulosum* type strain, a last group of isolates possess new
263 taxonomic characteristics that allowed the description of a new species named *P. subericola*
264 (Barreto et al., 2011a).
265 Most of the DNA sequences of the other isolates when compared with the type strain
266 sequences deposited in the CBS database gave high homologies, values equal and even
267 higher than 95%, being therefore classified in the respective species. However, some
268 isolates showed intraspecific variation namely those belonging *to P. citrinum, P.*
269 *citreonigrum, P. janczewskii* and *P. miczynskii.* Since their macromorphology shared many

characteristics with the type strains previously mentioned they were classified under the respective species names.

Samplings occurred in several seasons: winter (batch 59), autumn (batches 17 and 4) and summer (batch 38). In overall, batch 38 possessed more diversity and fungal quantity per g of cork than any one of the other batches (Table 1). The fungal diversity present in cork slabs was higher in raw cork independently of the cork geographic origin (Portugal or Spain). In fact, fungal diversity collected before the boiling stage (BB) ranged between 9 (batch 17) different species to 13 (batch 4) in opposition with the post-boiling stages. In almost all cases the diversity and counted fungi decreased along the manufacturing process of cork discs, mainly immediately after the boiling processes, except after the first boiling (batch 38) where six different fungal species were isolated from 27 CFU/g of cork. Most of the samples collected in the resting stage (PB) showed a slightly increase in both the number of isolated fungus and their diversity (Table 1), in comparison with the samples collected immediately after the first boiling stage. Still in some samplings, the fungal quantity showed to be similar (1B4) or even 100 fold increased (1B59) in those two stages. Non-treated cork discs stage possesses some fungal biodiversity although most of the isolated species belong to *Penicillium* (table 1). Even though, the number of isolated fungus in this stage is slightly higher in batch 17 having 2200 CFU/g of cork. Perhaps cork being a heterogeneous substrate did not possess the same quantity of fungal spores in all its structure.

The results presented in Table 1 were used to compute a principal component analysis (PCA). The samples were projected in the space defined by the first three coordinates as shown in fig. 1. Although in the first three axis only 50.55% of the sample variance is explained, the cophenetic correlation coefficient between the original distance and the distances implied in the first three principal coordinates is high (r=0.944). Consequently, the original distances were well preserved to allow this analysis tool to be applied in this study.

All the three dimensions showed that (Fig. 1) the BB samples collected in the before boiling stage were dispersed especially in the second and third dimensions, while the other manufacturing samples stages clustered together. The fungal species from group A that contributed to the positive part of the first axis were collected in the BB stages, mainly in the Portuguese (batches 4 and 38) and Spanish (batch 17) samples. Furthermore, the species that contributed to the negative part of the second dimension were mostly collected in the Spanish cork (batch 59), with exception of *P. toxicarium* (batches 4 and 17) and the black yeast (batch 4). Likewise, the fungal isolates that contributed both positively and negatively to the third dimension were only collected in the before boiling stage, except *E. herbarium* that was also collected in the first boiling stage (batch 38). The species that belong to group F were isolated only from batch 38 while the species from the group G were collected in the batches 4 and 17. All the BB samples possessed high diversity, nevertheless samples BB4 and BB17 grouped more closely than any of the other samples from the other batches.

Total Fungal population diversity i) DGGE technique

The objective of DGGE technique was to study the fungal diversity between two Portuguese batches collected in different seasons: autumn (batch 4) and summer (batch 38).

To assure the amplification of only fungal genomic DNA the forward primer used in the first PCR round was a universal primer specific for fungal amplification (ITS1F) together with a universal primer for eukaryotes (ITS4).

Fig. 2 shows the dendrogram and values using the Dice coefficient. The cophenetic correlation for each branch was calculated thus expressing the dendrogram robustness.

The reproducibility of this technique was assured; a duplicate randomly selected and represented 10% of the samples were used. This sample belongs to the 4BB stage and clusters with its duplicate at 100% similarity, as seen in fig. 2 confirming the reproducibility of this method.

320 The DGGE fingerprint profiles visualized in the gel possessed in each lane low number of
321 bands. This factor indicated the small fungal diversity observed on each sample. The
322 dendrogram analysis showed that all the samples from the batch 4 grouped together in a
323 separate cluster from the samples of batch 38. However, one cluster was constituted by
324 samples from both batches (4 and 38). This could indicate that the samples from the
325 different Portuguese batches shared some diversity, especially in the cork processing stages
326 after the first boiling.
327 The BB samples (batch 38) presents the most distinct fingerprinting profile since clusters
328 with the remaining samples at 28.5% similarity supported with a cophenetic value of 87.
329 Nevertheless, this was not verified for the samples collected in the before boiling stage
330 belonging to batch 4. In this case, sample BB4 clustered with 1B4 at high similarity (90.9%).
331 As a result, the fungal population present in these two samples was probably very similar
332 and distinct from the remaining samples. BB4 and 1B4 clusters with the remaining samples
333 from batch 4 and 38 at 37.1% of similarity, except with the PB38 and BB38 samples.
334 **Total Fungal population diversity ii) cloning technique**
335 Total fungal population profile dynamics of all manufacturing stages of one batch (batch 4)
336 was studied in more detail through cloning technique. This technique enables the
337 identification of the phylotypes present in each stage of the cork manufacturing process.
338 Fifty clones from each sample were analysed, with exception of samples from the first
339 boiling stage from which only 30 clones were obtained and sequenced. Clone screening
340 resulted in around 20 different restriction profiles for the BB sample and 3 and 8 for the
341 remaining samples. For each profile a clone was randomly choose to be sequenced and
342 analysed. The correspondent phylogenetic analysis is presented in Fig. 3 and the nearest
343 probable identification is given in the Table 2 as well as their homology percentage.
344 Most of the represented clones present in the BB samples belonged to uncultivable species
345 (40.6%), followed by *Ascomycete* (35.1%) fungi; some of them were present in the

346 mitosporic phase (e.g. *Cladosporium* sp.). Additionally, endophytes (24.3%) were also
347 present. The first boiling samples possessed only an uncultivable fungal phylotype. All the
348 remaining samples shared two restriction profiles: *C. sitophila*, and *Penicillium glabrum* .
349 Moreover, samples collected in the resting stage (PB) had one *Pezicula sp.* phylotype. Also,
350 in samples taken in the second boiling (2B) and nontreated cork discs (D) a
351 *Sporobolomyces* sp. phylotype was also present in both stages. The main diversity was
352 obtained in the BB samples.
353 These results were confirmed by the phylogeny (fig. 3) where the clones detected before the
354 boiling stage (BB) was in higher number and distributed along all the dendogram. Except
355 two clusters that grouped most of the clones from the cork resting stage (PB), the second
356 boiling (2B) and non-treated cork discs (D).
357 With exception of *Cladosporium* (clone 16), some cultivable phylotypes detected in small
358 frequencies like *Helicoma* (clone 19, 20), *Acanthostigma* (clone 5), *Gloniopsis* (clone 2) were
359 described as colonizing other substrates than cork. Most of them, being wood colonizers.
360 Clones 1, 4, 17, 18, 22 and 23 constituted one of those clusters and were related to a fungal
361 endophyte. Another group constituted by clone 6 was related to an uncultured *Ascomycete*.
362 Another phylotype that was probably an *Helicoma vacinii* is the cluster formed by clones 7,
363 19 and 20 which is near the cluster formed by the *Acanthostigma perpusil* related phylotype
364 detected in the 1B samples (clone 18).
365 The group constituted by clones 8, 14, 15 and 21 was related to a fungal endophyte and an
366 uncultivable *Pleosporales*, thus probably being uncultivable fungi.
367 A group constituted by clones 3 and 12 is probably related to a *Cryptococcus* sp. or an
368 uncultivable Basidiomycota, probably being Basidiomycota yeast. Clone 13 was also near
369 the uncultured fungus and *Auricularia auricula judae* and it is a Basidiomycete.
370 **Discussion**

371 To our knowledge, some fungal species reported in this work were not previously isolated on
372 cork substrate. Most of them were described as soil inhabitants (*E. amstelodami*, *E.*
373 *herbariorum*, *P. brasilianum*, *P. miczynskii*, *P. rubefaciens*, *P. venetum* and *P. westlingii*),
374 others colonized some foods (*P. crustosum*, *P. paneum*, *P. venetum*), while other were
375 present in indoor environments (*P. sumatrense*), or in water (*Cl. herbarum*). The remaining
376 isolated fungal species have been reported in other works to occur in cork substrate. Among
377 all species *P. glabrum* and *C. sitophila* were the most common fungi found in cork substrate
378 originated from several locations and collected in different seasons (Simpson and Lee, 1988,
379 Danesh et al., 1997, Álvarez-Rodriguez et al., 2002, Oliveira et al., 2003, Serra et al., 2008,
380 Prat et al., 2009a, Prat et al., 2009). Mostly they were reported to occur in the post-boiling
381 stages of the cork stoppers except *P. glabrum* that was also isolated in raw cork samples, as
382 well as in the factory environment (Lacey, 1973). Probably, both species are well adapted to
383 the cork substrate.
384 The combined phenotypic and molecular techniques employed to the isolates allowed us to
385 identify several different fungi to species level. Moreover, the use of specific PCR primers for
386 β-tubulin in all the *Penicillium* and *Aspergillus* isolates and calmodulin for the isolates belong
387 to the *Penicillium* series *Glabra*, as well as the identification of the fungal isolates using the
388 bioloMICS database resulted in a reliable taxonomic identification of the isolates. Probably if
389 the cloning technique was done using multi-primer sets the results could show several
390 subsets of the fungal community, as reported in other studies (Nagano et al., 2008).
391 The presence of *Eurotium* species isolated in some stages of the cork manufacturing discs
392 was not very surprising since most mitosporic species when subjected to high temperatures
393 become heat-resistant species (Houbraken et al, 2008). *Eurotium* species were previously
394 reported to be present in fungal DNA directly extracted from cork stoppers (*E. repens*) (Pratt
395 et al., 2009b). Probably these species originated in the tree and survived the boiling

processes of cork. They both appeared in Portuguese and Spanish corks, however in batch 59 they were only detected in raw cork (BB59).

Cork possessed high fungal diversity in the before boiling stages (BB), independently of the cork geographic origin (Portuguese and Spanish), as confirmed by the culture-dependent methods applied. The diversity decreases after the first boiling, which is supported by the results included in Table 1 and Fig.1. Batches 4 and 17 shared similar fungal diversity, especially in the BB stage, probably due to the fact that they were both sampled in the same season (autumn) even if the geographical origin of the cork was different (Portugal and Spain), according to PCA analysis (Fig. 1). Moreover, two fungal species (*P. spinulosum* and *P. janczewskii*) were only isolated in those cork samplings (Table 1).

The projections of the cork samples in the first three dimensions (Fig. 1) showed that the BB samples were dispersed mostly in the second and third dimensions, while the remaining samples collected in other manufacturing stages clustered together. The phylogenetic tree (Fig.3) also shows that three clusters are constituted by phylotypes isolated in the BB samples. Moreover, one cluster that possessed the phylotypes isolated in the first boiling samples (1B4) was closer to another cluster formed by the BB4 samples (Fig. 3). These results were also supported by the dendrogram (Fig. 2) where BB4 and 1B4 samples clustered together and shared 90.1% similarity. The mycobiota present in before boiling (BB) samples is diverse and mainly constituted by uncultivable fungi, followed by Ascomycetes and endophytes, respectively (Fig. 3). Their relative frequencies ranged from 2.7 % to 13.5 % (table 2). Additionally, when a culture-dependent method was employed several fungi that belonged mainly to *Penicillium*, *Eurotium* and *Chrysonilia* were isolated. Probably, some samples taken in the before boiling stage (BB) could possess low quantity of cultivable species that only germinated in enriched culture medium. Maybe, the use of other PCR primers in the cloning technique could detect other phylotypes present in the samples in low frequencies.

422 During the boiling stage, the mycobiota present in the slabs were subjected to high
423 temperatures and humidity levels. Consequently, due to those conditions some fungi already
424 present in the cork can be reduced mostly to <10 with no spores detected or 2 CFU/g of
425 cork. However, sample 1B38 possessed 27 CFU/g of cork and their diversity consisted of six
426 different fungal species (Table 1). This shows that most of the cork contained few fungal
427 spores. However in some cases due to its heterogeneity or the capacity to create
428 microhabitats inside its structure, cork can lodge some mycobiota that could survive the
429 boiling process.
430 When the cloning technique was employed the detected phylotype in the 1B sample
431 belonged to an uncultivable fungi (cluster 1B4) and none cultivable species were detected,
432 in accordance with results expressed by the cultivable method (<10 CFU/g of cork).
433 Probably the fungal concentration present in these samples was reduced and constituted
434 mainly by uncultivable fungal species. However, some cultivable fungi could be present in
435 low numbers and their DNA templates were not amplified by PCR. Some of them could
436 germinate when the cork was placed in enriched culture medium (Table 1), or in some cases
437 these cultivable fungal species could be absent from most of the analysed cork samples.
438 The remaining cork manufacturing stages (PB, 2B and D) shared similar fungal diversity, as
439 supported by the cluster that contained all the phylotypes isolated in those stages (Fig. 3)
440 and also in the DGGE dendogram where they clustered together (Fig. 2), even in samples
441 collected in different seasons (batches 4 and 38). The origin of the mycobiota present in
442 samples collected in these three manufacturing stages (PB, 2B and D) was probably mainly
443 originated from the factory environment. In fact, a survey made in the factory air of three
444 cork factories showed that around 10^3 viable particles/m^3 of air were present in most of the
445 Industrial facilities, increasing in the rooms where the resting stage occurred (Pires, 2000).
446 Furthermore, *P. glabrum* was also isolated in factory air (Lacey, 1973).

447 In the course of this work, environmental fungi present in each sampling place was
448 assessed. The obtained results showed the presence of four predominant fungi: *A. flavus*
449 and *C. sitophila*, especially during the resting stage and *P. glabrum* and *Penicillium* sp
450 (biverticillate) mostly in all the sampling stages (data not shown).
451 Two phylotypes amplified in the first boiling stage present in low relative frequency (1.14 %)
452 (Table 2) were detected. One phylotype (*Pezicula* sp.) was detected in the resting stage
453 (PB) and another one (*Sporobolomyces*) in the second boiling (2B) and non-treated cork
454 discs (D). Both of them probably originated in the cork tree since they were associated to
455 colonize plant material (Bai et al., 2002, Verkley et al., 2003) and could survived the whole
456 manufacturing process (fig. 3).
457 In conclusion, the combination of culture dependent and independent methods contributed
458 to complement the obtained results. In the manufacturing process of cork discs the humidity
459 levels play a central role in the shifts of the cork fungal communities. Before the boiling stage
460 mycobiota mainly dominated by tree colonization. When the cork is placed in the factory to
461 be processed into cork discs (stoppers), the mycobiota that dominates the following stages
462 is originated from the factory environment. Some fungal species seems to be well adapted to
463 this substrate (*P. glabrum* and *C. sitophila*) while others seems to colonize cork in low
464 frequencies (e.g. *P. brasilianum* and *P. rubefaciens*) in one or two stages, preferentially in
465 raw cork.
466 The knowledge of the mycobiota that colonizes cork during all the stages of the
467 manufacturing process of cork discs (stoppers) allow us to localize the phases in which the
468 colonization shifts occurs in order to eliminate mostly the presence of fungal isolates that
469 could endanger cork. However, studies done until now (Barreto et al, 2011a and b) showed
470 that the most common fungal species occurring in cork did not produce any chemical
471 compound susceptible to spoil the cork final product.
472

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Bibliography

Álvarez- Rodríguez, M. L., López-Ocaña, L., López-Coronado, J. M., Rodríguez, E., Martínez, M. J., Larriba, G., Coque, J.-J. R. (2002) Cork taint of wines: role of the filamentous fungi isolated from cork in the formation of 2,4,6-trichloroanisole by O methylation of the 2,4,6-trichlorophenol. *App. Env. Microb.* 68(12): 5860-5869.

Altschul, S. F., Gisher, W., Miller, W., Myers, E. W., Lipman, D. J. (2009) Basic local aligment search tool. J. Mol. Biol. 215: 403-410.

Bai, F.-Y., Zhao, J.-H., Takashima, M., Jia, J.-H., Boekhout, T., Nakase, T. (2002) Reclassification of the *Sporobolomyces roseus* and *Sporidiobolus paraseus* complexes, with the description of *Sporobolomyces phaffii* sp. nov. Int. J. Syst. Evol. Microb. 52: 2309-2314.

Barreto, M. C., Houbraken, J., Samson, R., Frisvad, J. C., San-Romão, M. V. (2011a) Taxonomic studies of the Penicillium glabrum complex and the description of a new species *P. subericola.* Fungal Diversity 49: 23-33.

498 Barreto, M. C., Frisvad, J. C., Larsen, T. O., Mogensen, J., San Romão, M. V. (2011b) Exo-
499 metabolome of some fungal isolates growing on cork based medium. Eur. Food Res.
500 Technol. 232: 575-582.
501
502 Barreto, M. C., Vilas Boas, L., Carneiro, L. C., San-Romão, M. V. (2011c) Volatile
503 compounds in samples of cork and produced by selected fungi. J. Agric. Food Chem.
504 59(12): 6568-6574.
505
506 Basílio, M. C., Gaspar, R., Silva Pereira, C., San Romão, M. V. (2006) *Penicillium glabrum*
507 cork colonizing isolates – preliminary analysis of their genomic similarity. Rev. Iberoam.
508 Micol. 23: 151-154.
509
510 Belloch, C., Villa-Carvajal, M., Álvaerz-Rodríguez, M. L., Coque, J. J. R. (2009) *Rhodotorula*
511 *subericola* sp. nov., an anamosphic basidiomycetous yeast species isolated from bark of
512 *Quercus suber* (cork oak). Inter J. Syst Evo. Microb. 57: 1668-1671.
513
514 Danesh, P., Velez Caldas, F. M., Figueiredo Marques, J. J., San Romão, M. V. (1997)
515 Mycobiota in Portuguese “normal” and “green” cork throughout the manufacturing process of
516 stoppers. J. App. Microb. 82: 689-694.
517
518 Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using bootstrap.
519 Evolution 39: 783-791.
520
521 Gadanho, M., Sampaio, J. P. (2004) Application of temperature gradient gel electrophoresis
522 to the study of yeast diversity in the estuary of the Tagus River. FEMS Yeast Res. 5: 253-
523 261.

524

525 Gadanho, M., Sampaio, J. P. (2006) Microeukaryotic diversity in extreme environments of
526 the Iberian Pyrite: a comparison between universal and fungi-specific primer sets,
527 temperature gradient gel electrophoresis and cloning. FEMS Microbiol Ecol. 57: 139-148.

528

529 Gardes, M., Bruns, T. D. (1993) ITS primer with enhanced specificity for Basidiomycetes –
530 application to the identification of mycorrhizae and rusts. Mol. Ecol. 2 (2): 113-118.

531

532 Glass, N. L., Donaldson, G.C. (1995) Development of primer sets designed for use with the
533 PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental
534 Microbiology 61: 1323-1330.

535

536 Hocking, A. D., Pitt, J. I. (1980) Dichloran glycerol medium for enumeration of xerophilic
537 fungi from low- moisture foods. Appl. Environ. Microbiol. 39: 488-492.

538

539 Houbraken, J., Due, M., Varga, J., Meijer, M., Frisvad, J. C., Samon, R. A. (2007)
540 Polyphasic taxonomy of *Aspergillus* section *Usti*. Stud. Mycol. 59: 107-128.

541

542 Houbraken J., Varga J., Rico-Munoz E., Johnson S., Samson, R. A. (2008) Sexual
543 reproduction as the cause of heat resistance in the food spoilage fungus *Byssochlamys*
544 *spectabilis* (anamorph: *Paecilomyces variotii*). Appl. Environ. Microbiol. 74:1613-1619.

545

546 Lacey, J. (1973) The air spora of a Portuguese cork factory. Ann. Occup. Hyg. 16: 223-230.

547

548 Linaldeddu, B. T., Sirca, C., Spano, D., Franceschini, A. (2009) Physiological responses of
549 cork oak and holm oak to infection by fungal pathogens involved in oak decline. For. Path.
550 39: 232-238.
551
552 Muyzer, G., de Waal, E. C., Uitierlinden, A. G. (1993) Profiling of complex microbial
553 populations by denaturing gel gradient electrophoresis analysis of polymerase chain
554 reaction-amplified genes coding for 16S rRNA. Appl. Env. Microbiol. 59(3): 695-700.
555
556 Nagano, Y., Nagahama, T., Hatada, Y., Nunoura, T., Takami, H., Miyazaki, J., Takai, K.,
557 Horikoshi, K. (2010) Fungal diversity in deep-sea sediments – the presence of novel fungal
558 groups. Fungal Ecology 3: 316-325.
559
560 Oliveira, A. C., Peres, C. M., Correia Pires, J. M., Silva Pereira, C., Figueiredo Marques,
561 Barreto Crespo, M. T., San Romão, M. V. (2003) Cork stoppers industry: defining
562 appropriate mould colonization. Microbiol. Res. 158: 117-124.
563
564 Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions
565 through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111-120.
566
567 Pereira, V. J., Fernandes, D., Carvalho, G., Benoliel, M. J., San Romão, M. V., Barreto
568 Crespo, M. T. (2010) Assessment of the presence and dynamics of fungi in drinking water
569 sources using cultural and molecular methods. Water Research 44: 4850-4859.
570
571 Pereira, H. (2007) Cork biology, production and uses. Elsevier. Amsterdam.
572

573 Pitt, J. I. (1979) The genus *Penicillium* and its teleomorphic states *Eupenicillium* and
574 Talaromyces. Academic, London.
575
576 Pitt, J., Hocking, A. D. (2009) Fungi and food spoilage. Springer, New York. 3rd edition, pp
577 540.
578
579 Pires, J. M. (2000) Relatório final do projecto de apoio à inovação tecnológica no sector
580 rolha de cortiça. Final report of Agronomic engineer University degree, ISA, UTL, Lisboa,
581 Portugal.
582
583 Pires, J. M., Pereira, H., San-Romão, M. V. (2007) Study of humidity and water activity of
584 cork slabs during cork stopper manufacturing process - preliminary results. Ciên. Tec. Vit
585 22(1): 15-20.
586
587 Prat, C., Ruiz-Rueda, O., Trias, R., Anticó, E., Capone, D., Sefton, M., Bañeras, L. (2009)
588 Molecular fingerprinting by PCR-Denaturing gradient gel electrophoresis reveals differences
589 in the levels of microbial diversity for musty-earthy tainted corks. App. Env. Microb. 75(7):
590 1922-1931.
591
592 Rohlf, J. F. (2000) Numerical taxonomy and multivariate analysis system. Version 2.1 Exeter
593 software. Setauket NY.
594
595 Saitou, N., Nei, M. (1987). The neighbor joining method: a new method for reconstructing
596 phylogenetic trees. *Mol Biol Evol* **4**: 406-42.
597

598 Samson, R. A., Houbraken, J., Thrane, U., Frisvad, J. C., Andersen, B. (2010) Food and
599 Indoor Fungi. CBS Manual Laboratory Manual Series 2. Centraalbureau voor
600 Schimmelcultures, Utrecht, The Netherlands.
601
602 Schadt, C. W., Martin, A. P., Lipson, D. A., Schmidt, S. K. (2003) Seasonal dynamics of
603 previously unknown fungal lineages in tundra soils. Science 301: 1359-1361.
604
605 Serra, R., Peterson, S., CTCOR, Venâncio, A. (2008) Multilocus sequence identification of
606 *Penicillium* species in cork bark during plank preparation for the manufacture of stoppers.
607 *Res. Microb*. 159: 178-186.
608
609 Silva Pereira, C., Pires, A., Valle, M. J., Vilas Boas, L., Figueiredo Marques, J. J., San
610 Romão, M. V. (2000) Role of *Chrysonilia sitophila* in the quality of cork stoppers for sealing
611 wine bottles. J. Ind. Microbiol. 24(4): 256-261.
612
613 Simpson, R. F., Lee, T. H. (1990) The microbiology and taints of cork and oak. In:
614 International Association for modern Winery Technology and Management E. V.
615 Proceedings of the Ninth International Oenological Symposium; 24-26 May 1990; Cascais,
616 Portugal. (Internationale Interessengemeinschaft fur modern Kellertechnik and
617 Betriebstuhrung e.v. Breisach) pp. 653-667.
618
619 Swofford, D. L. (2001). PAUP*. Phylogenetic. Analysis Using Parsimony (*and Other
620 Methods). Sinauer Associates, Sunderland, Massachusetts.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**: 4876-4882.

Verkley, G. J. M., Zijstra, J. D., Summerbell, R. C., Berendese, F. (2003) Phylogeny and taxonomy of root-inhabiting *Cryptosporiopsis* species and *C. rhizophila* sp. nov., a fungus inhabiting roots of several *Ericaceae*. Mycol. Res. 107(6): 689-698.

Vandenkoornhuyse P., Husband R., Fitter A.H., Young J.P.W. (2002) Arbuscular mycorrhizal community composition with co-occurring plant species from a grassland ecosystem. Molecular Ecology, 11: 1555-1564.

White, T. J., Bruns, T., Lee, S., Taylor, J. W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J. (Eds). PCR protocols: A guide to methods and applications. Academic Press, Inc., New York, N. Y., pp.315-322

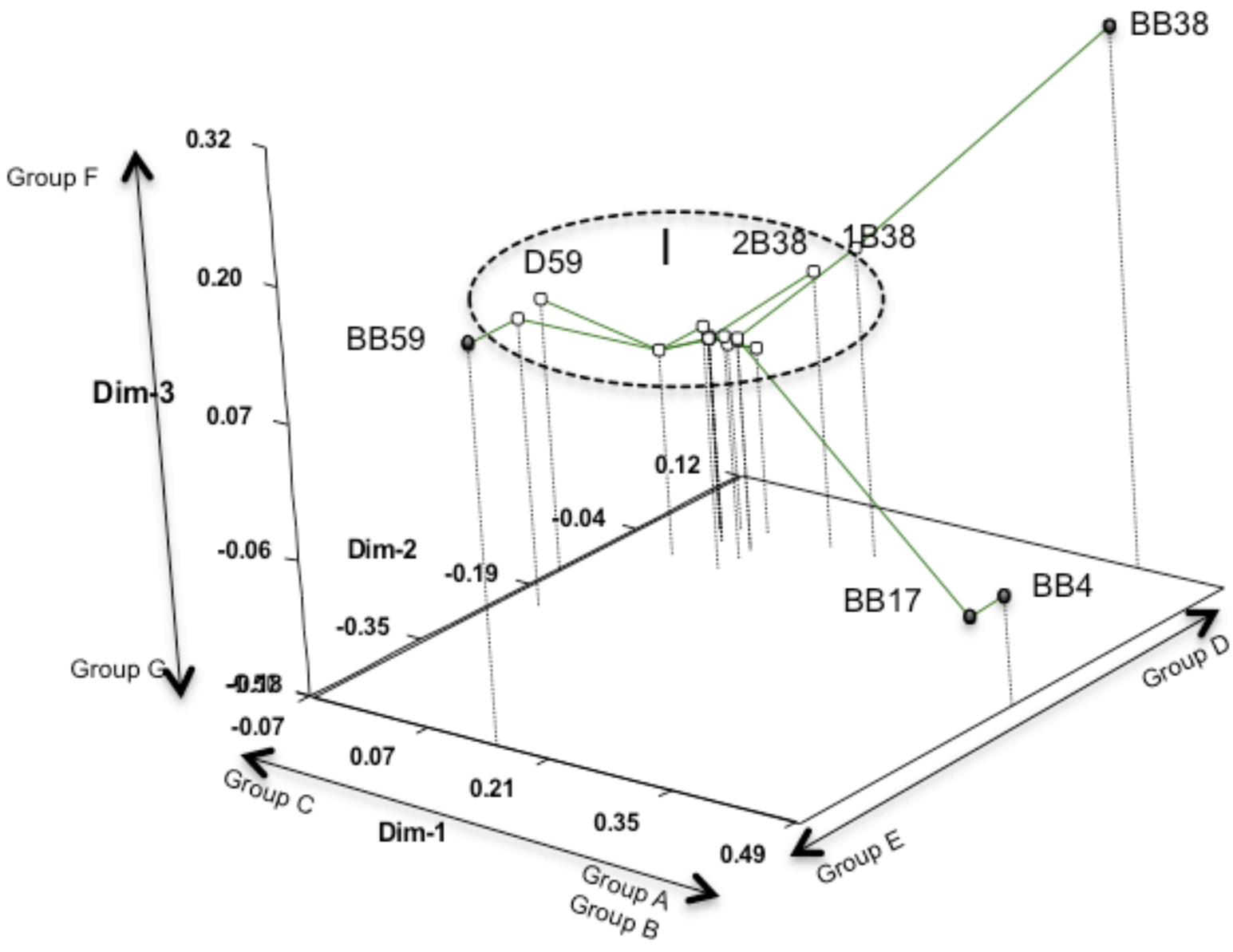

Fig. 1 – Plot of the projections in the first three dimensions of cork samples taken from several manufacturing stages onto the principal component analysis (PCA). The first dimension explained 20.5% of the total variance, the second 17.89% and the third 13.16%. I is constituted by the cork samples: 1B59, PB59, 2B59, 1B17, PB17, 2B17, D17, 1B4, PB4, 2B4, D4, PB38, D38.

	Fungal species	Eigenvalue
Group A	*T. longibracheatum*	0.83
	P. spinulosum	0.75
	P. janczewskii	0.75
Group B	*P. toxicarium*	0.66
	P. citreonigrum	0.65
	P. rubefaciens	0.64
	Unknown species	0.64
	Cl. herbarum	0.58
	P. brevicompactum	0.57
	Black yeast	0.50
	P. westlingii	0.50
	Penicillium sp.	0.50
	A. wentii	0.50
Group C	*M. plumbeus*	-0.40
	P. brasilianum	-0.26
	P. venetum	-0.26
Group D	*E. amstelodami*	0.37
	P. westlingii	0.36
	Penicillium sp.	0.36

	Fungal species	Eigenvalue
Group E	*P. crustosum*	-0.81
	P. miczynskii	-0.81
	Cryptococcus sp.	-0.78
	Hyphopodia sp.	-0.78
	Black yeast	-0.76
	P. glandicola	-0.76
	P. commune	-0.71
	P. toxicarium	-0.63
	C. sitophila	-0.60
Group F	*A. wentii*	0.71
	P. westlingii	0.71
	Penicillium sp.	0.71
	E. amstelodami	0.61
Group G	*P. janczewskii*	-0.61
	P. spinulosum	-0.61
	P. steckii	-0.52
	P. subericola	-0.52

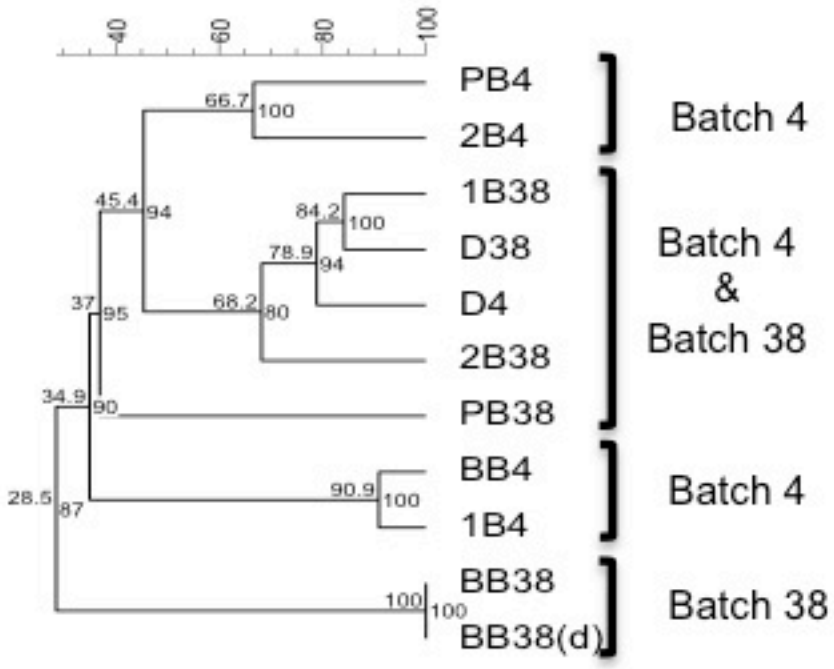

Fig. 2 – UPGMA dendrogram showing the diversity of the fungal population in two Portuguese samplings (batches 4 and 38) and using the Dice correlation of DGGE fingerprinting profiles. The similarity values (left) and cophenetic correlations values (right), are given for each branch.

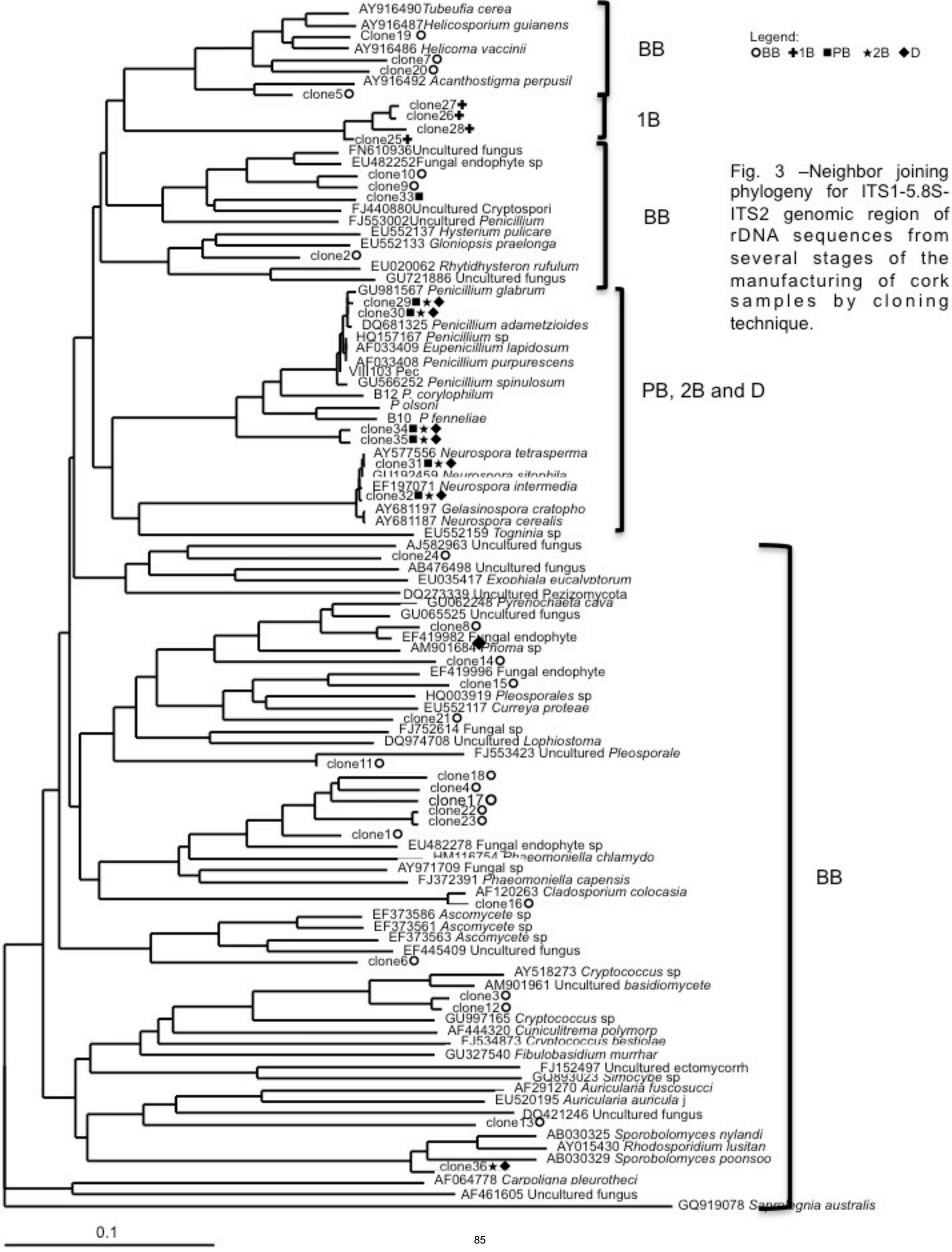

Fig. 3 –Neighbor joining phylogeny for ITS1-5.8S-ITS2 genomic region of rDNA sequences from several stages of the manufacturing of cork samples by cloning technique.

Table 1 – Occurrence and quantification of cultivable fungal species from two samplings of Spanish (Lt 59 – 1st sampling and Lt 17 – 2nd sampling) cork and Portuguese (Lt 4 – 1st sampling and Lt 38 – 2nd sampling) cork in the main stages of cork manufacturing discs using dichloran glycerol chloramphenicol (DGC18) culture medium.

		A. wentii	*Ch. sitophila*	*Cl. herbarum*	*Cladosporium sp.*	*E. amstelodami*	*E. herbariorum*	*E. rubrum*	*M. plumbeus*	*P. brevicompactum*	*P. brasilianum*	*P. chrysogenum*	*P. citreonigrum*	*P. citrinum*	*P. commune*	*P. crustosum*	*P. glabrum*	*P. glandicola*	*P. miczynskii*	*Pjanczewskii*	*P. paneum*	*P. rubefaciens*	*P. spinulosum*	*P. steckii*	*P. subericola*	*P. sumatrense*	*P. toxicarium*	*P. venetum*	*P. westlingii*	*Penicium sp.*	*T. longibracheatum*	unknown species	*Cryptococcus sp.*	*Hyphopidia sp.*	Black yeast	Total cultivable fungi CFU/g cork
Spanish cork	BB59		+					+		+					+	+	+	+	+								+						+	+	+	40
	1B59																																			<10
	PB59		+						+								+	+																+	+		132
	2B59																																				<10
	D59								+		+				+		+	+										+								65
	BB17						+	+					+				+			+			+	+	+		+									1044
	1B17							+	+																												<10
	PB17							+									+									+										80
	2B17						+																														<10
	D17																+																				2200
Portuguese cork	BB4		+	+			+	+		+							+			+		+	+				+					+	+			+	1445
	1B4							+									+																				<10
	PB4						+		+																												<10
	2B4																+																				<10
	D4		+							+							+				+																n.q.
	BB38	+		+		+	+	+		+			+				+												+	+	+					49950
	1B38					+	+	+		+		+					+																				27
	PB38		+						+								+																				55
	2B38			+				+		+		+		+																							150
	D38		+	+	+												+																				40

n.q. = not quantified

Table 2 - Percentage of sequence identity to the nearest relative in the GenBank database, elative frequency and their genbank number for the clones sequenced in the present study.

	Relative frequency (%)	Genbank nº	Homology	Probable identification
Clone 1	13.5	JN858112	96	Uncultivable Basidiomycete clone BF-OTU116
Clone 2	8.1	JN858113	92	*Gloniopsis praelonga* CBS 119332
Clone 3	2.7	JN858114	96	Uncultivable *Basidiomycetes*
Clone 4	2.7	JN858115	93	Fungal endophyte 5T2.10
Clone 5	2.7	JN858116	94	*Acanthostigma perpusillum*
Clone 6	8.1	JN858117	90	Uncultivable *Ascomycete* sp.
Clone 7	5.4	JN858118	95	*Helicoma vacinii* CBS 216.90
Clone 8	2.7	JN858119	95	Fungal endophyte isolate CAW 20
Clone 9	2.7	JN858120	96	*Pezicula* sp. 3 ICMP 18931
Clone 10	2.7	JN858121	94	*Pezicula* sp. 3 ICMP 18931
Clone 11	2.7	JN858122	92	Uncultivable *Pleoporales*
Clone 12	2.7	JN858123	94	Uncultivable *Basidiomycete*
Clone 13	2.7	JN858124	85	Uncultivable soil fungus clone 138-33
Clone 14	5.4	JN858125	98	Uncultivable *Pleurophoma* clone KL
Clone 15	2.7	JN858126	97	Fungal endophyte isolate 9194
Clone 16	5.4	JN858127	98	*Cladosporium colocasiae*
Clone 17	2.7	JN858128	93	Fungal endophyte 5T2.10
Clone 18	2.7	JN858129	94	Fungal endophyte 5T2.10
Clone 19	2.7	JN858130	94	*Helicoma vaccinii* CBS 216.90
Clone 20	5.4	JN858131	96	*Helicoma vaccinii* CBS 216.90
Clone 21	2.7	JN858132	96	Fungal endophyte isolate 9194
Clone 22	2.7	JN858133	94	Fungal endophyte 5T2.10
Clone 23	5.4	JN858134	94	Fungal endophyte 5T2.10
Clone 24	2.7	JN858135	91	Uncultivable fungus clone TLF 34-5
Clone 25	56.3	JN858136	86	Uncultivable soil fungus clone CS2M5c53P
Clone 26	3.1	JN858137	84	Uncultivable soil fungus clone CS2M5c53P
Clone 27	25	JN858138	86	Uncultivable soil fungus clone CS2M5c53P
Clone 28	15.6	JN858139	85	Uncultivable soil fungus clone CS2M5c53P
Clone 29	21.2	JN858140	99	*P. glabrum*
Clone 30	1	JN858141	99	*P. glabrum*
Clone 31	20.2	JN858142	100	*C. sitophila*
Clone 32	36.5	JN8581 43	99	*C. sitophila*
Clone 33	1	JN858144	88	*Pezicula* sp. 3 ICMP 18931
Clone 34	1.9	JN858145	98	*P. glabrum*
Clone 35	17.3	JN858146	97	*P. glabrum*
Clone 36	0.9	JN858147	99	*Sporobolomyces sp.*

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Taxonomic studies of the *Penicillium glabrum* complex and the description of a new species *P. subericola*

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Abstract A mycological survey of fungi, present in several stages of the manufacturing of cork discs for champagne stoppers in Portugal, was made. Sixty-nine strains belonging to the *Glabra* series of the genus *Penicillium* were isolated and subsequently grouped according to their partial β-tubulin gene sequences. Six groups with different partial β-tubulin gene sequences were observed, and a selection of isolates of each group was made. These selected isolates and various related ex-type strains were subjected to a taxonomical study using a polyphasic approach. This approach included analysis of macro- and microscopic features, the comparison of extrolite profiles and sequencing a part of the β-tubulin and calmodulin gene. The six β-tubulin types were reduced to three different species. One group of isolates was centred on the ex-type strain of *P. glabrum*, a second group accommodated the type strain of *P. spinulosum* and a third group contained isolates which were unique in their β-tubulin and calmodulin sequences, extrolite profiles and growth characteristics. This group of isolates is described as the new species *Penicillium subericola*. The type strain of *P. subericola* CBS 125096^{T} was isolated from Portuguese raw cork, but additional isolates were found from soil, air and lumen.

Keywords Taxonomy · Phylogeny · Tubulin · Cork

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Introduction

Cork is the outer bark of the cork oak tree (*Quercus suber*). It is the most suitable material for cork stoppers, due to its unique properties, such as elasticity, compressibility and impermeability to gas or liquids (Lopes et al. 2001; Mano 2002). During a survey of the colonizing mycobiota of cork slabs along the industrial manufacture of cork stoppers, numerous *Penicillium* isolates were isolated and identified using morphological characters. More than half of the isolates belonged to the *Glabra* series, and were present in all production stages. However, identification of the different isolates up to species level appeared to be difficult due the high similarities in macro- and micromorphology.

Raper and Thom (1949) placed *P. glabrum* (as *P. frequentans*), *P. spinulosum* and *P. purpurescens* in the *P. frequentans* series, and later this series was synonymised with the *Glabra* series by Pitt (1979). The *Glabra* series was created to accommodate the fast growing Penicillia with monoverticillate conidiophores and contains eight species (*P. chermesinum*, *P. sclerotiorum*, *P. donkii*, *P.*

decumbens, *P. thomii*, *P. glabrum*, *P. spinulosum* and *P. purpurescens*). Among those species, *P. glabrum* and *P. spinulosum* were morphologically similar and could be best differentiated based on conidial ornamentation. However, the morphological resemblance has caused much confusion and isolates are often misidentified or not differentiated by taxonomists using morphological and physiological techniques (Pitt et al. 1990).

Sixty-nine strains originating from cork and belonging to the *Glabra* series were grouped according to their partial β-tubulin gene sequences. A subset of these strains was selected for macro- and microscopic analysis, extrolite profiling and sequencing a part of the β-tubulin and calmodulin gene. In addition, ex-type strains of various related species were included in the analysis. Our polyphasic taxonomic approach shows that a group of isolates share peculiar differences with other known species, and a new species is proposed for this group of isolates.

Materials and methods

Fungal strains

For our taxonomic study, a selection of these sixty-nine strains isolated from cork, was made and supplemented with related (ex-type) strains (Table 1). Spore suspensions of the cultures were maintained in 20% glycerol at −80°C.

Sequencing and data analysis

The strains were grown for 2–3 days at 25°C on malt peptone medium. Genomic DNA was isolated using the Ultraclean™ Microbial DNA Isolation Kit (MoBio, Solana Beach, U.S.A.) according the manufacturer's instructions. Fragments, containing a part of the β-tubulin or calmodulin gene, were amplified and subsequently sequenced according the procedure previously described (Houbraken et al. 2007). The alignments and analyses were preformed as described by Samson et al. (2009). Newly obtained sequences were deposited in Genbank nucleotide sequence database under GQ367499-369547, GU372883-GU372894 and GU991606-GU991609.

Phenotypic identification

All strains were grown on malt extract agar (MEA, Oxoid), Czapek Yeast autolysate agar (CYA), creatine agar (CREA) and Yeast Extract Sucrose agar (YES) (Samson et al. 2010). These media were inoculated in a three-point position and incubated at 25°C for 7 days. In addition, CYA plates were incubated at 30°C and 37°C. After incubation, the culture characteristics were recorded. Microscopic characters were determined on MEA and CYA.

Extrolite extraction and analysis

A selection of ten cork isolates was made based on the results of the β-tubulin analysis, and subjected to extrolite profiling. In addition, various related ex-type strains were examined. The extrolite extractions from the culture media were preformed according to the methods described by Frisvad and Thrane (1987) and Smedsgaard (1997), using 500 μL ethylacetate/methanol/dichloromethane 3:2:1 (vol./vol./vol.) with 1% formic acid. The mixture was ultrasonicated in a bath for 60 min. The organic solvent was transferred to a new vial and evaporated in a fume hood for 24 h. The extract was re-dissolved in 400 μL methanol, analysed by HPLC with diode array detection (DAD) and the extrolites were identified by their UV spectra and retention times.

Results

Grouping of members of the Glabra series isolated from cork

The genetic variation within the strains isolated from cork was investigated using the partial β-tubulin sequences. The strains isolated from cork and four ex-type strains (*P. glabrum*, *P. frequentans*, *P. paczoskii* and *P. spinulosum*) were added to the dataset, and subjected to an UPGMA analysis (Sneath and Sokal 1973). The sum of branch length of the optimal tree was 0.1301 and the dendrogram is shown in Fig. 1. In total, 422 positions were present in the final dataset. Six groups could be identified among the cork isolates belonging to the *Glabra* series. The largest group (50 isolates) shared the same partial β-tubulin sequence with the type of *P. glabrum*, CBS 125543 (Group 1). One cork isolate (CBS 127703) appeared to have a unique partial β-tubulin sequence differing from other isolates in this clade (group 2). Group 4 was the second largest group and consisted of 14 isolates. This group was closely related with group 3 (3 isolates) and these two groups only differed by one base pair. Group 5 and 6 were deviating from the other groups and the β-tubulin data shows that members of group 6 share sequences with the type of *P. spinulosum*. Group 5 contained one isolate and this strain will be described here as a new species *P. subericola*. Each unique sequence type was compared by a BLAST search in the NCBI database with the *P. glabrum* strains identified by Serra et al. (2008). In total three *P. glabrum* sequences were deposited by Serra et al. (2008) and NRRL 35621 appeared to have identical sequences as "group 2", while the other two sequences (NRRL 35626 and NRRL 35684) were unique and not

Table 1 List of isolates belonging to Series *Glabra* and related Penicillia

CBS no.	Other no.	Name	Remarks
CBS 235.60	ATCC 18483=FRR 634	*E. pinetorum*	Ex-type of *P. silvaticum*; forest soil, USSR
CBS 295.62	ATCC 14770=CCRC 31517=DSM 2438= IFO 7743=IMI 094209=MUCL 31196= NRRL 3008	*E. pinetorum*	Ex-type; soil, conifer and hardwood forest, Wisconsin, USA
CBS 260.29	IMI 092242=NRRL 774=Thom4733.60	*P. glabrum*	Ex-type of *P. flavidorsum*; unrecorded source
CBS 213.28	FRR 770=IMI 092265=IMI 092265ii=NRRL 770	*P. glabrum*	Ex-type of *P. oledzskii*; soil under conifer, Poland
CBS 344.59	ATCC 18486=IFO 5359=IMI 068617=NRRL 3460	*P. glabrum*	Ex-type *P. spinuloramigenum*; butter, Japan
CBS 228.28	FRR 752=IMI 092232=MUCL 29114=NRRL 752	*P. glabrum*	Ex-type of *P. terlikowskii*; soil under conifer, Poland
CBS 229.28	FRR 751=IMI 092231=MUCL 29111=NRRL 751	*P. glabrum*	Ex type of *P. paczowskii*; soil under conifer, Poland
CBS 105.11		*P. glabrum*	Ex-type of *P. frequentans*; unknown substrate, Germany
CBS 127700		*P. glabrum*	Non-boiled cork
CBS 127701		*P. glabrum*	Cork, after the 1st boiling process
CBS 126333		*P. glabrum*	Cork discs
CBS 127702		*P. glabrum*	Non-boiled cork
CBS 127703		*P. glabrum*	Non-boiled cork
CBS 127704		*P. glabrum*	Non-boiled cork
CBS 127705		*P. glabrum*	Non-boiled cork
CBS 126336		*P. glabrum*	Non-boiled cork
CBS 125543	IBT 22658	*P. glabrum*	Ex-type; unrecorded source
CBS 687.77	IJFM 3745=IMI 253783	*P. grancanariae*	Ex-type of *P. grancanariae*; air, Gran Canaria, Spain
CBS 336.79	ATCC 38669=IJFM 3840=VKM F-2181	*P. palmense*	Ex-type; air, Gran Canaria, Spain
CBS 126.64		*P. purpurescens*	Soil, Erzurum, Turkey
CBS 366.48	ATCC 10485=IMI 039745=NRRL 720=QM 1959	*P. purpurescens*	Neotype; soil, Canada
CBS 328.48	ATCC 10444=IMI 040234=NRRL 1915	*P. spinulosum*	Ex-type of *P. trzebinskii*; forest soil, Poland
CBS 269.35	IMI 190574	*P. spinulosum*	Ex-type of *P. mucosum*; soil, beech forest; Germany
CBS 268.35	IMI 189582	*P. spinulosum*	Ex-type of *P. mediocre*; soil, pine forest; Germany
CBS 289.36	IMI 190573	*P. spinulosum*	Ex-type of *P. tannophagum*; tannin solution, Germany
CBS 271.35	IMI 190675	*P. spinulosum*	Ex-type of *P. tannophilum*; leaf litter, Germany
CBS 374.48	ATCC 10498=IMI 024316=MUCL 13910=MUCL 13911=NRRL 1750	*P. spinulosum*	Ex-type; culture contaminant, Germany
CBS 223.28		*P. spinulosum*	Unknown source
CBS 127698		*P. spinulosum*	Non-boiled cork
CBS 127699		*P. spinulosum*	Non-boiled cork
CBS 125096		*P. subericola*	Non-boiled cork, Portugal
CBS 127706	KAS 1289=IBT 22618	*P. subericola*	Lumber, Vancouver, BC, Canada
CBS 125097	IBT 23009	*P. subericola*	Air, margarine factory, Vejle, Denmark
CBS 125100	FRR 4914=IBT 30068	*P. subericola*	From dried grapes (sultanas, *Vitis vinifera*), Mildura, Vic, Australia
CBS 125099	IBT 20217	*P. subericola*	Acidified lake, Butte, Montana, USA
CBS 125098	IBT 20218	*P. subericola*	Acidified lake, Butte, Montana, USA
CBS 347.59	FAT 340=IFO 6031=IMI 068221	*P. thomii*	Ex-type of *P. thomii* var. *flavescens*; unrecorded substrate, Japan
CBS 350.59	ATCC 18333=FRR 3395=IFO 5362= IMI 068615	*P. thomii*	Ex-type of *P. yezoense*; butter, Japan

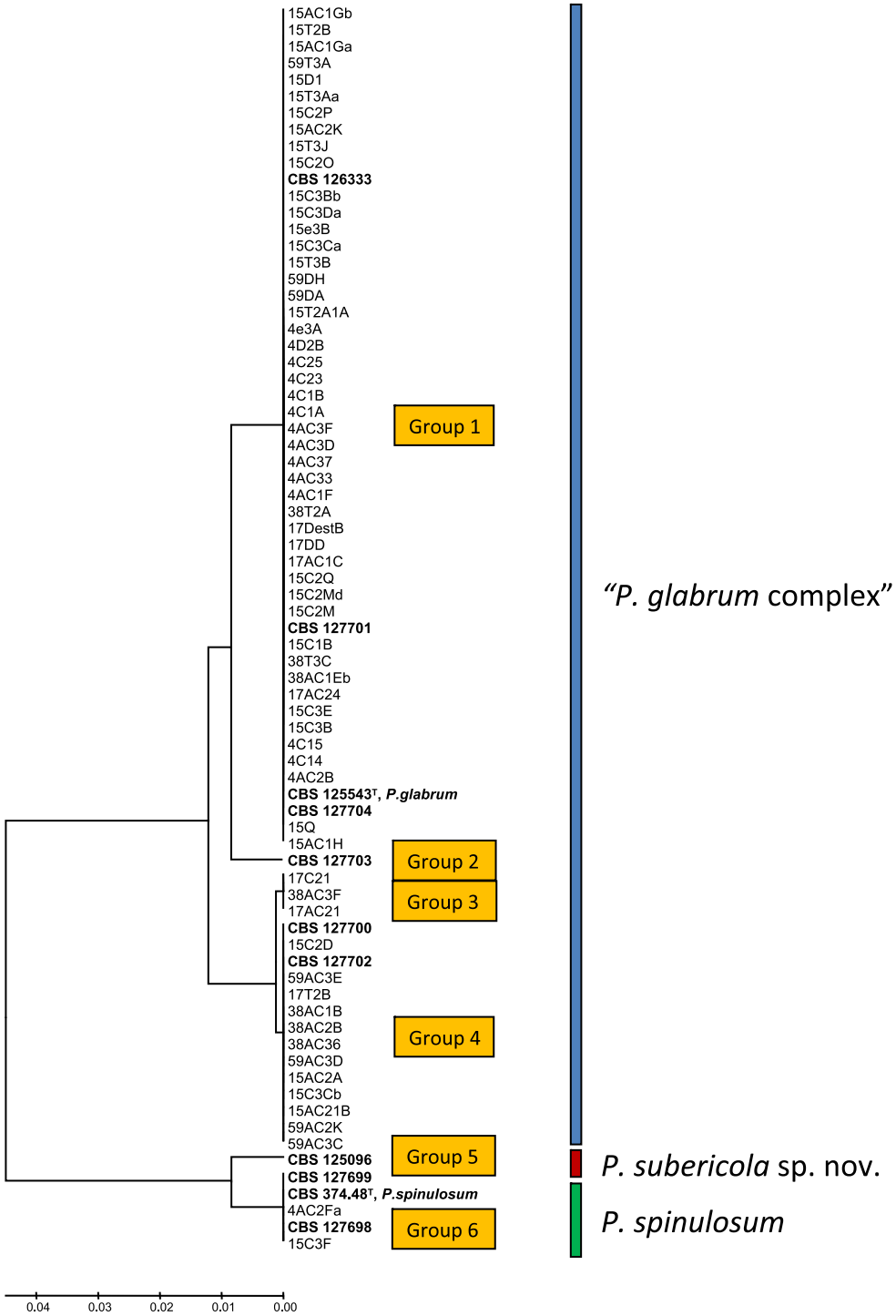

Fig. 1 Cladogram showing the results of the UPGMA analysis of the isolated cork strains belonging to *Penicillium* series *Glabra*. The strains presented in bold are used in the detailed phylogenetic analysis

assignable to any of our groups. A selection of strains was made and the isolates presented in bold in Fig. 1 were used for a detailed polyphasic study.

Phylogenetic analysis

A combined dataset with partial β-tubulin and calmodulin gene sequences was analysed using RAxML (Fig. 2). The alignment had 230 distinct patterns and the proportion of gaps and completely undetermined characters in the alignment was 0.0302. The phylogenetic analysis showed that there were two main well supported clades. In one clade *P. spinulosum*, *P. palmense* and *P. subericola* were present and in the other clade *P. glabrum*, and *P. purpurescens* were located. *Penicillium purpurescens* was basal to *P. glabrum* and the *P. glabrum* isolates were divided in two groups. In one group the majority of the cork isolates were located, together with the type strain of

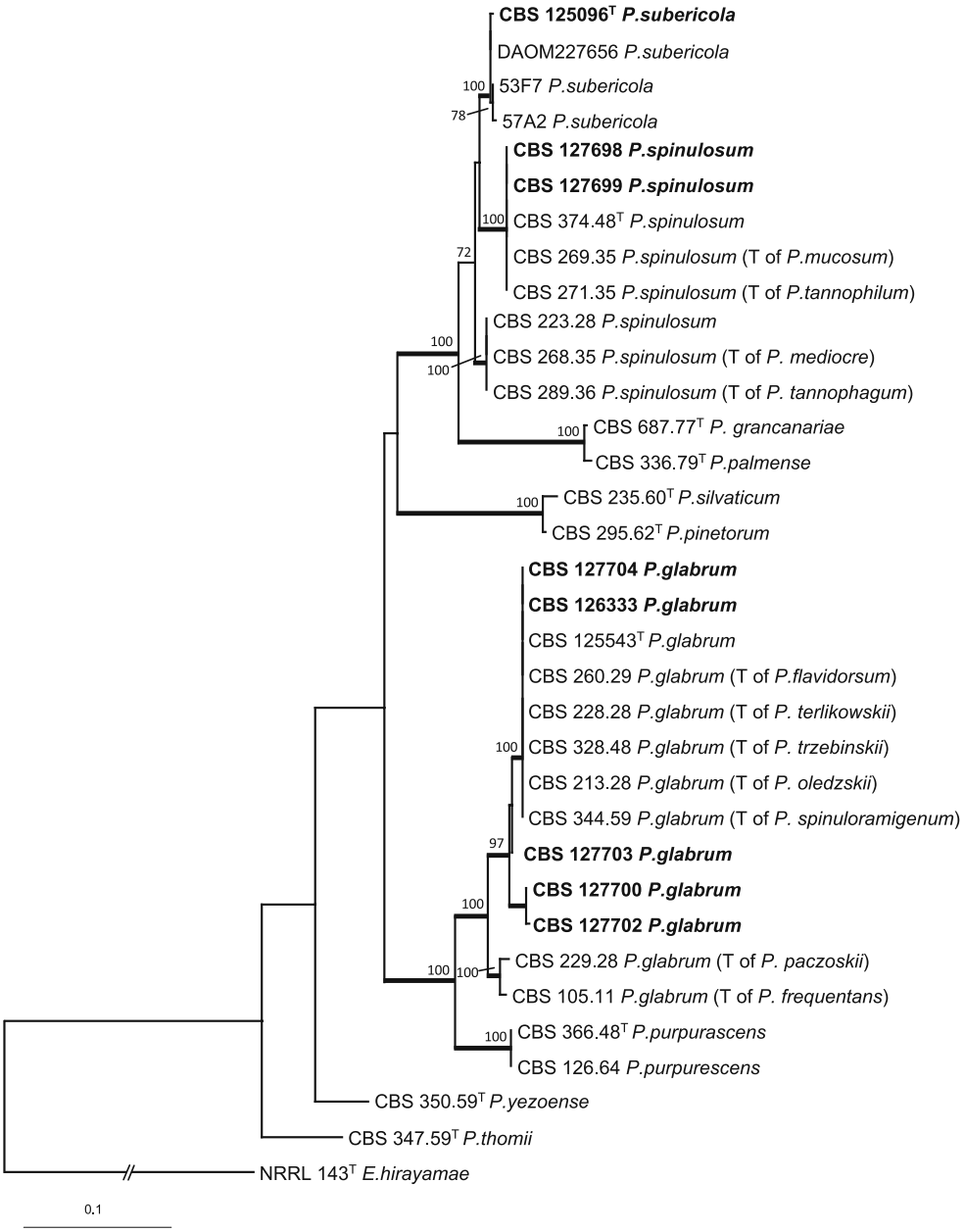

Fig. 2 Phylogram based on the combined dataset of partial β-tubulin and calmodulin gene sequences and analysed using RAxML. The strains in bold are isolated from cork

P. glabrum and the ex-type strains of *P. flavidorsum*, *P. spinuloramigenum*, *P. terlikowskii*, *P. trzebinskii* and *P. oledzskii*. The other group consisted of the type strains of *P. frequentans* and *P. paczowskii*. In the other clade, *P. palmense* was basal to *P. spinulosum* and *P. subericola*. The ex type of *P. palmense* clustered together with *P. grancanariae* CBS 687.77^{T}.

Penicillium spinulosum and *P. subericola* were on a branch with a fair bootstrap support (72%). Three groups were detected within this clade, but none of the phylogenetic relations between those groups were well supported. The isolates of *P. subericola* were on one branch. Interestingly, *P. spinulosum* was divided in two groups. One group compasses the type culture of this species and the type strains of *P. mucosum* CBS 269.35 and *P. tannophilum* CBS 271.35; the other group contained the type strains of *P. mediocre* CBS 268.35 and *P. tannophagum* CBS 289.36.

Phenotypic analysis

The strains isolated from cork were inoculated on the agar media MEA, CYA 25°C, CYA30°C, CYA 37°C, CREA and YES and were compared with the type strains of *P. glabrum*, *P. spinulosum*, *P. frequentans* and *P. paczoskii.* None of the examined strains were able to grow on CYA incubated at 37°C. In Fig. 3 an overview is shown of growth patterns on various agar media. There was a large variation in macromorphology among the *Glabra* strains.

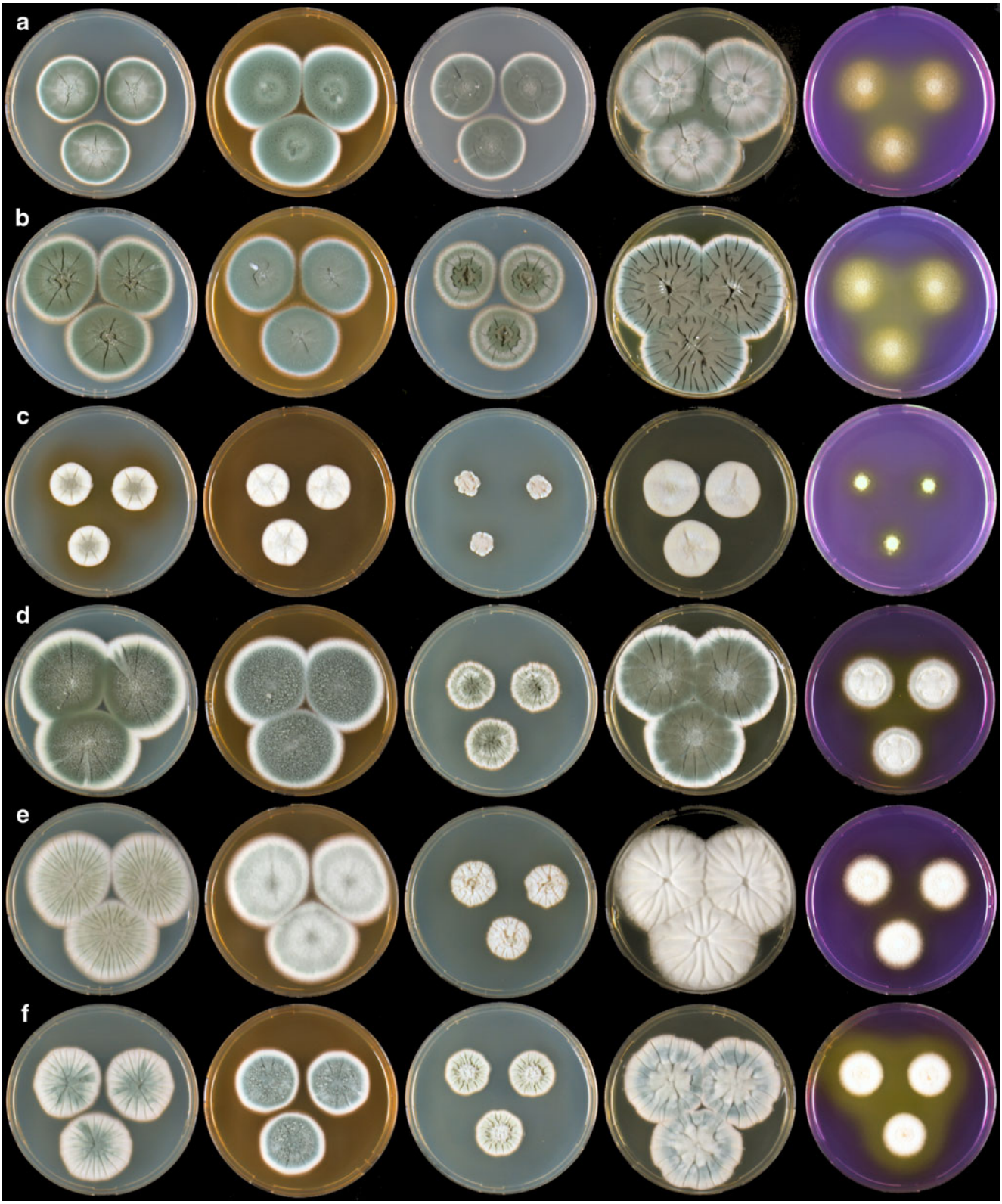

Fig. 3 Colonies incubated for 7 days. Columns, from left to right CYA at 25°C, MEA, CYA at 30°C, YES, creatine agar; rows, top to bottom, *Penicillium glabrum* CBS 127701, *P. glabrum* CBS 127702, *P. glabrum* CBS 125543^{T}, *P. spinulosum* CBS 127699, *P. spinulosum* CBS 374.48^{T}, *P. subericola* CBS 125096^{T}

The type strain of *P. glabrum* and *P. spinulosum* were deviating and showed reduced growth rates and weak sporulation. The reverse colours on CYA of the *Glabra* members were in shades of orange or orange brown, and occasionally in crème colours. The intensity of these colours varied per isolate and ranged from pale orange-brown to vivid orange or red-orange (in *P. spinulosum*). The variation observed among the *Glabra* cork isolates could not clearly be correlated to any of the six groups previously assigned with the partial β-tubulin data. No clear distinctive characters to differentiate between *P. glabrum*, *P. spinulosum* and the new species could be observed on CYA, MEA and YES. However, there was a striking difference on creatine agar. Isolates of *P. spinulosum* and the new species *P. subericola* grew moderate to good on this medium and the majority of both species produced base compounds after prolonged incubation. The colony diameter was generally larger than 25 mm, while *P. glabrum* isolates grew more restricted (often less than 25 mm) Fig. 3.

Microscopic analysis of the strains showed that *P. glabrum*, *P. spinulosum* and *P. subericola* sp. nov. were very similar to each other. All species were predominantly monoverticillate, with vesiculate conidiophores and 6–12 ampulliform phialides. The main microscopical difference was the conidia ornamentation, which was smooth to slightly rugose in *P. glabrum* and *P. subericola* sp. nov., and distinctly rugose in *P. spinulosum*. Moreover, the conidia of *P. subericola* tended to be more rugose than in *P. glabrum* and the conidiophores of this species occasionally were branched, a character not observed in *P. glabrum* and *P. spinulosum*.

Extrolites analysis

The majority of the strains assigned to *P. glabrum*, *P. spinulosum* and *P. subericola* produced a pattern of extrolites typical for each species (see Table 2). The *P. glabrum* isolates had a typical extrolites profile containing asterric acid, bisdechlorogeodin, sulochrin or citromycetin, while isolates of *P. spinulosum* produce asperfuran, palitantin and frequentin. Asperfuran, deoxybrevianamide E and unidentified compounds which were tentatively named AMF were found in the *P. subericola*. These AMF compounds are indols with an extended chromophore similar to penitremone. Two cork isolates which phylogenetically clearly belong to *P. glabrum* (CBS 126333 and 127701) were chemically weak and show no detectable extrolite production.

Discussion

The majority of cork isolates were identified as *P. glabrum* using the current taxonomical schemes. Four different sequence types of β-tubulin within *P. glabrum* could be detected. BLAST searches on the NCBI database and local databases of the CBS-Fungal Biodiversity Centre showed that many more sequence types are present in *P. glabrum*. This intra-species β-tubulin variation is in contrast with species in subgenus *Penicillium*, where various species share the same tubulin sequence (Samson et al. 2004). The large variability among *P. glabrum* isolates originating from cork is also observed using microsatellite primers (Basílio et al. 2006). Our analysis show that *P. flavidorsum*, *P. spinuloramigenum*, *P. terlikowskii*, *P. trzebinskii* and *P. oledzskii* are synonyms of *P. glabrum.*

Raper and Thom (1949) placed *P. glabrum* (*P. frequentans*), *P. spinulosum* and *P. purpurescens* in the *P. frequentans* series. Our data show that these three species are phylogenetic related. Pitt (1979) named this the *Glabra* series and expanded it with Penicillia, which have monoverticillate penicilli and a colony diameter on CYA larger than 30 mm after 7 days at 25°C. *Penicillium chermesinum*, *P. sclerotiorum, P. donkii, P. decumbens, P. thomii, P. glabrum, P. spinulosum* and *P. purpurescens* were included, but the phylogenetic analysis of the genus *Penicillium* by Peterson (2000) showed that the former four species were not closely related to *P. glabrum*. Furthermore, Peterson (2000) named this monophyletic clade “Group 2”, and showed that the species *E. pinetorum*, *P. asperosporum*, *P. lividum* and *E. lapidosum* were related to *P. glabrum*. These findings in a large extent supported in our study, but there are some differences. The taxonomic position of *E. lapidosum* warrants further attention. This species was not included in our phylogenetic study because the type strain of this species (CBS 343.48) is phylogenetically unrelated to the *Glabra* group (J. Houbraken, unpublished data). This is in contrast with the observation made by Peterson (2000), which stated that *E. lapidosum* was conspecific with *P. thomii*.

Our data show that *P. palmense* and *P. grancanariae,* both isolated from air in Gran Canaria, Spain (Ramirez et al. 1978), are synonymous. The type strains of *P. frequentans* and *P. paczowskii* were considered to be synonyms of *P. glabrum* and *P. spinulosum* respectively (Pitt, 1979). However, based on calmodulin, tubulin and RPB2 data (data not shown) both type strains are placed in a separate clade related to *P. glabrum,* suggesting that *P. frequentans*/*P. paczowskii* and *P. glabrum* are two distinct species. This evidence is also supported by the extrolites profiles of these species (Frisvad, unpublished data).

Phenotypical differences were observed between the type strains and the cultures isolated from the cork. This is probably due to the fact that the type strains are maintained in cultures collections for a considerable period. Gradual degeneration of various traits due to long-term maintenance and sub culturing are reported. Also degeneration could be due to the lyophili-

Table 2 Extrolite profile of the cork isolates and type or authentic isolates belonging to *Glabra* series on CYA, YES and OA after 7 days of incubation

Species	Isolates	Extrolites
P. glabrum	CBS 213.28	Asterric acid, bisdechlorogeodin, questin, sulochrin
	CBS 328.48=FRR 1915	Asterric acid, bisdechlorogeodin, citromycetin, PI-3, PI-4
	ATCC 42228=IBT 13946	Asterric acid, bisdechlorogeodin, sulochrin
	CBS 127703	Asterric acid, bisdechlorogeodin, PI-4, sulochrin
	CBS 127700	Asterric acid, bisdechlorogeodin, PI-4, sulochrin
	CBS 126336	Asterric acid, citromycetin, bisdechlorogeodin, PI-4, questin, questinol,sulochrin
	CBS 127702	Asterric acid, citromycetin, bisdechlorogeodin, PI-4, questin, questinol, sulochrin
	CBS 127704	Asterric acid, bisdechlorogeodin, PI-4, questinol, sulochrin
	CBS 126333	No metabolites expressed
	CBS 127701	No metabolites expressed
P. palmense	CBS 336.79=IBT 4912	4 chromophore types in common with *P. subericola*, and 4 chromophore types only found in this species
	ATCC 38669=IBT 16227	4 chromophore types in common with *P. subericola*, and 4 chromophore types only found in this species
P. spinulosum	NRRL 1750	Asperfuran
	DAOM 215366=IBT 22621	Asperfuran, palitantin, frequentin
	DAOM 227655=IBT 22622	Asperfuran, palitantin
	CBS 127698	2 chromophore types found in this isolate and CBS 127699
	CBS 127699	2 chromophore types found in this isolate and CBS 127698
P. subericola	CBS 125096	AMF[a], deoxybrevianamide E
	CBS 125100=FRR 4914=IBT 30068	AMF, deoxybrevianamide E
	IBT 23009 & IBT 23010	AMF
	DAOM 227656=IBT 22618	AMF, asperfuran, deoxybrevianamide E
	CBS 125099=IBT 20217	AMF, asperfuran
	CBS 125098=IBT 20218	AMF
	IBT 23016	AMF
E. pinetorum	WSF 15-c=IBT 22704	Asperfuran and 4 chromophore types on seen in this species
	RMF 9252=IBT 22795	Asperfuran and 4 chromophore types on seen in this species
	CBS 311.63=IBT 22192	Asperfuran and 4 chromophore types on seen in this species
P. purpurescens	CBS 366.48	5 chromophore types only seen in this species

[a] AMF compounds are not fully chemically identified indols with an extended chromophore similar to penitremone

zation process, and colony characteristics could be affected due to a lower survival of spores in lyophilised cultures, compared to the fresh cultures (Okuda et al. 1990). The main distinction between *P. glabrum* and *P. spinulosum* was the conidia wall texture, which was smooth to finely rugose in *P. glabrum* and finely roughened to distinctly spinose in *P. spinulosum*. Some isolates belonging to the *Glabra* series were difficult to identify correctly even by skilled taxonomists (Pitt et al. 1990). However, to overcome this problem molecular and chemical techniques combined with classical taxonomy were analysed together here, giving a more accurate answer to the taxonomic position of these closely related species. In this study we show that *P. glabrum* can be differentiated from *P. spinulosum* and *P. subericola* by its weak growth on creatine agar.

The concept of exo-metabolome was introduced by Thrane et al. (2007) to enclose all the metabolites produced by fungi in interaction with the environment. The cork isolates belonging to the *Glabra* series could be grouped in three different extrolite profiles. One similar to the type strain of *P. glabrum*, a second group produced extrolites in common with the type strain of *P. spinulosum* and a third one characteristic of *P. subericola*. Two isolates were chemically weak and did not produce any extrolites. This might be due to degeneration by long-term maintenance, sub-culturing or lack of selection pressure from the environment. The non-production of expected metabolites could also be due to some (point) mutations on the regulatory gene (Larsen et al. 2005). Moreover, *P. spinulosum* cork isolates produced also some metabolites that were not characteristic of the species, although some of them were described in some *P. spinulosum* isolates. Since the production of secondary metabolites is more or less genus or species specific (Frisvad et al. 1998,

2008) the existence of *P. glabrum* cork isolates that produced two different extrolite profiles indicated the existence of intraspecific variability.

The species concept, based not only on DNA sequences, but also in ecological, phenotypic characters and exometabolome profiles provide a more accurate and real classification, as verified by studies on *Penicillium* subgenus *Penicillium* (Samson and Frisvad 2004) and black Aspergilli (Samson et al. 2007). Applying this polyphasic approach, *P. spinulosum* and *P. subericola* can be regarded as two separate species. Hoff et al. (2008) suggested in their study of *P. chrysogenum* that closely related species could be mating types of the same biological species. However, no differences in extrolite patterns and phenotype could be observed in isolates of different mating types of *Paecilomyces variotii* (Houbraken et al. 2008, Samson et al. 2009). Furthermore, our studies showed that the two mating types discovered in *Aspergillus fumigatus* (O'Gorman et al. 2009) and *Penicillium chrysogenum* (Hoff et al. 2008) produced the same pattern of extrolites and are identical in their phenotype (Houbraken, Samson and Frisvad, unpublished data). In case of *P. subericola* we have observed differences in both growth patterns and extrolite production and hence the description of a new species is warranted.

The cork isolates now classified as *P. glabrum* species showed a high intraspecific variability. The macro- and micromorphologies, extrolites profiles and results of the sequencing of partial regions of the β-tubulin and calmodulin genes supported that variability. If the results were analyzed separately (e.g. the extrolite profile and β-tubulin sequencing) probably some of them could indicate the existence of at least two different species. The analysis of more isolates of this species isolated from different sources and from different geographic locations is needed to determine species boundaries in *P. glabrum* and related species.

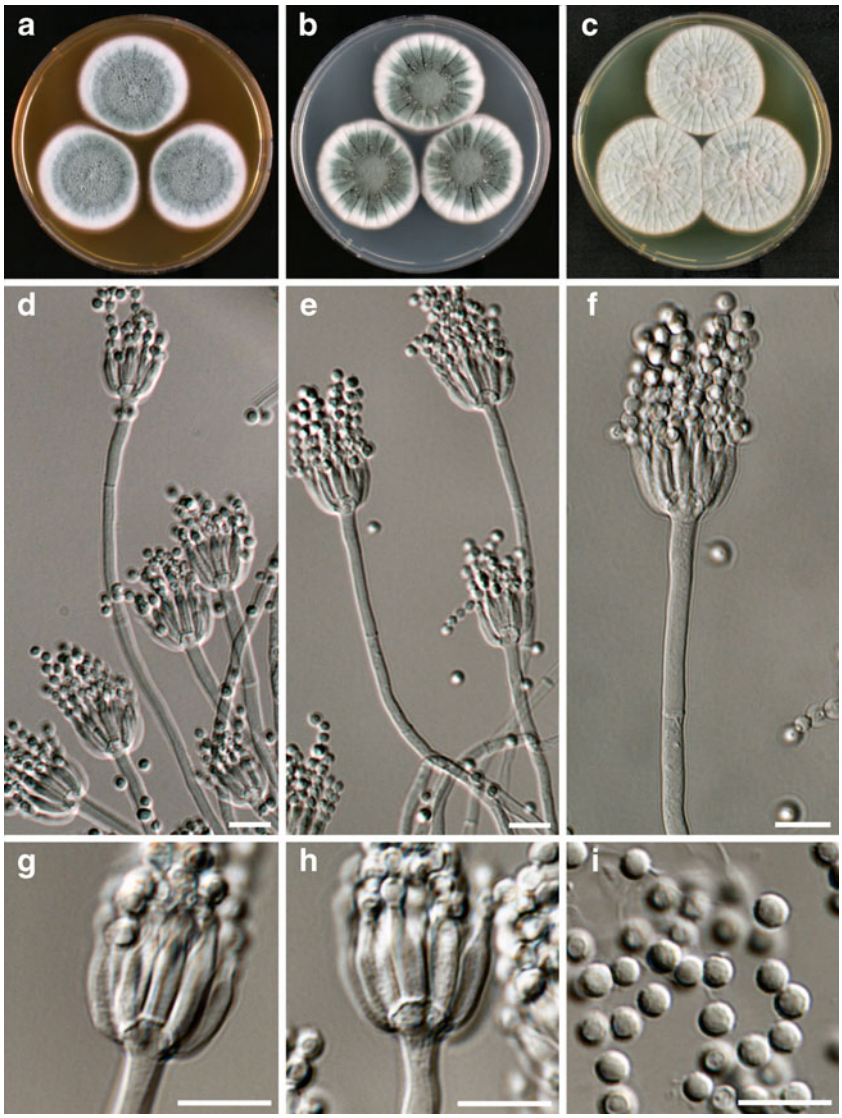

Fig. 4 *Penicillium subericola*, cultures incubated for 7 days at 25°C, A. MEA, B. CYA, C. YES. D-I. Conidiophores, phialides and conidia. Scale bar= 10 μm

Penicillium subericola Baretto, Frisvad & Samson, sp. nov.—Mycobank MB 517383 - Fig. 4.

Penicillio glabro simile, sed bene crescenti in agaro creatino et formatione mixtionis chemicae obscurae (sed in P. glabro non producenti) distinguitur.

Culture ex type: CBS 125096, ex raw cork, Portugal

Colony diameters at 7 days in mm: CYA at 25º C: 37–44; CYA at 30°C: 16–34; CYA at 37°C: no growth; MEA 35–42; YES 39–46; CREA 14–26, moderate to good growth with moderate to good acid production, base production after prolonged incubation (14 days).

Good sporulation on CYA, grey-green, velvety and floccose in centre, non sporulating margins 1–6 mm, few small hyaline exudates droplets present, reverse colour cream to brownish. Colonies on MEA grey-green, good sporulation, floccose some isolates with velvety colonies and/or velvety with floccose in the centre, exudate absent, reverse is orange brown. Colonies on YES in various shades of green-grey, none or weak sporulation, mycelium inconspicuous, white margins with 1–2 mm, exudates absent, reverse orange-brown to yellow-brown, strongly sulcated (wrinkled).

Conidiophores strictly monoverticillate, stipes vesiculate up to 6 μm, smooth, occasionally short 40 μm, majority longer, width 3.0–4.0, vesicles 4.5–7.0 μm, phialides flask shaped, 10–14×2.0–3.0 μm, conidia globose, finely roughened, 3–3.5 μm.

Extrolites: asperfuran, deoxybrevianamide E and unidentified compounds which are indols with an extended chromophore similar to penitremone.

Other isolates examined: CBS 127706 ex-lumber, Vancouver, BC, Canada; CBS 125100=IBT 30068, from dried grapes (sultanas, *Vitis vinifera*), Mildura, Vic, Australia; CBS 125099=IBT 20218 and CBS 125098=IBT 20217, both from acidified lake, Butte, Montana.

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References

Basílio MC, Gaspar R, Silva Pereira C, San Romão MV (2006) *Penicillium glabrum* cork colonising isolates—preliminary analysis of their genomic similarity. Rev Iberoam Micol 23:151–154

Frisvad JC, Thrane U (1987) Standardized high-performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV-VIS spectra (diode array detection). J Chromatogr 404:195–214

Frisvad JC, Thrane U, Filtenborg O (1998) Role and use of secondary metabolites in fungal taxonomy. In: Frisvad JC, Bridge PD, Arora DK (eds) Chemical fungal taxonomy. Marcel Dekker, New York, pp 289–319

Frisvad JC, Andersen B, Thrane U (2008) The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. Mycol Res 112:231–240

Hoff B, Pöggeler S, Kück U (2008) Eighty years after its discovery, Fleming's *Penicillium* strain discloses the secret of its sex. Eukaryotic Cell 7:465–470

Houbraken J, Due M, Varga J, Meijer M, Frisvad JC, Samson RA (2007) Polyphasic taxonomy of *Aspergillus* section *Usti*. Stud Mycol 59:107–128

Houbraken J, Varga J, Rico-Munoz E, Johnson S, Samson RA (2008) Sexual reproduction as the cause of heat resistance in the food spoilage fungus Byssochlamys spectabilis (anamorph: Paecilomyces variotii). Appl Environ Microbiol 74:1613–1619

Larsen T, Smedsgaard J, Nielsen K, Hansen M, Frisvad J (2005) Phenotypic taxonomy and metabolite profiling in microbial drug discovery. Nat Prod Rep 22:672–695

Lopes M, Barros A, Neto C, Rutledge D, Delgadillo I, Gil A (2001) Variability of cork from Portuguese *Quercus suber* studied by solid-state C-13-NMR and FTIR spectroscopies. Biopolymers 62:268–277

Mano J (2002) The viscoelastic properties of cork. J Mat Sci 37:257–263

O'Gorman CM, Fuller HT, Dyer PS (2009) Discovery of a sexual cycle in the opportunistic fungal pathogen Aspergillus fumigatus. Nature 457:471–474

Okuda T, Klich MA, Seifert K, Ando K (1990) Media and incubation effects on morphological characteristics of *Penicillium* and *Aspergillus*. In: Samson R, Pitt JI (eds) Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification. Plenum Press, New York, pp 83–99

Peterson SW (2000) Phylogenetic analysis of Penicillium species based on ITS and LSU-rDNA nucleotide sequences. In: Samson R, Pitt J (eds) Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification. Harwood, Reading, pp 163–178

Pitt JI (1979) The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. Academic, London

Pitt JI, Klich MA, Shaffer GP, Cruickshank RH, Frisvad JC, Mullaney EJ, Onions AHS, Samson RA, Williams AP (1990) Differentiation of *Penicillium glabrum* from *Penicillium spinulosum* and other closely related species: an integrated taxonomic approach. System Appl Microbiol 13:304–309

Ramirez C, Martinez AT, Ferrer S (1978) Three new species of *Penicillium*. Mycopathol 66:77–82

Raper KB, Thom C (1949) Manual of the Penicillia. Williams and Wilkins, Baltimore

Samson RA, Frisvad JC (2004) *Penicillium* subgenus *Penicillium*: new taxonomic schemes, mycotoxins and other extrolites. Stud Mycol 49:1–174

Samson RA, Seifert KA, Kuijpers AFA, Houbraken JAMP, Frisvad JC (2004) Phylogenetic analysis of *Penicillium* subgenus *Penicillium* using partial b-tubulin sequences. Stud Mycol 49:175–200

Samson RA, Noonim P, Meijer M, Houbraken J, Frisvad JC, Varga J (2007) Diagnostic tools to identify black Aspergilli. Stud Mycol 59:129–145

Samson RA, Houbraken J, Varga J, Frisvad JC (2009) Polyphasic taxonomy of the heat resistant ascomycete genus *Byssochlamys* and its *Paecilomyces* anamorphs. Persoonia 22:14–27

Samson RA, Houbraken J, Thrane U, Frisvad JC, Andersen B (2010) Food and Indoor Fungi. CBS Laboratory Manual Series 2. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

Serra R, Peterson S, CTCOR VA (2008) Multilocus sequence identification of *Penicillium* species in cork bark during plank preparation for the manufacture of stoppers. Res Microbiol 159:178–86

Smedsgaard J (1997) Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. J Chromatogr A 760:264–270

Sneath PHA, Sokal RR (1973) Numerical Taxonomy. Freeman, San Francisco

Thrane U, Andersen B, Frisvad J, Smedsgaard J (2007) The exometabolome in filamentous fungi in Topics in Current Genetics. Vol 18. In: Nielsen J, Jewett MC (eds), Metabolomics. pp 235–252

Chapter 3

Exo-metabolites produced by some fungal isolates in several media cultures

This chapter includes the study of the possible production of exo-metabolites by some isolated fungal species. The following culture media were employed: two semi-synthetic media, a cork-based medium and cork-based medium added with *Chrysonilia sitophila* remains.

The following scientific article constitutes this chapter:

- Exo-metabolome of some fungal isolates growing on cork-based medium (*European Food Research Technology*, 2011, 232:575-582)

The author performed the experimental work presented in this chapter. The HPLC, GC-MS and data analysis were done in collaboration with Center for Microbial biotechnology, Department of Systems, Technical University of Denmark, Lyngby, Denmark. The manuscript was written by the author and revised by the other co-authors.

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ORIGINAL PAPER

Exo-metabolome of some fungal isolates growing on cork-based medium

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Abstract Fungal species colonize the cork slabs during the manufacturing of cork stoppers process. The most important fungal species that colonizes cork slabs immediately after boiling is *Chrysonilia sitophila*. Other fungal species may germinate replacing the *C. sitophila* mycelium on the cork slabs when the slabs' water activity decreases below 0.9. The possible production of exo-metabolites or volatile compounds by some fungal species during the post-boiling stage was verified in pure cultures using three different media compositions. The results suggest that no deleterious exo-metabolites or mycotoxins are produced by the studied fungal species, both in cork medium or in cork medium added with *C. sitophila* extracts. However, the addition of *C. sitophila* extract to the cork medium enhanced the growth of the other studied fungal isolates and altered the respective exo-metabolome profile, leading to the assumption that in their natural *habitat*, the late cork colonizers like *Penicillium* spp. and *Aspergilus* spp. could take advantage from an earlier *C. sitophila* development as a result of its metabolism and/or mycelium remains. Fungal successions may thus not only be a function of time and substrate, but also they can be dependent of the remains of former colonizers. In fact, the production of the exo-metabolites by the studied fungal isolates suggests that, under the used experimental conditions, they appear to play an important role in fungal interactions amongst the cork mycoflora.

Keywords Cork fungi · Extrolites · Volatile compounds · Cork niche

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Introduction

The manufacturing of cork stoppers involves the boiling of cork slabs. The boiling step increases the humidity in cork leading to fungal mycelium growth on the slabs surface. *Chrysonilia sitophila* (Mont.) Arx is the principal colonizer in slabs when the water activity is above 0.9 value [1, 2, Barreto & Gaspar, unpublished results]). When water activity decreases below 0.9, cork fungal mycobiota shifts, which is characterized by the growth of other fungi, for instance *Penicillium* spp., *Aspergillus* spp. and *Trichoderma* spp. [3–5].

Since *C. sitophila* is only visible during the first days of cork slab resting period, after the boiling step, its establishment implies that this species can metabolize the available substrates on cork. Changes in the compounds present in cork due to *C. sitophila* metabolism and the decrease in water activity below 0.9 can lead to the establishment of the late cork fungal colonizers [6–8]. The late colonizers (e.g. *Penicillium*, *Aspergillus*) are fungal species known to produce exo-metabolites in semi-synthetic media culture [9, 10], also denominated as extrolites [9]. Fungal colonization

in cork slabs can be conditioned not only by the shifts in the humidity but also by the presence of diverse species living and interacting close together in the same niche.

The exo-metabolites are energetic costly chemical products, which are usually associated with fungal sporulation [11]. Some of these compounds are deleterious (e.g. mycotoxins), while others are favourable (e.g. antibiotics) to humankind. Their production depends on the substrate [12] and the interaction/competition with other organisms [13].

Some of the exo-metabolites produced by the fungi are volatiles, usually designed by volatile organic compounds (VOCs). These compounds normally contribute to the intense and characteristic odours of fungi. Some previous studies show that their production is consistent and related to the cultural conditions and abiotic environment [14]. VOC-mediated positive, negative or neutral interactions can occur between a very wide range of soil bacteria and fungi contributing to the soil microflora constitution. Many organisms are known to modify the environment in order to construct an adequate niche where a natural selection can take place [15].

Cork is a natural and biodegradable material produced from the outer bark of *Quercus suber* L. possessing specific chemical and physical composition [16], which makes it an excellent sealing device. On the other hand, it is a recalcitrant substrate that requires specific enzymes for its metabolization to occur, whether it is total or partial [17].

The exo-metabolites production by some fungal species that are present in the post-boiling stage is of the outmost importance since some of these chemical compounds can be deleterious to the final product, thus affecting its quality.

This study investigated the capacity of the most important fungal cork colonizers to produce exo-metabolites using different rich semi-synthetic culture media to know their exo-metabolites profile. The possible production of exo-metabolites in cork-based medium to mimic the cork natural substrate and in cork medium added with *C. sitophila* extracts to simulate the post-boiling stage of the cork slabs was also investigated. Finally, the possible influence of the fungal exo-metabolite and volatiles on the shifts of cork slab fungal colonization was also stressed.

Materials and methods

Fungal isolates and culture media

All fungal species used in this study were previously isolated in our laboratory [1, 2, Barreto unpublished results] and identified using phenotypic and molecular techniques at CBS (Utrecht, Holland) (Barreto, unpublished results).

Eleven fungal strains isolated after the boiling stage of cork slabs during the cork stopper manufacturing process were used. These strains are now deposited in international culture collection with the following deposit numbers: *Chrysonilia sitophila* DSM 16514 (DSMZ, Germany), *Eurotium amstelodami* (CBS 126335), *Eurotium repens* (CBS 126221), *Penicillium brevicompactum* (CBS 126334), *Penicillium citreonigrum* (CBS 126338), *Penicillium citrinum* (CBS 126219), *Penicillium chrysogenum* (CBS 126337), *Penicillium glabrum* (1) (CBS 126333), *Penicillium glabrum* (2) (CBS 126336), *Penicillium paneum* and *Aspergillus tubingensis* (CBS 126391).

Composition of the culture media used in this work

1. YES medium—yeast extract sucrose agar prepared according to manufacturer's instructions (Fluka, Saint Louis, MO, USA).
2. Composed medium 1 (CoM): 1 g YES medium, 5 g malt broth (Merck, Darmstadt).
3. Cork medium (CM): 30 g of cork powder, 0.5 g of K_2HPO_4 and 15 g of agar, per litre of distilled water. The medium was autoclaved at 121 °C for 15 min and 10 mL of a salt solution was added after filtration through a 0.45-μm filter (salt solution composition: $NaNO_3$ 30 g; KCl 5 g; $MgSO_4.7H_2O$ 5 g; $FeSO_4{\cdot}7H_2O$ 0.1 g; $ZnSO_4{\cdot}7H_2O$ 0.1 g; and $CuSO_4{\cdot}7H_2O$ 0.05 g per 100 mL of distilled water). The cork powder was previously treated with gamma radiation −32 KGy.
4. Cork medium added with *C. sitophila* extracts (CM 1): prepared according to CM medium and to which 3 g/L of *C. sitophila* extract was added before sterilization.

Preparation of the *C. sitophila* extract used in CoM2 medium culture

A suspension of hyphae and *C. sitophila* spores (10^6 spores/mL) was inoculated on DG18 culture medium (Oxoid Basingstoke, UK) for 7 days, at 25 °C in the dark. After growth, the spores and mycelium were completely scraped with a loop into a falcon tube and 3 g of the fungal debris was lyophilized. The extract was kept at −20 °C.

Incubation conditions

Inoculations in all four media were performed using spore suspensions that were stored at −80 °C: Inoculations on YES and CM 1 media were incubated at 25 °C in the dark for 14 days. Inoculations on the two other media were incubated for 21 days using the same conditions.

Exo-metabolite analysis

The extrolites were extracted from the culture media according to the methods described by Frisvad [18] and Smedsgaard[19]. The extracts were analysed by HLPC using a HP

1100 series (Hewlett Packard, Germany) equipped with one pump and an auto-injector (Hewlett Packard) maintained at room temperature. Detection was performed using a diode array detector (DAD) with a 6-mm flow cell collecting two UV spectra per second from 200 to 600 nm with a bandwidth of 4 nm and a fluorescence detector (FLP) with excitation at 230 nm and emission at 333 nm. Separations were made on a 100 × 2 mm Luna C18 cartridge column packed with 3-µm particles and using a guard column with the same material, maintained at 40 °C. Elution was done using a linear gradient starting with 50% (v/v) water (A) and 50% (v/v) acetonitrile (B) reaching 100% of acetonitrile in 20 min and maintaining the flow for 5 min. Both eluents contained 0.005% (v/v) of trifluoroacetic acid (TFA). The flow rate was 0.40 mL/min.

All experiments in this study were performed in duplicate

All chemicals used were Merck (Darmstadt, Germany) analytical grade if nothing different is referred. All solvents were prepared using MilliQ water.

Volatile analysis

The same eleven fungal strains previously used for exo-metabolites analysis were grown in two different culture media, YES and CM prepared as stated earlier.

Moreover, two sets of fungal mixtures were tested in CM and CM1 culture medium: (1) *C. sitophila*, *P. glabrum*, *P. brevicompactum* and *P. chrysogenum*. (2) *C. sitophila*, *P. citrinum*, *P. paneum* and *E. rubrum*.

Volatile metabolites were collected during 4 days for the strains inoculated in YES medium and 14 days in the case of the CM medium, since the growth and sporulation is slower in the case of the second culture medium. To collect the volatiles, a stainless steel Petri dish lid with a standard 1/4′′ Swagelock™ replaced the usual lid [20]. This lid possessed a standard 1/4′′ Swagelok fitting with PTFE insert in the centre that is used to hold a charcoal tube (SKC, 226-01). The collected volatiles were extracted from the charcoal tube with 1 mL of ether (5 × 200 µL). The samples were concentrated to approximately 100 µL using a nitrogen flow. Hundred microlitres of each sample was put in GC vials and analysed using a gas chromatography–mass spectrometry (GC–MS) (Finnigan Focus GC coupled to a Finnigan Focus DSQ mass selective detector).

The separation of the volatiles was done on a Supelco SLB™-5 MS capillary column, using He as carrier gas, at a 1.2 mL/min flux. The injection and detection time was set to 220 °C. One microlitre of each sample was injected into the GC–MS system.

Chromatographic conditions were set to an initial temperature of 35 °C for 1 min, raised at 6 °C/min to 220 °C and then 20 °C/min to 260 °C for 1 min. The separated compounds were characterized by their mass spectra generated by electron ionization (EI) at 70 eV at a scan range from *m*/*z* 35–300.

Data analysis

The exo-metabolite compounds were identified by comparison with alkylphenone retention indices and diode array UV–VIS detection as described by Frisvad and Thrane [18].

The mass spectra from the volatile compounds with identical retention times were compared with the ones available in the library database to determine their similarity. Identification of sesquiterpenes was made by comparison of the mass spectra and the fragmentation profile of each compound with spectra in the software library database and with the literature [21].

Results

The production of exo-metabolites from the eleven fungi is presented on Table 1.

Exo-metabolite production in rich culture media (YES and CoM)

The fungal metabolite production was similar in both culture media, with the exceptions of *E. repens P. brevicompactum*, *P. citreonigrum* and *P. citrinum*. *P. brevicompactum* produced a higher variety of compounds, while the other fungi appear to be restricted to the production of one or two compounds. As expected, the production of mycotoxins (citrinin and citreoviridin) by *P. citrinum and P. citreonigrum* could be observed in both media.

Exo-metabolite production in cork culture medium (CM)

The exo-metabolite production was almost inexistent in CM culture medium. As shown in Table 1, only *E. amstelodami*, *P. brevicompactum* and *P. citrinum* produced metabolites in this culture medium. Although *E. amstelodami* produced the same number of metabolites in this medium compared with CoM, one compound is different [e.g. echinulin (1298) in CM and flavoglaucin (1646) in CoM1]. However, the fungal growth in CM medium is scarce (Fig. 1a), probably because cork is a recalcitrant substrate.

Exo-metabolite production in cork-based media with *C. sitophila* extracts (CM1)

Comparing the fungal behaviour in CM and CM1 culture media, higher fungal growth was observed on CM1

Table 1 Identification of the exo-metabolites produced by some fungal species in four different culture media

	YES medium	CoM1 medium	CM medium	CoM2 medium
Aspergillus tubingensis	Naphto-γ-pyrones, "kotanin" (990)	Tensidol B (943), aurasperone B (1008), cf. kotanin (991), aspereynone (1467), asperazine (1201)	ND	Tensidol B (952), aurasperone B (1007)
Chrysonilia sitophila	ND	ND	ND	ND
Eurotium amstelodami	Flavoglaucin (1646).	Tetrahydroauroglaucin (1629), flavoglaucin (1646)	Echinulin (1298), tetrahydroauroglaucin (1517)	Two neoechinulins (897, 946), echinulin (1541) flavoglaucin (1646)
Eurotium repens	Flavoglaucin (1497)	Asperentin (845, 932, 1023), auroglaucin (1633)	ND	Asperentin (784) and andrastin E (1099)
Penicillium brevicompactum	Raistrick phenol (747), mycophenolic acid (1017), asperphenamate (1239), a pebrolide (1171) and brevianamide A (880)	Mycophenolic acid (1010), xanthoepocin (1169), brevianamide A (880) and asperphenamate (1239)	Brevianamide A (805)	Mycophenolic acid (1017) and brevianamide A (888)
Penicillium citreonigrum	Citreoviridin (1105)	Citreoviridin (1105), "met ø" (957)	ND	"met ø" (indol alkaloid) (957)
Penicillium citrinum	Quinolactacin (774), citrinin (1260)	Quinolactin (774), citrinin (1216)	Quinolactacin (774)	Quinolactacin (774),
Penicillium chrysogenum	Meleagrin (775)	Meleagrin (775)	ND	Meleagrin (775)
*Penicillium glabrum*1	"One unknown extrolite" (578)	Citromycetin (657), PI-4 (767)	ND	ND
Penicillium glabrum 2	Sulochrin (871) and "one unknown extrolite" (578)	Citromycetin (657)	ND	"Neoglabrol" (982)
Penicillium paneum	Citreoisocoumarin (742), marcfortine A (747) and roquefortine E (759)	A citreoisocoumarin derivative (1058)	ND	Marcfortine derivative (813)

The retention indexes of the metabolites are given in brackets. *ND* not detected

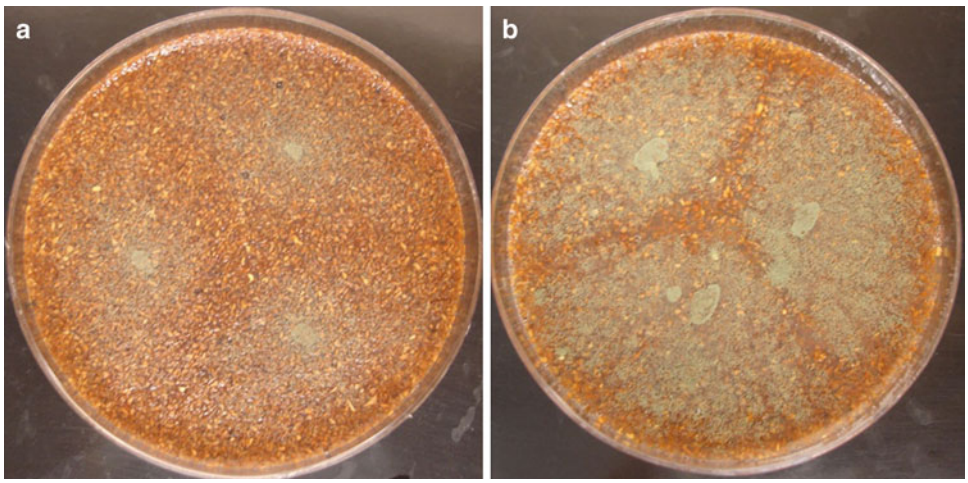

Fig. 1 *Penicillium paneum* isolate growing in two different culture media **a** cork medium and **b** cork medium with *Chrysonilia sitophila* extracts (CM1)

(Fig. 1a, b). As shown in Table 1, when *C. sitophila* extract was added to the cork medium (CM1), more compounds were detected in all analysed fungal extracts. Only *E. amstelodami*, *P. brevicompactum* and *P. citrinum* produced exo-metabolites in both culture media (CM and CM1). Moreover, *E. amestelodami* and *P. brevicompactum* show different exo-metabolite profiles. For instance, *P. brevicompactum* produced not only brevianamide A but

also mycophenolic acid (Fig. 2a, b). Furthermore, species that have not produced any exo-metabolite in CM when grown on CM1 produced at least one compound: *P. paneum* (marcfortine derivative) and *P. chrysogenum* (melagrin). Also *A. tubingensis* (tensidol B and aurasperone B) and *E. repens* (asperentin and andrastin E) produced two compounds. *P. citreonigrum* produced an indol alkaloid [R.I. of 957], possibly cividiclavin, formerly reported from *P. citreonigrum* [22], and *P. glabrum* 2 produced one compound, here named 'neoglabrol' [R.I. of 982].

Under the tested conditions, *C. sitophila* was the only fungal species that did not produce any metabolite in all of the assayed culture media.

Under the assayed conditions, no fungal isolate produced any mycotoxins either in cork-based medium or when *C. sitophila* extract was added.

Volatile production in YES and cork-based media (CM and CM1)

In YES culture medium only *P. paneum* produced sesquiterpenes that were identified by their retention time (RT) and the respective fragmentation pattern [21]. The most important sesquiterpenes were detected at RT between 15.51 and 17.96 min. The other fungal isolates did not produce any volatile compounds, under the assayed conditions. On the other hand, two sets of different fungal mixtures inoculated in CM + CM1 culture medium produced a non-identified volatile compound in all plates, but one (RT = 23.08 min) presenting as principal fragmentation masses m/z = 153.03; 43.91; 181.02 (Fig. 3a, b).

Discussion

Most of the exo-metabolites produced by the studied fungal species in YES medium were consistent with those described for the same species in earlier works when grown under similar conditions [9, 23–26]. According to previous works, some of the produced metabolites have taxonomic relevance for all fungi isolates in both synthetic culture media [9]. For example, citromycetin is produced by *P. glabrum*, which is in accordance with previous reports [28], or both brevianamide A and mycophenolic acid are produced by *P. brevicompactum* [9]. Their detection in this study contributes to better characterize the fungal isolates considered here.

The production of exo-metabolites is limited by the medium composition, culture conditions and genetic factors [9, 10, 23, 24]. The results obtained in this study are in accordance with Calvo et al. [11] who stated that the same environmental conditions required for sporulation are often also necessary for the secondary metabolite production, as exemplified in Fig. 1a, b and Table 1.

Furthermore, some studies confirmed the presence and activity of the enzymes necessary to the breakage of cork components into digestible compounds by some *Aspergillus*, *Penicillium* and *Trichoderma* species [28, 29]. Similarly, previous works showed that *C. sitophila* can also secrete some of the enzymes necessary to partially degrade cork components [17, 30].

In this study, a more differentiated exo-metabolite production is observed when the fungal isolates grow in CM1 in comparison with the metabolites profiles produced by the same fungi in CM culture medium. The growth in CM culture medium is scarce, and the fungal metabolism is probably targeted for the biomass production. The addition of *C. sitophila* remains that can serve as food source for the late cork colonizers will act as an additional nutrient source (e.g. nitrogen). This additional food source can stimulate the exo-metabolome change to increase their survival fitness [31]. The higher growth and sporulation of the several fungal isolates, exemplified in Fig. 1a, b, can induce the fungal exo-metabolome to produce metabolites to ensure a successful degradation and colonization of the substrate. This process can include the inhibition of some early fungal colonizers.

Under the assayed conditions, no fungal isolate produced any mycotoxins, although, mycophenolic acid and brevianamide produced by *P. brevicompactum* have been sometimes referred as mycotoxins. However, those compounds cannot be considered mycotoxins because they are not toxic to any vertebrate, according to the established concept of mycotoxin by Frisvad [32]. These exo-metabolites have pharmacological interesting capacities, like mycophenolic acid, which is an immunosuppressor used in organ-transplanted patients, and brevianamide A with insecticide properties [27, 33]. The fact that *C. sitophila* did not produce any chemical exo-metabolite under the studied conditions, which is in accordance with previous studies regarding the safety of *Neurospora* species (*Chrysonilia* teleomorph), concluded that the species belonging to this genus are neither pathogen nor toxin producers [34]. Moreover, "with the exception of carotenoid and melanin pigment synthesis, *Neurospora* has not been shown to possess secondary metabolism" [35]. This knowledge is relevant regarding that cork stoppers will be used to seal wine bottles.

Prat et al. [36] showed that the isolates of *P. glabrum* assayed in their work had different capacity of taint development since the volatile production was a strain-specific effect. In fact, in our study, both *P. glabrum* isolates had different exo-metabolite profile between them. This effect supports the high intraspecific heterogeneity that exists amongst isolates of this taxon, as previously mentioned in other works [37–39, Barreto et al., unpublished results]. It

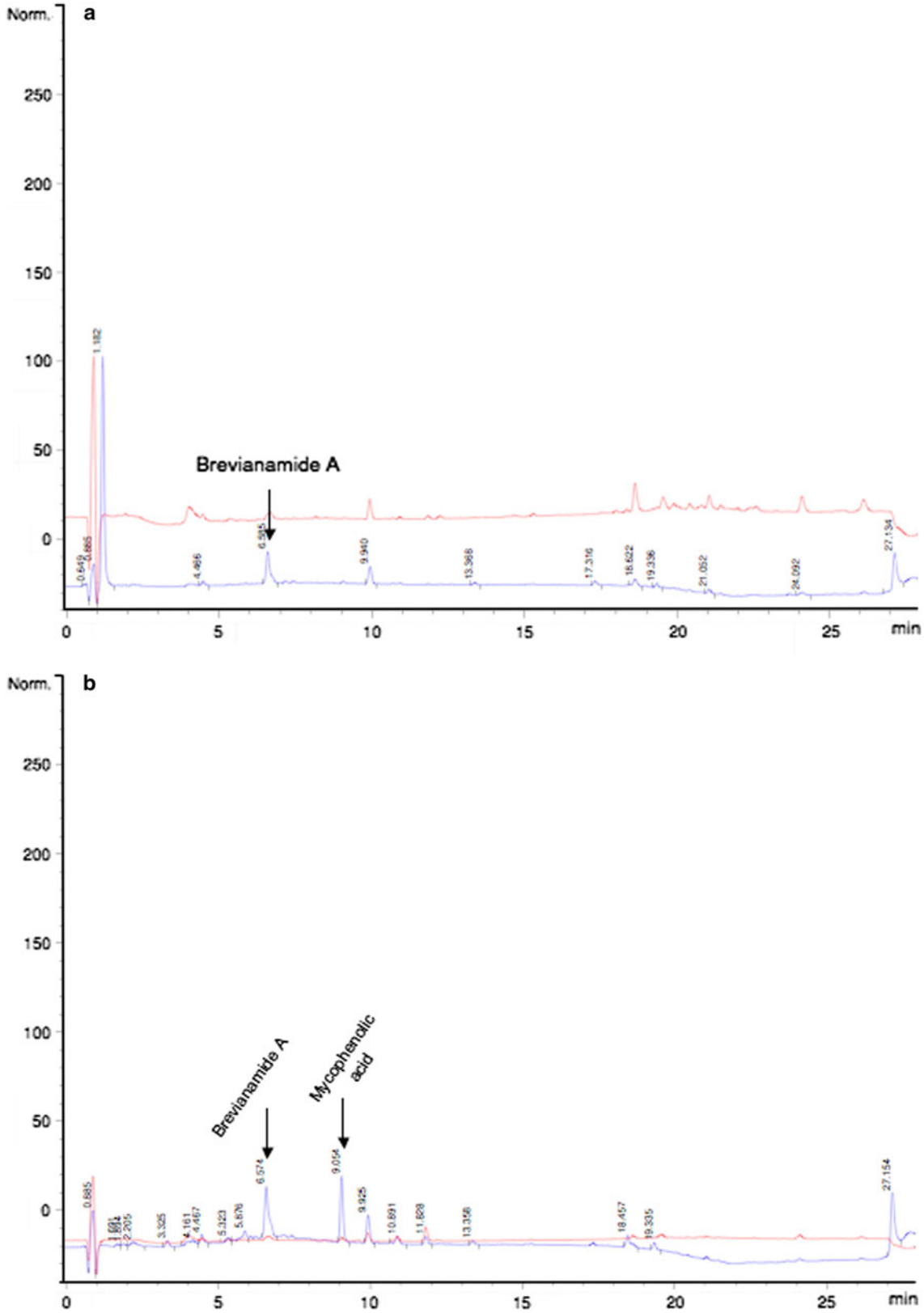

Fig. 2 UV-VIS chromatograms of the HPLC profile for *Penicillium brevicompactum* (the black line represent DAD 210 nm and the red line DAD 280 nm) in **a** cork medium culture and **b** cork medium culture added with *Chrysonilia sitophila* extracts (CM1)

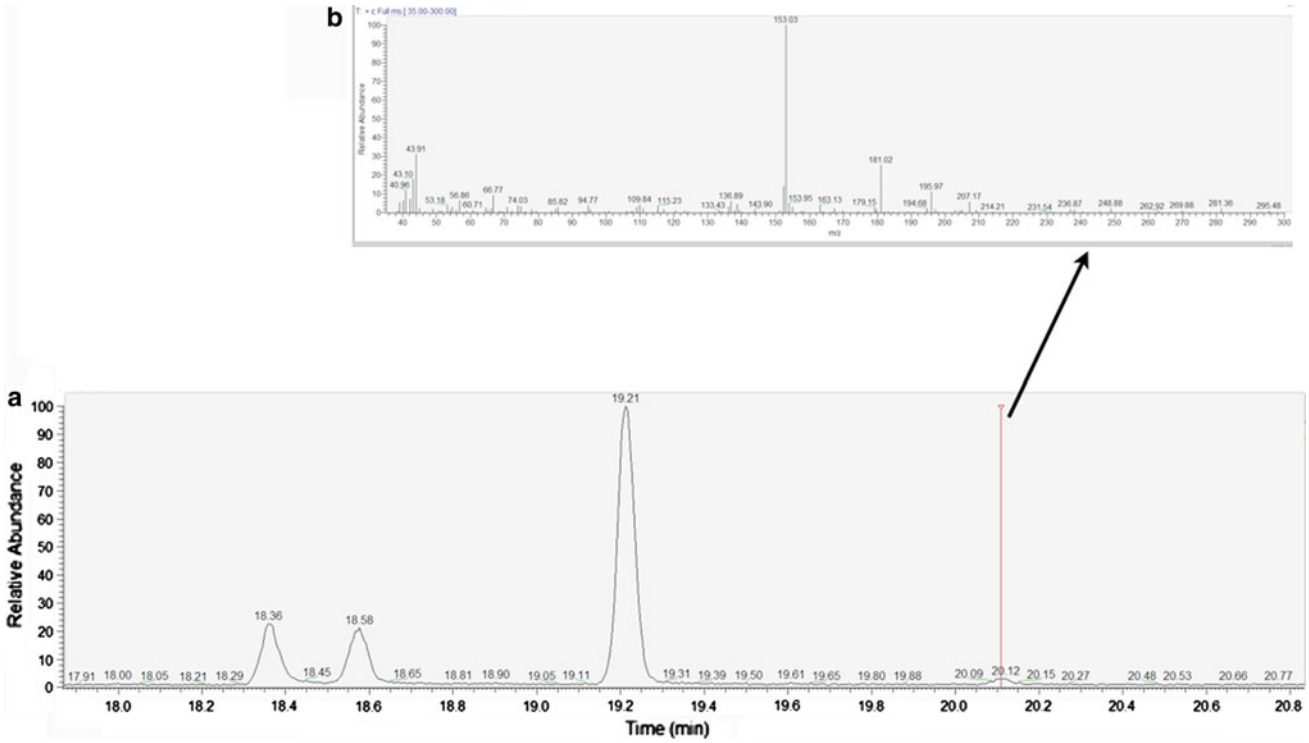

Fig. 3 **a** Chromatogram of the RT interval 17.91–20.80 for *Chrysonilia sitophila*, *Penicillium glabrum*, *Penicillium brevicompactum* and *Penicillium chrysogenum* in cork culture medium with *C. sitophila* extracts **b** mass spectrum for the peak at 20.12 RT 35.00–300.00 *m/z*

is probable that this characteristic can also be observed in some other fungal isolates and also justify some results observed in this work (Table 1).

Concerning the production of volatile compounds, sesquiterpene production by *P. paneum* in YES medium was also verified in earlier works that have reported them as exclusive for that species [20]. These compounds are known as having anti-fungal activity so they can interact with each other [40, 41] or act synergistically, therefore enhancing their effect [42]. The productions of a non-identified volatile compound by the two sets of fungal mixture confirm that presence of *C. sitophila* extract in the media modifies the fungal behaviour in a small niche. Besides that, the presence of different fungal species in co-culture can induce the production of a new chemical compound that was inexistent when the fungal isolates were inoculated in single culture in medium plates. In this case, the interaction amongst the interspecific fungal species could be responsible for the production of the volatile compound, as described by Evans [43].

In conclusion, the production of both exo-metabolites and volatile compounds by the studied fungi on CM and CM1 media cultures was low; however, the addition of *C. sitophila* extract to the culture medium not only enhances the fungal growth but also increases the variety of exo-metabolites produced by some fungal species, clearly suggesting that interactions may take place on cork slabs fungal colonizing communities. Probably the partial metabolization of the cork components after the cork boiling followed by the establishment of *C. sitophila* leaves the substrate more accessible to the late fungal colonizers.

These results point to the central role of *C. sitophila* earlier germination after the late fungal colonization of cork slabs. This fungal species will turn the substrate more accessible to the other fungi colonizers, since it is known to produce enzymes capable of degrading some cork components [3]. On the other hand, when other fungi germinate after partial cork degradation by *C. sitophila* and use its mycelium remains, they can produce exo-metabolites, which in turn can inhibit *C. sitophila* mycelium expansion. In fact, it was also observed in previous studies that *C. sitophila* growth in cork-based medium inhibits other fungi development (e.g. *Trichoderma*, *Penicillium* and *Mucor*) [44].

These results clearly suggest that the most predominant fungal species that are active in the post-boiling stage of the cork stoppers manufacture could be regarded as “non-deleterious” to the cork stoppers final product. These observations contribute to prove the statement that natural cork stoppers are the most adequate sealing device for wine bottles, especially concerning the necessary safety of the product that will be in contact with the stopper.

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References

1. Danesh P, Caldas FMV, Figueiredo Marques JJ, San Romão MV (1997) J Appl Microbiol 82:689–694
2. Pires JM, Pereira H, San Romão MV (2007) Ciência e Técnica Vitivinícola 22:15–20
3. Álvarez-Rodríguez ML, López-Ocana L, López-Coronado JM, Rodríguez E, Martinez MJ, Larriba G, Coque J-JR (2002) Appl Environ Microb 68:5860–5869
4. Oliveira AC, Peres CM, Correia Pires JM, Silva Pereira C, Vitorino S, Figueiredo Marques JJ, Barreto Crespo MT, San Romão MV (2003) Microbiol Res 158:117–124
5. Prak S, Gunata Z, Guiraud J-P, Schorr-Galindo S (2007) Food Microbiol 24:271–280
6. Centeno S, Calvo M (2001) Microbios 106:69–73
7. Riu H, Roig G, Sancho J (1997) Microbiologia 13:185–192
8. Vitorino S, Almeida-Vara E, Tenreiro R, San Romão MV (2002) In: AGROGES (eds) Congresso Mundial do Sobreiro e da Cortiça Proceedings Book. Lisboa
9. Frisvad J, Smeedsgard J, Larsen T, Samson R (2004) Stud Mycol 49:201–242
10. Larsen TO, Smedsgaard J, Nielsen KF, Hansen ME, Frisvad JC (2005) Nat Prod Rep 22:672–695
11. Calvo AM, Wilson RA, Bok JW, Keller NP (2002) Microbiol Mol Biol Rev 66:447–459
12. Borjesson T, Stollman U, Schnurer JL (1990) Appl Environ Microbiol 56:3705–3710
13. Korpi A, Pasanen A-L, Pasanen P (1998) Appl Environ Microbiol 64:2914–2919
14. Larsen TO (1998) In: Frisvad JC, Bridge PD, Arora DK (eds) Chemical fungal taxonomy. Marcel Dekker, New York
15. Brown SP, Inglis RF, Taddei F (2009) Evol App 2:32–39
16. Pereira H (1988) Chemical composition and variability of cork from *Quercus suber* L. Wood Sci Technol 22:211–218
17. Vitorino SI, Neves ESG, Gaspar F, Figueiredo Marques JJ, San Romão MV (2007) Ciência e Téc Vitiv 22:1–4
18. Frisvad JC, Thrane U (1987) J Chromatogr 404:195–214
19. Smedsgaard J (1997) J Chromatogr A 760:264–270
20. Larsen TO, Frisvad JC (1994) J Microbiol Meth 19:297–305
21. Karlshøj K, Larsen TO (2005) D J Agric Food Chem 53:708–715
22. Krumphanzl V (ed) (1982) Overproduction of microbial products. Academic Press, London
23. Samson RA, Houbraken JAMP, Kuijpers AFA, Frank JM, Frisvad JC (2004) Stud Mycol 50:45–61
24. Smetanina O, Kalinovskii A, Khudyakova Y, Slinkina N, Pivkin M, Kuznetsova T (2007) Chem Nat Compd 43:395–398
25. Thrane U, Andersen B, Frisvad JC, Smedsgaard J (2007) In: Nielsen J, Hewitt J (eds) Topics in Current Genetics, vol 18. Springer, Berlin
26. Fox EM, Howlett BJ (2008) Curr Opin Microbiol 11:481–487
27. Evans GE, Staunton J (1988) J Chem Soc Perk T 1988:755–761
28. Fang J, Qu Y, Gao P (1997) Biotechnol Tech 11:195–197
29. Krogh KBR, Morkeberg A, Jorgensen H, Frisvad JC, Olsson L (2004) Appl Biochem Biotech 114:389–401
30. Durán N, Ferrer I, Rodríguez J (1987) Appl Biochem Biotech 16:157–167
31. Williams DH, Stone MJ, Hauck PR, Rahman SK (1989) J Nat Prod 52:1189–1208
32. Frisvad JC, Thrane U (2004) In: Samson RA, Hoekstra ES, Frisvad JC (eds). Introduction to food and airborne fungi, 7th edn. Centraalbureau voor Schimmelcultures. Utrecht
33. Paterson RRM, Simmonds MJS, Kemmelmeier C, Blaney WM (1990) Mycol Res 94:538–542
34. Perkins DD, Davis RH (2000) Appl Environ Microbiol 6:5107–5109
35. Galagan JE, Calvo SE, Borkovich KA, Selker EU, Read ND, Jaffe D, FitzHugh W, Ma L, Smirnov S, Purcell S, Rehman B, Elkins T, Engels R, Wang S, Nielsen CB, Butler J, Endrizzi M, Qui D, Ianakiev P, Bell-Pedersen D, Nelson MA, Werner-Washburne M, Selitrennikoff CP, Kinsey JA, Braun EL, Zelter A, Schulte U, Kothe GO, Jedd G, Mewes W, Staben C, Marcotte E, Greenberg D, Roy A, Foley K, Naylor J, Strange-Thomann N, Barrett R, Gnerre S, Kamal M, Kamvysselis M, Mauceli E, Bielke C, Rudd S, Frishmen D, Krystofova S, Cogoni C, Macino G, Catcheside D, Li W, Pratt RJ, Osmani SA, DeSouza CPC, Glass L, Orbach MJ, Berglund JA, Voelker R, Yarden O, Plamann M, Seiter S, Dunlap J, Radford A, Aramayo R, Natrig DO, Alex LA, Marinhaupt G, Ebbole DJ, Freitag M, Paulsen I, Sachs MS, Lander ES, Nusbaum C, Birren B (2003) Nature 422:859–868
36. Pratt C, Trias R, Culleré L, Escudero A, Anticó E, Bañeras L (2009) J Agric Food Chem 57:7473–7479
37. Pitt JI, Klich MA, Schaffer GP, Cruickshank RH, Frisvad JC, Mullaney EJ, Onions AHS, Samson RA, Williams AP (1990) Syst Appl Microbiol 13:304–309
38. Basílio MC, Gaspar R, Silva Pereira C, San Romão MV (2006) Rev Iberoam Micol 23:151–154
39. Serra R, Peterson S, CTCOR, Venâncio A (2008) Res Microbiol 159:178–186
40. Kubo I, Fujita K, Lee SH (2001) J Agric Food Chem 49:1607–1611
41. Arif T, Bhosale JD, Mandal TK, Bendre RS, Lavekar GS, Dabur R (2009) J Asian Nat Prod Res 11:621–638
42. Lorito M, Peterbauer C, Hayes CK, Harman GF (1998) Microbiology 140:623–629
43. Evans JA, Eyre CA, Rogers HJ, Boddy L, Muller CT (2008) Fungal Ecol 1:57–68
44. San Romão MV (2009) Ciência Téc Vitivi 24:81–90

Chapter 4

Volatile compounds produced by cork mycobiota

In an attempt to assess the production of chemical compounds that can be harmful to the wine, the volatile compounds produced by microbiota existing in different cork samples collected during the manufacturing of cork discs was studied. Furthermore, the releasable volatile compounds and 2,4,6-trichloroanisole (TCA) produced by some previously isolated fungal species were analyzed by gas chromatography coupled with mass spectrometry (GC-MS).

The following scientific article is included in this chapter:

- Volatile compounds in samples of cork and also produced by selected fungi (*Journal of Agricultural and Food Chemistry*, 2011, 59: 6568-6574).

The author performed the experimental work presented in this chapter. The GC-MS and data analysis were done in collaboration with Analytical Chemistry Laboratory, ITQB, IBET, Oeiras. The manuscript was written by the author and revised by the other co-authors.

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Volatile Compounds in Samples of Cork and also Produced by Selected Fungi

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Supporting Information

ABSTRACT: The production of volatile compounds by microbial communities of cork samples taken during the cork manufacturing process was investigated. The majority of volatiles were found in samples collected at two stages: resting after the first boiling and nontreated cork disks. Volatile profiles produced by microbiota in both stages are similar. The releasable volatile compounds and 2,4,6-trichloroanisole (TCA) produced in cork-based culture medium by five isolated fungal species in pure and mixed cultures were also analyzed by gas chromatography coupled with mass spectrometry (GC-MS).The results showed that 1-octen-3-ol and esters of fatty acids (medium chain length C8−C20) were the main volatile compounds produced by either pure fungal species or their mixture. Apparently,*Penicillium glabrum* is the main contributor to the overall volatile composition observed in the mixed culture. The production of releasable TCA on cork cannot be attributed to any of the assayed fungal isolates.

KEYWORDS: fungi, cork, 2,4,6-trichloroanisole, volatile compounds

INTRODUCTION

Cork is the material best-suited for sealing wine bottles, due to its unique physical and chemical properties.[1] During the manufacturing process of cork stoppers, a mycoflora develops, resulting from either cork colonization or factory environment.[2−5]

The germination of mycospora fungi can enable the metabolite production resulting from the available substrates' metabolism or produced as a response to environmental conditions.[6] The production of some volatile compounds by fungi in cork slabs, namely, chloroanisoles, is considered to be the most frequent cause of organoleptic defects of wines.[4,7,8] Although other compounds can contribute to a musty taint in wines, for example, 2,4,6-tribromoanisole (TBA)[9] and 2-methoxy-3,5-dimethylpyrazine,[10] 2,4,6-trichloroanisole (TCA) was recognized to be present in 80−85% of cork-tainted wines.[8] Due to its very low detection olfactory threshold (30−300 pg L^{-1} in water and 1.5−3 ng L^{-1} in alcoholic solution/wine)[11] and low perception threshold for humans, TCA was considered to be the main cause for unpleasant corky flavors.[12] The presence of these compounds can be at the origin of important losses in both wine and cork-stopper industries, endangering the sustainability of the cork stoppers industry because since the 1990s some alternative sealing devices have begun to be developed, especially in countries that are not producers of cork.

In this work, the volatile composition profile of cork samples taken during the manufacturing process of cork disks was investigated. There was also an attempt to establish a relationship with the released volatile compounds produced by a set of fungal species isolated during the process and inoculated (individually and as a mixture) in a cork-based medium. Moreover, releasable TCA was quantified in the same samples tested in the laboratory to assess the possible contribution of the selected fungi to produce TCA in conditions typical of cork-stopper factories.

MATERIALS AND METHODS

Determination of the Total Volatiles Released from Cork Samples Collected at Different Stages of the Cork Disk Manufacturing Process. *Sampling Plan.* The cork slabs are normally disposed in stacks inside the factory, each stack having three levels of slabs. A piece of cork was taken in the upper part of each level, and each sample is made of three cork pieces with approximately 20 cm side, taken diagonally across the stack. All of the samples were taken during the whole processing of the same batch of cork slabs. All of the cork samples used in this experiment were collected in the following manufacturing stages: nonboiled cork; immediately after the first boiling in water, which takes about 1 h; during the resting phase after boiling; immediately after the second boiling (normally 20−30 min); and nontreated cork disks.

Samples Preparation. Each cork sample was ground to powder of 0.1 mesh. Each sample (0.12 g) was weighed into a 20 mL GC screw-capped vial (20 mL La-Pha-Pack, Werner Reifferscherdt GmbH, Langerwehe, Germany). Four milliliters of a culture medium (containing 0.004 g of K_2HPO_4 previously autoclaved and added to 0.04 mL of a sterile salt solution containing, per 100 mL of distilled water, $NaNO_3$, 30 g; KCl, 5 g; $MgSO_4 \cdot 7H_2O$, 5 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; $ZnSO_4 \cdot 7H_2O$, 0.1 g; and $CuSO_4 \cdot 7H_2O$, 0.05 g) was poured in each vial. The different vials were

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incubated at 27 °C in the dark and agitated on an Innova 2300 rotary shaker (New Brunswick Scientific, Edison, NJ) during 3 months. Blanks containing only the culture medium and 0.12 g of cork sterilized by γ radiation (32 kGy)[13] were also run. All of the experiments were done in duplicate.

SPME-GC-MS Analysis of the Volatile Compounds. After the incubation period, the different samples were analyzed using a GC-MS system: autosampler AOC-5000 autoinjector, gas chromatograph−mass spectrometer Shimadzu GCMS-QP2010 (Shimadzu Corp., Kyoto, Japan), equipped with a capillary column DB-5MS (J&W Scientific, Folsom, CA), 28 m × 0.32 mm and 0.25 μm phase thickness.

A solid phase microextraction fiber DVD/CAR/PDMS (50/30 μm) (Supelco, Bellefonte, PA) was exposed to the sample headspace for 60 min at 45 °C with an agitation speed 250 rpm and then transferred to the GC injector at 250 °C to desorb during 2.5 min (injection in splitless mode).

The column temperature program started at 40 °C for 5 min, was raised at 5 °C min^{-1} to 170 °C and then at 30 °C min^{-1} to 250 °C, and held for 4 min. The carrier gas (helium) was kept at a constant flow (50 cm s^{-1}). Analyses were performed in full-scan mode in the range m/z 30−300 at a scan speed of 540 au s^{-1}.

Analyses of Releasable Volatile Compounds and Quantification of TCA Produced by Fungal Isolates Growing on Cork Culture Medium. *Fungal Isolates and Culture Conditions.* Five fungal strains previously isolated from cork slabs and identified using phenotypic and molecular techniques at CBS Fungal Biodiversity Centre (Utrecht, The Netherlands) were used, both in pure and mixed cultures. These strains are now deposited in international culture collections and designated *Chrysonilia sitophila* DSM 16514 (DSMZ, Germany), *Eurotium rubrum* CBS 126220 (CBS, The Netherlands), *Penicillium brevicompactum* CBS 126334, *Penicillium glabrum* CBS 126333, and *Penicillium paneum* CBS 126218.

A spore suspension containing 5 μL of 6 × 10^5 spores mL^{-1} of each species was used to inoculate a culture medium contained in 500 mL glass flasks.The fungal mixture was prepared using 1 μL of the same spore suspension of each of the five fungal species previously mentioned (5 μL total) and was inoculated in the culture medium. The medium was constituted by 7.5 g of sterile cork, 0.25 g of K_2HPO_4, and 2.5 mL of sterile salt solution to a final volume of 250 mL. The culture medium was prepared as previously mentioned. A blank assay containing only the culture medium was also prepared.

All of the inoculated media and blanks were incubated at 27 °C, agitated on the rotary shaker (60 rpm), in the dark. Two different culture periods were considered: 7 days and 4 weeks. All of the experiments were done in triplicate.

Analyses of Releasable Volatiles. *Sample Preparation.* After the fungal growth period, the sample preparation was done according to the procedure described in International Standard ISO 20752 for the determination of the releasable TCA. Briefly, after the incubation period, for each sample, the culture medium was discarded; the cork pieces were placed in a glass jar filled to the top with a 12% hydroalcoholic solution and maintained at room temperature (ca. 22 °C) during 24 h.

From each jar was taken 7 mL of the hydroalcoholic solution and transferred into a GC vial containing 3 g of NaCl. Each vial was agitated with a vortex shaker (type REAX 2000, Heidolph Instruments GmbH, Schwabach, Germany) for 2 min and transferred to the SPME-GC-MS system for analysis.

A blank containing the 12% hydroalcoholic solution was also analyzed.

SPME-GC-MS Analysis. The different samples were analyzed using the same GC-MS equipment and conditions referred to above, except that the column used was a Factor Four VF-5 m, 30 m × 0.25 mm and 0.25 μm phase thickness (Varian Inc., Lake Forest, CA).

Releasable TCA Quantification. The used method followed the International Standard ISO 20752 and was previously validated.[14]

Briefly, the sample was prepared as described before (under Sample Preparation), and the TCA analysis was done using the GC-MS equipment and column referred to above. A PDMS fiber (100 μm) (Supelco) was exposed to the sample headspace for 15 min at 40 °C with an agitation speed of 250 rpm and then transferred to the GC injector at 250 °C to desorb during 2 min (injection in splitless mode). The samples were analyzed according to the program and conditions described as follows: the column temperature started at 60 °C for 2 min, was raised at 25 °C min^{-1} to 205 °C and then at 30 °C min^{-1} until 265 °C, and held for 1 min. The carrier gas (helium) had a constant flow at 51 cm s^{-1}. Analyses were performed in the SIM mode for m/z 217, 215, 212, 210, 199, and 195. The acquisition data were taken every 0.20 s, and the limit threshold was 500.

The amount of released TCA in the various samples was calculated using a TCA calibration curve (0.5, 1, 2, 3, 4, 5, 6, 8, and 10 ng L^{-1} prepared from a stock solution of TCA 5 × 10^{-7} g L^{-1}. Pentadeuterated 2,4,6-TCA (d_5-TCA) (Cambridge Isotope Laboratories, Inc., Andover, MA) was used as internal standard: a solution 5 × 10^{-5} g L^{-1} was added to each vial containing calibration solutions or sample extracts to have a concentration close to 50 ng L^{-1}.

A blank containing the 12% (v/v) hydroalcoholic solution was analyzed under the same conditions.

The analyses of calibration standards were run as duplicates and the samples as triplicates.

Data Analysis. *Identification of the Compounds.* The identification of compounds from mass spectra obtained in scan mode was done by comparison of the mass spectra with spectra available in the data system libraries (NIST12, NIST27, NIST62, NIST147, and WILEY229). Shimadzu software GCMSsolution was used for chromatogram data acquisition, comparison of chromatograms, integration of peaks, and calculation of similarity indices on comparison of acquired mass spectra with those of the data system libraries.

The linear retention index (LRI) was calculated for each volatile compound detected in the samples and compared with published data.[15,16,17]

Principal Coordinates Analysis (PCOORDA). A qualitative table (presence/absence) of the identified volatile compounds produced by the studied fungi in pure and mixture cultures in both incubation periods was constructed. The Unweighted Pair-Group Method Using Arithmetic Averages (UPGMA) was applied to analyze the results. A similarity/dissimilarity matrix was obtained using Jaccard's similarity coefficient. A PCOORDA of the similarity matrix was computed, and the minimum spanning tree was calculated.

The system of programs NTSYS-pc[18] was used in all statistical treatments by multivariate analyses.

TCA Quantification. The validation for the releasable TCA quantification was done in two steps: qualification and quantification. The areas of the three TCA peaks (212, 210, and 195) and those of d_5-TCA (217, 215, and 199) were measured. The qualification of peaks for quantitative analysis is done by calculating the ratios between peak areas (A) and comparing with the expected values: A_{195}/A_{210} = 1.44, A_{195}/A_{212} = 1.51, and A_{210}/A_{212} = 1.06 for TCA; A_{215}/A_{217} = 1.0, A_{215}/A_{199} = 0.70, and A_{210}/A_{212} = 1.06 for d_5-TCA. Peak areas obtained for m/z 215 of d_5-TCA (internal standard) were found to be suitable for quantitative analysis because no interferences were observed.

A linear regression treatment was applied to the calibration curve; the linear equation and the correlation coefficients were determined for the ratio of measured area of each TCA peak in relation to the peak of the internal standard (m/z 215). A Grubbs test was used to establish if there were deviant values in the analysis of samples (triplicates).

RESULTS AND DISCUSSION

Analysis of Volatile Compounds Released from Cork Samples Collected during the Manufacturing Process. The volatile compounds detected in nonboiled cork were completely

Table 1. Qualitative Analysis (Presence/Absence) of Volatile Compounds of Cork Samples Collected during the Manufacturing Process of Cork Disks Incubated at 27 °C during 3 Months

	retention index	cork before boiling	cork after boiling	cork slabs resting stage after boiling	cork after second boiling	cork disks
Alcohols						
dodecanol	1042.52	−	−	+	−	+
tridecanol	1647.98	−	−	+	−	+
6,11-dimethyl-2,6,10-dodecatrien-1-ol	2064.20	−	−	+	−	+
Esters						
trans-methyldihydrojasmonate (like[a])	1631.60	−	−	+	−	+
isopropyl myristate	2026.45	−	−	+	−	+
Alkanes						
6-methyloctadecane	1639.91	−	−	+	−	+
2-methylnonadecane (like[a])	1691.04	−	−	+	−	+
3-methylheptadecane (like[a])	1693.93	−	−	+	−	+
1,1-bis-hexadecane (like[a])	2004.97	−	−	+	−	+
eicosane	2009.99	−	−	+	−	+
nonadecane	2014.11	−	−	+	−	+
1-(ethenyloxy)octadecane	2022.05	−	−	+	−	+
squalene	2485.47	−	−	−	+	+
hentriacontane	2514.08	−	−	−	+	+
Aldehydes						
hexadecanal	1613.36	−	−	+	−	+
3,7-dimethyloctanal (like[a])	1681.34	−	−	+	+	+
Chlorated Alkanes						
1-chloro-octadecane (like[a])	1661.88	−	−	+	−	+
1-chloro-8-heptadecene	2064.20	−	−	+	−	+
Terpenoid						
camphor	1076.92	−	+	+	−	+
Aromatic Hydrocarbon						
naphthalene-1,2,4a,6,8a-hexahydro-4,7-dimethyl-1 (1-methylethyl) (like[a])	1059.65	+	−	−	−	−
Ketone						
3-butyl-4,5-hexadien-2-one (like[a])	1041.88	+	−	−	−	−

[a] A homology below 85% corresponds to an identification of the compounds to be probable ("like").

different from those detected in cork samples from other processing stages. As expected, in the stages before boiling and immediately after the first and second boilings, few volatile compounds were detected. Under the conditions used in this study, volatile compounds were mostly detected in the cork resting stage after boiling and in cork disks without any treatment (Table1). This can be explained by fungal development over the humid cork slabs occurring during the resting stage after the boiling step.

Usually, cork slabs are rested for 4 days inside the factory until they attain adequate humidity to be processed. During this period the slabs become completely covered by fungal mycelium from several species, mainly *Penicillium*, *Aspergillus*, *Chrysonilia*, and *Trichoderma*.[4,19] At this stage, these species are active and consequently are able to produce volatile compounds and other exo-metabolites, as a result of the biodegradation of the cork constituents. Interactions between the microbial populations existing in the cork slabs can condition the metabolic processes and consequently the formed products.[20] Most of the detected volatile compounds mentioned in Table 1 can result from the substrate fatty acid oxidation or by microbial degradation of aliphatic alcohols (e.g., dodecanol, tridecanol), aliphatic aldehydes (e.g., hexadecanal), aliphatic ketones (e.g., 3-butyl-4,5-hexadien-2-one), and alkanes (e.g., nonadecane, 6-methyloctadecane) as previously reported.[16] Cork contains in its constitution suberin, which is a complex polymer of long-chain fatty acids and phenolic residues.[21] The degradation by fungi of suberin can be suggested by the occurrence of some compounds such as tridecanol. The isopropyl myristate, hexadecanal, dimethyloctane, dodecanol, tridecanol, and 6,11-dimethyl-2,6,10-dodecatrien-1-ol could result from the degradation of the fatty acid chains composing either the wax-like fraction of the extractives or the suberin layer. Furthermore, the produced alkanes could originate from the degradation of hydrocarbons of the aliphatic chains from both extractives and suberin layers.

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Table 2. Qualitative Analysis (Presence/Absence) of the Releasable Volatile Compounds Produced by Five Isolated Fungi and a Fungal Mixture Grown during 7 Days and 4 Weeks

		C. sitophila		*P. glabrum*		*P. brevicompactum*		*P. paneum*		*E. rubrum*		fungal mixture	
	retention index	7 days	4 weeks	7 days	4 weeks	7 days	4 weeks	7 days	4 weeks	7 days	4 weeks	7 days	4 weeks
Alcohols													
1-octen-3-ol	884.37	+	+	+	+	−	+	+	+	−	+	+	+
2-octen-1-ol	965.20	−	−	+	−	−	−	−	−	−	−	−	−
2-methylhexadecan-1-ol	1849.88	−	−	−	−	−	−	−	−	+	−	−	−
9-hexadecen-1-ol	2044.04	−	−	−	−	+	−	−	−	−	−	−	−
Esters													
ethyl *n*-caproate	1000.99	+	−	−	−	−	−	−	−	−	−	−	−
ethyl nonanoate	1249.74	−	−	+	−	−	−	−	−	−	−	+	−
ethyl decanoate	1297.66	−	−	+	−	−	−	−	−	+	−	+	−
ethyl undecanoate	1448.75	−	−	+	−	−	−	−	−	−	−	+	+
ethyl laurate	1497.00	−	−	+	−	+	−	−	−	+	−	+	−
nethyl dihydrojasmonate	1634.24	+	−	+	−	+	−	−	−	−	−	+	−
ethyl pentadecanoate	1698.68	−	−	+	−	−	−	+	−	−	−	+	−
ethyl palmitate	1899.14	−	−	+	+	+	+	+	−	+	+	+	+
ethyl stearate	2099.06	−	−	−	+	−	−	−	−	−	−	+	+
Sesquiterpene Compounds													
unknown compound (like[a])	1442.05	−	−	−	−	−	−	−	+	−	−	−	−
sesquiterpenoid compound (like[a])	1477.60	−	−	−	−	−	−	−	+	−	−	−	−
Ether													
1,3-dimethoxybenzene	1085.46	−	−	−	+	−	−	−	−	−	+	−	+
Hydrocarbon													
3-eicosene	1849.65	−	−	−	−	−	−	+	−	−	−	−	−

[a] A homology below 85% corresponds to an identification of the compounds to be probable ("like").

Saturated hydrocarbons were also detected, which is in accordance with previous findings.[16,22] However, in the present work the detected hydrocarbons have much longer aliphatic chains (≥C18) than those previously found (≥C8). A possible explanation for the observed deeper degradation of cork tissues can be the constitution of the microbial population, a longer incubation time, or the combination of both factors.

Both cork after the second boiling and cork disks contained other volatile compounds. The presence of two hydrocarbons (squalene and hentriacontane) should be pointed out. These compounds are found in a variety of plants, the last one being involved in stimulation of fungal spore germination.[23]

Although many volatile compounds were detected during the resting stage after the first boiling of cork slabs and in nontreated cork discs, they will not influence negatively the cork stoppers final quality since none of these volatile compounds are known to contribute for the so-called wine cork taint. Furthermore, some of them like fatty acids and terpenes are volatile components of wine.[24]

Even if these volatile compounds were detected in the untreated cork disks, some of their contents would be reduced or even disappear due to the final treatment of the cork disks consisting of washing and drying. Therefore, the final product quality will not be impaired.

Analysis of Releasable Volatile Compounds Produced by Some Fungal Species. Qualitative results are presented in Table 2 showing the presence/absence of releasable volatiles produced by five fungal species in pure cultures and a mixture containing all of them. The samples were taken 7 days and 4 weeks after inoculation.

The results presented in Table 2 were used to construct a similarity/dissimilarity matrix using the Jaccard coefficient (r = 0.868), and a cluster analysis was performed. A PCOORDA was computed and Figure 1 shows the samples projected on the space defined by the three first principal coordinates that explain ~50% of the accumulated variance, providing a representation with the greatest variability of the obtained results. The minimum spanning tree has been superimposed on the projections to show where distortion is more evident.

Figure 1a shows that the group of samples incubated during 7 days is separated from the isolates incubated during 4 weeks along the second axis. This apparent separation can be explained by the differences of volatile profiles produced by the samples in each incubation period. Moreover, with regard to the samples incubated during 7 days, they show a diverse volatile production. The majority of the studied fungi produced 1-octen-3-ol (except *P. brevicompactum* and *E. rubrum*, which produced 9-hexadecen-1-ol and 2-methylhexadecan-1-ol, respectively), and diverse esters, mainly with short and medium chains (e.g., ethyl caproate and laurate) were also formed. Besides those compounds, *C. sitophila*, *P. brevicompactum*, and *P. glabrum* also produced methyl dihydrojasmonate. Moreover, *P. paneum* was the only isolate to produce 3-eicosene, which, to our knowledge, had not been reported to be a fungal metabolite. However, the culture medium is mainly constituted by cork, and it is known that the

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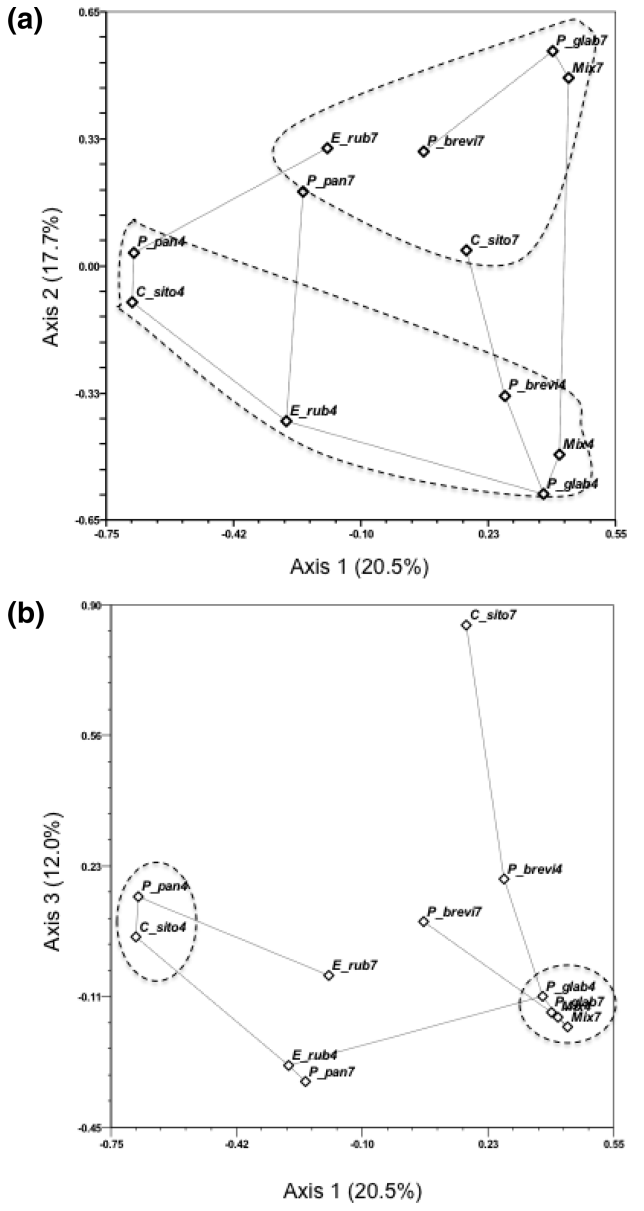

Figure 1. Plot of projections of 12 samples onto the principal coordinates axes: (a) plane of the first and second axes; (b) plane of the first and third axes. The minimum-length spanning tree is superimposed, and the variance (%) explained by the three first principal coordinates is displayed.

volatile compound production is highly influenced by the respective substrate composition and length of fungal growth.[25] These factors may reflect on the distribution of the samples along the third axis with *P. paneum* at the bottom and *C. sitophila* at the upper part of the graph (Figure 1b).

The samples incubated during 4 weeks show in general fewer volatile compounds. The five studied fungi consistently produced 1-octen-3-ol, in agreement with previous studies.[16,25] The esters formed by the fungal isolates were mostly ethyl palmitate (with exception of *C. sitophila* and *P. paneum*) and ethyl stearate (*P. glabrum* and fungal mixture), although the fungal mixture also produced ethyl undecanoate. Moreover, 1,3-dimethoxybenzene is a compound known to be an intermediate product of lignin degradation[22] and was produced by *E. rubrum*, *P. glabrum*, and the fungal mixture. The presence of this volatile compound suggests a more extensive degradation of cork by the fungal species incubated for 4 weeks.

These results may indicate that there is a greater similarity of volatile profiles for samples incubated during 4 weeks. This fact is shown by the distribution of the isolates along the third axis, where the isolates are located at the bottom and middle parts of the graph (Figure 1b). The fungal isolates *P. paneum* and *C. sitophila* seem to be exceptions, both producing the aliphatic alcohol 1-octen-3-ol, and *P. paneum* produced one unidentified sesquiterpene and one sesquiterpenoid-like compound. This fact is in accordance with earlier works that describe *P. paneum* as a terpenoid producer.[17] These fungal isolates are located at the left part of the graph.

The high similarity among the volatiles produced by *P. glabrum* and the mixed fungal cultures observed in both incubation periods (Figure 1) clearly suggests the greater contribution of that species to the overall volatile composition of fungal colonized cork. These observations support data collected over the years concerning the study of cork mycobiota[3] that the predominant fungal species during the cork stopper and disk manufacturing stages is *P. glabrum*.[19] Furthermore, *C. sitophila* samples produced one or two volatile compounds that will not contribute significantly to the overall volatile composition. It is known that *C. sitophila* mycelium completely covers the cork slabs immediately after the boiling stage,[3] and these results highlight again its innocuous presence on the cork substrate.[3,5,7]

Methyl dihydrojasmonate, a linoleic acid derived molecule, was the only compound detected in some chromatographic profiles obtained: both in the analysis of samples of cork with the natural microflora as in cork samples inoculated with previously isolated fungi. This volatile compound was described to be a signaling molecule, which mediated plant responses to environmental stress such as injury and insect or pathogen attack.[26] Its production by fungi has been reported earlier,[27] although its biological function in the fungal community is not clear yet. A rapid decline of this compound was observed in vivo, suggesting its fast metabolism.[28] Also in the present work, it was detected only in samples incubated for 7 days, being absent in the samples incubated during 4 weeks. However, this compound was also detected in samples of cork from two manufacturing stages incubated during 3 months.

Samples from some cork boiling waters were analyzed (Barreto et al., data not shown). In those samples many compounds usually described in the literature as plant-associated compounds were detected (e.g., sesquiterpenes, monoterpenes, and essential oil constituents). Some of the compounds detected in the boiling water were also found in the cork medium inoculated with some fungi: 1-octen-3-ol produced by all of the studied fungal species; 1,3-dimethoxybenzene produced by *P. glabrum*, *E. rubrum*, and the fungal mixture; and ethyl laurate produced by *P. glabrum*, *P. brevicompactum*, *E. rubrum*, and the fungal mixture. Moreover, some compounds detected in the cork samples incubated during 3 months were present in the boiling water: 1-tridecanol and eicosane produced during the cork resting stage after boiling and by cork disks.

This can be considered additional evidence that the fungal community is installed inside the cork structure[29] and is able to produce some volatile compounds, using cork constituents as substrates, which can be released into the water during the cork slab boiling process.

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Quantification of Releasable TCA Produced by Fungal Isolates and a Fungal Mixture. The content of releasable TCA from samples inoculated with some fungal isolates in pure and mixed cultures was determined. The analysis conditions were very similar to those used in the analysis of cork stoppers in industrial quality control. The estimated detection limit (LD) and quantification limit (LQ) were, respectively, 1.6 and 5.4 ng L^{-1}.

The noninoculated cork media contained 1.90 and 1.78 ng L^{-1} of extractable TCA after 7 days and 4 weeks of incubation, respectively. TCA is formed by the *O*-methylation of the corresponding chlorophenol precursor.[6] Previous studies showed that at least some of the fungal species isolated from the cork possess the *S*-adenosyl-L-methionine (SAM)-dependent chlorophenol-*O*-methyltransferase (CPOMT) enzyme, which in the presence of TCP can metabolize TCA.[30] To understand the origin of theTCA values detected in our blank samples, the chlorophenol contents of the cork were determined. The analysis showed that cork contained an average of 2.7−3.3 ng g^{-1} of TCA, 5.2−6.7 ng g^{-1} of 2,4,6-trichlorophenol (TCP), 0.6−1.3 ng g^{-1} of 2,3,4,6-tetrachlorophenol and 0.7−1.1 ng g^{-1} of 2,4,6-tribromophenol. These results show that TCA was detected in noninoculated cork media and should have originated either from the corresponding trichlorophenol or from other chlorophenols also present in the cork.

Chlorophenols are common pollutants present in the environment due to earlier environmental contamination, and their presence was previously detected in cork.[31]

Under the conditions of our study, the TCA content determined in the cork samples inoculated with the fungal isolates was similar to that of the noninoculated samples. Moreover, the extracts of cultures, both of 7 days and 4 weeks incubation time, showed similar values of releasable TCA. Applying the variance analysis with 5% significance level (Supporting Information), in any case the TCA values obtained from the analysis of the inoculated samples could not be differentiated from those obtained from the noninoculated samples. It appears that under the conditions of analysis, the releasable TCA on cork cannot be attributed to any of the assayed fungal isolates.

To evaluate if cork dipped in the extracting medium could retain part of the TCA eventually produced by fungi, a hydroalcoholic solution (12% v/v) containing 800 ng L^{-1} of TCA and d_5-TCA was placed in contact with cork granules at ca. 22 °C. The relative concentrations of TCA present in the solution after different agitation times (5, 10, and 94 min) were evaluated by measurements of peak areas of TCA and d_5-TCA in total ion chromatograms (TIC) obtained by GC-MS analysis. The results showed that after 5 min of contact only ∼20% of the TCA content remained in the hydroalcoholic solution. After 94 min, only ∼11% of the initial TCA remained in the hydroalcoholic solution. No exchange of TCA between the extracting solution and the TCA originally present in cork was detected because no significant variation was observed in the ratio TCA/d_5-TCA. This experiment confirmed that cork had fixed most of the added TCA, which is in agreement with other studies that have shown that only ca. 3−5% of TCA contained in cork stoppers was released to the wine.[31,32] The amount of TCA adsorbed onto the cork granules depends on the cork surface, temperature, and time of exposure. In this study cork granules were used, which corresponds to a higher contact area between solution and cork than when entire cork stoppers are used in similar assays.

Chlorophenol precursors present in the cork tree can be converted into chloroanisoles by the existent colonizing fungal species in a chemical reaction catalyzed by the SAM-dependent *O*-methyltransferase. However, the levels of chlorophenols in the cork forests are not very high, as seen by the cork analyses done in our study. Under these studied conditions it is improbable that quantities of TCA produced by the fungal species present in cork can be released into the wine to produce a significant contamination.

To conclude, the levels of chlorophenols usually existing in cork slabs in an industrial environment are not high enough to induce biosynthesis of TCA by the existing fungi, even when grown in more favorable conditions provided by laboratory tests.

■ ASSOCIATED CONTENT

Supporting Information. Tables of data for the quantification of releasable TCA. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Pereira, H. Chemical composition and variability of cork from *Quercus suber* L. *Wood Sci. Technol.* **1988**, *22*, 211–218.

(2) Lacey, J. The air spora of a Portuguese cork factory. *Ann. Occup. Hyg.* **1973**, *16*, 223–230.

(3) Silva Pereira, C.; Pires, A.; Valle, M.; Vilas-Boas, L.; Figueiredo Marques, J.; San Romão, M. Role of *Chrysonilia sitophila* on the quality for cork stoppers for sealing wine bottle. *J. Ind. Microbiol. Biotechnol.* **2000**, *24*, 256–261.

(4) Alvarez-Rodríguez, M. L.; López-Ocaña, L.; López-Coronado, J. M.; Rodríguez, E.; Martínez, M. J.; Larriba, G.; Coque, J.-J. R. Cork taint of wines: role of the filamentous fungi isolated from cork in the formation of 2,4,6-trichloroanisole by *O*-methylation of 2,4,6-trichlorophenol. *Appl. Environ. Microbiol.* **2002**, *68*, 5860–5869.

(5) Prak, S.; Gunata, Z.; Guiraud, J. P.; Schorr-Galindo, S. Fungal strains isolated from cork stoppers and the formation of 2,4,6-trichloroanisole involved in the cork taint of wine. *Food Microbiol.* **2007**, *24*, 271–280.

(6) Thrane, U.; Andersen, B.; Frisvad, J. C.; Smedsgaard, J. The exometabolome in filamentous fungi. *Metabolomics. Topics in Current Genetics*; Nielsen, J., Hewitt, J., Eds.; Springer: Berlin, Germany, 2007; Vol. 18, pp 235−252.

(7) Silva Pereira, C.; Figueiredo Marques, J.; San Romão, M. V. Cork taint in wine: scientific knowledge and public perception: a critical review. *Crit. Rev. Microbiol.* **2000**, *26*, 147–162.

(8) Sefton, M. A.; Simpson, R. F. Compounds causing cork taint and the factors affecting their transfer from natural cork closures to wine — a review. *Aust. J. Grape Wine Res.* **2005**, *11*, 226–240

(9) Chatonnet, P.; Bonnet, S.; Boutou, S.; Labadie, M. D. Identification and responsability of 2,4,6-tribromoanisole in musty, corked odors in wine. *J. Agric. Food Chem.* **2004**, *52*, 1255–1262.

(10) Simpson, R. F.; Capone, D. L.; Sefton, M. A. Isolation and identification of 2-methoxy-3,5-dimethylpyrazine, a potent musty compound from wine corks. *J. Agric. Food Chem.* **2004**, *52*, 5425–5430.

(11) Culleré, L.; Cacho, J.; Ferreira, V. Comparative study of the aromatic profile of different kinds of wine cork stoppers. *Food Chem.* **2009**, *112*, 381–387.

(12) Pollnitz, A. P.; Pardon, K. H.; Liacoupos, D.; Skouroumounis, G. K.; Sefton, M. A. The analysis of 2,4,6-trichloroanisole and other chloroanisole in tainted wines. *Aust. J. Grape Wine Res.* **1996**, *2*, 184–190.

(13) Botelho, M. L.; Almeida, E.; Tenreiro, R.; Andrade, M. E. Searching for a strategy to γ sterilize Portuguese cork stoppers — preliminary studies on bioburden radioresistance and sterility assurance. *Int. J. Radiat. Appl. Instrum. C: Radiat. Phys. Chem.* **1988**, *31*, 775–781.

(14) Dias, J. A. *Análises Cromatográficas de Compostos Organoclorados no Controlo e Garantia de Qualidade de Rolhas de Champanhe*. M.Sc. Dissertation, Faculdade de Ciências, Universidade de Lisboa, 2008.

(15) Pherobase, available at http://www.pherobase.com (accessed Jan 20, 2011).

(16) Rocha, S.; Delgadillo, I.; Ferrer Correia, A. J. GC-MS of volatiles of normal and microbiologically attacked cork from *Quercus suber* L. *J. Agric. Food Chem.* **1996**, *44*, 865–871.

(17) Karlshof, K.; Larsen, T. O. Differentiation of species from the *Penicillium roqueforti* group by volatile metabolite profiling. *J. Agric. Food Chem.* **2005**, *53*, 708–715.

(18) Rohlf, J. F. *NTSYS-pc — Numerical Taxonomy and Multivariate Analysis System*, version 2.1; Exeter Software: Setauket, NY, 2000.

(19) Soares, G. A. M.; Basílio, M. C.; Tenreiro, R.; San Romão, M. V. Diversity of *Penicillium* spp. colonizing cork slabs: a classical and molecular approach. *Biological Resource Centres and Use of Microbes — XXII ECO Meeting Proceedings Book*; Lima, N., Smith, D., Eds.; MUM: Braga, Portugal, 2003; pp 161–170.

(20) Peiris, D.; Dunn, W. B.; Brown, M.; Kell, D. B.; Roy, I.; Hedger, J. N. Metabolite profiles of interacting mycelial fronts differ for pairings of the wood decay basidiomycete fungus, *Stereum hirsutum* with its competitors *Coprinus micaceus* and *Coprinus disseminatus*. *Metabolomics* **2008**, *4*, 52–62.

(21) Bernards, M. A. Demystifying suberin. *Can. J. Bot.* **2002**, *80*, 227–240.

(22) Javor, T.; Buchberger, W.; Tanzcos, I. Determination of low-molecular-mass phenolics and non-phenolic lignin degradation compounds in wood digestion solutions by capillary electrophoresis. *Microchim. Acta.* **2000**, *135*, 45–53.

(23) Tessman, D.; Dianese, J. C. Hentriacontrane: a leaf hydrocarbon from *Syzygium jambos* with stimulatory effects on the germination of urediniospores of *Puccinia psidii*. *Fitopatol. Bras.* **2002**, *27*, 538–542.

(24) Tao, Y.; Li, H.; Wanga, H.; Zhang, L. Volatile compounds of young Cabernet Sauvignon red wine from Changli County (China). *J. Food Compos. Anal.* **2008**, *21*, 689–694.

(25) Borjesson, T.; Stollman, U.; Schnurer, J. Volatile metabolites and other indicators of *Penicillium aurantiogriseum* growth on different substrates. *Appl. Environ. Microbiol.* **1990**, *56*, 3705–3710.

(26) Wasternack, C. Jasmonates: an update on biosynthesis. Signal transduction and action in plant stress response, growth and development. *Ann. Bot.* **2007**, *100*, 1–17.

(27) Miersch, O.; Porzel, A.; Wasternack, C. Microbial conversion of jasmonates — hydroxylations by *Aspergillus niger*. *Phytochemistry* **1999**, *50*, 1147–1152.

(28) Mueller, M. J.; Brodschelm, W.; Spannagl, E.; Zenk, M. H. Signaling in the elicitation process is mediated through the octadecanoid pathway leading to jasmonic acid. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7490–7494.

(29) Silva Pereira, C.; Soares, G. A. M.; Oliveira, A. C.; Rosa, M. E.; Pereira, H.; Moreno, N.; San Romão, M. V. Effect of fungal colonization on mechanical performance of cork. *Int. Biodeterior. Biodegrad.* **2006**, *57*, 244–250.

(30) Coque, J.-J.; Álvarez-Rodríguez, M. L.; Larriba, G. Characterization of an inducible chlorophenol*O*-methyltransferase from *Trichoderma longibracheatum* involved in the formation of chloroanisoles and determination of its role in cork taint of wines. *Appl. Env. Microbiol.* **2003**, *69*, 5089–5095.

(31) Alvarez-Rodriguez, M. L.; Recio, E.; Coque, J. J. R. The analysis of natural cork in transversal sections as an effective tool to determine the origin of the taint by 2,4,6-trichloroanisole. *Eur. Food Res. Technol.* **2009**, *230*, 135–143.

(32) Juanola, R.; Subirá, D.; Salvadó, V.; Regueiro Garcia, J. A.; Anticó, E. Migration of 2,4,6-trichloroanisole from cork stoppers to wine. *Eur. Food Res. Technol.* **2005**, *220*, 347–352.

Supporting information

Quantification of releasable TCA. Analysis of variance (ANOVA)

Results (triplicates) of analysis of TCA for samples incubated during 7 days are presented in Table 1 and were treated by ANOVA to determine whether there were significant differences between blank and samples and also between samples.

The results of calculations necessary for analysis of variance were included in table 1: mean and variance for each triplicate and square of diferences between the mean of each sample and the mean for all samples.

The F-test was applied to values estimated for the variance between samples (2.2645) and variance within samples (1.7033) (ng/L).

Table 1

7 days	triplicates			Mean	Variance	Square
Blank	0.7083	1.9137	2.7700	1.7973	1.0728	0.1780
C. sitophila	3.9245	1.7651	2.0267	2.5721	1.3889	0.1245
P. glabrum	0.6610	2.3867	0.6457	1.2311	1.0015	0.9764
P. brevicompactum	1.6803	3.0726	0.8855	1.8795	1.2255	0.1155
P. paneum	3.1725	4.6009	1.3306	3.0346	2.6880	0.6649
E. rubrum	4.5808	0.9191	2.4334	2.6444	3.3854	0.1808
Fungal mixture	1.1667	3.2347	2.7257	2.3757	1.1611	0.0245
				2.2193	**1.7033**	**2.2645**
				average	average	sum

F=2.2645/1.7033= **1.329** < **2.848** (critical value of F for a one-tailed test)

Probability (P=0.05) df(between samples)=6

df=degrees of freedom df(within samples)=14

The calculated value for F (1.329) is smaller than the critical value of F (2.848) (obtained from the tables of t-test): therefore no significant differences were observed in the results of analysis obtained for blank and for the various samples (ng/L).

The same statistical treatment was applied to the samples incubated during 4 weeks.

Table 2

4 weeks	triplicates			Mean	Variance	Squares
Blank	3.4186	1.8893	0.0399	1.7826	2.8624	0.1858
C. sitophila	3.5541	3.6682	1.7255	2.9826	1.1885	0.5913
P. glabrum	1.2259	2.2230	3.6183	2.3557	1.4441	0.0202
P. brevicompactum	2.5605	1.1630	0.9557	1.5597	0.7619	0.4277
P. paneum	2.9761	0.5203	1.2211	1.5725	1.6004	0.4111
E. rubrum	4.1644	0.3128		2.2386	7.4175	0.0006
Fungal mixture	2.8960	0.4676	5.6731	3.0122	6.7844	0.6377
			2.2137	2.2137	**3.1513**	**2.2743**
				average	average	sum

F=3.151/2.274= **1.386** < **2.915** (critical value of F for a one-tailed test)

(P=0.05) df(between samples)=6

df(within samples)=13

The calculated value for F (1.386) is smaller than the critical value of F (2.915): therefore no significant differences were observed in the results of analysis obtained for blank and for the various samples (ng/L).

The set of all samples (7 days and 4 weeks) was also considered and the same statistical treatment was applied.

Table 3 contains the complete collection of results obtained in the analysis of releasable TCA as well as the results of calculations necessary for analysis of variance .

Table 3

7 days	triplicates			Means	Variances	Squares
Blank	0.7083	1.9137	2.7700	1.7973	1.0728	0.1098
C. sitophila	3.9245	1.7651	2.0267	2.5721	1.3889	0.1966
P. glabrum	0.6610	2.3867	0.6457	1.2311	1.0015	0.8057
P. brevicompactum	1.6803	3.0726	0.8855	1.8795	1.2255	0.0621
P. paneum	3.1725	4.6009	1.3306	3.0346	2.6880	0.8207
E. rubrum	4.5808	0.9191	2.4334	2.6444	3.3854	0.2660
Fungal mixture	1.1667	3.2347	2.7257	2.3757	1.1611	0.0610
4 weeks						
Blank	3.4186	1.8893	0.0399	1.7826	2.8624	0.1198
C. sitophila	3.4186	1.8893	0.0399	1.7826	2.8624	0.1198
P. glabrum	1.2259	2.2230	3.6183	2.3557	1.4441	0.0515
P. brevicompactum	2.5605	1.1630	0.9557	1.5597	0.7619	0.3238
P. paneum	2.9761	0.5203	1.2211	1.5725	1.6004	0.3094
E. rubrum	4.1644	0.3128		2.2386	7.4175	0.0121
Fungal mixture	2.8960	0.4676	5.6731	3.0122	6.7844	0.7805
				2.1287	**2.5469**	**4.0388**
				average	average	sum

F=4.0387/2.5469 **1.5858 <** **2.1068** (critical value of F for a one-tailed test)
(P=0.05) df(between samples)=13
df(within samples)=27

The calculated value for F (1.5858) is smaller than the critical value of F (2.1068): therefore no significant differences were observed in the results of analysis obtained for blank and for the various samples

Chapter 5

Discussion

To address the study of the natural fungal community present in the cork slabs one culture dependent and two culture independent methods were employed. The isolation method provided indications of the fungal quantity and diversity present in the samples taken in some stages of the cork discs manufacturing. The methods showed that the fungal concentrations present in each sample have different quantities depending mainly on the stage of the cork sample. Additionally, the mycobiota diversity is conditioned first by the season on which the sample was made and at a later stage the cork geographic origin. The diversity is higher in non-boiled cork. Other works performed earlier to study the cork mycobiota isolated in several stages of the cork manufacturing of stoppers gave similar results although the fungal load present in those samples were different of the ones considered in our work. Another difference encountered is the fact that the fungal quantity present in cork samples collected in the resting stage is higher than the amount present in the raw cork samples (Alvarez-Rodriguez, 2002). This is probably due to the fact that the period of time that the cork slabs remained in the storeroom under conditions favoring the fungal growth could be higher than the ones experienced in this work (Alvarez-Rodriguez, 2002).

The culture-dependent technique combined the phenotypic and molecular methods to identify the fungi isolated in this work. Most of the fungal isolates belong to *Penicillium* and *Aspergillus* genera and the most predominant species were *P. glabrum* and *C. sitophila*. *P. glabrum* was isolated in most of the studied samples, while *C. sitophila* appeared mostly in the resting stage, covering all the cork slabs in that phase. In this work, this species was isolated in one sample from the Spanish cork batch and in another from the

Portuguese cork batch; those samples were collected in the stages before boiling and non-treated cork discs. These two fungal species colonizing cork substrate were reported by other authors (Davis, 1981; Daly, 1984; Lee, 1993; Danesh, 1997; Alvarez-Rodriguez, 2002; Basílio, 2006).

The use of PCR specific primers, β-tubulin gene for all the *Penicillium* and *Aspergilus* genera and calmodulin for the isolates that belong to the *Glabra* series, allow the correct taxonomic identification to species rank of most of the isolates. In addition a more perceptive taxonomic study in the *Glabra* series was done and the description of a new fungal species *P.subericola* was obtained (Barreto, 2011b). Cork is an unique substrate that possible allows new fungal species some of them already described (Belloch, 2007).

The combination of two culture-independent methods to study the fungal population resulted in a more precise knowledge of the composition of this population. The fungal diversity that begins to be known by the isolation technique was more unveiled using these techniques. The cloning method revealed the existence of a variety of fungi mostly in the raw cork composed mainly of uncultivable fungi, *Ascomycetes* and endophytes. Most of them are present in low relative frequencies. Cork mycobiota in samples taken immediately after the first boiling was probably composed by uncultivable species. Probably, the mycobiota present in these stages are mainly composed by fungal species that colonised the cork tree in the forest, as already being remarked in other studies (Serra, 2008). However, at this stage one of the Portuguese batches (38) was successful in isolating six different fungal species.

Finally in at the remaining manufacturing stages the mycobiota population present is similar to all of them. This population is composed mainly by cultivable fungal species belonging to *Penicillium*, *Aspergillus* and *Neurospora* species. Reinforcing the fact that most of the fungal population present at these stages originates from the environmental mycobiota present in the factory and after the boiling have the necessary conditions (temperature and humidity) to colonize the slabs and dominate the fungal colonization. Although, the presence of at least one or two phylotypes (*Sporobolomyces* sp. and *Pezicula* sp.) usually associated to the plant material and that could be originated from the cork tree and survived the whole manufacturing process was detected.

The mycospores could be lodged inside the cork lenticels and due to the cork's impermeability to high temperatures and humidity levels that occur during the boiling process does not reach all the cork structure. Thus, a microhabitat is created inside the cork structure and enables the survival of some microbiota that were previously present.

The concern of cork quality was the starting point to study the exo-metabolites and volatiles produced by some fungal species isolated from the cork substrate. Some of these species are known to produce exo-metabolites in semi-synthetic culture media; some of them when growing in culture media are even mycotoxins producers (e.g. *P. citrinum*, *P. citreonigrum*). The possible production of exo-metabolites and volatiles by *C. sitophila* was studied since this fungal species dominates the cork slabs mostly during their resting stage.

The studied fungal isolates produced some exo-metabolites specially when growing in the semi-synthetic media. When growing on cork-based culture medium most of the isolates do not produce any metabolite, while some (*E. amstelodami*, *P. brevicompactum* and *P. citrinum*) produce few exo-metabolites. However, if *C. sitophila* extracts are added to the cork-based culture medium only two of the studied fungal isolates did not produce any metabolite in this medium. This situation tries to mimic the cork at the resting stage where the water activity (a_w) decreases to values below 0.9. Until this value *C. sitophila* mycelium completely covered the cork slabs. Bellow 0.9 a_w this fungus was replaced by the mycelium of other fungal species (e.g. *Penicillium*, *Aspergillus*, *Trichoderma*). This late mycobiota develops in the cork slabs that have the remains of *C. sitophila.* The presence of this mycelium on the cork slabs enhances the exo-metabolome of the late fungal colonizers leading to a production of more metabolites (Barreto, 2011a). *C. sitophila,* as expected did not produce any metabolite in any of the used culture media, which is in accordance with previous studies made on the safety of *Neurospora crassa* (Perkins, 2000).These results suggested that the most predominant mycoflora present in the resting stage of cork could be regarded as non-deleterious to the cork discs (or cork stoppers) final product.

It appears that studies concerning the interactions that could exist among cork colonizing fungi in such specific substrate as cork are crucial. Also the different metabolic mechanisms used by those fungal species to colonize/ survive in such environment should be a subject of study.

The genome of some related species that colonize cork (e.g. *Neurospora crassa* and *Trichoderma viride*) are fully sequenced.

This fact could be the starting point to some further research of the mechanisms of degradation of some main cork constituents by the respective fungal species.

Furthermore, the production of volatile compounds was studied for a) cork samples from one batch taken during several manufacturing process and b) some fungal isolates growing in cork-based culture medium, in both pure and mixed culture.

Most of the volatile compounds were produced by the mycobiota that exists in two manufacturing stages: resting stage after the first boiling and non-treated cork discs. Additionally, the volatile compounds produced by the fungal isolates, either in pure or mixed culture consisted mainly of 1-octen-3-ol and esters of fatty acids (medium chain length C8 –C20). *P. glabrum* seems to be the main contributor to the overall volatile composition observed in the mixed culture. In general, no volatile compounds detected in this work will influence negatively the cork's final product (Barreto, 2011c).

The presence of chemical compounds that can produce organoleptic defects on the wine is a major concern in the cork Industry. These compounds can be present in very small quantities (range of ng/L^{-1}) and spoil the wine.

2,4,6–Trichloroanisole (TCA) was present in most of the cases of "cork taint" in wines. TCA contaminates cork by many processes, but usually results from the transformation of polychlorophenols, used in some wood preservatives and in some pesticides, by the action of fungal metabolism, e.g. *Penicillium* and *Aspergillus*.

Some fungal isolates, in pure and mixed culture, were screened to investigate their capacity to produce TCA in cork-based medium. To perform this experiment, Industrial conditions were considered as well as the chlorophenols levels that usually exist in cork slabs.

The results show that the amount of TCA present in the non-inoculated medium culture could not be distinguished from the levels obtained in the studied samples, even if analysis of variance with 5% significance level was applied (Supporting Information in (Barreto, 2011c)).

Moreover, in the presence of a hydroalcoholic solution containing known concentrations of TCA and 5d-TCA, the cork used in the culture medium, retained most of the added chloroanisoles. After the first 5 min of contact only ~20% of the added TCA remained in the hydroalcoholic solution. No exchange of TCA between the extracting solution and the TCA originally present in the cork was observed (Barreto, 2011c).

These results can be the starting point to study the potential role of cork granules in the treatment of polluted environments, namely waters. Additionally, cork being an unique material with distinct chemical constitution and exclusive physical properties can also serve to investigate the growth and development of microbiota that need to survive to extreme conditions to be used in bioremediation situations. For instance, pentachlorophenol (PCP) is a chemical compound widely used as herbicide, biocide and pesticide and thus contaminating the terrestrial and aquatic environments. Recently, few fungal species previously isolated from cork substrate showed the capacity to fully degrade PCP (Carvalho, 2009). These results reinforce the demand to study microbiota that colonizes recalcitrant substrates and investigate their capacity to decontaminate polluted environments.

The Industry needs to maintain the control of humidity of the cork slabs (around 0.9 a_w) during the slabs resting stage, to avoid the germination of fungal species that can contribute to off-odors.

However, the results obtained in this work point out that if the humidity levels are controlled and the hygienic practices respected (e.g. no contamination of the cork slabs from the environmental and soil factory), no unpleasant sensory properties can be imputed to the cork final product.

Chapter 6

Bibliography

AFGC (2007). Organohalogen taints in foods. In *Supplement to food Australia*.

Alexopoulos, C. J., Mims, C. W., Blackwell, M. (1996). *Introductory Mycology*, 4th edn. New York: Wiley.

Álvarez-Rodríguez, M. L., Belloch, C., Villa, M., Uruburu, F., Larriba, G., Coque, J-J R. (2003). Degradation of vanillic acid and production of guaiacol by microorganisms isolated from cork samples. *FEMS Microbiology Letters* **220**, 49-55.

Alvarez-Rodriguez, M. L., Lopez-Ocana, L., Lopez-Coronado, J. M., Rodriguez, E., Martinez, M. J., Larriba, G., Coque, J. J. (2002). Cork taint of wines: role of the filamentous fungi isolated from cork in the formation of 2,4,6-trichloroanisole by o methylation of 2,4,6-trichlorophenol. *Appl Environ Microbiol* **68**, 5860-5869.

Alvarez-Rodriguez, M. L., Recio, E., Coque, J. J. R. (2009). The analysis of natural cork in transversal sections as an effective tool to determine the origin of the taint by 2,4,6 – trichloroanisole. *EUR Food Res Technol* **230**, 135-143.

Amon, J. M., Vandepeer, J. M., Simpson, R. F. (1989). Compounds responsable for the cork taint in wine. *Wine Industry Journal* **2**, 62-69.

Anderson, I. C., Campbell, C. D., Prosser, J. I. (2003a). Potential bias of fungal 18S rDNA and internal transcribed spacer

polymerase chain reaction primers for estimating fungal biodiversity in soil. *Environ Microbiol* **5**, 36-47.

Anderson, I. C., Campbell, C. D., Prosser, J. I. (2003b). Diversity of fungi in organic soils under a moorland- Scots pine (*Pinus sylvestris* L.) gradient. *Environ Microbiol* **5**, 1121-1132.

APCOR Other applications of cork. available at http://www.cork.pt/other-applications-of-cork.html (acessed May 8, 2011)

Ávila, R., Lacey, J. (1974). The role of *Penicillium frequentans* in suberosis (respiratory disease in workers in the cork industry). *Clinical Allergy* **4**, 109-117.

Barreto, M. C., Frisvad, J. C., Larsen, T. O., Mogensen, J., San-Romão, M. V. (2011a). Exo-metabolome of some fungal isolates growing on cork-based medium. *EUR Food Res Technol* **232**, 575-582.

Barreto, M. C., Houbraken, J., Samson, R., Frisvad, J. C., San-Romão, M. V. (2011b). Taxonomic studies of the *Penicillium glabrum* complex and the description of a new species *P. subericola*. *Fungal Diversity*.

Barreto, M. C., Vilas Boas, L., Carneiro, L. C., San Romão, M. V. (2011c). Volatile compounds in samples of cork and also produced by selected fungi. *J Agric Food Chem* **59**, 6568-6574.

Basílio, M. C. G., R.; Silva Pereira & San Romão, M. V. (2006). *Penicillium glabrum* cork colonising isolates - preliminary analysis of their genomic similarity. *Rev Iberoam Micol* **23**, 151-154.

Belloch, C., Villa-Carvajal, M., Álvarez-Rodríguez, M. L., Coque, J. J. (2007). *Rhodotorula subericola* sp. nov., and anamorphic basidiomycetous yeast speceis isolated from bark of *Quercus suber* (cork oak). *Int J Syst Evol Microbiol* **57**, 1668-1671.

Bennet, J. W., Feibelman, T. (2001). Fungal bacteria interacteractions. In *The Mycota* pp. 229 - 242. Edited by F. A. Hock. Heidelberg, Berlin: Springer-Verlag.

Brown, S. P., Inglis, R. F., Taddei, F. (2009). Evolutionary ecology of microbial wars: within-host competition and (incidental) virulence. *Evol Appl* **2**, 32-39.

Buser, H. R., Zanier, C., Tanner, H. (1982). Identification of 2,4,6-trichloroanisole as a potent component causing cork taint in wine *J Agric Food Chem* **30**, 359-362.

Caldentey, P., M.D. Fumi, Mazzoleni, V., Careri, M. (1998). Volatile compounds produced by microorganisms isolated from cork. *Flavour Fragr* **13**, 185-188.

Calvo, A., Wilson, R. A., Bok, J. W., Keller, N. P. (2002). Relationships between secondary metabolism and fungal

development. *Microbiol Mol Biol Rev* **66**, 447-459.

Capone, D. L., Skouroumounis, G. K., Sefton, M. A. (2002). Permeation of 2,4,6-trichloroanisole through cork closures in wine bottles. *Australian Journal of grape and wine research* **8**, 196-199.

Carlile, M. J., Watkinson, S. C., Gooday, G. W. (2001). *Fungal diversity*, 2nd edition edn. London, UK: Academic Press.

Carvalho, M. B., Martins, I., Leitão, M. C., Garcia, H., Rodrigues, C., San-Romão, V., Hursthouse, A., Silva Pereira, C. (2009). Screening pentachlorophenol degradation ability by environmental fungal strains belonging to the phyla Ascomycota and Zygomycota. *J Ind Microbiol Biotechnol* **36**, 1249-1256.

Celiège (2006). International code of cork stopper manufacturing practice.

Chatonnet, P., Bonnet, S., Bouton, S., Labadie, M-D. (2004). Identification and responsibility of 2,4,6 - tribromoanisole in musty, corked odors in wine. *Journal of agricultural and food chemistry* **52**, 1255-1262.

Chen, D. M., Cairney, J. W. G. (2002). Investigation of the influence of prescribed burning on ITS profiles of ectomycorrhizal and other soil fungi at three Australian sclerophyll forest sites. *Mycol Res* **106**, 532-540.

Coque, J. J., Álvarez-Rodriguez, M. L., Larriba, G. (2003). Characterization of an inducible chlorophenol *O*-methyltransferase from *Trichoderma longibracheatum* involved in the formation of chloroanisoles and determination of its role in cork taint of wines. *Appl Environ Microbiol* **69**, 5089-5095.

Coque, J. J., Rodríguez, M.L., Goswami, M., Martinez, R.F. (2006). *Causes and origins of wine contamination by haloanisoles (chloroanisoles and bromoanisoles)*. León: Asecor - Agrupación Sanvicenteña Empresarios del Corcho.

Coquet, C., Bauza, E., Oberto, G., Berghi, A., Farnet, A. M., Ferré, E., Peyronel, D., Dal Farra, C., Domloge, N. (2005). *Quercus suber* cork extract displays a tensor and smoothing effect on human skin: an *in vivo* study. *Drugs Exptl Clin Res* **31**, 89-99.

Cork facts available at http://www.corkfacts.com/nchoice1.htm). (acessed July 8, 2011)

Curtis, R. F., Dennis, C., Gee, J. M., Gee, M. G., Griffths, M. N., Land, D. G., Peel, J. L., Robinson, D. (1974). Chloroanisoles as a cause of musty taint in chickens and their microbiological formation from chlorophenols in broiler house liter. *Journal Science Food Agriculture* **25**, 811-828.

Daly, N. M. L., T. H., Fleet, G. H. (1984). Growth of fungi on wine corks and its contribution to cork taints in wine. *Food Technology*

in Australia **36**, 22-24.

Danesh, P., Velez Caldas, F. M., Figueiredo Marques, J. J., San Romão, M. V. (1997). Mycobiota in portuguese "normal" and "green" cork throughout te manufacturing process of stoppers. *J Appl Microbiol* **82**, 689-694.

Darriet, P., Pons, M., Lamy, S., Dubourdieu, D. (2000). Identification and quantification of geosmin, an earthy odorant contaminating wines. *J Agric Food Chem* **48**, 4835-4838.

Davis, C. R., Fleet, G. H., Lee, T. H. (1981). The microflora of wine corks. *Aust Grapegrower winemaker* **208**, 42-44.

Dias, A. (2008). Análises cromatográficas de compostos organoclorados no controlo e garantia de qualidade de rolhas de champanhe. M.Sc. Dissertation, Faculdade de Ciências, Universidade de Lisboa.

Dickie, I. A., Xu, B., Kolde, R. T. (2002). Vertical distribution of ectomycorrhizal hyphae in soil as shown bt T-RFLP analysis. *New Phytol* **156**, 527-535.

Duncan, B. C., Gibson, R. L., Obradovic, D. (1997). 2,4,6-trichloroanisole and cork production. *Wine Industry Journal* **12**, 180-184.

Engel, G., Ernest von Milczewski, K., Prokopek, D., Teuber, M. (1982). Strain-specific synthesis of mycophenolic acid by

Penicillium roquefortii in blue-veined cheese. *App Env Microbiol* **43**, 1034-1040.

Filtenborg, O., Frisvad, J. C., Thrane, U. (1990). The significance of yeast extract composition on metabolite production in *Penicillium*. In *Modern concepts in Penicillium and Aspergillus classification*, pp. 433-440. Edited by R. Samson, Pitt, J. New York: Plenum.

Flodin, C., Whitfield, F. B. (2000). Brominated anisoles and cresols in the red alga *Polysiphonia sphaerocarpa Phytochemistry* **53**, 77-80.

Fortes, M. A., Rosa, M. E., Pereira, H. (2004). *A cortiça*. Lisboa: IST Press.

Frisvad, J. C., Filtenborg, O., Thrane, U. (1989). Analysis and screening for mycotoxins and other secondary metabolites in fungal cultures by thin-layer chromatography and high-performance liquid chromatography. *Arch Environ Contam Toxicol* **18**, 331-335.

Frisvad, J. C., Thrane, U., Filtenborg, O. (1998). Role and use of secondary metabolites in fungal taxonomy. In *Chemical fungal taxonomy*, pp. 289-319. Edited by J. C. Frisvad, Bridge, P. D., Arora, D. K. New York: Marcel Dekker.

Frisvad, J. C. S., R. A. (2004). Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food

and air-borne terverticillate Penicilia and their mycotoxins. *Stud Mycol* **49**, 173.

Gil, A. M., Lopes, M. H., Neto, C. P., Callaghan, P. T. (2000). An NMR microscopy study of water absorption in cork. *J Mater Sci* **35**, 1891-1900.

Gil , L. (1996). Densification of black agglomerate cork boards and study of densified agglomerates. *Wood Science and Technology* **30**, 217-223.

Gil, L. (1998). *A cortiça - Produção, Tecnologia e Aplicação*. Lisboa: INETI.

Gil, L. (2007). Cork composites: a review. *Materials* **2**, 776-789.

Gourlay, I. D., Pereira, H. (1998). The effect of bark stripping on wood production in cork oak (*Quercus suber*) and problems of growth ring definition. In *Cork oak and cork European Conference on cork oak and cork 5 -7 May 1997*, pp. 99-107. Edited by H. Pereira. Lisboa: CEF - Centro de Estudos Florestais - Instituto Superior de Agronomia.

Haas, H., Galler, H., Habib, J., Melkes, A., Schlacher, R., Buzina, W., Friedl, H., Marth, E., Reinthaler, F. F. (2010). Concentrations of viable fungal spores and trichloroanisole in wine cellars. *Int J Food Microbiol* **114**, 126-132.

Hawksworth, D. L. (1991). The fungal dimension of biodiversity:

magnitude, significance and conservation. *Mycol Res* **95**, 641-655.

Hawksworth, D. L., Kirk, P. M., Sutton, B. C., Pegler, D. L. (1995). *Ainsworth and Bisby's Dictionary of the fungi*, 8th edn. Wallingford: CAB International.

Hibbet, D. S. (1992). Ribosomal RNA and fungal systematics. *Trans Mycol Soc Japan* **33**, 533-556.

Hill, J. L., Hocking, A. D., Whitfield, F. B. (1995). The role of fungi in the production of chloroanisoles in general purpoose freight containers. *Food Chemistry* **54**, 161-166.

Hitchcock, C. J., Chambers, S. M., Anderson, I. C., Cairney, J. W. G. (2003). Development of markers for simple sequence repeat-rich regions that discriminate between *Pisolithus albus* and *P. microcarpus*. *Mycol Res* **107**, 699-706.

Hoffman, A., Sponholz, W. R., David, F., Sandra, P. (2000). Corkiness in wine-trace analysis of 2,4,6-trichloroanisole by stir bar sorptive extraction (SBSE) and thermal desorption GC/MS. *AppNote* **3**, 1-5.

Houbraken, J. V., J.; Rico-Munoz, E.; Johnson, S. & Samson, R. (2008). Sexual reproduction as the cause of heat resistance in the food spoilage fungus Byssochlamys spectabilis (Anamorph Paecilomyces variotii). *Applied Environmental Microbiology* 74, 7.

Howland, P. R., Pollnitz, A. P., Liacopoulos, D., Mclean, H. J., Sefton, M. A. (2008). The location of 2,4,6-trichloroanisole in a batch of contaminated wine corks. *Aust J Grape Wine Res* **3**, 1-5.

International, P. G. f. P. (2010). PAN International list of highly hazardous pesticides (PAN list of HHP) available at www.pan-germany.org/download/PAN_HHP-List_101216.pdf (accessed July 8, 2010).

Juanola, R. S., D.; Salvado, V.; García Regueiro, J. A. & Antico, E. (2005). Migration of 2,4,6-trichloroanisole from cork stoppers to wine. *EUR Food Res Technol* **220**, 347-352.

Karahadian, C., Josephson, D. B., Lindsay, R. C. (1985). Volatile compounds from *Penicillium* sp. contributing musty-earthy notes to brie and camembert cheese flavors. *J Agric Food Chem* **33**, 339-343.

Karbowiak, T., Gougeon, R. D., Alinc, J.-B., Brachais, L., Debeaufort, F., Voilley, A., Chassagne, D. (2010). Wine oxidation and the role of cork. *Crit Rev Food Sci Nutr* **50**, 20-52.

Karlovsky, P. (2008). Secondary metabolites in soil ecology. In *Soil Biology series*, pp. 1-19. Edited by A. Varma: Springer.

Lacey, J. (1973). The air spora of a Portuguese cork factory. *Ann Occup Hyg* **16**, 223-230.

Landeweert, R., Leeflang, P., Smit, E., Kuyper, T. W. (2004).

Diversity of an ectomycorrhizal fungal community studied by a root tip and total soil DNA approach. *Mycorrhiza* **15**, 1-6.

Larsen, T. O. (1998). Volatiles in fungal taxonomy. In *Chemical fungal taxonomy*, pp. 263-288. Edited by J. C. Frisvad, Bridge, P. D., Arora, D. K. New York: Marcel Dekker.

Larsen, T. O., Frisvad, J. C. (1995a). Characterization of volatile metabolites from 47 *Penicillium* taxa. *Mycol Res* **99**, 1153-1166.

Larsen, T. O., Frisvad, J. C. (1995b). Chemosystematics of Penicillium based on profiles of volatile metabolites. *Mycol Res* **99**, 1167-1174.

Larsen, T. O., Smedsgaard, J., Nielsen, K. F., Hansen, M. E., Frisvad, J. C. (2005). Phenotypic taxonomy and metabolite profiling in microbial drug discovery. *Nat Prod Rep* **22**, 672-695.

Lee, T. H., Simpson, R. F. (1993). Microbiology and chemistry of cork taints in wine. In *Wine, microbiology and Technology*. Edited by G. H. Fleet: Harwood Academic Publishers.

Lefebvre, A., Riboulet, J. -M., Boidron, J.-N. & Ribéreau-Gayon, P. (1983). Incidence des micro-organismes du liège sur les altérations olfactives du vin. *Science des Aliments* **3**, 13.

Lopes, P., Saucier, C., Teissedre, P.-L, Glories, Y. (2007). Main routes of oxygen ingress through different closures into wine bottles. *J Agric Food Chem* **55**, 5167-5170.

Macku, C., Gonzalez, L., Schleussner, C., Mesquita, A. C., Herwatt, J. W., Kirch, L. C., Schwartz, R. J. (2009). Sensory screening for large-format natural corks by "dry soak" testing and its correlation to headspace solid-phase microextraction (SPME) gas chromatography/mass spectrometry (GC/MS) releasable trichloroanisole (TCA) analysis. *J Agric Food Chem* **57**, 7962-7968.

Maga, J. A., Puech, J. L. (2005). Cork and alcoholic beverages. *Food Reviews International* **21**, 53-68.

Maggi, L., Mazzoleni, V., Fumi, M. D., Salinas, M. R. (2008). Transformation ability of fungi isolated from cork and grape to produce 2,4,6-trichloroanisole from 2,4,6-trichlorophenol. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* **25**, 265-269.

Mills, N., Lopes, P., Cabral, M. (2006). Oxygen brings new life to closure debate. *The Australian & New Zealand Grapegrower & Winemaker*, 112-114.

Muyzer, G. (1999). DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr Opin Microbiol* **2**, 317-322.

Muyzer, G., Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek* **73**, 127-141.

Nannipieri, P., Ascher, J., Ceccherini, M. T., Landi, L., Pietramellara, G., Renella, G. (2003). Microbial diversity and soil functions. *Eur J Soil Science* **54**, 655-670.

Nielsen, D. S., Hønholt, S., Tano-Debrah, K., Jespersen, L. (2005). Yeast populations associated with Ghanaian cocoa fermentations analysed using denaturing gradient gel electrophoresis (DGGE). *Yeast* **22**, 271-284.

Oliveira, A. C., Peres, C. M., Correia Pires, J. M., Silva Pereira, C., Vitorino, S., Figueiredo Marques, J. J., Crespo, M. T. B., San Romão, M. V. (2003). Cork stoppers Industry: defining appropriate mould colonization. *Microb Res* **158**, 117-124.

Oliveira, M. A., Oliveira, L. (2000). *A cortiça*. Casais de Mem Martins, Rio de Mouro: Corticeira Amorim.

Pereira, C., Gil, L., Carriço, L. (2007a). Reduction of the 2,4,6-trichloroanisole content in cork stoppers using gamma radiation. *Radiat Phys Chem* **76**, 729-732.

Pereira, H. (2007b). *Cork: Biology, production and uses*. Amsterdam: Elsevier.

Pereira, M., Gil, L. (2006). O problema do odor a mofo nas rolhas de cortiça e processos para a sua redução/eliminação. *Silva Lusitana* **14**, 101-111.

Perkins, D. D., Davis, R. H. (2000). Evidence for safety of *Neurospora* speceis for academic and commercial uses. *Appl Environ Microbiol* **66**, 5107-5109.

Pires, J. M. C. P., H., San- Romão, M. V. (2007). Study of humidity and water activity of cork slabs during cork stopper manufacturing process - preliminary results. *Ciência Téc Vitiv* **22**, 15-20.

Pitt, J. J., Hocking, A. D. (1997). *Fungi and food spoilage*. London: Blackie Academic & Professional, Chapman & Hall.

Plutowska, B., Wardencki, W. (2008). Application of gas chromatography-olfactometry (GC-O) in analysis and quality assessment of alcoholic beverages - A review. *Food Chemistry* **107**, 449-463.

Pollnitz, A. P. P., K. H.; Liacopoulos, D.; Skouroumounis, G. K., Sefton, M. A. (1996). The analysis of 2,4,6-trichloroanisole and other chloroanisoles in tainted wines and corks. *Aust J Grape Wine Res* **2**, 184-190.

Prak, S., Gunata, Z., Guiraud, J. -P, Schorr-Galindo, S. (2007). Fungal strains isolated from cork stoppers and the formation of 2,4,6-trichloroanisole involved in the cork taint of wine. *Food Microbiol* **24**, 271-280.

Prat, C., Bañeras, L., Anticó, E. (2008). Screening of musty-

earthy compounds from tainted cork using water-based soaks followed by headspace solid -phase microextraction and gas chromatography-mass spectrometry. *EUR Food Res Technol* **227**, 1085-1090.

Prat., C., Trias, R., Culleré, L., Escudero, A., Anticó, E., Banñeras, L. (2009). Off-odor compounds produced in cork by isolated bacteria and fungi: a gas chromatography-mass spectrometry and gas chromatography-olfatometry study. *J Agric Food Chem* **57**, 7473-7479.

Ranjard, L., Poly, F., Nazaret, S. (2000). Monitoring complex bacteria communities using culture-independent molecular techniques: application to soil environment. *Res Microbiol* **151**, 167-177.

Riboulet, J. M. (1982). Contribution a l'etude chimique et microbiologique des "gouts de bouchon" dans le vins: Universite de Bordeaux II.

Rigaud, J., Issanchou, S., Sarris, J., Langlois, D. (1984). Incidence des composés volatils issus du liège sur le "goût de bouchon" des vins. *Science des Aliments* **4**, 81-93.

Sahgal, N., Monk, B., Wasil, M., Magan, N. (2006). Trycophyton species: use of volatile fingerprints for rapid identification and discrimination. *British J Dermatol* **155**, 1209-1216.

Saxby, M. J. (1996). A survey of chemicals causing taints and

off-flavours in food. In *Food and taints and off-flavors*, pp. 326. Edited by M. J. Saxby. London: Blackie Academic & Professional.

Sefton, M., Simpson, R. (2005). Compounds causing cork taint and factors affecting their transfer from natural cork closures to wine - a review. *Aust J Grape Wine Res* **11**, 226-240.

Serra, R., Peterson, S. W., Centro Tecnológico da Cortiça, Venâncio, A. (2008). Multilocus sequence identification of *Penicillium* species in cork bark during plank preparation for the manufacture of stoppers. *Res Microb* **159**, 178-186.

Silva Pereira, C., Figueiredo Marques, J. J., San Romão, M. V. (2000a). Cork taint in wines: Scientific knowledge and public perception - a critical review. *Critical Rev Microbiol* **26**, 147-162.

Silva Pereira, C., Pires, A., Valle, M. J., Vilas Boas, L., Figueiredo Marques, J. J., San Romão, M. V. (2000b). Role of *Chrysonilia sitophila* in the quality of cork stoppers for sealing wine bottles. *J Ind Microbiol Biotechnol* **24**, 256-261.

Silva, S. P., Sabino, M. A., Fernandes, E. M., Correlo, V. M., Boesel, L. F., Reis, R. L. (2005). Cork: properties, capabilities and applications. *International Material Revue* **50**, 345-365.

Simon, L., Levesque, R. C., Lalonde, M. (1993). Identification of endomycorrhizal fungi colonising roots by fluorescent single.strand conformation polymorphism-polymerase chain

reaction. *App Environ Microbiol* **59**, 4211-4215.

Simpson, R. F., Lee, T. H. (1990). *The microbiology and taints of cork and oak*: E. and Figlestahler, E., Eds. International Association for modern Winery Technology and Management.

Simpson, R. F., Sefton, M. A. (2007). Origin and fate of 2,4,6-trichloroanisole in cork bark and wine corks. *Aust J Grape Wine Res* **13**, 106-116.

Skouroumounis, G. K., Kwiatkowski, M. J., Francis, I. L., Oakey, H., Capone, D. L., Duncan, B., Sefton, M. A., Waters, E. J. (2005). The impact of closure type and storage conditions on the composition, colour and flavour properties of a Riesling and a wooded Chardonnay wine during five years' storage. *Aust J Grape Wine Res* **11**, 369-377.

Soleas, G. J., Yan, J., Seaver, T., Goldberg, D. M. (2002). Method for the gas chromatographic assay with mass selective detection of trichloro compounds in corks and wines applied to elucidate the potential cause of cork taint. *J Agri Food Chem* **50**, 1032-1039.

Soriano, J. M., Dragacci, S. (2004). Ocurrence of fumosins in foods. *Food Res Int* **37**, 985-1000.

Sousa, A. F., Pinto, P. C. R. O., Silvestre, A. J. D., Neto, C. P. (2006). Triterpenic and other lipophilic components from industrial ork byproducts. *J Agric Food Chem* **54**, 6888-6893.

Spadone, J.-C., Takeoka, G., Liandron, R. (1990). Analytical investigation of Rio off-flavour in green coffee. *J Agr Food Chem* **38**, 226-233.

Sweeney, M. J., Dobson, A. D. (1998). Mycotoxin production by *Aspergillus*, *Fusarium* and *Penicillium* species. *Int J Food Microbiol* **43**, 141-158.

Teixeira, M., San Romão, M. V., Bronze, M. R. , Vilas Boas, L. (2006). 2,4,6-Trichloroanisole a consumer panel evaluation. *Ciência Téc Vitiv* **21**, 53-65.

Thrane, U., Andersen, B., Frisvad, J. C., Smedsgaard, J. (2007). The exo-metabolome in filamentous fungi. In *Metabolomics: A powerful tool in systems biology*, pp. 235-252. Edited by J. Nielsen, Hewitt, J. Berlin: Springer.

Toit, W. J., Marais, J., Pretorius, I. S., Toit, M. (2006). Oxygen in must and wine: a review. *S Afr J Enol Vitic* **27**, 76-94.

Vainio, E. J., Hantula, J. (2000). Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified DNA. *Mycol Res* **104**, 927-936.

van Elsas, J. D., Duarte, G. F., Keijzer-Wolters, A., Smit, E. (2000). Analysis of the dynamics of fungal communities in soil via fungal-specific PCR of soil DNA followed by denaturing gradient gel electrophoresis. *J Microb Meth* **43**, 133-151.

Vandenkoornhuyse, P., Baldauf, S. L., Leyval, C., Straczek, J., Young, J. P. W. (2002). Extensive fungal diversity in plants. *Science* **15**, 2051.

Vandenkoornhuyse, P., Ridgway, K. P., Watson, I. J., Fitter, A. H., Young, P. W. (2003). Co-existing grass species have distictive arbuscular mycorrhizal communities. *Mol Ecol* **12**, 3085-3095.

Villa-Carvajal, M., Coque, J. J., Álvarez-Rodriguez, M. L., Uruburu, F., Belloch, M., (2004). Polyphasic identification of yeasts isolated from bark of cork oak during the manufacturing process of cork stoppers. *FEMS Yeast Research* **4**, 745-750.

Watanabe, I., Kashimoto, T., Tatsukawa, R. (1983). Polybrominated anisoles in marine fish, shellfish and sediments in Japan. *Arch Environ Contam Toxicol* **12**, 615-619.

Weindenboerner, M., Wieczorek, C., Appel, S., Kunz, B. (2000). Whole wheat and white wheat flour- the mycobiota and potencial mycotoxins. *Food Mycotoxins* **17**, 103-107.

WWF (2006). Cork screwed? Environmental and economic impacts of the cork stoppers market. In *Report*. Rome: WWF/MEDPO

(http://archive.audubonmagazine.org/features0701/habitat.html (accessed May 8, 2011).

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