Fungos endofíticos de *Arbutus unedo* L.: diversidade, propriedades antimicrobianas e composição volátil

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Índice

Resumo

As plantas medicinais têm sido reconhecidas como um repositório de fungos endófitos com novos metabólitos de importância antimicrobiana. *Arbutus unedo* L. é uma planta endémica do Mediterrâneo amplamente utilizada na medicina popular. Este estudo tem como objetivo avaliar a diversidade de fungos endófitos associados às partes desta planta mais frequentemente utilizadas para fins medicinais (raiz, folha, caule e casca) e de explorar o seu potencial antimicrobiano. Os compostos voláteis produzidos por endófitos foram identificados por cromatografia gasosa/espectrometria de massa e ainda correlacionadas com a atividade antimicrobiana. Foi obtido um total de 288 isolados pertencentes a 118 espécies. *Cryptosporiopsis diversispora* foi a espécie mais comum, representando 26% do total dos isolados, com uma taxa de colonização global de 3,4%. A diversidade e a frequência de colonização de fungos endófitos foram significativamente maiores na casca, face às outras partes da planta. A composição das espécies mostrou ser dependente do tipo de órgão. Entre as cinco espécies selecionadas para avaliar a sua atividade antimicrobiana, apenas *C. diversispora* e *Penicillium* sp. 3, inibiram significativamente agentes patogénicos humanos quando comparado com os medicamentos comerciais. *Cryptosporiopsis diversispora* foi o mais eficaz na inibição de leveduras (até 1,9 vezes quando comparado com o fluconazol, 25 μg/mL) e de bactérias gram-negativas (até 2,4 vezes quando comparado com o cloranfenicol, 30 μg/mL), enquanto *Penicillium* sp. 3 foi mais eficaz na inibição de ambas as bactérias gram-negativas e gram-positivas (até 2,0 vezes e 2,5 vezes quando comparado com o cloranfenicol, 30 μg/mL, respectivamente). A composição volátil do fungo que apresentou a maior (*C. diversispora*) e menor (*Penicillium glabrum*) atividade antimicrobiana revelou a presença de 22 compostos pertencentes a diferentes classes químicas (10 sesquiterpenos, 7 álcoois, 3 ésteres e 2 cetonas). A comparação do perfil volátil dessas duas espécies associadas com a análise de componentes principais sugeriu que a atividade antimicrobiana apresentada por *C. diversispora* pode ser atribuída à sua composição volátil, em particular ao seu elevado teor em voláteis antimicrobianos 3-metil-1-butanol e álcool fenetílico. Além de fornecer novas prespetivas sobre fungos endófitos de *A. unedo*, o presente trabalho mostrou o potencial desses fungos para produzir compostos antimicrobianos.

Palavras-chave: medronheiro, órgãos da planta, *Cryptosporiopsis diversispora*, bactérias, leveduras, HS-SPME/GC/MS.

Abstract

Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of antimicrobial importance. *Arbutus unedo* L. is a Mediterranean endemic plant widely employed in folk medicine. This study aims to assess the diversity of fungal endophytes inhabiting the parts most often used for medicinal purposes (root, leaf, twig and bark) of this plant and to explore their antimicrobial potential. The volatile compounds produced by endophytes were identified by gas chromatography/mass spectrometry and further correlated with the antimicrobial activity. A total of 288 endophyte isolates belonging to 118 fungal species were isolated. *Cryptosporiopsis diversispora* was the most common specie, accounting 26% of the total isolates, with an overall colonization rate of 3.4%. The diversity and the colonization frequency of endophytic fungi were significantly greater on bark in comparison to the other plant parts. The species composition was found to be dependent on the organ type. Among the five species selected for screen their antimicrobial activity, only *C. diversispora* and *Penicillium* sp. 3, inhibit significantly human pathogens when compared to commercial drugs. *Cryptosporiopsis diversispora* was the most effective in inhibiting yeasts (up to 1.9-fold when compared to fluconazole, 25 µg/mL) and gram-negative bacteria (up to 2.4-fold when compared to chloramphenicol, 30 µg/mL), whereas *Penicillium* sp. 3 was most effective in inhibiting both gram-negative and gram-positive bacteria (up to 2.0 fold and up to 2.5-fold when compared to chloramphenicol, 30 µg/mL, respectively). The volatile composition of the fungi that displayed the highest (*C. diversispora*) and the lowest (*Penicillium glabrum*) antimicrobial activity revealed the presence of 22 compounds belonging to different chemical classes (10 sesquiterpenes, 7 alcohols, 3 esters and 2 ketones). The comparison of the volatile profile of these two species associated to the principal component analysis suggested that the antimicrobial activity displayed by *C. diversispora* may be ascribed to their volatile composition, in particular to the high content in the antimicrobial volatiles 3-methyl-1-butanol and phenylethyl alcohol. In addition to provide new insights into fungal endophyte of *A. unedo*, the present work showed the potential of these fungi to produce antimicrobial compounds.

Key-words: Strawberry tree, plant organs, *Cryptosporiopsis diversispora*, bacteria, yeasts, HS-SPME/GC/MS.

Capítulo 1

Enquadramento e Objetivos

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A bioprospeção de fungos endofíticos de plantas medicinais constitui uma atividade promissora na pesquisa e deteção de novos compostos com atividade biológica de interesse a nível farmacêutico e medicinal. Este facto deve-se sobretudo por estes fungos representarem uma fonte rica e diversa de compostos bioativos naturais, contribuindo para a produção de uma vasta gama de metabolitos biologicamente ativos, tais como antibióticos, compostos antitumorais, imunossupressores, antivirais, agentes antiparasíticos e compostos inativadores de enzimas (Gunatilaka 2006; Olano et al. 2008; Donnez et al. 2009). A pesquisa destes microrganismos em plantas medicinais tem sido alvo de intensos estudos nos últimos anos por se acreditar que algumas das propriedades terapêuticas atribuídas a estas plantas possam estar relacionadas com a produção de metabolitos secundários pelos fungos endofíticos.

O medronheiro (*Arbutus unedo* L.) é um arbusto nativo da região mediterrânica, encontrando-se disseminado por todo o território nacional, inclusive em Trás-os-Montes. São várias as partes desta planta que têm reconhecida aplicação fitoterapêutica, destacando-se em especial as folhas, frutos, cascas e raízes (Oliveira et al., 2011).

Tanto quanto é do nosso conhecimento, estudos sobre a diversidade de fungos endofíticos associados ao medronheiro e consequentemente das suas propriedades bioativas nunca foram realizados até ao momento. Estudos neste âmbito são de extrema importância uma vez que os fungos endofíticos podem também ser os responsáveis pelas propriedades medicinais atribuída ao medronheiro. O desconhecimento da diversidade fúngica associada às suas potencialidades bioativas, sugere a existência de uma grande quantidade de compostos bioativos naturais que se encontram ainda por explorar. Até ao momento foram descritos mais de 20 000 compostos naturais novos e bioativos obtidos de fungos endofíticos (Ownley et al., 2010), e 51% destes apresentam estruturas inéditas (Yang et al., 2012).

Neste contexto, o presente trabalho teve como objetivo geral isolar fungos endofíticos de diversos órgãos do medronheiro e avaliar o seu potencial antimicrobiano. Especificamente pretendeu-se:

1- Isolar e avaliar a diversidade de fungos endofíticos de folhas, ramos, raízes e casca do medronheiro;
2- Caracterizar molecularmente os isolados fúngicos com maior abundância, pela sequenciação da região espaçadora transcrita interna (ITS) do rDNA;
3- Avaliar a atividade antimicrobiana dos isolados fúngicos mais abundantes;
4- Identificar potenciais compostos voláteis que possam ser responsáveis pela atividade antimicrobiana exibida pelos endófitos, por GC/MS.

Espera-se que este estudo seja um contributo para a identificação de espécies de fungos endofíticos com propriedades antimicrobianas, que possam no futuro vir a ser exploradas no desenvolvimento de novos antibióticos/antifúngicos.

A dissertação foi elaborada no formato de artigo científico (capítulo 3), ao qual se incluiu um capítulo inicial introdutório (capítulo 2) e, no fim, um capítulo de conclusões gerais (capítulo 4). Na introdução será feita referência aos fungos endofíticos (definição e sua importância a nível medicinal, em especial ao seu potencial antimicrobiano) e ao procedimento a seguir na bioprospeção de metabolitos bioativos produzidos por estes fungos. São ainda referidas as propriedades medicinais atribuídas às diferentes partes do medronheiro (fruto, folha, raiz e casca).

Capítulo 2

Introdução

2.1. Fungos endofíticos

2.1.1. Definição

A palavra endófito significa Endo: dentro e Fito: planta. Desta forma, os fungos endofíticos caracterizam-se por colonizarem os tecidos internos dos órgãos das plantas (como folhas, ramos, raízes, frutos, sementes e flores), sem aparentemente causarem quaisquer danos no hospedeiro (Hyde e Soytong, 2008). Neste tipo de associação o fungo recebe nutrientes e proteção da planta hospedeira. Por sua vez o fungo confere à planta hospedeira resistência / tolerância a stresses bióticos (patogénicos, herbívoros, entre outros) e abióticos (secura, salinidade, metais pesados, entre outros) (Soliman et al., 2013). O carácter assintomático da interação planta – fungo endófito resulta de um equilíbrio que varia do mutualismo ao antagonismo, havendo sempre um grau de virulência por parte do microrganismo e, ao mesmo tempo, defesa por parte da planta hospedeira (Schulz e Boyle, 2005). Nesta interação ocorre a produção de metabolitos que vão desempenhar funções importantes em ambos os intervenientes: os fungos secretam enzimas e outros metabolitos necessários ao processo de infeção; por sua vez, a planta produz metabolitos responsáveis pela contenção da infeção (Schulz e Boyle, 2005). Desta forma, durante a interação os fungos endofíticos induzem alterações morfológicas, fisiológicas e bioquímicas na planta hospedeira melhorando o seu desempenho em condições de stresse e estimulam o seu crescimento (Schulz e Boyle, 2005).

Os fungos endofíticos são ubíquos tendo sido observada a sua presença em todas as espécies de plantas estudadas até ao momento. Por exemplo, foi já descrita a sua presença em plantas pertencentes ao grupo das pteridófitas (Del Olmo-Ruiz e Arnold, 2014), angiospérmicas (Santiago et al., 2012), briófitas (U'Ren et al., 2011) e gimnospérmicas (Soca-Chafze et al., 2011). A maioria das espécies fúngicas endofíticas colonizadoras destas plantas pertence ao filo Ascomycota e, em menor número, aos filos Basidiomycota e Deuteromycota.

De acordo com Kumar e Hyde (2004), os fungos endofíticos estão associados aos mais diversos órgãos e tecidos vegetais, incluindo folhas, ramos, caules, raízes e estruturas florais como pólen, ovário, anteras e estames. A comunidade fúngica presente numa determinada planta hospedeira difere entre os vários tecidos e órgãos (Moricca et al., 2012). Este facto poderá estar relacionado com diferenças de

composição química entre os diferentes órgãos que, ao constituírem microhabitats distintos, permitem apenas o desenvolvimento de uma determinada espécie fúngica endofítica.

Estima-se que a diversidade de fungos endofíticos seja enorme, cerca de 1,5 milhões de espécies. Deste total, apenas 10% foram descobertas e estudadas até ao momento, e apenas 1% foram alvo de estudo quanto à sua capacidade de produção de metabolitos secundários (Guo et al., 2008). Estudos mais recentes, propõem que a diversidade de fungos seja muito superior, ou seja, de aproximadamente 5,1 milhões de espécies (Blackwell, 2011). Normalmente, numa única planta ocorrem dezenas de espécies fúngicas endofíticas, tendo sido verificado a ocorrência de mais do que uma espécie por 2 mm^2 de tecido foliar (Gamboa et al., 2002).

2.1.2. Importância de fungos endofíticos como produtores de compostos antimicrobianos

Durante a interação fungo endofítico – planta hospedeira ocorre a produção de metabolitos que favorecem o crescimento vegetativo e a competitividade do hospedeiro. Prevê-se que a diversidade destes metabolitos seja elevada dada a multiplicidade de vias biossintéticas que podem ocorrer durante a associação mutualista (Carter, 2011). A variabilidade de fatores bióticos (tais como as espécies envolvidas na interação) e abióticos (como por exemplo fatores ambientais) contribuem também para o incremento da diversidade de metabolitos produzidos durante a interação fungo endofítico-planta (Soliman et al., 2013). Face ao exposto, os fungos endofíticos constituem uma importante fonte de compostos bioativos naturais com aplicação em diversas áreas, nomeadamente na farmacêutica. A bioprospeção de novos compostos antimicrobianos ao nível de fungos endofíticos reveste-se, neste âmbito, de enorme importância. Na atualidade, muitos dos antibióticos e antifúngicos disponíveis no mercado têm-se mostrado ineficazes no tratamento de infeções causadas por bactérias e fungos, respetivamente. O aumento do aparecimento de estirpes bacterianas multirresistentes a antibióticos, devido sobretudo ao seu uso irracional, é a principal causa da ineficácia destes agentes terapêuticos (ECDC, 2012). Até o momento, são considerados, pela comunidade científica internacional, patogénicos multirresistentes as espécies *Enterococcus* spp., *Staphylococcus* spp., *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, e Enterobactérias, destacando-se entre estas a *Klebsiella pneumoniae* e as espécies de *Enterobacter* spp. (Boucher et

al., 2009). A dificuldade de eliminar infeções provocadas pelos microrganismos *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* e *Enterobacter* spp., devido aos diversos mecanismos de escape que apresentam, fez com que se atribuísse a este grupo de microrganismos o acrónimo de ESKAPE (Boucher et al., 2009). Este termo também pretende alertar para a necessidade da descoberta de novos compostos antimicrobianos capazes de eliminar as infeções por eles causadas. Apesar da necessidade da descoberta de novos medicamentos antimicrobianos para o tratamento de infeções resistentes, o sector farmacêutico não tem investido o suficiente nesta área. Para fazer face a esta problemática, a *Infectious Diseases Society of America* (IDSA), lançou uma iniciativa que apoia o desenvolvimento de 10 novas classes de antibióticos até 2020 (Wright, 2012). Os fungos endofíticos constituem uma enorme fonte de compostos naturais que poderiam ser explorados no desenvolvimento de novos compostos antimicrobianos.

Os diversos estudos efetuados ao nível da prospeção de metabolitos secundários em fungos endofíticos permitiram identificar, até ao momento, mais de 20 000 compostos bioativos (Ownley et al., 2010). Estes compostos pertencem a várias classes tais como alcalóides, terpenóides, flavonóides, esteróides, terpenos, isocumarinas, quinonas, fenilpropanóides, lignanas, ácidos fenólicos, entre outros (Zhang et al., 2006; Yu et al., 2009). As atividades biológicas apresentadas por estes compostos são muito diversas incluindo, por exemplo, antimicrobiana, antiparasitária, neuroprotetiva, antioxidante, antidiabética, imunossupressora, antiviral, anticolinesterásica, antineoplásicos e citotóxica (Ondeyka et al., 1997 ; Zhang et al., 1999 ; Guo et al., 2000 ; Zhang et al., 2006 ; Shweta et al., 2010 ; Aly et al., 2010, 2011; Wang et al., 2012).

No que concerne especificamente aos metabolitos secundários produzidos por fungos endofíticos e relacionados com atividade antimicrobiana, destacam-se sobretudo os compostos fenólicos (e.g. quinonas e flavonóides), alcalóides, peptídeos, terpenóides e esteróides (Yu et al., 2009) e, ainda, compostos voláteis tais como ésteres, lípidos, álcoois, ácidos orgânicos, cetonas, entre outros (Banerjee et al., 2010; Kudalkar et al., 2012).

Os compostos fenólicos são metabolitos secundários aos quais se encontram associados propriedades bioativas, sendo as propriedades antimicrobianas um dos exemplos. São vários os autores a evidenciar estas propriedades em metabolitos

produzidos por fungos endofíticos. Hoffman et al. (2008) verificaram que um grupo de compostos fenólicos (designados por ácido úsnico, cercosporamida e Phomodione) produzidos pelo fungo endofítico *Phoma sp.* isolado da planta medicinal *Saurauia scaberrinae* exibia atividade antibacteriana contra as bactérias *Staphylococcus aureus* e *Escherichia coli*. Similarmente Han et al. (2008) identificaram dois novos compostos fenólicos com atividade antimicrobiana contra *Staphylococcus aureus* resistentes a meticilina, produzidos pelo fungo endofítico *Penicillium sp*. isolado de *Cerbera martghas*. De entre os compostos fenólicos destacam-se os flavonóides. Um número alargado de famílias de compostos pertencentes aos flavonóides foi isolado do fungo endofítico *Nodulisporium sp.* de *Juniperus cedre* e observada a sua ação antimicrobiana contra *Bacillus megaterium*, *Microbotryum violaceum, Septoria tritici* e *Chlorella fusca* (Dai et al., 2006). Aly et al. (2008) identificaram novos metabolitos antimicrobianos da classe das quinonas no fungo endofítico *Ampelomyces sp*., isolado da planta medicinal *Urospermum picroides*. Estas quinonas apresentavam atividade antibacteriana contra *Staphylococcus aureus, S. epidermidis* e *Enterococcus faecalis*.

Os alcalóides são metabolitos secundários produzidos vulgarmente por fungos endófitos e, alguns dos quais, apresentam atividade antimicrobiana. Alguns exemplos de compostos alcalóides com esta propriedade incluem: chaetoglobosinas A e C, produzido pelo endófito *Chaetomium globosum* isolado de *Ginkgo biloba* que mostrou atividade antibacteriana contra *Mucor miehei* (Qin et al., 2009); pirrocidinas A e B, produzido pelo fungo *Acremonium zeae* isolado de grãos de milho (*Zea mays*), que apresentou atividade antifúngica contra *Aspergillus flavus, Fusarium verticillioides* e *Candida albicans* e antibacteriana contra várias estirpes de bactérias gram-positivas, incluindo estirpes multirresistentes a antibióticos (Wicklow et al., 2005; Wicklow e Poling, 2009); *Phomoenamide* sintetizado pelo fungo *Phomopsis sp*. isolado de *Garcinia dulcis*, que apresentou atividade antimicrobiana significativa contra *Mycobacterium tuberculosis* (Rukachaisirikul et al., 2008).

Os peptídeos produzidos por fungos endófitos têm igualmente exibido atividade antimicrobiana. A título de exemplo destaca-se a cryptocandina, lipopeptídeo antimicótico, produzido pelo fungo *Cryptosporiopsis quercina* isolado de *Tripterigium wiflordii* que mostrou inibir *C. albicans* (Strobel et al., 1999). Um grupo de peptídeos da classe das equinocandinas (A, B, C, D, H) produzidos pelo fungo *Cryptosporiopsis sp*. e *Pezicula sp*. isolados a partir de *Pinus sylvestris* e *Fagus sylvatica* mostraram atividade antifúngica contra leveduras (Noble et al., 1991). A

partir da cultura do fungo endofítico *Penicillium* sp. isolado de *Acrostichum aureum*, Cui et al. (2008), identificaram 2 novos peptídeos cíclicos, (Pro-Thr) e (Pro-Tyr), que mostraram ação antimicrobiana contra *Staphylococcus aureus* e *Candida albicans*.

Os sesquiterpenos, diterpenos e triterpenóides são os principais terpenóides isolados de fungos endófitos (Yu et al., 2009). Os quatro antibióticos diterpenóides novos, designados por guanancastepene A, guanacastepene, periconicin A e perieoniein B, são apenas alguns exemplos de terpenóides produzidos por fungos endofíticos e com reconhecida atividade antimicrobiana (Brady et al., 2000, 2001; Kim et al., 2004). Similarmente foi observada a ação antimicrobiana de cinco sesquiterpenos (cadinenos) produzidos pelo fungo endofítico *Phomopsis cassiae* isolado de *Cassia spectabilis* (Silva et al., 2006).

Alguns compostos produzidos por fungos endofíticos pertencentes à classe química dos esteróides tem exibido ação antimicrobiana contra fungos e bactérias. De entre os compostos produzidos e com atividade antimicrobiana destacam-se o ergosterol e 5α, 8α-epidioxyergosterol isolado do fungo *Nodulisporium sp.* colonizador de *Juniperus cedre* (Dai et al., 2006). Foi ainda identificada uma série de esteróides novos na espécie fúngica *Colletotrichum sp*. isolada de *Artemisia annua* que apresentou uma significativa atividade antimicrobiana contra os fitopatogénicos *Phytophthora capisici*, *Rhizoctonia cerealis*, *Gaeumannomyces graminis var. tritici* e *Helminthosporium sativum* (Lu et al., 2000).

No que concerne aos compostos voláteis, Mitchell et al. (2010) verificaram que o fungo endófito *Muscodor crispans* isolado de *Ananas ananassoides* produzia uma mistura de voláteis, a maioria ácido propanóico, 2-metil-metil éster, 2-metil-ácido propanóico, 3-metil-1 butanol, acetato de 3-metil-1-butanol e etanol, que inibiam o crescimento de microrganismos fitopatogénicos e patogénicos de humanos, incluindo *Yersinia pestis*, *Mycobacterium tuberculosis* e *Staphylococcus aureus*. De igual modo, foi observado que a mistura de compostos voláteis, na sua maioria naftaleno, 2-metil-ácido propanóico e éster metílico do ácido propanóico, produzido pelo endófito *Muscodor* sp. inibia diversos microrganismos fitopatogénicos e patogénicos de humanos, como *Escherichia coli* (Zhang et al., 2010).

De uma maneira geral, estes estudos demonstram o potencial antimicrobiano dos fungos endofíticos. Alguns autores demonstraram que a percentagem de isolados endofíticos com propriedades antimicrobianas poderia ser, em determinados casos, superiores a 30% (Mussi-Dias et al., 2012). Por exemplo, Vaz et al. (2009) avaliaram

a atividade antimicrobiana de fungos endofíticos associados à *Orchidaceae* e verificaram que 33% das espécies isoladas inibiam pelo menos um dos microrganismos analisados.

2.2. Bioprospeção de metabolitos com atividade antimicrobiana em fungos endofíticos

A bioprospeção de metabolitos com atividade antimicrobiana em fungos endofiticos envolve geralmente quatro etapas: seleção da planta hospedeira para isolamento dos fungos endofíticos, isolamento e identificação de fungos endofíticos, avaliação da sua atividade antimicrobiana e finalmente elucidação da natureza química do metabolito (ou metabolitos) responsável por esta propriedade. Cada uma destas etapas é a seguir discriminada.

2.2.1. Seleção da planta para isolamento de fungos endofíticos

Muitas propriedades que eram atribuídas à produção de substâncias ativas pelas plantas medicinais, foram recentemente verificadas que estão na realidade relacionadas com os endofíticos que as colonizam (Ji et al., 2005; Kusari et al., 2012). O composto antitumoral, maytansinoid (ansamitocin), é um exemplo tendo sido originalmente isolado de plantas pertencentes às famílias *Celastraceae*, *Rhamnaceae* e *Euphorbiaceae*, mas cuja produção parece estar relacionada com um endofítico (Yu et al., 2002). Vários estudos têm, igualmente, demonstrado a capacidade dos fungos endofíticos de sintetizarem metabolitos secundários idênticos ou semelhantes aos produzidos pelas plantas hospedeiras. Por exemplo, o paclitaxel (Taxol®), uma substância isolada de plantas do género *Taxus* e utilizada na terapia do cancro da mama e do útero, já foi identificado em vários géneros de fungos que colonizam espécies vegetais produtoras desse metabolito (Zhou et al., 2010). Estas descobertas são extremamente importantes do ponto de vista biotecnológico e ecológico uma vez que para extrair 1 kg de Taxol a partir das cascas da planta, são necessárias cerca de 1000 árvores com 100 anos de idade, o que levou quase à extinção desta importante planta medicinal (Stinson et al., 2003). Os fungos endofíticos, com capacidade de sintetizar os compostos das suas plantas hospedeiras, sob condições ótimas de cultura, pode ser um meio para a obtenção de compostos bioativos a baixo-custo, ecológico, reprodutível e compatível com a sua exploração industrial (Kusari et al., 2012). Esta

capacidade do fungo endofítico em biossintetizar metabolitos originalmente produzidos pela planta hospedeira pode dever-se à transferência de genes da planta para o fungo ou vice-versa (Kusari e Spiteller, 2011).

O facto das atividades biológicas e os metabolitos produzidos pelos fungos endofíticos se encontrar associado à planta hospedeira levou vários autores a adotar uma nova abordagem na pesquisa de atividade das plantas medicinais (Kusari et al., 2008). Nesta pesquisa, para além dos extratos vegetais, também os microrganismos endofíticos presentes na planta medicinal deverão ser estudados (Kusari et al., 2008). Face ao exposto, os endófitos e, em especial os colonizadores de plantas medicinais, por sintetizarem metabolitos secundários semelhantes às suas plantas hospedeiras, são considerados recursos naturais com grande potencial para a descoberta de novos compostos bioativos (Kusari et al., 2008). Assim, quando se têm em vista a exploração dos metabolitos de fungos endofíticos na área medicinal é aconselhável que estes sejam isolados de plantas que apresentem um histórico etnobotânico, ou seja, que sejam plantas medicinais. Strobel e colaboradores (2004), sugerem que devem estar também incluídas nesta seleção, plantas de ambientes peculiares, especialmente aquelas que apresentam estratégias de sobrevivência pouco comuns; plantas endémicas de determinadas regiões que apresentam longevidade incomum e que estão localizadas em ambientes ancestrais; plantas cujo desenvolvimento ocorre em áreas de grande biodiversidade, como florestas temperadas e tropicais; e plantas infetadas por fitopatogénicos e que não demonstrem sintomatologia. Estas plantas encontram-se colonizadas por fungos endofíticos que produzem compostos antimicrobianos. Por exemplo, Tuntiwachwuttikul et al. (2008) verificaram que o fungo endofitico *Colletotrichum musae* exibia atividade antimicrobiana contra agentes fitopatogénicos.

2.2.2. Isolamento e identificação de fungos endofíticos

Os fungos endofíticos podem ser isolados dos diferentes órgãos da planta (folha, caule, raiz, casca, entre outros). O material vegetal, depois de colhido no campo, é lavado em água corrente, fragmentado e submetido a um processo de esterilização (Hallmann et al., 2006). Neste processo, o material vegetal é exposto a um agente de desinfeção, sendo os mais vulgares etanol e lixívia, seguida por lavagens em água destilada estéril (Qadri et al., 2013). As concentrações, bem como o tempo de

exposição são variáveis de acordo com o material vegetal. Os fragmentos que foram sujeitos ao processo de desinfeção são colocados em caixas de Petri contendo meio Batata Dextrose Agar (PDA), visto que este é o meio que normalmente se utiliza para o isolamento de fungos, podendo também ser isolado em outros meios. No meio pode-se ainda adicionar um antibiótico (Hallmann et al., 2006). A fim de promover o crescimento de espécies fúngicas especificas da planta, por vezes, são adicionados ao meio de cultura, tecidos ou extratos vegetais da planta hospedeira (Arnold et al., 2003). Posteriormente, as caixas são incubadas numa estufa a 25ºC (no escuro), vigiadas diariamente para avaliação do crescimento fúngico. Aquando do desenvolvimento das colónias, estas são repicadas até se obter culturas puras, em meio PDA (Hallmann et al., 2006).

A identificação de fungos endofíticos é feita com base na avaliação das características morfológicas das colónias fúngicas, micélio e estruturas reprodutivas (Devie e Prabakaran, 2014), complementado com a caracterização molecular (Huang et al., 2009; Lu et al., 2012). O método molecular mais utilizado baseia-se na amplificação da região espaçadora transcrita interna (ITS, *Internal transcribed spacer*) do DNA que contém o conjunto de genes que codificam o RNA ribossómico (rRNA), seguida da sua sequenciação (Huang et al., 2009; Lu et al., 2012). O conjunto de genes de rRNA existe em múltiplas cópias e encontram-se alinhados em *tandem* (Martin e Rygiewicz, 2005). Cada unidade repetitiva do rDNA é constituída por (i) regiões codificantes conservadas, correspondentes aos três genes 18S, 5,8S e 26S; (ii) regiões não codificantes, que correspondem aos espaçadores internos transcritos (ITS) e espaçadores intergénicos não transcritos (IGS), que separam as diferentes unidades de transcrição (Figura 1) (Martin e Rygiewicz, 2005). De entre as regiões codificantes, o gene 26S apresenta sequências menos conservadas face aos genes 18S e 5,8S. O par de iniciadores nucleotídicos frequentemente usados para amplificar a região ITS em fungos, recorrendo à reação da polimerase em cadeia (PCR), é ITS1 (5'-TCCGTAGGTGAACCTGCGG-3′) e ITS4 (5′-TCCTCCGCTTATTGATATGC-3) (Martin e Rygiewicz, 2005). Esta região corresponde a um fragmento do rDNA que inclui o gene 5,8S e as regiões ITS1 e ITS2 (Huang et al., 2009; Lu et al., 2012), e foi recentemente aceite como a região "barcoding" (código de barras do DNA) de fungos (Schoch et al., 2012). As diferenças na região ITS são detetadas normalmente por sequenciação dos produtos

amplificados, utilizando neste processo o mesmo par de iniciadores nucleotídicos usados na amplificação.

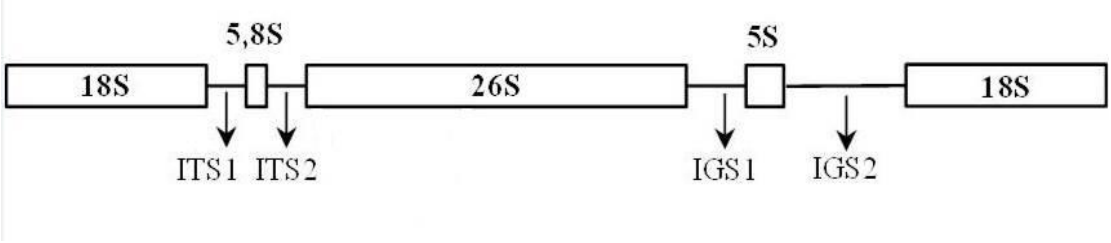

Figura 1 - Representação esquemática da região de rDNA, que contém as unidades repetitivas dos genes que codificam o RNA ribossómico nuclear. Os retângulos representam os genes com sequências conservadas, enquanto as linhas representam as regiões espaçadoras mais variáveis.

2.2.3. Avaliação da atividade antimicrobiana

A composição do meio de cultura e as condições ambientais onde o fungo é posto a crescer afeta significativamente a produção de metabolitos secundários, uma vez que pode favorecer ou não a produção de substâncias de interesse (Berdy et al, 2005). Assim sendo, a avaliação de qualquer propriedade bioativa de fungos endofíticos requer a otimização prévia das condições de crescimento do fungo. As condições ótimas para o crescimento e produção de metabolitos variam muito de espécie para espécie. A fonte de carbono e azoto do meio de cultura, assim como a temperatura, arejamento, tempo de cultura, são exemplos de alguns factores que influenciam o crescimento e a produção de metabolitos secundários por fungos endofíticos (Scherlach e Hertweck, 2009; Tayung et al., 2011; VanderMolen et al., 2013). Alguns fungos requerem ainda elicitores para poderem biossintetizar certos compostos de interesse. Por exemplo, muitas vezes é adicionado ao meio de cultura extratos da planta hospedeira com o intuito de incrementar a produção de metabolitos bioativos pelo fungo endofítico (Yenn et al., 2012).

Existe uma grande variedade de métodos que podem ser utilizados para avaliar a atividade antimicrobiana de fungos endofíticos ou dos seus extratos. De entre estes, os mais utilizados são o método de difusão em agar e o método da microdiluição. O primeiro método é considerado qualitativo, uma vez que normalmente é utilizado para demonstrar a presença ou ausência de compostos com atividade antimicrobiana. Por

sua vez, o método da microdiluição é considerado quantitativo por permitir a determinação da concentração mínima inibitória (CMI).

O método de difusão em agar baseia-se na deposição sobre meio de cultura gelificado, previamente inoculado com bactérias ou leveduras, de um papel de filtro embebido em extrato fúngico endofítico preparado a partir do meio de cultura ou do micélio (Lu et al., 2000; Hoffman et al., 2008). Alternativamente, os extratos podem ser depositados em poços efetuados no meio de cultura sólido, realizados com cilindros de 6-8 mm de diâmetro (Krohn et al. 2009; Xing et al., 2011). A difusão do extrato fúngico leva à formação de um halo de inibição de crescimento microbiano, cujo diâmetro é indicativo da atividade antimicrobiana. O valor de halo estimado é sempre comparado com um controlo positivo, onde se utiliza antibióticos ou antifúngicos, de acordo com o microrganismo testado (bactéria ou fungo/levedura, respetivamente). Como controle negativo utiliza-se o solvente utilizado para a dissolução dos extratos, normalmente dimetilsulfóxido (DMSO).

Na microdiluição utilizam-se microplacas com 96 poços, onde é colocado um volume de meio de cultura (entre 0,1 e 0,2 mL), e um volume do extrato a analisar de forma a obter um gradiente crescente de concentrações de extrato (Hu et al., 2010; Arivudainambi et al., 2011; Qadri et al., 2013). Cada poço é, em seguida, inoculado com o microrganismo (normalmente à concentração de 10^4 UFC/poço). São ainda reservados alguns poços da placa para efetuar o controlo positivo e negativo. As placas são postas a incubar a uma temperatura controlada, normalmente a 37ºC para bactérias e 27ºC para leveduras/fungos, durante 24 a 48h. Findo este tempo procede-se à avaliação do crescimento microbiano num leitor de microplacas a um determinado comprimento de onda.

2.2.4. Extração e identificação do metabolito bioativo

Para avaliação das possíveis aplicações biotecnológicas dos metabolitos que exibiram atividade antimicrobiana, é necessário proceder ao seu isolamento e identificação. Só desta forma se pode contribuir para a descoberta de novas moléculas com atividade antimicrobiana.

A elucidação da natureza química dos metabolitos produzidos pelo fungo endofítico envolve a sua extração seguida da sua identificação. Devido ao desconhecimento da natureza química dos metabolitos bioativos produzidos pelo

fungo endofítico, não é possível estabelecer-se uma técnica especifica que garanta a extração de todos os constituintes da mistura. Desta forma, a melhor abordagem consiste na extração destes compostos do meio de cultura liquido (metabolitos extracelulares) e/ou do micélio fúngico (metabolitos intracelulares) através do uso de solventes orgânicos com diferentes polaridades. Os solventes mais comuns utilizados no processo de extração, em ordem crescente de polaridade, são o hexano (Gao et al., 2011), acetato de etilo (Tayung et al., 2011) e metanol (Rukachaisirkul et al., 2008).

Após a extração os metabolitos são concentrados, normalmente por evaporação, e a sua atividade antimicrobiana é testada. Em caso de possuir atividade antimicrobiana contra microrganismos teste (bactérias e/ou leveduras), o composto é identificado e patenteado, podendo ser utilizado na forma de fármacos. No processo de identificação os metabolitos contidos no extrato são separados por técnicas cromatográficas, como por exemplo Sephadex ou gel de sílica (Jiao et al., 2013), ou mais frequentemente por cromatografia líquida de alta pressão (HPLC) (Sileshig et al., 2013) ou mesmo cromatografia gasosa (GC) (Tayung et al., 2011). Após separação, os metabolitos são identificados, recorrendo para tal a diversas técnicas, sendo as mais comuns a espectroscopia de infravermelho, ressonância magnética nuclear (Wang et al., 2012) e espectrometria de massa (MS) (Tayung et al., 2011).

2.3. Propriedades medicinais do medronheiro

O *Arbutus unedo L.*, é uma árvore de fruto que pertencente à família Ericacea, e ao género *Arbutus*. Encontra-se distribuída por vários países, nomeadamente todo o sul da Europa, norte de África e Palestina, podendo ainda ser encontrada em países como a Irlanda e Macaronésia (Canárias) (Celikel et al., 2008). Em Portugal, esta árvore existe a sul do rio Tejo nomeadamente na região das Serras do Caldeirão e Monchique (Algarve), podendo estar disseminada por todo país inclusive em Trás-os-Montes (Pedro, 1994).

Desde os tempos ancestrais que esta espécie é utilizada na medicina tradicional em países mediterrâneos, onde infusões e decocções de diferentes partes do medronheiro (folhas, raiz e casca) eram aplicadas contra variados problemas de saúde (Oliveira et al., 2011). As folhas desta planta são utilizadas em infusões, devido às suas propriedades antioxidantes, adstringentes, diuréticas, antissépticas, antidiarreicas e depurativas. As folhas são também muito importantes para o

tratamento de doenças como hipertensão, diabetes e problemas inflamatórios (Ziyyat e Boussairi, 1998; Afkir et al., 2008; Mariotto et al., 2008). Na medicina popular, aproveitam-se também as cascas e as raízes, para o tratamento de distúrbios gastrointestinais e urológicos e de problemas dermatológicos (Novais et al., 2004; Leonti et al., 2009). O fruto (medronho) é também muito utilizado na medicina popular devido às suas propriedades diuréticas, antissépticas das vias urinárias e laxativas (Ziyyat et al., 1997; Ziyyat e Boussairi, 1998; Mariotto et al., 2008, Afkir et al., 2008; Pallauf et al., 2008; Oliveira et al., 2011). São ainda utilizados na terapia de diversas doenças, como por exemplo distúrbios gastrointestinais, dermatológicos, urológicos, cardiovasculares (Leonti et al., 2009), doenças renais (El Hilaly et al., 2003) e gastrite (Cornara et al., 2009).

Bibliografia

Afkir, S., Nguelefack, T.B., Aziz, M., Zoheir, J., Cuisinaud, G., Bnouham, M., Mekhfi, H., Legssyer, A., Lahlou, S., Ziyyat, A. (2008). *Arbutus unedo* prevents cardiovascular and morphological alterations in L-NAME-induced hypertensive rats. Part I: Cardiovascular and renal hemodynamic effects of *Arbutus unedo* in L- NAME-induced hypertensive rats. Journal of Ethnopharmacology, 116: 288–295.

Aly, A.H., Debbab, A., Kjer, J., Proksch, P. (2010). Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Divers, 41:1–16.

Aly, A.H., Edrada-Ebel, R., Wray, V., Muller, W.E.G., Kozytska S, Hentschel U, et al. (2008). Bioactive metabolites from the endophytic fungus *Ampelomyces sp.* isolated from the medicinal plant *Urospermum picroides*. Phytochemistry, 69: 1716–25.

Arivudainambi, U.S., Anand, T.D., Shanmugaiah, V., Karunakaran, C., Rajendran, A. (2011). Novel bioactive metabolites producing endophytic fungus *Colletotrichum gloeosporioides* against multidrug-resistant *Staphylococcus aureus*. FEMS Immunology and Medical Microbiology, 61: 340–345.

Arnold, A.E., Mejía, L.C., Kyllo, D., Rojas, E.I., Maynard, Z., Robbins, N., and Herre, E.A. (2003). Fungal endophytes limit pathogen damage in a tropical tree. PNAS, (100 (26)): 15649-15654.

Banerjee D., Strobel G., Geary B., Sears J., Ezra D., Liarzi O., Coombs J. (2010) Muscodor albus strain GBA, an endophytic fungus of *Ginkgo biloba* from the United States of America, produces volatile antimicrobials. Mycology, 1:179–186.

Berdy, J. (2005). Bioactive microbial metabolites. J Antibiot (Tokyo), 58: 1–26.

Blackwell, M. (2011). The fungi: 1, 2, 3 … 5.1 Million Species? American Journal of Botany, 98(3): 426–438.

Boucher, H.W., Talbot, G.H., Bradley, J.S., et al. (2009). Bad Bugs, No Drugs: no ESKAPE! an update from the Infectious Diseases Society of America. Clinical Infectious Diseases, 48(1): 1-12.

Brady, S.F., Bondi, S.M, Clardy J. (2001). The Guanacastepene: A highly diverse

family of secondary metabolites produced by an endophytic fungus. Journal of the American Chemical Society, 123: 9900–1.

Brady, S.F., Singh, M.P., Janso, J.E., Clardy, J.J. (2000). Guanacastepene, a fungal derived diterpene antibiotic with a new carbon skeleton. Journal of the American Chemical Society, 122: 2116–7.

Carter, G.T. (2011). Natural products and Pharma 2011: strategic changes spur new opportunities. Nat. Prod. Rep, 28: 1783.

Celikel, G., Demirsoy, L., Demirsoy, H. (2008). The strawberry tree (*Arbutus unedo L.*) selection in Turkey. Scientia Horticulture, 118: 115–119.

Cornara, L., La Rocca, A., Marsili, S., Mariotti, M. (2009). Traditional uses of plants in the Eastern Riviera (Liguria, Italy). Journal of Ethnopharmacology, 125: 16-30.

Cui, H.B., Mei, W.L., Miao, C.D., Lin, H.P., Hong, K., Dai, H.F. (2008). Antibacterial constituents from the endophytic fungus *Penicillium sp.*0935030 of mangrove plant *Acrostichum aureurm*. Chemical Journal of Chinese Universities, 33: 407–10.

Dai, J.Q., Krohn,K., Flörke, U., Draeger, S., Schulz, B., Kiss-Szikszai, A., et al. (2006). Metabolites from the endophytic fungus *Nodulisporium* sp. from *Juniperus cedre*. European journal of organic chemistry, 3498–506.

Del Olmo-Ruiz, M., and Arnold A.E. (2014) Interannual variation and host affiliations of endophytic fungi associated with ferns at La Selva, Costa Rica. Mycologia, 106: 8-21.

Devi, NN., and Prabakaran, JJ. (2014). Bioactive metabolites from an endophytic fungus Penicillium sp. isolated from Centella asiatica. Current Research in Environmental & Applied Mycology, 4(1): 34-43.

Donnez, D., Jeandet, P., Clément, C., Courot, E. (2009). Bioproduction of resveratrol and stilbene derivatives by plant cells and microorganisms. Trends in Biotechnology, 27(12): 706 – 713.

ECDC - European Centre for Disease Prevention and Control (2012). Antimicrobial resistance surveillance in Europe 2011. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net), Stockholm: ECDC.

El-Hilaly, J., Hmammouchi, M., Lyoussi, B. (2003). Ethnobotanical studies and economic evaluation of medicinal plants in Taounate province (Northern

Morocco). Journal of Ethnopharmacology, 86: 149-158.

Gamboa, M.A., Laureano, S., Bayman, P. (2002). Measuring diversity of endophytic fungi in leaf fragments: does size. Matter Mycopathologia, 156: 41–45.

Gunatilaka, L. (2006). Natural Products from Plant-Associated Microorganisms: Distribuition, Structural Diversity, Bioactivity and Implications of their occurrence. Journal of Natural Products, 69: 509-526.

Guo, B., Dai J.R., Ng, S., Huang, Y., Leong, C., Ong, W., and Carte, B.K. (2000). Cytonic acids A and B: novel tridepside inhibitors of hCMV protease from the endophytic fungus *Cytonaema* species. J Nat Prod, 63: 602–604981.

Guo, B., Wang, Y., Sun, X., Tang, K. (2008). Bioactive Natural Products from Endophytes: A Review. Applied Biochemistry and Microbiology, 44(2): 136-142.

Hallmann, J., Berg, G., Schulz, B. (2006). Isolation Procedures for Endophytic Microorganisms. In Schulz B., Boyle C., Sieber T.N. (Eds.) Soil Biology, 9: 299-319.

Han, Z., Mei, W.L., Cui, H.B., Zeng, Y.B., Lin, H.P., Hong, K., et al. (2008). Antibacterial constituents from the endophytic fungus *Penicillium* sp. of mangrove plant *cerbera manghas*. Chemical Journal of Chinese Universities, 29(4): 749–752.

Hoffman, A.M., Mayer, S.G., Strobel, G.A., Hess, W.M., Sovocool, G.W., Grange, A.H., et al. (2008). Purification, identification and activity of phomodione, a furandione from an endophytic *Phoma* species. Phytochemistry, 69: 1049–56.

Hu, Z.Y., Li, Y.Y., Lu, C.H., Lin, T., Hu, P., Shen, Y.M. (2010). Seven novel linear polyketides from *Xylaria* sp. NCY2. Helvetica Chimica Acta, 93: 925–933.

Huang, W.Y., Cai, Y.Z., Surveswaran, S., Hyde, K.D., Corke, H., Sun, M. (2009). Molecular phylogenetic identification of endophytic fungi isolated from three *Artemisia* species. Fungal Diversity, (36): 69-88.

Hyde, K., Soytong, K. (2008). The fungal endophyte dilemma. Fungal Diversity, 33: 167 – 173.

Ji, Z.Q., Wu, W.J., Wang, M.A., Gu, A.G. (2005). Identification of fungicidal compounds from endophytic fungi *Fusarium proliferatum* in *celastrus angulatus*. Journal of North- west Sci-Tech University of Agriculture and Forest (Nat. Sci. Ed.), 33(5): 61–4.

Jiao, Y., Zhang, X., Wang, L., Li, G., Zhou, J.C., Lou, H.X. (2013). Metabolites from *Penicillium* sp., an endophytic fungus from the liverwort Riccardia multifida (L.) S. Gray. Gray, Phytochemistry Letters, 6: 14-17.

Kim, S., Sin, D.S., Lee, T., Oh, J.B. (2004). Periconicins two new fusicoccane diterpenes produced by an endophytic fungus *Periconia* sp. with antibacterial activity. Journal of Natural products, 67: 448–50.

Krohn, K., Kouam, S.F., Kuigoua, G.M., Hussain, H., Cludius-Brandt, S., Florke, U., Kurtan, T., Pescitelli, G., Di Bari, L., Draeger, S., Schulz, B. (2009). Xanthones and oxepino[2,3-b]chromones from three endophytic fungi. Chemistry – A European Journal, 15: 12121–12132.

Kudalkar, P., Strobel, G., Riyaz-Ul-Hassan, S., Geary, B., Sears, J. (2012). Muscodor sutura, a novel endophytic fungus with volatile antibiotic activities. Mycoscience, 53: 319–325.

Kumar, D.S.S., and Hyde, KD. (2004). Biodiversity and tissue-recurrence of endophytic fungi in *Tripterygium wilfordii*. Fungal Diversity, 17: 69-90.

Kusari, S., and Spiteller, M. (2011). Are we ready for industrial production of bioactive plant secondary metabolites utilizing endophytes? Nat. Prod. Rep, 28: 1203–1207.

Kusari, S., Hertweck, C,. and Spiteller, M. (2012). Chemical Ecology of Endophytic Fungi: Origins of Secondary Metabolites. Chemistry & Biology, 19: 792–798.

Kusari, S., Lamshoft, M., Zuhlke, S., Spiteller, M. (2008). An endophytic fungus from *Hypericun perfolatum* that produces hypericin. Journal of Natural Products, 71.

Leonti, M., Casu, L., Sanna, F., Bonsignore, L. (2009). A comparison of medicinal plant use in Sardinia and Sicily-De Materia Medica revisited? Journal of Ethnopharmacology, 121: 255-267.

Lu, H., Zou, W.X., Meng, J.C., Hu, J., Tan R.X. (2000) New bioactive metabolites produced by *Colletotrichum sp*., an endophytic fungus in *Artemisia annua*. Plant Science, 151: 67–73.

Lu, Y., Chen, C., Chen, H., Zhang, J., Chen, W. (2012). Isolation and Identification of Endophytic Fungi from *Actinidia macrosperma* and Investigation of Their Bioactivities. Evidence-Based Complementary and Alternative Medicine. (Article ID 382742), 8 pages.

Mariotto, S., Esposito, E., Di Paola, R., Ciampa, A., Mazzon, E., Carcereri de Prati,

A., Darra, E., Vincenzo, S., Cucinotta, G., Caminiti, R., Suzuki, H., Cuzzocrea, S. (2008). Protective effect of *Arbutus unedo* aqueous extract in carrageenaninduced lung inflammation in mice. Pharmacological Research, 57: 110–124.

Martin, K.J., and Rygiewicz, P.T. (2005). Fungal-specific PCR primers develop for analysis of the ITS region of environmental DNA extracts. BMC Microbiology, 5: 28.

Mitchell, A.M., Strobel, G.A., Moore, E., Robison, R., Sears, J. (2010). Volatile antimicrobials from *Muscodor crispans*, a novel endophytic fungus. Microbiology, 156: 270-277.

Moricca, S., Ginetti, B., Ragazzi, A. (2012) Species and organ specificity in endophytes colonizing healthy and declining Mediterranean oaks. Phytopathologia Mediterranea, 51: 587−598.

Mussi-Dias, V et al. (2012). Fungos endofíticos associados a plantas medicinais. Sociedade Brasileira de Plantas Medicinais, 14(2): 261-266.

Noble, H.M., Langley, D., Sidebottom, P.J., Lane, S.J., Fisher, P.J. (1991). An echinocandin from an endophytic *Cryptosporiopsis* sp. and *Pezicula* sp. in *Pinus sylvestris* and *Fagus sylvatica*. Mycological Rearch, 95: 1439–40.

Novais, M., Santos, I., Mendesa, S., Pinto-Gomes, C. (2004). Studies on pharmaceutical ethnobotany in Arrabida Natural Park (Portugal). Journal of Ethnopharmacology, 93: 183-195.

Olano, C., Lombó, F., Ménez, C., Salas, J. (2008). Improving production of bioactive secondary metabolites in actinomycetes by metabolic engineering. Metabolic Engineering, 10(5): 281 – 292.

Oliveira, I., Batista, P., Bento, A., Pereira, J.A. (2011). *Arbutus unedo* L. and its benefits on human health. Journal of Food and Nutrition Research, 50: 73-85.

Ondeyka, J.G., Helms G.L., Hensens, O.D., Goetz, M.A., Zink, D.L., Tsipouras, A., Shoop, W.L., Slayton, L., Dombrowski, A.W., Polishook J.D., Ostlind, D.A., Tsou, N.N., Ball, R. G., and Singh, S.B. (1997). Nodulisporic acid A, a novel and potent insecticide from a *Nodulisporium sp.* Isolation, structure determination, and chemical transformations. Journal of the American Chemical Society, 119: 8809-8816.

Ownley, B.H., Gwinn, K.D., Vega, F.E. (2010). Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution. BioControl, 55: 113.

Pallauf, K., Rivas-Gonzalo, J.C., Castillo, M.D., Cano, M.P., Pascual-Teresa, S. (2008). Characterization of the antioxidant composition of strawberry tree (*Arbutus unedo* L.) fruits. Journal of Food Composition Analysis, 21: 273-281.

Pedro, J. (1994). Carta da distribuição de figueira e medronheiro – Notícia Explicativa. Ministério do Ambiente e Recursos Naturais, Direcção Geral do Ambiente. Lisboa.

Qadri, M., Johri, S., Shah, B.A., Khajuria, A., Sidiq, T., Lattoo, S.K., Abdin, M.Z., Riyaz-Ul-Hassan S. (2013). Identification and bioactive potential of endophytic fungi isolated from selected plants of the Western Himalayas. SpringerPlus, 2(8): 2-14.

Qin, J.C., Zhang, Y.M., Gao, J.M., Bai, M.S., Yang, S.X., Laatschb, H., et al. (2009). Bioactive metabolites produced by *Chaetomium globosum*, an endophytic fungus isolated from *Ginkgo biloba*. Bioorganic & Medicinal Chemistry Letters, 19(6): 1572–4.

Rukachaisirikul, V., Sommart, U., Phongpaichit, S., Sakayaroj, J., Kirtikara, K. (2008). Metabolites from the endophytic fungus *Phomopsis* sp. PSU-D15. Phytochemistry, 69: 783–7.

Santiago, I.F., Alves, T.M., Rabello, A., Sales Junior, P.A., Romanha, A.J., Zani, C.L., Rosa, C.A., Rosa, L.H. (2012). Leishmanicidal and antitumoral activities of endophytic fungi associated with the Antarctic angiosperms Deschampsia antarctica Desv. and Colobanthus quitensis (Kunth) Bartl. Extremophiles, 16: 95-103.

Scherlach, K., and Hertweck, C. (2009). Triggering cryptic natural product biosynthesis in microorganisms. . Org. Biomol. Chem, (7): 1753-1760.

Schoch, CL., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W. (2012). Fungal Barcoding Consortium. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. PNAS, 109(16): 6241–6246.

Schulz, B., and Boyle, C. (2005). The endophytic continuum. Mycol Res, 109: 661-686.

Shweta, S., Züehlke, S., Ramesha, B.T., Priti, V., Kumar, P.M., Ravikanth, G.,

Spiteller, M., Vasudeva, R., Shaanker, R.U. (2010). Endophytic fungal strains of *Fusarium solani*, from *Apodytes dimidiata* E. Mey. ex Arn (*Icacinaceae*) produce camptothecin, 10-hydroxycamptothecin and 9-methoxycamptothecin. Phytochemistry, 71: 117–122.

Sileshi, G.W., Schmidt, J.S., Wiese, S., and Staerk, D. (2013). Combined with HPLC-HRMS-SPE-NMR for Identification of Potential Health-Promoting Constituents in Sea Aster and Searocket—New Nordic Food Ingredients. J. Agric. Food Chem, 61 (36): 8616–8623.

Silva, G.H., Teles, H.L., Zanardi, L.M. (2006). Cadinane sesquiterpenoids of *Phomopsis cassiae*, an endophytic fungus associated with *Cassia spectabilis* (Leguminosae). Phytochemistry, 67: 1964–9.

Soca-Chafre, G.; Rivera-Oruña, F.N.; Hidalgo-Lara, M.E.; Hernandez-Rodriguez, C.; Marsch, R.; Flores-Cotera, L.B.(2011). Molecular phylogeny and paclitaxel screening of fungal endophytes from *Taxus globosa*. Fungal Biology, 115**:** 143-156.

Soliman, S.S.M., Trobacher, C.P., Tsao, R., Greenwood, J.S., Raizada, M.N. (2013). A fungal endophyte induces transcription of genes encoding a redundant fungicide pathway in its host plant. BMC Plant Biology, 13(93): 2-10.

Stinson, M., Ezra, D., Hess, W.M., Sears, J., and Strobel, G. (2003). An endophytic *Gliocladium* sp. of Eucryphia cordifolia producing selective volatile antimicrobial compounds, Plant Science, 165: 913-922.

Strobel, G., Daisy, B., Castillo, U., Harper, J. (2004). Natural products from endophytic microorganisms. Journal of Natural Products, 67: 257–68.

Strobel, G.A., Miller, R.V., Martinez-Miller, C., Condron, M.M., Teplow, D.B., Hess, WM. (1999). Cryptocandin a potent and antimycotic from the endophytic fungus *Cryptosporiopsis* cf. *quercina*. Microbiology, 145: 1919–26.

Tayung, K., Barik, B.P., Jha, D.K., Deka, D.C. (2011). Identification and characterization of antimicrobial metabolite from an endophytic fungus, *Fusarium solani* isolated from bark of *Himalayan yew*. Mycosphere, 2(3): 203–213.

Tuntiwachwuttikul, P., Taechowisan, T., Wanbanjob, A., Thadaniti, S., Taylor, W.C. (2008). Lansai A-D, secondary metabolites from *Streptomyces* sp. SUC1. Tetrahedron, 64: 7583–6.

U'rem, J.M., Dalling, J.W., Gallery, R.E., Maddison, D.R., Davis, E.C., Gilbsin, C.M., Arnold, A.E. (2011). Diversity and evolutionary origins of fungi associated with seeds of a neotropical pioneer tree: a case study for analyzing fungal environmental samples. Mycological Research, 113: 432-449.

VanderMolen, K.M., Raja, H.A., El-Elimat, T., Oberlies, N.H. (2013). Evaluation of culture media for the production of secondary metabolites in a natural products screening program. AMB Express, (3): 71.

Vaz, A.B.M., Mota, R.C., Bomfim, M.R.Q., Zani, C.L; Rosa, C.A., Rosa, L.H. (2009). Antimicrobial activity of endophytic fungi associated with *Orchidaceae* in Brazil. Can. J. Microbiol, 55(12): 1381-1391.

Wang, Y.H., Xu, L., Ren, W.M., Zhao, D., Zhu, Y.P., Wu, X.M. (2012). Bioactive metabolites from *Chaetomium globosum* L18, an endophytic fungus in the medicinal plant *Curcuma wenyujin.* Phytomedicine, 19: 364–368.

Wicklow, D.T., and Poling, S.M. (2009). Antimicrobial Activity of Pyrrocidines from *Acremonium zeae* Against Endophytes and Pathogens of Maize. Biological Control, 99: 109-115.

Wicklow, D.T., Roth, S., Deyrup, S.T., Gloer, J.B. (2005). A protective endophyte of maize: *Acremonium zeae* antibiotics inhibitory to *Aspergillus flavus* and *Fusarium verticillioides*. Mycological Research, 109(5): 610–8.

Wright, GD. (2012) Antibiotics: A New Hope. Chemistry & Biology, 19: 3-10.

Xing, YM., Chen, J., Cui, JL., Chen, XM., Guo, SX. (2011). Antimicrobial activity and biodiversity of endophytic fungi in *Dendrobium devonianum* and *Dendrobium thyrsiflorum* from Vietman. Current Microbiology, 62. 1218–1224.

Yang X.-L., Zhang J.-Z., Luo D.-Q. (2012). The taxonomy, biology and chemistry of the fungal *Pestalotiopsis* genus. Natural Product Reports, 29: 622–641.

Yenn, T.W., Lee, C.C., Ibrahim, D., Zakaria, L. (2012). Enhancement of anti-candidal activity of endophytic fungus *Phomopsis* sp. ED2, isolated from *Orthosiphon stamineus* Benth, by incorporation of host plant extract in culture medium. Journal of Microbiology, 50: 581-585.

Yu, T.W., Bai, L., Clade, D., Hoffmann, D., Toelzer, S., Trinh, K.Q., Xu, J., Moss, S.J., Leistner, E., and Floss, H.G. (2002). The biosynthetic gene cluster of the maytansinoid antitumor agent ansamitocin from *Actinosynnemapretiosum*. PNAS. USA, 99: 7968–7973.

Yu. H., Zhang, Z., Li, L., Zheng, C., Guo, L., Li. W., Sun, P., Qin, L. (2009). Recent

developments and future prospects of antimicrobial metabolites produced by endophytes. Microbiological Research, 32(165): 437-449.

Zhang, B., Salituro, G., Szalkowski, D., Li, Z., Zhang,Y., Royo, I., Vilella, D., Dez, M., Pelaez, F., Ruby, C., Kendall, R.L., Mao, X., Griffin, P., Calaycay. J., Zierath, J.R., Heck, J.V., Smith, R.G., and Moller, D.E. (1999). Discovery of small molecule insulin mimetic with antidiabetic activity in mice. Science, 284:974.

Zhang, C.L., Wang, G.P., Mao, L.J., Komon-Zelazowska, M., Yuan, ZL., Lin, FC., Druzhinina, IS., Kubicek, CP. (2010). *Muscodor fengyangensis* sp. nov. from southeast China: morphology, physiology and production of volatile compounds. Fungal Biol, 114: 797-808.

Zhang, L.X., Liu, H.P., Han, J.C., Gao, J.M., Cheng, M.F. (2006). Isolation and screening of endophytic antifungal fungi from tomato. Journal of Shanxi Agricultural University, 25: 30–3.

Zhou, X., Zhu, H., Liu, L., Lin, J., Tang, K. (2010). A review: Recent advances and future prospects of taxol-producing endophytic fungi. Microbiol Biotechnol, 86: 1707-17.

Ziyyat, A., and Boussairi, E. (1998). Cardiovascular effects of *Arbutus unedo* L. in spontaneously hypertensive rats. Phytotherapy Research, 12: 110-113.

Ziyyat, A., Legssyer, A., Mekhfilt, A., Dassouli, A., Serhrouchni, M. and Benjelloun, W. (1997). Phytotherapy of hypertension and diabetes in oriental Morocco. Journal of Ethnopharmacology, 58: 45–54.

Capítulo 3

Fungal endophyte of *Arbutus unedo* L.: diversity, antimicrobial proprieties and volatile composition

Fungal endophyte of *Arbutus unedo* L.: diversity, antimicrobial proprieties and volatile composition

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Abstract

Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of antimicrobial importance. *Arbutus unedo* L. is a Mediterranean endemic plant widely employed in folk medicine. This study aims to assess the diversity of fungal endophytes inhabiting the parts most often used for medicinal purposes (root, leaf, twig and bark) of this plant and to explore their antimicrobial potential. The volatile compounds produced by endophytes were identified by gas chromatography/mass spectrometry and further correlated with the antimicrobial activity. A total of 288 endophyte isolates belonging to 118 fungal species were isolated. *Cryptosporiopsis diversispora* was the most common specie, accounting 26% of the total isolates, with an overall colonization rate of 3.4%. The diversity and the colonization frequency of endophytic fungi were significantly greater on bark in comparison to the other plant parts. The species composition was found to be dependent on the organ type. Among the five species selected for screen their antimicrobial activity, only *C. diversispora* and *Penicillium* sp. 3, inhibit significantly human pathogens when compared to commercial drugs. *Cryptosporiopsis diversispora* was the most effective in inhibiting yeasts (up to 1.9-fold when compared to fluconazole, 25 µg/mL) and gram-negative bacteria (up to 2.4-fold when compared to chloramphenicol, 30 µg/mL), whereas *Penicillium* sp. 3 was most effective in inhibiting both gram-negative and gram-positive bacteria (up to 2.0 fold and up to 2.5-fold when compared to chloramphenicol, 30 µg/mL, respectively). The volatile composition of the fungi that displayed the highest (*C. diversispora*) and the lowest (*Penicillium glabrum*) antimicrobial activity revealed the presence of 22 compounds belonging to different chemical classes (10 sesquiterpenes, 7 alcohols, 3 esters and 2 ketones). The comparison of the volatile profile of these two species associated to the principal component analysis suggested that the antimicrobial activity displayed by *C. diversispora* may be ascribed to their volatile composition, in particular to the high content in the antimicrobial volatiles 3-methyl-1-butanol and phenylethyl alcohol. In addition to provide new insights into fungal endophyte of *A. unedo*, the present work showed the potential of these fungi to produce antimicrobial compounds.

Key-words: Strawberry tree, plant organs, *Cryptosporiopsis diversispora*, bacteria, yeasts, HS-SPME/GC/MS

Introduction

Today, one of the major threats to public health in Europe, and globally, is the increasing resistance to antimicrobials (Carlet et al., 2012). This is particularly notice in gram-negative rods such as *Escherichia coli*, *Salmonella* spp., *Klebsiella* spp., *Pseudomonas aeruginosa* and *Acinetobacter* spp., which are resistant to almost all currently available antibiotics (Carlet et al., 2012). One possible option to tackle the problem, in the longer term, is the development of new antibiotics. Keeping this in mind, the Infectious Diseases Society of America launched the "10×20 Initiative" in 2010 with the main objective to produce 10 new antibiotics by the year 2020 (IDSA, 2010). Fungal endophytes are considered a potential source for many novel secondary metabolites, and thus may have been interesting for the discovery of new antibiotics (Yu et al., 2010). These fungi are a very diverse polyphyletic group of microorganisms characterized to colonise the plant tissues internally, for at least a part of their life cycle, without causing any visible manifestation of disease (Hyde and Soytong, 2008). They are distributed throughout the host in all plant organs such as leaves, stems, roots, fruits, seeds and flowers (Arnold, 2007). Fungal endophytes confer fitness benefits to their host plants by producing a plethora of secondary metabolites that provide protection and ultimately survival value to the plant (Strobel et al., 2004). Some of these compounds possess unique structures and have been shown to exhibited antimicrobial properties against a vast array of plant and human pathogens (Yu et al., 2010; Mousa and Raizada, 2013), which reinforce the role of fungal endophytes as an important source of compounds with potential to enter the field of drug development. Antimicrobials metabolites of various reported fungal endophytes are very diverse and belonging to several chemical classes, including alkaloids, peptides, steroids, terpenoids, phenols, phenylpropanoids, aliphatic compounds, polyketides, quinones and flavonoids (Yu et al., 2010; Mousa and Raizada, 2013), as well as volatile organic compounds (VOCs) such as esters, lipids, alcohols, acids, ketones, among others (Banerjee et al., 2010; Kudalkar et al., 2012).

Some fungal endophytes have been shown the ability to produce the same and/or similar bioactive chemicals with therapeutic value as those originated from their host plants (Kusari et al., 2008, 2009, 2012a). Gene transference from the plant to the endophyte and vice-versa is suggests to happen allowing common secondary

metabolites production (Kusari and Spiteller, 2011). Motivated by this discovery, many scientists have become increasingly interested in studying fungal endophytes associated with traditionally used medicinal plants for the discovery of new substances for human therapeutics, including antibiotics (Kaul et al., 2012). Such plants, with established ethnobotanic values, would be more promising sources of endophytes producing novel biologically active secondary metabolites (Strobel and Daisy, 2003). Exploitation of endophytic fungi, especially those with capacity to produce host plant compounds, for pharmaceutical purposes would be cost-effective, reproducible and environmental friendly due to the preservation of the medicinal plant (Kusari et al., 2012b).

Arbutus unedo L., commonly known as strawberry tree, is an evergreen shrub or small tree, belonging to the Ericaceae family, endemic to Mediterranean region (Celikel et al., 2008). Different parts of this plant have been used in traditional medicine since ancient times (Oliveira et al., 2011). For instances, the leaves have been used for their astringent, diuretic, urinary anti-septic, antidiarrheal, depurative and in the therapy of hypertension, diabetes, and inflammatory diseases; while the roots and barks have been used on gastrointestinal disorders, as well as for urological and dermatologic problems (reviewed by Oliveira et al., 2011). The notable medicinal properties mentioned make this plant as a good candidate for screening of fungal endophytes with potential to produce novel bioactive compounds. Endemic plants, such as *A. unedo*, are also more likely to lodge endophytes with active natural products than other plants (Strobel and Daisy, 2003). To our knowledge, studies on the diversity of endophytic fungi of *A. unedo* and consequently their bioactive properties have never been made. Only, Qin et al. (2011) have isolate and identified four new compounds, named as pestalotheols E–H, from ethyl acetate extracts of the culture of an unidentified Ascomycete isolated from *A. unedo*. Those extracts have shown strong antifungal activity and good antibacterial and antialgal activities.

Therefore, the main aim of this study was to isolate and assess fungal endophyte diversity in different *A. unedo* organs (leaves, stems, roots and bark) and screen the antimicrobial activity of the most frequent species against bacteria and yeasts pathogens. In addition, the VOCs produced by the tested fungi were identified by gas chromatography/mass spectrometry (GC/MS) and further correlated with the antimicrobial activity with an attempted to identified potential components of the antimicrobial activity. It is expected that the screening of endophytic fungi from *A.*

unedo for antimicrobial activities will serve as a good basis for discovering new antibiotic agents.

Materials and Methods

Plant sample collection

Seven healthy trees were randomly selected in natural populations of *Arbutus unedo* L. located in Bragança (Northeast of Portugal). From each tree, was randomly harvested one branch with leaves, one sample of roots and of barks, in January 2014. The plant material was placed in individual plastic bags, labelled, and transported to the laboratory in an icebox. All samples were stored at 4ºC and processed within one day.

Surface sterilization and isolation of endophytic fungi

All the plant material collected was firstly rinsed in water. After that, the leaves were removed from the branches, and those with no obvious lesions, were selected for assessment. Each twig, root and bark was further cut into 4 cm long segments before surface sterilization. Five segments each of roots, twigs, barks and five leaves per tree were randomly selected, and further surface sterilized through sequential immersion in 70% (v/v) ethanol for 2 min, 3-5% (v/v) sodium hypochlorite for 3 min (for leaves and twigs) or 5 min (for roots and barks), 70% (v/v) ethanol for 1 min and rinsed three times (1 min each) with sterile distillate water. After being dried in sterile filter paper, each root and twig were cut in five segments of approximately 4-5 mm, and for each leaf, four segments of *ca*. 5 x 5 mm from the lamina and one from the petiole of approximately 4-5 mm were excised. The bark was also cut in five segments of *ca*. 5 x 5 mm. These tissue segments were immediately transferred to 9 cm diameter Petri plates, containing 10 ml of sterile potato-dextrose agar (PDA) medium and incubated at 25 ± 2 ºC in the dark. Each plate contained 5 tissue fragments for a total of 25 fragments assay per plant organ and tree. Therefore, in total, 700 segments (7 trees x 4 plant organs x 25 tissue segments) were used in this study. Efficiency of the surface sterilization procedure was ascertained by imprinting onto PDA Petri plate randomly selected surface sterilized leaves, roots, barks and twigs segments. The fungi growing out of the tissue segments were recorded as

endophytic fungi and were sub-cultured on individual PDA plates to obtain pure isolates for subsequent identification. Pure cultures of each isolate were deposited in the culture collection of the Polytechnic Institute of Bragança (School of Agriculture).

Identification of fungal isolates

A combination of morphological and molecular approach was used to identify fungal isolates. At the first, isolates from pure cultures were grown on PDA medium and maintained at 25 ± 2 ºC in the dark for 1-3 weeks, depending on their growth rate. These fungal cultures were examined periodically, and groups of strains were formed according to their morphological similarity. Isolates were divided based on the morphology of the fungal culture colony (e.g. color, shape and texture) or hyphae, the characteristics of the spores (e.g. size and shape) and reproductive structures, mainly according to Yarrow (1998). One representative strain for each morphotype was selected for further molecular identification using the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA). This is the recommended genomic region to be used as the DNA barcode marker for fungi (Schoch et al., 2012).

Genomic DNA was extract from spores or mycelial mat following the protocol of Oliveira et al. (2012). The ITS region (ITS1, 5.8S, ITS2) was amplified using the universal *ITS1* and *ITS4* primers (White et al., 1990), in a PCR protocol formerly described by Oliveira et al. (2012). Amplified products (~600 pb) were purified using JETQUICK PCR Product Purification Spin Kit (Genomed) following the manufacturer´s instructions. Clean PCR products were sequenced using the STABVida services (Oeiras, Portugal). The obtained DNA sequences were analysed with DNASTAR v.2.58 software, and fungal identification was performed using the NCBI database (http://www.ncbi.nlm.nih.gov) and the BLAST algorithm. Blast results were sorted according to the higher identity score and the lowest E-value and sequence similarity was only accepted for maximal identity values >95% and ≅ 0.0 E-values.

Endophyte community analysis

Species diversity

The diversity of fungal endophytes within tissues samples (root, leaf, twig, bark and total plant organs) was evaluated at the level of their richness (number of different taxa) and abundance (number of isolates per taxa), and also by computing the most widely used indices of species diversity, such as Simpson's Reciprocal Index (1/D) and Shannon–Wiener (H). Both indices combine species richness and abundance, in different ways (Magurran, 2004). Both indices were computed in Species Diversity and Richness v. 4.0 (Seaby and Henderson, 2006), and were presented as the mean of seven independent experiments displaying the respective SD values or as total number (value for all samples lumped together).

Overall colonization

Measurement of fungal occurrence in each plant organ surveyed was established by calculating the frequency of colonization and relative abundance. The frequency of colonization (FC, %) was calculated as the total number of plant tissue segments colonized by each endophyte divided by the total number of plant segments surveyed (Suryanarayanan et al., 2003). The relative abundance (RA, %) of a fungal taxon was determined as the total number of isolates of a taxon divided by the total number of isolates of all taxa.

Similarity

Non-metric multidimensional scaling (NMDS) was carried out to explore the similarity of fungal endophytes community among plant organs (bark, twig, root and leaf). This analysis ranks fungal endophytes of each plant organ (represented by points) in ordination space in a way that the distance between two points is inversely proportional to their similarity (Kruskal, 1964). Therefore, points closer together are more similar in microorganism species composition than those further apart. The correspondence of the ordination diagram to the distances is described by a stress value (Kruskal's stress), with values less than 0.2 representing good ordination plots and greater than 0.3 provides a poor representation (Clarke, 1993). NMDS was

performed using the Bray-Curtis similarity matrice (Magurran, 2004). This index compares taxa presence or absence and abundance among samples. This coefficient ignores cases in which the species is absent in both community samples, and is strongly influenced by the abundant species so rare species add very little influence (Bray and Curtis, 1957). The ordination analyse was conducted on Ln(x+1) transformed data.

Screening of fungal endophytes for antimicrobial activity

The screening of fungal endophytes for antimicrobial activity was only performed for those species with greatest frequency of colonization and those that are easily grown in artificial media. According to these criteria's, five species were selected: *Penicillium glabrum* (Wehmer) Westling, *Penicillium crustosum* Thom, *Penicillium commune* Thom and *Cryptosporiopsis diversispora* Robak, all isolated from bark, and *Penicillium* sp. 3 isolated from roots (please see results and discussion section).

Microbial strains

The antimicrobial activity of endophytes was evaluated against three gram-positive bacteria (*Bacillus cereus*, *Bacillus subtilis* and *Staphyloccocus aureus*), two gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and three yeasts (*Candida albicans*, *Candida glabrata* and *Candida parapsilosis*). All the microorganisms were obtained from the Biology Department - University of Minho (Braga, Portugal). Yeast strains were maintained at 4°C in YEPDA medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar], and sub-cultured periodically. Growth was promoted aerobically at 28°C. Bacterial stocks cultures were maintained at 4ºC on LB agar [tryptone 1% (w/v), yeast extract 0.5% (w/v), NaCl 1% (w/v) and agar 2% (w/v)], being sub-cultured periodically at 37°C.

Antimicrobial activity by bioassay method

The screening of fungal endophytes for antimicrobial activity was performed by using the bioassay method developed by Pereira et al. (2013). This method has the advantage of being less time consuming and allows the direct detection of

antimicrobial activity of growing fungi against single cell microorganisms without the prior isolation of the active substance(s). Also, this method allows to study the dynamics of antimicrobial compounds production by filamentous fungi.

Petri dishes (9 cm diameter) containing PDA medium were centrally inoculated with 10 µL of a spore suspension ($1x10^6$ spore/mL) of each *P. glabrum*, *P. crustosum*, *P. commune*, *Penicillium* sp. 3 and *C. diversispora* fungus. Spore suspensions were obtained by flooding fungal cultures growing on PDA medium with 2 mL of sterile aqueous solution of 0.02% (v/v) Tween 80. The spore concentration was adjusted to $1x10^6$ spore/mL with a Neübauer haemacytometer, under light microscope (Leica, CTR 5000). After incubation at 25°±1ºC, in the dark, for 3 (*P. glabrum*, *P. crustosum*, *P. commune* and *Penicillium* sp. 3) and 7 days (*C. diversispora*), the inoculated plates were overlaid with the sensitive indicator strain. To prepare the sensitive microbial suspension, yeast and bacteria biomass were scraped from a 24h YPDA or LB culture plate, respectively, and suspended in 1 ml of NaCl 0.85% (w/v). Suspensions of the microorganisms were prepared and mixed with melted-agar 0.8% (w/v) in order to obtain 10^6 CFU (colony forming units)/ml. A volume of 3 ml of the mixture was then seeded as a lawn onto the surface of the plates previously inoculated with endophytic fungi. The plates were incubated at 25ºC / 48h and 32ºC / 24h for yeasts and bacteria, respectively. Standard discs of chloramphenicol (30 µg/ml) and fluconazole (25 µg/ml) were obtained from Oxoid Ltd. and served as positive controls for antibacterial and antifungal activity, respectively. Antimicrobial activity was observed when the fungal inoculum was surrounded by a clear zone of growth inhibition and evaluated by determining both the diameter of the fungal colony and the diameter of the inhibition halo. Values were used to calculate the areas occupied by the fungus and by the inhibition halo formed by its antimicrobial activity. Data are presented as the mean of 3 to 4 independent experiments, with 3 replicas each. The corresponding standard error values are displayed.

Analysis of volatile compounds

Secondary metabolites presents in the volatile fraction of the endophytic fungus specie that showed the highest antimicrobial activity (i.e. *C. diversispora*, please see results and discussion section) were studied, as an attempt to identify compounds

responsible for such activity. This study was performed in fungus growing in PDA medium, in order to get a more realistic picture of the microbial volatile organic compounds released from the endophyte. The fungus specie that showed the lowest antimicrobial activity (i.e. *P. glabrum*, please see results and discussion section) was used as negative control. The comparison of the volatile profile of the two fungal species will allow a more accurate identification of volatile antimicrobials compounds.

In vitro fungal cultures

The fungi were grown in 50 mL flasks (Duran Gaines Synth, Bioblock), containing 10 mL of PDA medium, sealed with a polypropylene cap with PTFE/silicon septum (Duran) (Fig. S1, Supporting information). The inoculation of culture medium was performed by transferring 10 µL of a spore suspension ($1x10^6$ spore/mL) of each *P. glabrum* and *C. diversispora* fungus. Four replicates per fungal species were performed; and flasks containing exclusively PDA medium were also included as control. The cultures were incubated at 25 ºC in the dark, for 3 (*P. glabrum*) and 7 days (*C. diversispora*), and further used to analyse the volatile compounds.

Extraction of volatile compounds by headspace solid-phase microextraction

The extraction of volatile compounds from *P. glabrum* and *C. diversispora* growing under *in vitro* conditions was performed by headspace solid-phase microextraction (HS-SPME). The volatiles were extracted using a fiber coated with divinylbenzene/carbonex/polydimethylsiloxane (DVB/CAR/PDMS 50/30 µm) (Supelco, Bellefonte, USA). The entire procedure was carried out in a horizontal position in a gas emission system (isolation) at 40ºC (Fig. S1, Supporting information). The flasks with the respective fungus were placed for 10 minutes in an oven at 40ºC for an effective release of the volatile compounds. After this period, the SPME fiber was exposed during 60 minutes for the compounds adsorption in the headspace. The fiber was collected and inserted into the injection port of the gas chromatography system. The HS-SPME procedure was performed in quadruplicate.

The same procedure was performed with a control sample containing only PDA medium.

Gas Chromatography-Mass Spectrometry (GC/MS) analysis

The retained compounds were eluted from the fiber by thermal adsorption for 1 minute. For cleaning and conditioning of further analyzes the fiber was maintained during 10 minutes at 220 ºC in the injector port of the chromatography system. The volatile compounds detection and quantification were performed in a gas chromatographer Shimadzu GC-2010 Plus equipped with a mass spectrometer Shimadzu GC/MS-QP2010 SE detector. A TRB-5MS (30 m × 0.25mm × 0.25µm) column (Teknokroma, Spain) was used. The injector was set at 220 °C and the manual injections were made in splitless mode. The mobile phase consisted in helium (Praxair, Portugal) at a linear velocity of 30 cm/s and a total flow of 24.4 mL/min. The oven temperature was fixed at 40 °C during 1 minute and then raised 2 °C per minute to 220 °C, and held at this temperature during 30 minutes. The ionization source was maintained at 250 °C with an ionization energy of 70 eV, and with a ionization current of 0.1 kV. All mass spectra were acquired by electron ionization. The ionization was left off during the first 3 minutes. The MS spectra fragments were compared with those obtained from a database (NIST 11).

For quantification purposes, each sample was injected in quadruplicate, and the areas of the chromatographic peaks were determined integrating the re-constructed chromatogram from the full scan chromatogram using for each compound the ion base (m/z intensity 100%). Data from each volatile (as peak area / 1000) were respectively reported as the mean of four independent experiments with the respective standard deviation.

Data analysis

Principal component analysis (PCA)

Principal components analysis (PCA) was applied for reducing the number of variables regarding the volatile composition of *C. diversispora* and *P. glabrum* growing under *in vitro* as well as their antimicrobial activity (diameter of the inhibition halo) against several microorganisms (22 variables corresponding to

volatile components, and 8 variables corresponding to inhibition halo values; with a total of 30 variables) to a smaller number of new derived variables (principal component or factors) that adequately summarize the original information, i.e., the *in vitro* volatile composition of both fungi in study and their capability to inhibit microbial growth. Moreover, it allowed recognizing patterns in the data by plotting them in a multidimensional space, using the new derived variables as dimensions (factor scores). PCA was performed by using SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.).

Other statistical analysis

Significant differences among samples were determined by analysis of variance (ANOVA), using SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.) and averages were compared using Tukey's ($p<0.05$).

Results and discussion

The screening of antimicrobial compounds from fungal endophytes is currently being widely studied to meet the increasing threat of drug-resistant strains of human pathogens. Studies focusing antimicrobial compounds derived particularly from fungal endophytes of medicinal plants have attracted much interest, since endophytes have been shown the capacity to synthesize the same metabolites produced by the host plant (Kusari et al., 2008, 2009, 2012a). Therefore, the current study was undertaken to isolate endophytic fungi from the medicinal plant *A. unedo*, and screen them for the presence of antimicrobial activity. Fungi were isolated from roots, leaves, twigs and barks, because they are the plant's most used organs in folk medicine (Oliveira et al., 2011). To our knowledge, there are no previous reports on the isolation and cultivation of endophytes from *A. unedo*. The composition of the volatile organic compounds of endophytes is also presented aiming to relate with their antimicrobial activities.

Composition of fungal endophytes in strawberry tree

The results indicate a great diversity and richness of fungal endophytes associated to this specie. All the seven strawberry trees sampled harbored fungal

endophytes, with a mean of 20.6 fungal taxa/tree and 41.1 fungal isolates/tree (Table 1). A total of 118 morphospecies were identified from 288 isolates. The high diversity found was corroborated by the Simpson and Shannon-Wiener indices estimate (Table 1). This high diversity could be due to the greater number of rare species present and low number of individuals. Studies in the Mediterranean region for other woody plant species, such as *Quercus cerris*, *Quercus pubescens* (Moricca et al., 2012) and *Quercus suber* (Linaldeddu et al., 2011), have reported lower number of endophyte species. For plant species of the same family as *A. unedo* (Ericaceae), such as *Rhododendron* (Okane et al., 1998; Purmale et al., 2012), *Enkianthus perulatus* and *Pieris japonica* (Okane et al., 1998), was also reported a lower number of fungal species compared to the present study. This variation may be due to differences in the number of plant organs surveyed. In fact, in all of those studies the surveyed of endophytic fungi was done in few plant organs, just on leaves and twigs (Moricca et al., 2012), or twigs, branches and trunk (Linaldeddu et al., 2011) or solely on leaves (Okane et al., 1998; Purmale et al., 2012). In the present study, the sampling of several plant organs (roots, leaves, twigs and barks) was performed with the intention of isolating as many endophyte species as possible from *A. unedo*. Another reason could be related with differences on plant species and sampling sites, as previously reported (Sebastianes et al., 2013). In addition, the study of culturable endophytes is recognise to be a method-dependent process (Hyde and Soytong, 2008), being the identity and number of isolates obtained influenced by several experimental variables that can affect the comparability of endophytes datasets. The mean overall colonisation of endophytes from *A. unedo* was 41%, which is very similar to that reported for other woody plant species elsewhere in the Mediterranean region (Linaldeddu et al., 2011), as well as elsewhere in the world (Guo et al., 2008; Sun et al., 2012).

The identity of the species with greatest frequency of colonization was further confirmed by their ITS-rDNA sequences (Table S1). Among the 33 taxa selected for this analysis, only 15 taxa were identified to the species level (Table 2). The ITS sequence of the remained species (18 taxa/OTUs) were not specified to the species level or even to the genus level in the GenBank fungal sequence database to name them to species. The species molecularly identified belonged to 10 families and 11 genera, being *Penicillium* the genera with greatest number of species (10) followed by *Umbelopsis* (3 species). The remaining genera were represented by only one taxa. The

endophytic assemblages of *A. unedo* comprised a number of taxa belonging to ubiquitous genera. Fungi belonging to the genera *Alternaria*, *Cladosporium* and *Penicillium* (Linaldeddu et al., 2011; Sun et al., 2012; Moricca et al., 2012) as well as some isolates identified as *Umbelopsis* spp. (Hoff et al., 2004), *Aureobasidium pullulans* (Pancher et al., 2012) and *Microsphaeropsis olivacea* (Li et al., 2007) have commonly been cited as endophytes of several woody plant species. Similarly, the species identified as *Stemphylium globuliferum* (Debbab et al., 2010) and *Allantophomopsis lycopodina* (Shiono et al., 2010), were previously described as endophytes and found to be producers of new secondary metabolites with potent antimicrobial and cytotoxic activity, respectively. Curiously, one of the isolates identified as *Discostroma* sp. has been previously found to be one of the most common fungal endophytes inhabiting ericaceous plants, namely *Rhododendron indicum*, *Rhododendron macrosepalum* and *Rhododendron obtusum* (Okane et al., 1996).

Cryptosporiopsis diversispora was the most common, accounting 26% of the total isolates, with an overall colonization rate of 3.4% (Table 2). There are few reports about this specie. Only Eo et al. (2014) have identified this fungus as endophyte of the woody plants *Acer tagmentosum*, *Larix kaempferi*, *Abies holophylla* and *Pinus koraiensis*.

Table 1 – Richness, diversity and frequency of colonization of fungal endophytes by strawberry tree organ. The results are present as the mean value ± SD (n=7) and as total number (in brackets). Different superscript lower case letters denote statistically significant differences (p<0.05) between plant organs.

Parameters	**Root**	**Twig**	**Leaf**	**Bark**	**Total**
Total number of isolates	73	32	41	142	288
Average number of isolates per tree	10.4 ± 1.9^{c}	4.6 ± 4.3^{d}	5.9 ± 3.1^{d}	20.3 ± 4.4^{b}	41.1 ± 9.3^{a}
Total number of taxa	39	22	13	48	118
Average number of taxa per tree	6.1 ± 2.5^{b}	3.7 ± 1.6^{c}	3.3 ± 1.3^{c}	7.6 ± 2.5^{b}	20.6 ± 4.8^{a}
Simpson's Reciprocal Index (1/D)	9.1 ± 6.7^{b} (39.8)	8.9 ± 4.2^{b} (27.6)	5.2 ± 4.7^{b} (6.4)	6.9 ± 3.6^{b} (20.9)	18.0 ± 8.2^{a} (59.1)
Shannon-Wiener index (H)	1.6 ± 0.5^{b} (3.5)	0.9 ± 0.4^{b} (2.9)	1.0 ± 0.5^{b} (2.1)	1.7 ± 0.4^{b} (3.4)	2.7 ± 0.3^{a} (4.4)
Frequency of colonization (%)	41.7 ± 7.6^{b}	18.3 ± 16.4^{c}	23.4 ± 12.7^{c}	81.1 ± 17.5^{a}	41.1 ± 9.3^{b}

Table 2 – Frequency of colonization (FC, %) and relative abundance (RA, %) of the most common fungal endophyte isolated from roots, twigs, barks and leaves of *Arbutus unedo*. The RA is shown in brackets.

Family, genera and species	**Root**	**Twig**	**Leaf**	**Bark**	**Total**
	FC (RA)%	FC (RA)%	FC (RA)%	FC (RA)%	FC (RA)%
Amphisphaeriaceae					
Discostroma sp.	0 (0)	0.57 (5.0)	0 (0)	0 (0)	0.14 (1.1)
Cladosporiaceae					
Cladosporium sp.	0 (0)	1.71 (15.0)	0.57 (20.0)	0 (0)	0.57 (4.3)
Dermateaceae					
Cryptosporiopsis					
C. diversispora Robak	0 (0)	0 (0)	0 (0)	13.7 (43.6)	3.43 (25.8)
Dothioraceae					
Aureobasidium					
A. pullulans (de Bary) G. Arnaud	0 (0)	0 (0)	0 (0)	0.57 (1.8)	0.14 (1.1)
Montagnulaceae					
Microsphaeropsis					
M. olivacea (Bonord.) Höhn.	0 (0)	0 (0)	0 (0)	0.57 (1.8)	0.14 (1.1)

Table 2 – Continuation.

Family, genera and species	Root	Twig	Leaf	Bark	Total
	FC (RA)%	FC (RA)%	FC (RA)%	FC (RA)%	FC (RA)%
Pezizaceae					
Chromelosporium					
C. carneum (Pers.) Hennebert	0 (0)	0 (0)	0.57 (20.0)	0 (0)	0.14 (1.1)
Phacidiaceae					
Allantophomopsis					
A. lycopodina (Höhn.) Carris	0 (0)	0 (0)	0 (0)	0.58 (1.8)	0.14 (1.1)
Pleosporaceae					
Alternaria					
A. alternata (Fr.) Keissl.	0 (0)	0.57 (5.0)	0 (0)	0 (0)	0.14 (1.1)
Stemphylium					
S. globuliferum (Vetergr.) E. G. Simmons	0 (0)	0 (0)	0.57 (20.0)	0 (0)	0.14 (1.1)
Trichocomaceae					
Penicillium					
P. brevicompactum Dierckx	0 (0)	0 (0)	0 (0)	0.57 (1.8)	0.14 (1.1)
P. commune Thom	0 (0)	0 (0)	0 (0)	2.9 (9.1)	0.71 (5.4)
P. crustosum Thom	0 (0)	0 (0)	0 (0)	2.9 (9.1)	0.7 1(5.4)
P. glabrum (Wehmer) Westling	0 (0)	0 (0)	0 (0)	0.57 (1.8)	0.14 (1.1)

Table 2 – Continuation.

Family, genera and species	Root	Twig	Leaf	Bark	Total
	FC (RA)%	FC (RA)%	FC (RA)%	FC (RA)%	FC (RA)%
P. griseolum G. Sm.	1.14 (15.4)	0 (0)	0 (0)	0 (0)	0.29 (2.2)
P. sanguifluum (Sopp) Biourge	0.57 (7.7)	0 (0)	0 (0)	0 (0)	0.14 (1.1)
P. thomii Maire	0 (0)	0 (0)	0 (0)	0.6 (1.8)	0.14 (1.1)
Penicillium sp. 1	1.1 (15.4)	0 (0)	0 (0)	0 (0)	0.29 (2.2)
Penicillium sp. 2	0 (0)	0.6 (5.0)	0.57 (20.0)	1.1 (3.6)	0.57 (4.3)
Penicillium sp. 3	1.71 (23.1)	0 (0)	0 (0)	0 (0)	1.7 (3.2)
Umbelopsidaceae					
Umbelopsis					
U. vinacea (Dixon-Stew.) Arx	0 (0)	0.57 (5.0)	0 (0)	0 (0)	0.14 (1.1)
Umbelopsis sp. 1	0.57 (7.7)	0 (0)	0 (0)	0 (0)	0.14 (1.1)
Umbelopsis sp. 2	0.67 (7.7)	0 (0)	0 (0)	0 (0)	0.14 (1.1)
Unidentified species					
Fungal sp. 1	0 (0)	0.57 (5.0)	0 (0)	0 (0)	0.14 (1.1)
Fungal sp. 2	0 (0)	0 (0)	0 (0)	0.57 (1.8)	0.14 (1.1)
Uncultured Ascomycota	0 (0)	0.57 (5.0)	0.57 (20.0)	0 (0)	0.29 (2.2)
Uncultured Cladosporium clone	0 (0)	0 (0)	0 (0)	0.57 (1.8)	0.14 (1.1)
Uncultured fungus clone 1	0 (0)	5.71 (50.0)	0 (0)	0 (0)	1.41 (10.8)

Table 2 – Continuation.

Family, genera and species	**Root**	**Twig**	**Leaf**	**Bark**	**Total**
	FC (RA)%	FC (RA)%	FC (RA)%	FC (RA)%	FC (RA)%
Uncultured fungus clone 2	0 (0)	0 (0)	0 (0)	0.57 (1.8)	0.14 (1.1)
Uncultured Rhizoctonia	0 (0)	0 (0)	0 (0)	5.71 (18.2)	1.41 (10.8)
Helotiales sp.	0.57 (7.7)	0 (0)	0 (0)	0 (0)	0.14 (1.1)
Pezizomycetes sp.	0 (0)	0.57 (5.0)	0 (0)	0 (0)	0.14 (1.1)
Phialocephala sp.	0.57 (7.7)	0 (0)	0 (0)	0 (0)	0.14 (1.1)
Trichocomaceae sp.	0.57 (7.7)	0 (0)	0 (0)	0 (0)	0.14 (1.1)
Total	7.43 (100)	11.4 (100)	2.86 (100)	31.4 (100)	13.3 (100)

Endophyte community variations between plant organs

Various *A. unedo* parts differed significantly in the diversity and richness of fungal endophytes. The fungal community from the bark was most diverse (48 taxa) and rich (142 isolates), followed by the roots (39 taxa, 73 isolates), twigs (22 taxa, 32 isolates) and leaves (13 taxa, 41 isolates) (Table 1). The average number of fungal taxa and isolates obtained from each organ per tree was also statistically significant, being highest in barks (Table 1). Differences in the assemblages of endophytic fungal species between tree organs have also been reported in previous studies (Fisher et al., 1993; Moricca et al., 2012; Qi et al., 2012; Sun et al., 2012; Sebastianes et al., 2013). For example, in *Eucalyptus nitens* was observed more endophytic fungal isolates in barks than in leaves (Fisher et al., 1993). Similarly, Qi et al. (2012) recovered more fungal endophyte taxa and isolates in barks than in annual twigs of *Acer ginnala*. However, there was no statistically significant difference in Simpson and Shannon diversity indices between the various *A. unedo* parts.

The four organs of *A. unedo* also differed significantly on endophytic colonization rate (Table 1). Overall colonization rate were highest in barks, as reflected by the highest mean colonization rate values (81%), followed by roots (42%), leaves (23%) and twigs (18%). Differences in the colonization frequency of endophytes between tree organs have also been reported for other plant species (Collado et al., 2000; Nalini et al., 2005; Guo et al., 2008; Moricca et al., 2012; Sun et al., 2012). For example, Collado et al. (2000) revealed much higher colonization rates of endophytic fungi from bark (65%) than leaves (25%) of *Quercus ilex* and from bark (75.6%) than leaves (36.3%) of *Quercus faginea* Lam., respectively. Similarly, in *Pinus tabulaeformis*, the colonization frequency was recorded much higher in bark (ranging from 53-96%) than in needles (ranging from 9-36%) (Guo et al., 2008).

Different endophyte taxa dominated the four organs of *A. unedo* (Table 2). In barks, *C. diversispora* (43.6%), Uncultured *Rhizoctonia* (18.2%), *Penicillium commune* (9.1%) and *Penicillium crustosum* (9.1%), were the dominant species and were isolated only from this organ. The roots were dominated by species of the genus *Penicillium*, namely *Penicillium* sp.3 (23.1%), *P. griseolum* and *Penicillium* sp.1 (15.4% each), and were isolated only from this organ. Uncultured fungus clone 1 (50.0%) and *Cladosporium* sp. (15.0%) were the most dominant species in twigs, being the first one exclusively isolated from twigs whereas the former was also occurred in leaves. The

fungal endophyte community of the leaves was co-dominated by *Cladosporium* sp., *Chromelosporium carneum*, *Stemphylium globuliferum*, *Penicillium* sp.2 and Uncultured Ascomycota (20.0% each). Among these species, *C. carneum* and *S. globuliferum*, were isolated only from leaves. These results indicate organ- or tissue-specificity in endophytes which are in agreement with previous studies (Nalini et al., 2005; Guo et al., 2008; Moricca et al., 2012; Sun et al., 2012). These differences in the species composition of endophytes recovered from different organs might be a reflection of tissue preference of individual taxa which may be attributed to the distinct substrate utilization patterns developed by the endophytic fungi (Petrini et al., 1992). This indicates that distribution of endophytic fungi depends on adaptation to particular tissue chemistry of host plant organs (Petrini et al., 1992).

Cluster analyses of fungal endophyte community based on Bray-Curtis similarity presented in two dimensional NMDS plots corroborate the differences found on endophytic assemblage between organs (Fig. 1). In this analysis was observed that, in general, the endophytes clustered according to the plant organ from which have been isolated. This is particularly noticed for leaf and roots endophytes. The separation of endophyte communities is mainly due to the presence of both dominant and exclusively species in each organ. The confidence of this pattern is very high due to the low Kruskal's stress value (<0.01) obtained in this analysis.

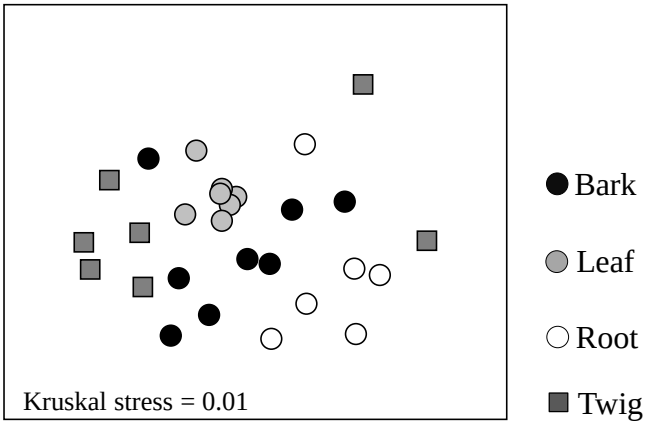

Figure 1 - Non-metric multidimensional scale (NMDS) plots corresponding to the clustering of fungal endophytes grouped by plant organ (bark, leaf, root and twig). Clustering analysis was performed with Bray-Curtis coefficient by using Ln(x+1) transformed data. Kruskal's stress values inferior to 0.2 represent good ordination plots.

Antimicrobial activity of endophytes

Five fungal endophytes, *P. glabrum*, *P. crustosum*, *P. commune* and *C. diversispora*, all isolated from bark, and *Penicillium* sp. 3 isolated from roots, were screened for their antimicrobial activity against gram-positive and gram-negative bacteria and yeasts. These species were chosen due to their high abundance and frequency of colonization. Results of antimicrobial activity are presented in Figure 2. Only two endophytes, *C. diversispora* and *Penicillium* sp. 3, displayed significant antimicrobial activity against the tested microorganisms when compared to control. *Cryptosporiopsis diversispora* was the most effective in inhibiting microorganisms with a widest spectrum. This fungus was the only one out of five endophytes with capacity to inhibit significantly the yeasts than the control. When compared with the fluconazole, *C. diversispora* reduced significantly the growth of *C. glabrata* (up to 1.9-fold), *C. albicans* (up to 2.7-fold) and *C. parapsilosis* (up to 3.1-fold). This endophyte was also the most effective in inhibiting the gram-negative bacteria *E. coli* and *P. aeruginosa* (up to 2.4-fold and 4.5-fold, respectively when compared to chloramphenicol). There are few reports about *C. diversispora* including of their antimicrobial proprieties. Only Hwang et al. (2011) reported that *C. diversispora* isolated from lichen displayed antifungal activity against *C. albicans*. Several bioactive metabolites, including antimicrobial compounds, have been previously isolated from the Genus *Cryptosporiopsis* and its teleomorph, *Pezicula* (Strobel et al., 1999; Verkley, 1999; Zilla et al., 2013). Therefore, the present study provides, for the first time, evidences on the antimicrobial potential of *C. diversispora*, which exhibited promising and broad spectrum of antagonistic activity against clinical microorganisms, including *E. coli* and *P. aeruginosa*. This is of much importance due to the increasing resistance to antibiotics of these two gram-negative bacteria, including in Europe, especially in Portugal and Italy (Nordberg et al., 2013). The distinctive feature of gram-negative bacteria is the presence of a double membrane surrounding each bacterial cell. This outer membrane excludes certain drugs and antibiotics from penetrating the cell, partially accounting for why gram-negative bacteria are generally more resistant to antibiotics than are gram-positive bacteria (Nordberg et al., 2013). The endophyte *Penicillium* sp.3 was also shown to be efficient in inhibiting these two gram-negative bacteria and also one gram-positive bacterium (Fig. 2). Therefore, this specie could also be of considerable interest to be exploited as a source of novel antimicrobials compounds to overcome the

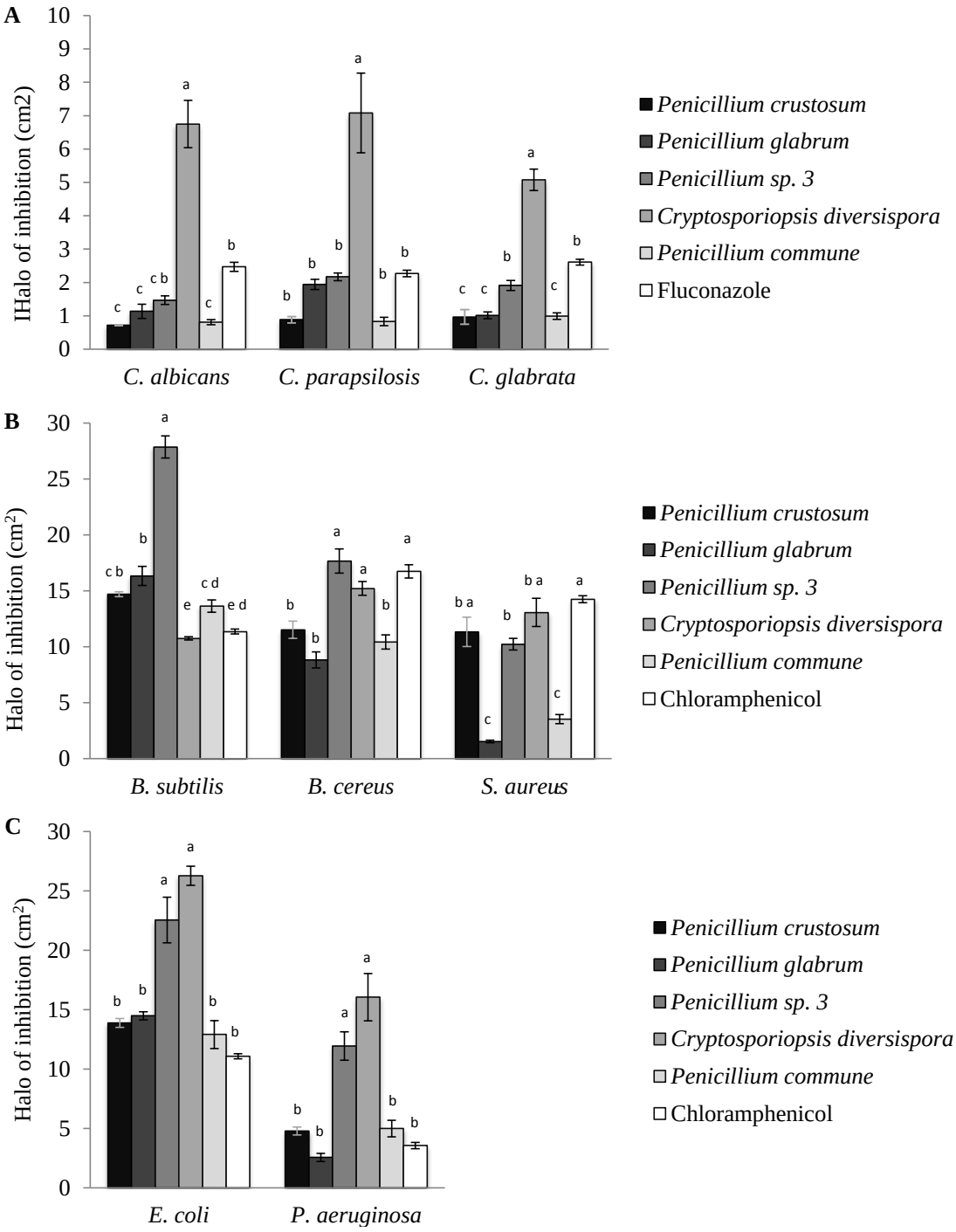

Figure 2 - Antimicrobial activity (halo of inhibition, in area) against yeasts (A), gram-positive bacteria (B) and gram-negative bacteria (C) displayed by the endophytic fungi *Penicillium glabrum*, *Penicillium crustosum*, *Penicillium commune*, *Cryptosporiopsis diversispora,* and *Penicillium* sp. 3. Each value is expressed as mean ± standard error of 3 independent experiments performed in triplicate. Bars with different letters indicate values with significant differences at $p<0.05$, within each microorganism. Chloramphenicol (30 µg/ml) and fluconazole (25 µg/ml) were used as positive controls for antibacterial and antifungal activity, respectively.

resistance from time to time. When compared with the control chloramphenicol, *Penicillium* sp.3 reduced significantly the growth of *E. coli* (up to 2.0-fold), *P. aeruginosa* (up to 3.3-fold) and *B. subtilis* (up to 2.5-fold). Species belonging to *Penicillium* genus are well known for their capacity to produce a vast array of bioactive secondary metabolites (Nicoletti et al., 2014), including antimicrobial compounds (Yu et al., 2010).

Analysis of volatile compounds

Results of antimicrobial activity showed that among the five endophytes analysed, *C. diversispora* was the most effective, whereas *P. glabrum* was one of the fungi with lowest activity against bacteria and yeasts. Therefore, the composition of volatile organic compounds (VOCs) of both fungi growing in PDA medium under *in vitro* conditions was analysed by GC/MS and the profiles obtained will be compared in an attempt to identify the compounds potentially responsible for the antimicrobial activity displayed by *C. diversispora*. Controls consisted of uninoculated PDA medium. The compounds appearing in the control were subtracted from those acquired from the *C. diversispora* and *P. glabrum* growing in PDA medium.

With the HS-SPME technique, 22 volatiles were extracted and identified by GC/MS in the two endophytic species. The volatiles identified belong to different chemical classes: 10 sesquiterpenes; 7 alcohols; 3 esters; and 2 ketones (corresponding to those reported in Table 3 and represented in Figure 3). In *C. diversispora* was identified a total of six volatiles compounds, being the most abundant phenylethyl alcohol and 3-methyl-1-butanol, representing 46% and 33% of the total volatile fraction, respectively (Table 3; Figure 3). The remaining volatile compounds were exclusively found in this fungus and comprised two esters (acetic acid, 2-phenylethyl ester and 3,7,12-tridecatrienoic acid, methyl ester), one ketone (ochracin) and one sesquiterpene (β-himachalene).

In *P. glabrum* was identified highest number of volatiles (18), being the sesquiterpene α-guaiene the most abundant, accounting 69% of the total volatile fraction identified in this specie (Table 3; Figure 3). This compound was exclusively identified in this fungus. Also, present exclusively in this specie were a series of sesquiterpenes (β-elemene, ylangene, (E)-β-farnesene, α-guaiene, valencene, α-selinene, β-selinene and two more sesquiterpene-like compounds not identified), alcohols (juniper camphor, and three more alcohol-like compounds not identified), one ketone-like compound and one ester (acetic acid, 2-phenylethyl ester), altogether accounting 31% of the total volatile fraction identified in this specie. Both the alcohols compounds, phenylethyl alcohol and 3-methyl-1-butanol, were also identified in *P. glabrum*, but in lower amounts when compared to *C. diversispora* (0.7-fold lower, each).

In an attempted to elucidate the contribution of the several identified volatile compounds on the antimicrobial activity displayed by the two endophytes, a PCA was

performed by using the data of volatile compounds of the two fungus as well as their inhibition halos (in cm^2) against bacteria and yeasts. The PCA obtained was able to distinguish the two species (Figure 4) explaining in two principal components 85.51% of the total variance captured. The first principal component clearly separate *C. diversispora* from *P. glabrum*, due to the higher content of the first specie on 3-methyl-1-butanol and phenylethyl alcohol. As already stated these two compounds were part of the volatile fraction of both fungi, but in considerable higher amounts in *C. diversispora* ($p<0.001$). The remaining volatiles characterized *P. glabrum*. An important information retained from PCA is that 3-methyl-1-butanol and phenylethyl alcohol are associated with *C. diversispora*, as well as the higher inhibition halos verified for all microorganisms (with exception of *B. subtilis*). Such information suggested the involvement of these two volatile compounds in the inhibition displayed by *C. diversispora* against these microorganisms. The inhibition displayed by *P. glabrum* against *B. subtilis*, could be similarly related with the production of volatiles compounds as highlighted in the figure 4. These hypotheses require however confirmation by performing bioassays with synthetic standards of the respective identified VOCs. Despite this, previous studies have been reported the antimicrobial potential of 3-methyl-1-butanol. For example, the VOCs produced by the endophyte *Muscodor crispans*, with 3-methyl-1-butanol as the major compound, have been shown to inhibited the growth of several phytopathogens (Mitchell et al., 2010). Similarly, Fialho et al. (2011) have reported the high antimicrobial effect of the 3-methyl-1-butanol against an array of phytopathogens under *in vitro* conditions. Phenylethyl alcohol is a well-known bacteriostatic agent that, at low concentrations, can inhibit the growth of gram-negative bacteria, including *Salmonella*, *Shigella*, *Aerobacter*, *Klebsiella*, *Escherichia*, *Pseudomonas* and *Proteus* (Naz et al., 2013).

Table 3 – Relative percentage of volatile compounds of the endophytic fungi *Penicillium glabrum* and *Cryptosporiopsis diversispora* growing under *in vitro* conditions (mean ± standard deviation, n=4). Compounds found in the control PDA are not included in this table. Different superscript lower case letters denote statistically significant differences ($p<0.05$) between fungal species.

	Compound	RT[1]	Characteristic ions (m/z)	QI (m/z 100%)[2]	Molecular weight	Molecular formula	*P. glabrum*	*C. diversispora*
1	3-Methyl-1-butanol	4.705	42/***55***/70	55	88	$C_5H_{12}O$	4.01±0.73[a]	32.75±1.97[b]
2	Butanoic acid, 2-methyl-, 3-methyllbutyl ester	23.575	43/57/***70***/85	70	172	$C_{10}H_{20}O_2$	0.63±0.13	_
3	Phenylethyl alcohol	24.05	***91***/92/122	91	122	$C_8H_{10}O$	5.33±2.50[a]	45.83±2.26[b]
4	Acetic acid, 2-phenylethyl ester	34.065	43/***104***	104	164	$C_{10}H_{12}O_2$	_	2.94±0.94
5	β-elemene	43.14	68/***81***/93/107	81	204	$C_{15}H_{24}$	9.06±2.04	_
6	Ylangene	46.535	93/***105***/119/161	105	204	$C_{15}H_{24}$	0.21±0.05	_
7	(E)-β-farnesene	47.4	41/***69***/93	69	204	$C_{15}H_{24}$	0.20±0.05	_
8	Sesquiterpene-like compound	47.82	***105***	105	204	$C_{15}H_{24}$	0.33±0.10	_
9	Sesquiterpene-like compound	48.28	91/105/***133***/189	133	204	$C_{15}H_{24}$	0.32±0.11	_
10	α-Guaiene	49.02	93/***105***/107/147/189	105	204	$C_{15}H_{24}$	68.55±1.75	_
11	Valencene	49.355	79/93/107/***161***	161	204	$C_{15}H_{24}$	2.72±0.62	_
12	α-Selinene	49.445	81/93/107/***189***/204	189	204	$C_{15}H_{24}$	0.86±0.22	_
13	β-Himachalene	49.57	41/105/***119***/134/204	119	204	$C_{15}H_{24}$	_	2.71±0.63
14	Ochracin	51.895	***134***/160/178	134	178	$C_{10}H_{10}O_3$	_	6.57±0.61
15	3,7,12-Tridecatrienoic acid, methyl ester	57.39	55/***67***	67	222	$C_{14}H_{22}O_2$	_	9.20±1.28
16	Alcohol-like compound	57.52	***43***/69/81/95/109/161	43	_	_	0.34±0.05	_
17	β-selinene	58.51	41/81/***107***/161/189/204	107	204	$C_{15}H_{24}$	4.16±0.50	_
18	Juniper camphor	58.66	43/***161***/189/204	161	222	$C_{15}H_{26}O$	0.22±0.11	_
19	Alcohol-like compound	58.945	***41***/81/91/107/131/145	41	_	_	0.11±0.03	_
20	Alcohol-like compound	62,225	43/79/105/119/147/***162***	162	_	_	0.13±0.04	_
21	Unknown compound	63.995	***135***/150	135	_	_	2.62±0.46	_
22	Ketone-like compound	66.43	147/161/***218***	218	_	_	0.24±0.03	_

[1]Retention time; [2]Quantification ions.

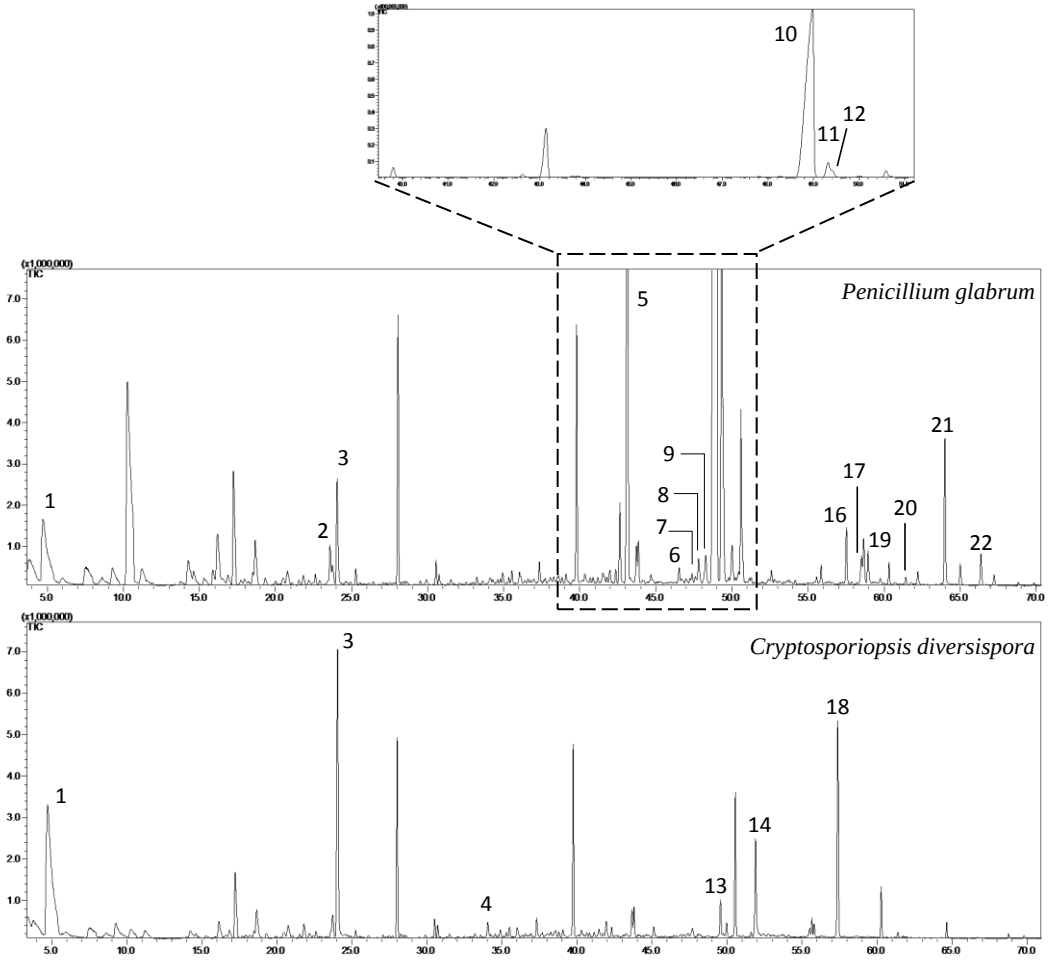

Figure 3 - Full scan chromatographic profile obtained from the endophytic fungi *Penicillium glabrum* and *Cryptosporiopsis diversispora* growing *in vitro* by HS-SPME using DVB/CAR/PDMS fiber (identification numbers correspond to those compounds reported in Table 3).

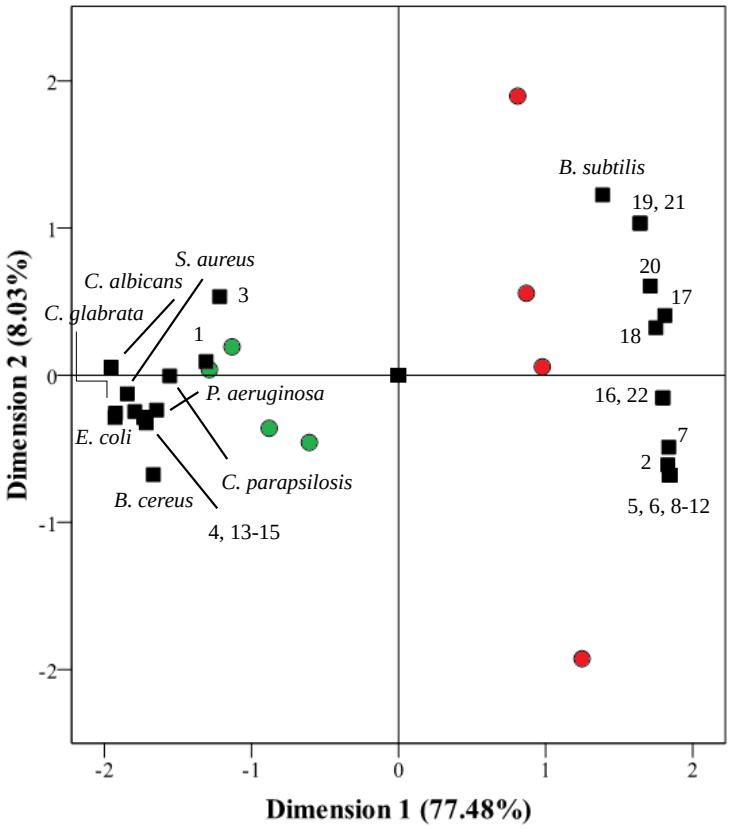

Figure 4 - Principal component analysis obtained using the volatile composition of *Penicillium glabrum* (●) and *Cryptosporiopsis diversispora* (●) growing i*n vitro* and antimicrobial activity displayed by these fungi against bacteria and yeasts, evaluated as halo inhibition (in cm^2). The PCA factors explain 85.51% of the total variance. The numbers represented correspond to those compounds presented in Table 3.

Conclusion

The results presented in this work show appreciable fungal diversity recovered from *A. unedo* that are relatively unexplored as hosts for fungal endophytes. The considerable high number of fungal endophytes observed combined with the apparent organ specificity exhibited makes this plant specie suitable in search for novel metabolites. The evaluation of capability of five fungal endophytes from *A. unedo* to produce compounds with antibacterial or antifungal activity indicates that both *C. diversispora* and *Penicillium* sp. 3 have great potential for exploitation in the development of new drugs. The antimicrobial activity displayed by *C. diversispora* may be ascribed to the volatile compounds 3-methyl-1-butanol and phenylethyl alcohol, which requires confirmation. Further investigations will be planned to identify and characterize active principles, and assess toxicity by laboratory assays.

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References

Arnold, A.E. (2007). Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biology Reviews, 21: 51–66.

Banerjee, D., Strobel, G., Geary, B., Sears, J., Ezra, D., Liarzi, O., Coombs, J. (2010). *Muscodor albus* strain GBA, an endophytic fungus of *Ginkgo biloba* from the United States of America, produces volatile antimicrobials. Mycology, 1:179–186.

Bray, J.R., Curtis, J.T. (1957). An ordination of the upland forest communities of Southern Wisconsin. Ecological Monographies, 27:325-349.

Carlet, J., Jarlier, V., Harbarth, S., Voss. A., Goossens, H., and Pittet, D. (2012). Ready for a world without antibiotics? The Pensières Antibiotic Resistance Call to Action. Antimicrobial Resistance and Infection Control, 1:11-13.

Celikel G, Demirsoy L, Demirsoy H. (2008). The strawberry tree (*Arbutus unedo* L.) selection in Turkey. Scientia Horticulturae, 118: 115-119.

Clarke, J (1993) Non-parametric multivariate analysis of changes in community structure. Aust J Ecol, 18:117–143.

Collado, J., Platas, G., Peláez, F. (2000). Host specificity in fungal endophytic populations of *Quercus ilex* and *Quercus faginea* from central Spain. Nova Hedwigia, 71: 421–430.

Debbab, A., Pretsch, A., Edrada-Ebel, R., Wray, V., Proksch, P. (2010). New antibiotic metabolites from the fungal endophyte Stemphylium globuliferum isolated from Mentha pulegium. Planta Med, 76 - 401.

Eo, J.K., Lee, B.H., Eom, A.H. (2014). A New Report on *Oidiodendron flavum* Isolated from Field Soil in Korea. The Korean Journal of Mycology, 42: 239-242.

Fialho, M.B., Moraes, M.H.D.D., Tremocoldi, A.R., Pascholati, S.F. (2011). Potential of antimicrobial volatile organic compounds to control *Sclerotinia sclerotiorum* in bean seeds. Pesquisa Agropecuária Brasileira, 46(2): 137-142.

Fisher, P.J., Petrini, O., and Sutton, B.C. (1993). A comparative study of fungal endophytes in leaves, xylem and bark of *Eucalyptus nitens* in Australia and England. Sydowia, 45(2): 338-345.

Guo, L.D., Huang, G.R., Wang, Y. (2008).Seasonal and tissue age influences on endophytic fungi of *Pinus tabulaeformis* (Pinaceae) in the Dongling Mountains, Beijing. Journal of Integrative Plant Biology, 50: 997–1003.

Hoff, J.A., Klopfenstein, N.B., McDonald, G.I., Tonn, J.R., Kim, M.S., Zambino, P.J., Hessburg, P.F., Rogers, J.D., Peever, T.L., and Carris, L.M. (2004). Fungal endophytes in woody roots of Douglas-fir (*Pseudotsuga menziesii*) and ponderosa pine (*Pinus ponderosa*). For. Pathol, 34: 255.

Hwang, H.G., Kim, Y.N., Baik, K.S., Choi, S.K. (2011). Isolation and Characteristics of Endolichenic Fungi Producing Antifungal Compound. The Korean Journal of Microbiology, 47(1): 97-101.

Hyde, K.D., and Soytong, K. (2008). The fungal endophyte dilemma. Fungal Diversity, 33: 163 – 173.

IDSA - Infectious Diseases Society of America. (2010). The 10 × ‘20 initiative: pursuing a global commitment to develop 10 new antibacterial drugs by 2020. Clin Infect Dis, 50:1081-1083.

Kaul, S., Gupta, S., Ahmed, M., Dhar, M.K. (2012). Endophytic fungi from medicinal plants: a treasure hunt for bioactive metabolites. Phytochem Rev, 11(4): 487–505.

Kruskal, J. (1964). Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. Psychometrika, 29: 1–27.

Kudalkar, P., Strobel, G., Riyaz-Ul-Hassan, S., Geary, B., Sears, J. (2012). *Muscodor sutura*, a novel endophytic fungus with volatile antibiotic activities. Mycoscience, 53: 319–325.

Kusari, S., and Spiteller, M. (2011). Are we ready for industrial production of bioactive plant secondary metabolites utilizing endophytes? Nat. Prod. Rep, 28: 1203–1207.

Kusari, S., Hertweck, C., and Spiteller, M. (2012). Chemical Ecology of Endophytic Fungi: Origins of Secondary Metabolites. Chemistry & Biology, 792-798.

Kusari, S., Lamshöft, M., and Spiteller, M. (2009). *Aspergillus fumigatus* Fresenius, an endophytic fungus from *Juniperus communis* L. Horstmann as a novel source of

the anticancer pro-drug deoxypodophyllotoxin. Jounal Applied Microbiology, 107: 1019–1030.

Kusari, S., Lamshöft, M., Zühlke, S., and Spiteller, M. (2008). An endophytic fungus from *Hypericum perforatum* that produces hypericin. Journal of Natural Products, 71: 159–162.

Kusari, S., Verma, VC., Lamshöft, M., and Spiteller, M. (2012). An endophytic fungus from *Azadirachta indica* A. Juss. that produces azadirachtin. World J. Microbiol. Biotechnol, 28: 1287–1294.

Li, G.H., Yu, Z.F., Li, X., Wang, X.B., Zheng, L.J., and Zhang, K.Q. (2007). Nematicidal Metabolites Produced by the Endophytic Fungus *Geotrichum* sp. Chemistry and Biodiversity, 4: 1520-1524.

Linaldeddu, B.T., Sirca, C., Spano, D., Franceschini, A. (2011). Variation of endophytic cork oak-associated fungal communities in relation to plant health and water stress. Forest Pathol, 41: 193–201.

Magurran AE (2004) Measuring biological diversity.

Moricca, S., Ginetti, B., Ragazzi, A. (2012). Species and organ specificity in endophytes colonizing healthy and declining Mediterranean oaks. Phytopathologia Mediterranea, 51: 587−598.

Mousa, W.K.; and Raizada, M.N. (2013). The diversity of anti-microbial secondary metabolites produced by fungal endophytes: an interdisciplinary perspective. Frontiers in microbiology, 4: 65.

Nalini, M.S., Mahesh, B., Tejesvi, M.V., Prakash, H.S., Subbaiah, V., Kini, K.R., and Shetty, H.S. (2005). Fungal endophytes from the three-leaved caper, Crataeva magna (Lour.) DC. (Capparidaceae). Mycopathologia, 159: 245–249.

Naz, S., Cretenet, M., & Vernoux, J. P. (2013). Current knowledge on antimicrobial metabolites produced from aromatic amino acid metabolism in fermented products. Microbial pathogens and strategies for combating them: science, technology and education (A. Méndez-Vilas, Ed.) FORMATEX, 337-346.

Nicoletti, R., Fiorentino, A., and Scognamiglio, M. (2014). Endophytism of *Penicillium* Species in Woody Plants. Open Mycology Journal, 8: 1-26.

Nordberg, P., Monnet, D.L., Cars, O. (2013). Background Paper, BP 6.1 Antimicrobial resistance. Boston University.

Okane, I., Nagakiri, A., Ito, T. (1995).Discostroma tricellulaire, a new endophytic ascomycete with a *Seimatosporium* anamorph isolated from Rhodendron. Can. J. Bot., 74: 1338–1344.

Okane, I., Nakagiri, A., Ito, T. (1998). Endophytic fungi in leaves of ericaceous plants. Canadian Journal of Botany, 76(4): 657-663.

Oliveira, I., Batista, P., Bento, A., Pereira, JA. (2011). *Arbutus unedo* L. and its benefits on human health. Journal of Food and Nutrition Research, 50: 73-85

Oliveira, I., Pereira, J.A, Lino-Neto, T., Bento, A., Baptista, P., 2012. Fungal Diversity Associated to the Olive Moth, *Prays Oleae* Bernard: A Survey for Potential Entomopathogenic Fungi. Microbial Ecology, 63(4): 964-74.

Pancher, M., Ceol, M., Corneo, P.E., Longa, C.M.O., Yousaf, S., Pertot, I., and Campisano, A. (2012). Fungal endophytic communities in grapevines (*Vitis vinifera* L.) respond to crop management. Appl Environ Microbiol, 78: 4308–4317.

Pereira, E., Santos, A., Reis, F,. Tavares M.R., Baptista, P., Lino-Neto, T., Almeida-Aguiar, C. (2013). A new effective assay to detect antimicrobial activity of filamentous fungi, Microbiological Research, 168: 1-5.

Petrini, O., Sieber, T.N., Toti, L., and Viret, O. (1992). Ecology, metabolite production and substrate utilization in endophytic fungi. Natural Toxins, 1: 185-196.

Purmale, L., Apine, I., Nikolajev, V., Granti, L., Verkly, G., and Tomsone, S. (2012). Endophytic fungi evergreen rhododendrons cultivated in vitro and in vivo. Environ Exp Biol, 10: 1-7.

Qi, F., Jing, T., Zhan, Y. (2012). Characterization of Endophytic Fungi from *Acer ginnala* Maxim. in an Artificial Plantation: Media Effect and Tissue-Dependent Variation. PLOS ONE, 7(10): e46785.

Schoch, CL, Seifert, K., Huhndorf, S., Robert, V., Spoug, J.L., Levesque, C.A., Chen, W. (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. PNAS, 109: 6241–6.

Seaby RM, Henderson PA (2006) Species Diversity and Richness Version 4.

Sebastianes, F.L.S., Romão-Dumaresq, A.S., Lacava, P.T., Harakava, R., Azevedo, J.L., Soares de Melo, I., Pizzirani-Kleiner, A.A. (2013). Species diversity of culturable endophytic fungi from Brazilian mangrove forests. Curr Genet, 59:153–166.

Shiono, Y., Yokoi, M., Koseki, T., Murayama, T., Aburai, N., and Kimura, K. (2010). Allantopyrone A, a new α-pyrone metabolite with potent cytotoxicity from an

endophytic fungus, *Allantophomopsis lycopodina* KS-97. The Journal of Antibiotics, 63: 251–253.

Strobel GA, Daisy B, 2003. Bioprospecting for microbial endophytes and their natural products. Microbiology and Molecular Biology Reviews, 67: 491–502

Strobel, G., Daisy, B., Castillo, U., Harper, J. (2004). Natural products from endophytic microorganisms. Journal of Natural Products, 67: 257-268.

Strobel, G.A., Miller, R.V., Miller, C., Condron, M., Teplow, D.B., Hess, W.M. (1999). Cryptocandin, a potent antimycotic from the endophytic fungus *Cryptosporiopsis* cf. *quercina*. Microbiology, 145: 1919–1926.

Sun, X., Ding, Q., Hyde, K.D., Guo, L.D. (2012). Community structure and preference of endophytic fungi of three woody plants in a mixed forest. Fungal Ecol, 5: 624-632.

Suryanarayanan, TS., Venkatesan, G., Murali, T.S. (2003). Endophytic fungal communities in leaves of tropical forest trees: diversity and distribution patterns. Current Science 85: 489-493

Verkley, G.J.M. (1999). A monograph of the genus Pezicula and its anamorphs. Studies in mycology, 44: 180.

White, T.j., Bruns, T., Lee, S., Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M., Gelfand, D., Shinsky, J., White, T.J. (eds), PCR Protocols: A Guide to Methods and Applications, Academic Press, San Diego, 315-322.

Yu, H., Zhang, L., Li, L., Zheng, C., Guo, L., Li, W., Qin, L. (2010). Recent developments and future prospects of antimicrobial metabolites produced by endophytes. Microbiological research, 165(6): 437-449.

Zilla, M.K., Qadri, M., Pathania, A.S., Strobel, G.A., Nalli, Y., Kumar, S., Guru, S.K., Bhushan, S., Singh, S.K., Vishwakarma, R.A., Riyaz-Ul-Hassan, S., Asif Ali, A. (2013). Bioactive metabolites from an endophytic Cryptosporiopsis sp. inhabiting Clidemia hirta. Phytochemistry, 95: 291–297.

Supporting information

Table S1 - Fungal taxa isolated in this study and the summary of BLAST results, showing the coverage of the sequences and sequence similarities with the most closely related organisms.

Isolated (GenBank Acc. no. of the ITS sequence)	Query coverage	Sequence similarity	E-value*	Organism with the highest sequence identity, GenBank Acc. no.
Allantophomopsis lycopodina (submitted)	81%	95%	$3{,}00E^{-164}$	JX981469
Alternaria alternata (submitted)	86%	96%	0.0	JN867471
Aureobasidium pullulans (submitted)	100%	99%	0.0	KC897669
Chromelosporium carneum (submitted)	99%	88%	$4{,}00E^{-112}$	JF440586
Cladosporium sp. (submitted)	99%	100%	0.0	KJ598873
Cryptosporiopsis diversispora (submitted)	99%	98%	0.0	JF714251
Discostroma sp. (submitted)	99%	96%	$8{,}00E^{-133}$	JN545795
Fungal sp. (submitted)	100%	100%	0.0	KC867746
Fungal sp. (submitted)	100%	99%	0.0	HM123559
Helotiales sp. (submitted)	100%	98%	0.0	KC180699
Microsphaeropsis olivacea (submitted)	99%	99%	0.0	JX681101
Penicillium brevicompactum (submitted)	100%	99%	0.0	KF876834
Penicillium commune (submitted)	80%	99%	0.0	KF938402
Penicillium crustosum (submitted)	64%	96%	0.0	LN482509
Penicillium glabrum (submitted)	100%	99%	0.0	AY373915

Penicillium griseolum (submitted)	95%	99%	0.0	EF422848
Penicillium sanguifluum (submitted)	100%	100%	0.0	JX140866
Penicillium sp (submitted)	99%	95%	$7{,}00E^{-103}$	JF429680
Penicillium sp. (submitted)	96%	99%	0.0	KF367506
Penicillium thomii (submitted)	100%	100%	0.0	JX140779
Pezizomycetes sp. (submitted)	100%	97%	0.0	JQ759165
Phialocephala sp. (submitted)	100%	99%	0.0	KJ425309
Stemphylium globuliferum (submitted)	93%	99%	0.0	KF871456
Trichocomaceae sp. (submitted)	100%	100%	0.0	KC007327
Umbelopsis sp. (submitted)	100%	100%	0.0	JQ912671
Umbelopsis sp. (submitted)	100%	99%	0.0	KC816011
Umbelopsis vinacea (submitted)	100%	97%	$3{,}00E^{-127}$	GU183114
Uncultured Ascomycota (submitted)	96%	99%	0.0	FR682256
Uncultured Cladosporium clone (submitted)	94%	88%	$1{,}00E^{-143}$	JF449738
Uncultured fungus clone (submitted)	100%	99%	0.0	HM044625
Uncultured fungus clone (submitted)	100%	100%	$5{,}00E^{-35}$	JF720038
Uncultured Rhizoctonia (submitted)	100%	99%	0.0	DQ061931

*BLAST expected value represents the number of sequence matches expected by random chance (the smaller the value, the better the match to the reported NCBI database sequence)

Figure S1 – *In vitro* fungal culture flasks (50 mL) sealed with a cap containing a silicone septum. This system was used to evaluate the production of volatile organic

Capítulo 4

Conclusão

Conclusão

A bioprospeção de metabolitos com atividade antimicrobiana em fungos endofíticos isolados de plantas medicinais tem despertado a atenção dos investigadores, pelo facto de sintetizarem metabolitos secundários semelhantes às suas plantas hospedeiras. Os fungos endofíticos, com capacidade de sintetizar os compostos das suas plantas hospedeiras, sob condições ótimas de cultura, pode ser um meio para a obtenção de compostos bioativos a baixo-custo, ecológico, reprodutível e compatível com a sua exploração industrial. O medronheiro (*Arbutus unedo* L.) é uma árvore fruteira endémica da região mediterrânica, pertencente à família Ericacea e ao género *Arbutus*. Em Portugal existe preponderantemente a sul do rio Tejo podendo contudo, encontrar-se difundido por todo o país, inclusive em Trás-os-Montes. Desde os tempos ancestrais que diversas partes desta árvore, em especial folhas, raízes, casca e fruto, são utilizadas na medicina tradicional. Tanto quanto é do nosso conhecimento, a comunidade fúngica endofítica associada a esta espécie bem como o potencial antimicrobiano destes fungos nunca foi explorado. Assim, neste trabalho avaliou-se pela primeira vez a comunidade fúngica endofítica associada às diversas partes desta planta utilizadas vulgarmente na medicina tradicional (folhas, ramos, raízes e cascas) e o seu potencial antimicrobiano contra bactérias gram-positivas e gram-negativas, e leveduras patogénicas humanas. A composição volátil de alguns endófitos foi ainda analisada e correlacionada com a atividade antimicrobiana exibida pelos endófitos.

A comunidade fúngica endofítica associada ao medronheiro demonstrou ser muito abundante e diversa. Dos 700 segmentos vegetais analisados, foi obtido um total de 288 isolados pertencentes a 118 taxa. Todas as árvores analisadas encontravam-se colonizadas por fungos endofíticos, apresentando cada árvore uma média de 20,6 taxa e 41,1 isolados. A sequenciação da região ITS do rDNA das 33 espécies mais abundantes, permitiu a identificação até à espécie de 15 taxa. As restantes 18 taxa não foram identificadas até à espécie pelo fato de não se encontrarem especificadas na base de dados GenBank. As espécies identificadas molecularmente pertencem a 10 famílias e 11 géneros, sendo o género *Penicillium* (10 taxa) e *Umbelopsis* (3 taxa) os mais diversificados. A espécie *Cryptosporiopsis diversispora* foi a mais comum, representando 26% do total de isolados e com uma frequência de colonização de 3,4%. As diferentes partes da planta analisadas apresentaram diferenças em termos de composição, riqueza e diversidade de fungos endofíticos. As cascas apresentaram um

maior número de espécies (48) e de isolados (142), comparado com as raízes (39 taxa, 73 isolados), ramos (22 taxa, 32 isolados) e folhas (13 taxa, 41 isolados). A frequência de colonização também foi superior nas cascas (81%) face às raízes (42%), folhas (23%) e ramos (18%). A comunidade fúngica endofítica variou ainda de acordo com o órgão da planta, verificando-se diferenças de predominância de determinadas espécies entre os órgãos amostrados. Este resultado reflete a preferência individual das espécies endofiticas para um determinado órgão/tecido. Este fato pode estar relacionado com diferenças de composição química entre os diferentes órgãos da planta permitindo o desenvolvimento de determinada espécie fúngica.

De entre as espécies identificadas selecionaram-se as cinco mais abundantes para avaliar a sua capacidade antimicrobiana contra bactérias gram-positiva e gram-negativa e leveduras. As espécies selecionadas foram *Penicillium glabrum*, *Penicillium crustosum*, *Penicillium commune* e *C. diversispora,* isoladas das cascas, e *Penicillium* sp. 3 isolada da raiz. De entre estas, apenas *C. diversispora* e *Penicillium* sp. 3, mostraram capacidade para inibir significativamente os microorganismos testados face aos antibióticos / antifúngicos comerciais. A espécie *C. diversispora* apresentou um amplo espetro, sendo a única que apresentou capacidade em inibir significativamente as leveduras *Candida glabrata*, *Candida albicans* e *Candida parapsilosis* mais de 1,2 vezes face ao antifúngico fluconazole (25 μg/ml). Esta espécie também mostrou ser a mais eficaz contra as bactérias gram-negativas *Escherichia coli* e *Pseudomonas aeruginosa*, inibindo significativamente o seu crescimento em mais de 2,4 vezes face ao antibiótico cloranfenicol (30 μg/ml). Por sua vez, *Penicillium* sp.3 demonstrou ser mais eficaz do que os antibióticos comerciais contra bactérias gram-positiva e gram-negativa.

A avaliação dos compostos voláteis por GC/MS dos fungos que apresentaram maior (*C. diversispora*) e menor (*P. glabrum*) atividade antimicrobiana, em crescimento sob condições *in vitro*, permitiu identificar um total de 22 compostos pertencentes a quatro classes químicas: sesquiterpenos (10), álcoois (7), éstere (3), e cetonas (2). Na espécie *C. diversispora* foi identificado um total de seis voláteis sendo os mais abundantes, o fenil-etil álcool e o 3-metil-1-butanol, representando 46% e 33% do total da fração volátil, respectivamente. Por sua vez, na espécie *P. glabrum* foi identificado um número superior de compostos voláteis (18 no total), sendo o sesquiterpeno α-guaiene o mais abundante representando cerca de 69% do total da fração volátil. Identificou-se, nesta espécie, a produção exclusiva de 16 compostos voláteis. A maioria

dos compostos voláteis identificados encontram-se descritos como possuindo propriedades antimicrobianas o que poderá explicar, em parte, a atividade antimicrobiana exibida pelos dois endófitos. A comparação do perfil volátil de *C. diversispora* e *P. glabrum* associada a uma análise de componentes principais demonstrou o envolvimento dos compostos voláteis na atividade antimicrobiana exibida pelas espécies endófitas. Esta analise evidenciou o envolvimento de 3-metil-1-butanol e fenil-etil álcool, produzido por *C. diversispora*, no processo de inibição deste fungo contra as leveduras e as bactérias gram-negativas.

Os resultados obtidos evidenciam, pela primeira vez, o potencial antimicrobiano de espécies fúngicas endofiticas do medronheiro. A espécie *C. diversispora* parece ser a mais promissora como fonte de antibióticos/antifúngicos naturais que poderão, no futuro, ser exploradas ao nível da indústria farmacêutica. No entanto, os resultados obtidos abrem novas perspetivas para o estudo mais detalhado da comunidade fúngica associada ao medronheiro e a bioprospeção de outros isolados obtidos. É ainda necessário proceder-se à elucidação da natureza química dos metabolitos produzidos e da avaliação da sua capacidade antimicrobiana.