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João Filipe da Silva Martins

BREEDING OF *ARBUTUS UNEDO* L.: USE OF CONVENTIONAL AND BIOTECHNOLOGICAL TOOLS TO OBTAIN TOLERANT GENOTYPES AGAINST ABIOTIC AND BIOTIC STRESSES

Tese no âmbito do Doutoramento em Biociências especialidade em Biotecnologia, orientada pelo Professor Doutor Jorge Manuel Pataca Leal Canhoto e pela Professora Doutora Glória Catarina Cintra da Costa Pinto e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Outubro de 2021

Faculdade de Ciências e Tecnologia
da Universidade de Coimbra

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Resumo

Arbutus unedo L. (medronheiro, Ericaceae) é uma árvore perene com uma distribuição circum-mediterrânica. Devido à sua resiliência a stresses abióticos e bióticos, tem grande relevância ecológica nas florestas do sul da Europa. As suas bagas vermelhas são comestíveis e usadas na produção de produtos tradicionais, incluindo um destilado de grande valor económico. Além disso, diversos compostos com atividade biológica têm sido usados na indústria farmacêutica e cosmética. De uma espécie negligenciada, o medronheiro tem vindo gradualmente a converter-se numa espécie de grande valor, com uma crescente área de plantação. Devido ao aumento da procura de plantas com características melhoradas por parte dos produtores, a implementação de um programa de melhoramento para a espécie é crucial. Apesar da intensa atividade de investigação realizada nos últimos anos em medronheiro, existem ainda muitas lacunas no conhecimento que terão de ser preenchidas, tais como o conhecimento da biologia da planta, otimização de protocolos de propagação e dos mecanismos por detrás da resistência aos stresses abiótico e biótico do medronheiro.

Assim, três linhas principais de investigação foram seguidas nesta tese: i) desenvolvimento e otimização de protocolos de micropropagação (embriogénese somática, proliferação de meristemas e organogénese em meio líquido), avaliação da capacidade de tolerância ao stress hídrico das plantas micropropagadas e estudo do microbioma das plantas *in vitro*; ii) estudo da biologia reprodutiva, cruzamento e seleção de genótipos resistentes ao stress hídrico, e estudo dos mecanismos de tolerância em condições de stress hídrico; e iii) caracterização do microbioma, caracterização química, e a sua relevância na defesa da planta.

A indução de embriogénese somática revelou-se particularmente eficaz com 2 mg L^{-1} de 6-benzilaminopurina (BAP) e 2 mg L^{-1} de ácido 1-naftalenoacético (NAA), com taxas de indução de 97,22% ± 4,81. No entanto, as taxas de indução variam em função do genótipo. Apesar de a fase de indução ser eficaz, a conversão de embriões em plantas é ainda limitada, o que poderá estar relacionado com a ocorrência de anomalias. A proliferação de meristemas em meio líquido revelou-se um método bastante eficaz assim como através de organogénese. Apesar de apresentaram sinais de vitrificação, as plantas recuperam o seu fenótipo normal e a sua performance em stresse hídrico, avaliada através de parâmetros fisiológicos, não é afetada. No que diz respeito à análise do microbioma, 79 géneros de bactérias pertencentes a 7 filos e apenas um género de Archaea foram identificados. O filo Actinobacteriota foi o mais abundante e diverso (48%), seguido pelo filo Proteobacteria (43%). Foram encontradas diferenças entre genótipos na composição do microbioma nas plantas *in vitro*. No entanto, essas diferenças diluíram-se quando as plantas foram aclimatizadas e um aumento significativo de diversidade foi igualmente observado. Um estudo morfológico das estruturas reprodutoras e da fenologia da planta permitiu a realização de

cruzamentos controlados e obtenção de híbridos, que quando submetidos a condições de défice hídrico revelarem grande plasticidade fenotípica. Além da importância do genótipo na tolerância ao stresse hídrico, verificou-se que a proveniência das plantas poderá também ter a sua contribuição. Este trabalho confirma o fato de o medronheiro apresentar um comportamento tipicamente isohídrico em condições de stresse hídrico, através de um rigoroso controlo dos estomas. Um perfil metabólico específico foi encontrado num genótipo tolerante e várias hormonas chave (*e.g.*, ácido abscísico e salicílico) mostraram estar regulados positivamente em plantas sob stresse hídrico. No que diz respeito à comunidade de endófitos cultivável, foram isoladas e identificadas diversas espécies de fungos (*e.g.*, *Aureobasidium pullulans* e *Trichoderma atroviride*) e bactérias (ex. *Bacillus cereus* e *Paenibacillus humicus*). Alguns destes microrganismos produzem várias enzimas, tais como celulases e proteases, sideróforos, e outros compostos que podem desempenhar um papel essencial nos mecanismos de defesa da planta e reduzir o efeito dos mais relevantes patógenos do medronheiro, entre eles *Phytophthora cinnamomi*. Em particular, *Trichoderma atroviride* e *Bacillus cereus* revelaram ser antagonistas de vários fitopatógenos. Finalmente, através de análises químicas, foram identificados 54 compostos na folha, e o perfil químico mostrou variações entre genótipos e também sazonais. O extrato foliar da planta, assim como alguns compostos fenólicos isolados (arbutina e hidroquinona), mostraram ter atividade antifúngica, o que revela um complexo e intrincado mecanismo de defesa contras patógenos.

Na sua globalidade, este trabalho interdisciplinar, realizado com a colaboração de diferentes laboratórios, representa um considerável avanço no conhecimento sobre a biologia de *A. unedo*. Assim, foi possível otimizar protocolos de micropropagação, compreender aspetos relacionados com a reprodução sexuada, analisar o papel de metabolitos secundários em termos de resposta ao stresse e perceber a interação da espécie com microrganismos. Além disso, os resultados obtidos abriram também caminho para novas linhas de investigação que poderão ajudar a promover a fileira do medronho e tornar ainda mais percetível a passagem desta espécie de negligenciada a espécie de interesse agrícola, especialmente em zonas onde outras espécies arbóreas ou arbustivas são difíceis de cultivar. As áreas de investigação consideradas prioritárias no futuro são também destacadas, tais como a seleção de plantas, o desenvolvimento de métodos rápidos de seleção através de marcadores metabólicos, a avaliação da introdução de endófitos em plantas produzidas em viveiro ou diretamente no campo, assim como o desenvolvimento de projetos de sequenciação genómica que permitam realizar estudos de biologia fundamental e a seleção com base em marcadores genómicos.

Palavras-chave: controlo biológico, hibridização, metabolómica, microbioma, micropropagação, seleção, stresse hídrico

Abstract

Arbutus unedo L. (strawberry tree, Ericaceae) is a perennial tree with a circum-Mediterranean distribution. It has a great ecological relevance in south Europe forests due to its resilience against abiotic and biotic stresses. The edible red berries are used in the production of traditional products, including a high-value spirit. Furthermore, several compounds with bioactive properties have been used by the cosmetic and the pharmaceutical industries. From a neglected species, strawberry tree has been gradually converted into a high valuable crop with an increased cultivation area in Southern European and Northern African countries. Due to an increasing demand for plants with improved features by farmers and other stakeholders, it is crucial to establish a breeding program for this species. Despite the intense research activity carried out in recent years in strawberry tree, some major knowledge gaps still need to be filled, such as our understanding of plant biology, optimization of propagation protocols and the mechanisms behind abiotic and biotic stress resistance.

Thus, three main lines of investigation were followed in this thesis: i) development and optimization of micropropagation protocols (somatic embryogenesis, shoot proliferation and organogenesis in liquid medium), evaluation of the water stress tolerance capacity of micropropagated plants and study of the microbiome of plants *in vitro*; ii) study of reproductive biology, breeding and selection of genotypes resistant to water stress, and study of tolerance mechanisms under water stress conditions; and iii) microbiome characterization, chemical fingerprint, and their relevance for plant defence.

Induction of somatic embryogenesis was particularly effective with 2 mg L^{-1} of 6-benzylaminopurine (BAP) and 2 mg L^{-1} of 1-naphthaleneacetic acid (NAA), with induction rates of 97.22% ± 4.81. However, induction rates vary depending on the genotype. Although the induction phase is effective, the conversion of embryos into plants is still limited, which could be related to the anomalies observed in several somatic embryos. The proliferation of meristems in liquid medium proved to be a very effective method as well as through organogenesis. Despite showing signs of hyperhydricity, the plants recover their normal phenotype and their performance under water stress, evaluated through physiological parameters, is not affected. Regarding the microbiome analysis, 79 bacterial genera belonging to 7 phyla and only one Archaea genus were identified. The phylum Actinobacteriota was the most abundant and diverse (48%), followed by the phylum Proteobacteria (43%). Differences between genotypes were found in the composition of the microbiome in plants *in vitro*. However, these differences were diluted when the plants were acclimatized and a significant increase in diversity was also observed. A morphological study of the reproductive structures and of the plant's phenology allowed the realization of controlled crossings and the obtainment of hybrids, which when subjected to conditions of water deficit reveal

great phenotypic plasticity. In addition to the importance of the genotype in tolerance to water stress, it was found that the origin of the plants could also play an important role. This work confirms the fact that the strawberry tree presents a typical isohydric behaviour in water stress, through a rigorous control of the stomata and adds new scientific knowledge. A specific metabolic profile was found in the tolerant genotype and several key hormones (*e.g.*, abscisic and salicylic acid) were up-regulated in plants under water stress. With regard to the cultivable endophyte community, several species of fungi (*e.g.*, *Aureobasidium pullulans* and *Trichoderma atroviride*) and bacteria (*e.g.*, *Bacillus cereus* and *Paenibacillus humicus*) were isolated and identified. Some of these microorganisms produce various enzymes, such as cellulases and proteases, siderophores, and other compounds that can play an essential role in the plant defence mechanisms and reduce the effect of the most relevant pathogens of strawberry tree, such as *Phytophthora cinnamomi*. In particular, *Trichoderma atroviride* and *Bacillus cereus* have been shown to be antagonists of several phytopathogens. Finally, through a chemical analysis, 54 compounds were identified in the leaf, and the chemical profile showed variations between genotypes and also seasonal. The plant leaf extract, as well as some isolated phenolic compounds (arbutin and hydroquinone) were shown to have antifungal activity, which reveals a complex and intricate defence mechanism against pathogens.

Overall, this interdisciplinary work, carried out in collaboration with different laboratories, represents a considerable advance in the knowledge about the biology of *A. unedo*. Thus, it was possible to optimize micropropagation protocols, understand aspects related to sexual reproduction, analyze the role of secondary metabolites in terms of stress response and understand the interaction of the species with microorganisms. In addition, the results obtained also opened the way for new lines of research that could help to promote even further the conversion of this once neglected species into a plant of agricultural interest, especially in areas where other tree or shrub species are difficult to cultivate. Research areas considered to be priorities for the future are also highlighted, such as plant selection, the development of rapid selection methods using metabolic markers, the evaluation of the introduction of endophytes in nursery or directly on the field, as well as the development of genomic sequencing projects that allow for fundamental biology studies and selection based on genomic markers.

Keywords: biological control, hybridization, metabolomics, microbiome, micropropagation, selection, water stress

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Introduction

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1. *Arbutus unedo* L.

Arbutus unedo L., commonly known as strawberry tree, has a huge economic potential. The edible fruits are commonly used in the manufacture of traditional products such as jam, jelly, and an alcoholic beverage, which is probably the most valuable derived product. Each tree produces on average 7 to 10 kg of fruits and 10 kg of fruits are usually required to produce 1L of spirit (Gomes and Canhoto 2009), which is obtained following fermentation and distillation. Strawberry tree honey is another typical product of some Mediterranean regions (Tuberoso et al. 2010), very appreciated due to its bitter flavor, intense odor and amber color, being rich in amino acids and with antioxidant properties (Rosa et al. 2011). Its price is usually much higher than that of sweet honey since the production is hard and reduced because blooming occurs during the falling season (Tuberoso et al. 2010). Strawberry tree has also been used as a source of biomass for energy production and in the floriculture sector to compose floral bouquets due to the appealing light green color of its young leaves (Metaxas et al. 2004). The large amount of tannins present in the bark make the species useful for tanning (Gomes et al. 2010). Moreover, several chemical compounds with bioactive properties have been identified on different parts of the plant, with applications on pharmaceutical, cosmetic and food industries (Migas and Krauze-Baranowska 2015).

A. unedo was once considered a neglected species and plants were almost exclusively found growing in the wild. However, phytosanitary problems with species once considered more interesting, such as *Pinus pinaster* and *Eucalyptus globulus*, as well as the intensity and frequency of forest fires associated with forest monocultures, have led to a renewed interest in the species, not only by policy authorities but also by farmers and other stakeholders (Garrido and Silveira 2020; Silveira 2020). Consequently, many orchards have been installed and the production area has been constantly increasing over the years, especially in Portugal, that is currently the largest world producer of this small berry (Garrido and Silveira 2020). Nevertheless, in order to obtain higher revenues, producers are looking for plants with improved traits such as high fruit production, tolerance to pathogens and drought stress, that are currently unavailable on the market. In order to fulfill such demands, it is essential to implement a strawberry tree breeding program, by selecting plants with interesting characteristics and to obtain cultivars with improved traits. Thus, the implementation of a traditional plant breeding program for strawberry tree with four major steps is proposed (Figure 1): (1) characterization of the wild populations, (2) selection of plants with interesting characteristics, (3) breeding using different techniques and (4) conservation and propagation of the selected genotypes. Population analysis should be conducted not only at a genetic and chemical level but also by the characterization of the associated microbiome. Such

information is essential to define a criterion for plant selection and further conservation and multiplication. Several breeding techniques can also be used to obtain new genetic combinations and plants with improved traits.

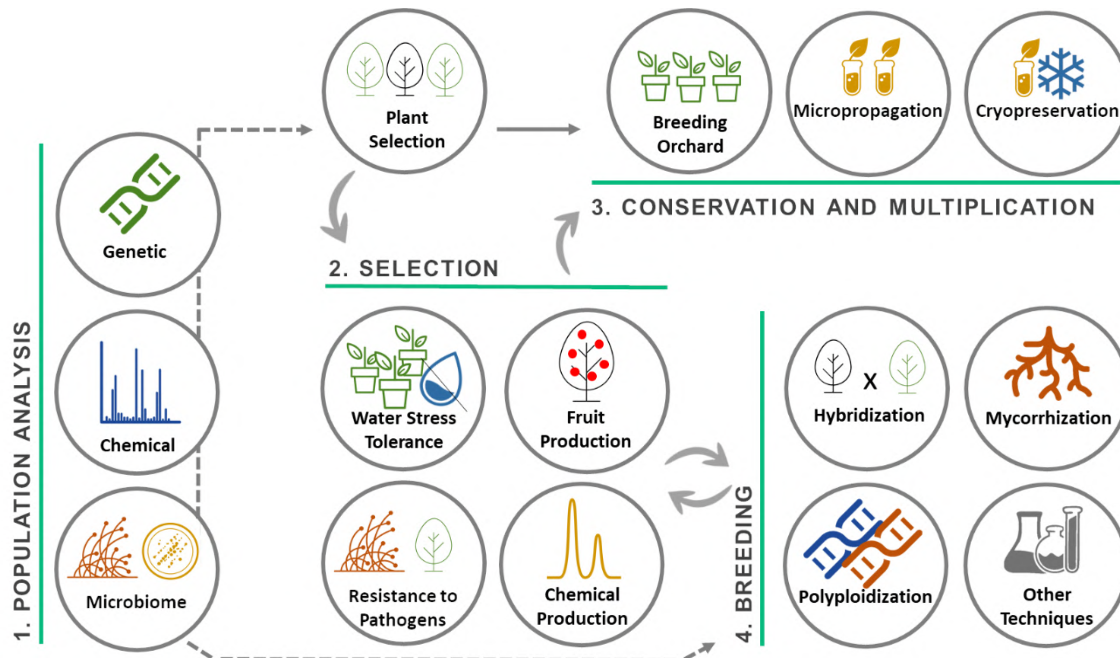

Figure 1. Scheme of a breeding program for strawberry tree, with four major steps: (1) population analysis, (2) selection, (3) conservation and multiplication and (4) breeding.

Information about the innumerous techniques successfully applied on strawberry tree that can be of help on a future breeding program, including in the selection, conservation and propagation steps will be discussed in the following sections. An overview of strawberry tree potential as a forest and crop species for several industrial applications is also provided by bringing together the information available about its botanical and chemical features. In spite of the large amount of literature available, the work done so far has failed to address major areas that might be crucial in the development of a successful long-term breeding program, thus compromising its feasibility. Some of these limitations are discussed on this review as well as the benefits and challenges of a future breeding program for strawberry tree.

1.1. Distribution, botanical description and phenology

Arbutus belongs to the cosmopolitan Ericaceae family, which represents 2% of all the eudicotyledons. It includes about 20 species, distributed along the US West Coast, Central America, Western Europe, Mediterranean Basin, North Africa and the Middle East (Heywood 1993; Stevens 2001). Some of the species, such as *Arbutus canariensis* (Lindl.) and *Arbutus menziesii* (Pursh.) display typical Mediterranean characteristics (Piotto et al. 2001). One of the most important economical species of the genus is *Arbutus unedo* L. (Gomes and Canhoto 2009), a small perennial tree (Figure 2 A) that can stand low temperatures, until -12 ºC (Mereti et al. 2002), and is tolerant

to drought (Munné-Bosch and Peñuelas 2004). Considered a relic of the warm period previous to the last Würm glaciation (Ribeiro et al. 2019), strawberry tree usually grows on acidic, rocky and well-drained soils, along the Mediterranean Basin, from Spain to Turkey, some regions in North Africa, Mediterranean Islands and the Atlantic coast of France, Ireland and Portugal (Figure 2 B, (Torres et al. 2002). It usually grows on shrub communities or forests associated with species of the genera *Quercus* and *Pinus* (Prada and Arizpe 2008).

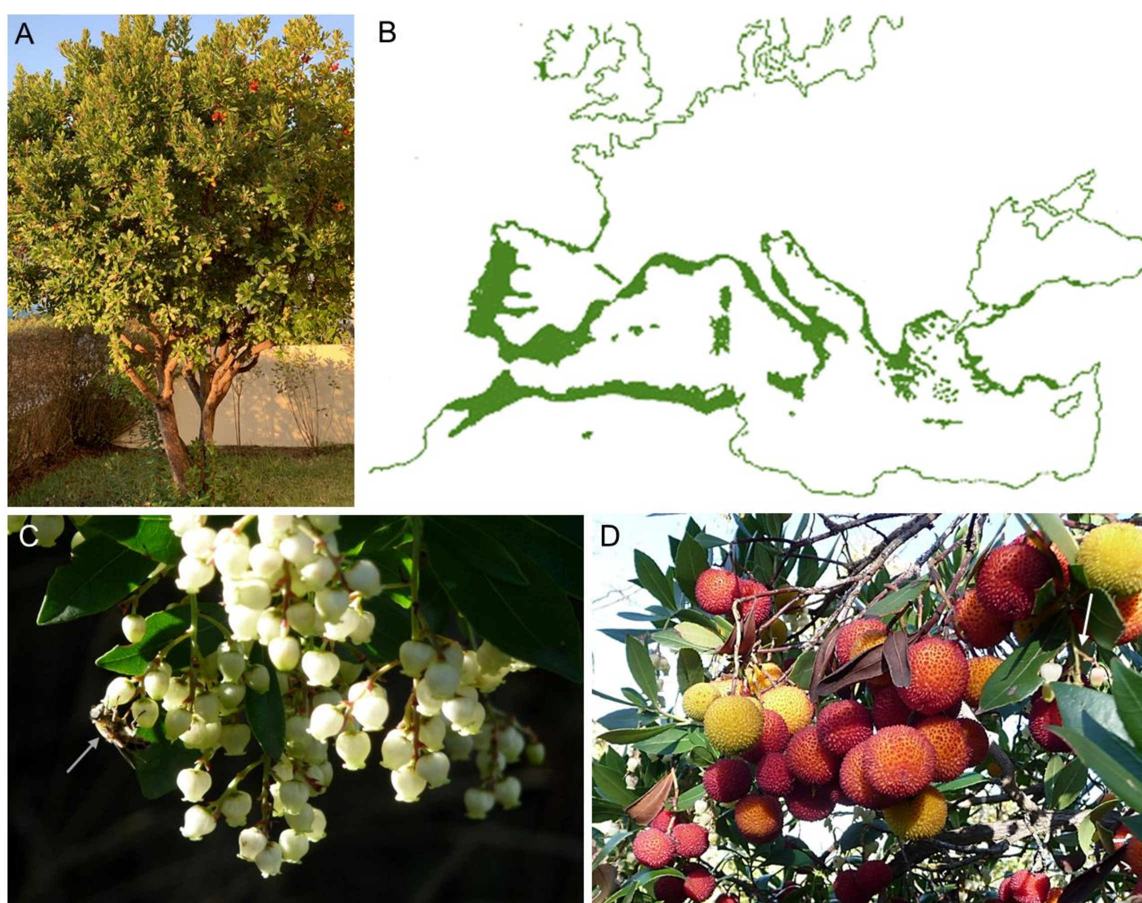

Figure 2. *Arbutus unedo* plant (A); strawberry tree distribution map around the Mediterranean basin and Atlantic coast of Portugal, Spain, France and Ireland (B); bell-shaped flowers being pollinated by a honeybee (*Apis melifera*, arrow) (C); and fruits at different ripening stages and flowers (arrow) (D).

The leaves are petiolate, oblong-lanceolate and usually sawn, with an intense light green color when juvenile and darker when mature. They display a lauroid morphology, inherited from the ancestors who colonized Europe in a subtropical environment, during the Tertiary (Silva 2007). The hermaphrodite flowers are bell-shaped (Figure 2 C), whitish to slightly pink and grouped in hanging panicles (up to 30 flowers). The corolla is sympetalae and the pollination is entomophilous (Prada and Arizpe 2008). The fruit, a spherical berry (Figure 2 D), about 20 mm diameter, is covered with conical papillae and harbors 10 to 50 small seeds, which are dispersed by endozoochory (Piotto et al. 2001). *A. unedo* naturally hybridizes with *A. andrachne* L. resulting in

the hybrid *A. andrachnoides* Lint. Other recognized hybrid is *A.* x *androsterilis* (Salas, Acebes & Arco; (Salas Pascual et al. 1993), which is obtained artificially by crossing of *A. unedo* with *A. canariensis* (Torres et al. 2002; Prada and Arizpe 2008).

The phenological cycle of strawberry tree is quite slow, lasting for almost two years. During this period, three distinct stages can be observed: flower buds, blooming and fruiting. The first flower buds usually begin to form in June and remain in a state of apparent quiescence for several months. Flower anthesis begins in October and may last until January. After fertilization, the long fruit development process starts, taking at least 9 months to be completed. The color of the fruit markedly changes during fruit development, passing through green, yellow, orange and red when mature. In autumn flowers and fruits can be found simultaneously on the same tree (Figure 2 D, (Villa 1982). A pentalocular ovary encloses several ovules (Takrouni and Boussaid 2010), that according to (Villa 1982) are already present in the flower buds formed in June but not yet completely organized. The differentiation of the nucellus occurs only in September when the ovules assume their final position inside the ovary with the micropyle in front of the placenta – anatropous ovules. After fertilization, the zygote remains in a state of apparent quiescence for approximately six months. After this period, the first division of the zygote occurs and the embryo development proceeds at a rather slow pace and several months are needed for the embryo to be completely formed (Villa 1982).

1.2. Ecological importance

Strawberry tree is a key species of Mediterranean ecosystems, especially in marginal lands where the thermal amplitudes are high and water is scarce during summer, and where other trees face difficulties to thrive. *A. unedo* provides food and shelter to fauna and helps to stabilize soils, avoiding erosion and promoting water retention. Furthermore, it has a great regeneration ability after forest fires (Piotto et al. 2001), a feature that makes the species interesting for reforestation programs. This is particularly important in southern European countries, such as Portugal, Spain and Greece where fires are a routine during the hot dry summer months. Strawberry tree has also shown potential to be used in phytostabilization programs, due to its tolerance to heavy metals (Godinho et al. 2010). Some phenolic compounds produced by strawberry tree as secondary metabolites might be involved in the regulation of the nitrogen cycle, keeping low concentrations of nitrates in the soil. The inhibition of nitrification would also block NO_2, a gas responsible for the greenhouse effect (Castaldi et al. 2009).

2. Population analysis and plant selection

In a tree species with a long-life cycle such as strawberry tree, selection and breeding can be time consuming. Thus, a good characterization of wild populations and development of molecular markers is essential to speed up the selection process. Molecular markers can also be useful for *in situ* and *ex situ* germplasm conservation, as insights about population evolution and

diversity can be obtained. Studies of genetic diversity and population structure provide essential knowledge for forest management and selection of individuals and/or populations for breeding and conservation purposes.

A chemical characterization of the plants is equally important to identify specific chemotypes especially as a plant selection criterion. Other important component of the plant that should not be neglected is the microbiome. These organisms play a crucial role in the resistance of their host against adverse conditions including pathogen attacks, either by direct competition and antagonism or by promoting plant defense mechanisms (Turner et al. 2013). Additionally, endophytes produce a large amount of compounds with interesting properties that can be used for numerous applications, including the treatment of human diseases (Kusari et al. 2012), widening the spectrum of possible applications for endophytes. Finally, strawberry tree orchards are usually installed on marginal lands and dry areas where plants are prone to drought stress. In addition, extreme climate events are expected to occur more frequently in the future, leading to a reduction of precipitation and temperature rising (Nardini et al. 2014; Forner et al. 2018; Polle et al. 2019). In this scenario, changes on strawberry tree distribution are expected to occur due to habitat loss on southern regions (Ribeiro et al. 2019). Thus, resistance to drought is certainly one of the vital traits to be pursued on strawberry tree selection and breeding. The knowledge of stress tolerance mechanisms of strawberry tree and characterization of their microbiome might be essential for plant survival and to ensure productivity of the orchards. Such information can be useful for plant selection, and to develop forest and agricultural management strategies that ameliorate plant growth and production under stress conditions.

2.1. Genetic characterization

Population genetic studies and the development of molecular markers are essential to significantly reduce the necessary time in the selection and breeding of new cultivars. The genetic structure of strawberry tree population has been studied using different approaches and several molecular markers have been developed, including RAPD, ISSR, cpSSR and AFLP (Takrouni and Boussaid 2010; Lopes et al. 2012; Takrouni et al. 2012; Gomes et al. 2013a; Santiso et al. 2016; Ribeiro et al. 2017). A low to moderate level of diversity was generally found in the studied populations, attributed to a high gene flow caused by the long seed distance dispersal. Furthermore, cluster analysis of the populations indicates there is no relationship to bioclimatic or geographical origin of the trees demonstrating that differentiation occurs at a local space scale (Santiso et al. 2016; Ribeiro et al. 2017). Thus, a higher level of genetic variation is usually found within population than among populations.

Supported by these findings, researchers have proposed an *ex situ* and/or *in situ* conservation strategy and genotype selection that takes into account the genetic diversity within populations and based on the selection of a large number of individuals from the same population (Takrouni and Boussaid 2010; Lopes et al. 2012; Takrouni et al. 2012; Gomes et al. 2013a).

2.2. Chemical fingerprint

Strawberry tree is a source of bioactive compounds and several studies have been carried out providing a chemical fingerprint from different parts of the plant, and collected at different seasons and locations, such as Portugal, Spain, Italy, Turkey and Algeria. The analysis of the distillate and strawberry tree honey was also carried out. From the enormous variety of compounds produced by strawberry tree some were found to have interesting bioactive properties, and have the potential to be used by pharmaceutical, cosmetic and food industries, thus increasing the economic value of the species. Such information can also be relevant for the selection of plants with specific chemical properties for propagation and breeding.

2.2.1. Fruits

Phenolic acids, galloyl derivatives, flavonols, flavan-3-ols and anthocyanins have been identified as main fruit components (Ayaz et al. 2000; Pawlowska et al. 2006; Pallauf et al. 2008; Fortalezas et al. 2010; Mendes et al. 2011; Guimarães et al. 2013). Unsaturated fatty acids (α-Linolenic, linoleic and oleic acids) and saturated fatty acids (palmitic acid) (Barros et al. 2010; Morales et al. 2013), vitamin E (Pallauf et al. 2008; Barros et al. 2010; Oliveira et al. 2011a; Morales et al. 2013) and vitamin C (Alarcão-e-Silva et al. 2001; Pallauf et al. 2008; Şeker and Toplu 2010; Ruiz-Rodríguez et al. 2011; Morales et al. 2013) have also been identified, as well as several organic acids, as fumaric, lactic, malic, suberic, citric, quinic and oxalic acids (Ayaz et al. 2000; Alarcão-e-Silva et al. 2001; Ruiz-Rodríguez et al. 2011; Morales et al. 2013). The fruits have high amounts of fructose and glucose and low levels of sucrose and maltose (Ayaz et al. 2000; Alarcão-e-Silva et al. 2001; Şeker and Toplu 2010; Ruiz-Rodríguez et al. 2011) and several minerals in its composition including calcium, potassium, magnesium, sodium, phosphorus and iron (Özcan and Haciseferogullan 2007; Şeker and Toplu 2010; Ruiz-Rodríguez et al. 2011). Composition and concentrations of some compounds, such as vitamins, organic acids and sugars might depend on fruit ripening stage, season and location (Alarcão-e-Silva et al. 2001; Ruiz-Rodríguez et al. 2011).

2.2.2. Leaves

Polyphenol profiles of *A. unedo* leaves are mainly composed by arbutin, hydroquinone and their derivatives, gallic acid derivatives, tannins and flavonoids like quercetin, kaempferol and myricetin derivatives (Pavlović et al. 2009; Tavares et al. 2010; Mendes et al. 2011; Maleš et al. 2013).

2.2.3. Volatile compounds and essential oil

Several volatiles from the aerial parts of the plant have been identified, being nonanal and decanal the most abundant (Owen et al. 1997). Alcohols such as (Z)-3-hexen-1-ol, 1-hexanol, hexanal, (E)-2-hexenal and (Z)-3-hex-enyl acetate are the main volatile compounds found in fruits at different ripening stages, followed by aldehydes and esters (Oliveira et al. 2011b). The essential

oil of leaves was also studied. Among the 37 constituents found by (Kivcak et al. 2001), (E)-2-decenal, α- terpineol, hexadecanoic acid and (E)-2-undecenal were the major constituents, while palmitic acid, linoleic acid and p-cresol, 2,6-di-tert-butyl were the major constituents identified by (Bessah and Benyoussef 2012).

2.2.4. Bioactive properties

The traditional use of the leaves as a diuretic, urinary antiseptic, depurative and as antihypertensive has been reported in the literature. In fact, several bioactive properties have been described, such as the antioxidant activity of strawberry tree extracts from leaves (Pabuçcuoğlu et al. 2003; Oliveira et al. 2009; Pavlović et al. 2009; Orak et al. 2011; Malheiro et al. 2012; Boulanouar et al. 2013) and fruits (Barros et al. 2010; Fortalezas et al. 2010; Şeker and Toplu 2010; Oliveira et al. 2011a; Akay et al. 2011; Morales et al. 2013; Guimarães et al. 2014), as well as the antimicrobial activity against some bacteria and molds (Orak et al. 2011; Malheiro et al. 2012; Ferreira et al. 2012; Dib et al. 2013). Other activities as anti-inflammatory (Carcache-Blanco et al. 2006; Mariotto et al. 2008b), antitumoral (Carcache-Blanco et al. 2006; Mariotto et al. 2008a), vasorelaxant and antihypertensive (Legssyer et al. 2004; Afkir et al. 2008) have also been described. Due to their antioxidant properties, some compounds have the potential to be used by food industries, such as in meat processed products (Armenteros et al. 2013; Ganhão et al. 2013).

Because of the several bioactivities identified, these natural compounds can be exploited for pharmaceutical and chemical applications, in the prevention or treatment of several human diseases. In fact, arbutin is already used by cosmetic industries as a skin whitening agent and along with its precursor hydroquinone is used in the formulation of commercial remedies for the treatment of urinary infections (Migas and Krauze-Baranowska 2015; Jurica et al. 2017). Due to the economic value of arbutin, our group has been working on the development of a biotransformation protocol in order to produce arbutin from its precursor hydroquinone (data not published) trough glycosylation (Figure 3 A). Using cell suspensions (Figure 3 B) and/or shoot segments *in vitro*, high amounts of this phenolic can be obtained (Figure 3 C).

2.2.5. Distillate and honey

Chemical surveys of the distillate have been carried out (Soufleros et al. 2005) and different microorganisms were identified during the fermentation of strawberry tree berries (Cavaco et al. 2007; Santo et al. 2012) which contribute to the chemical characteristics of the distillate.

Strawberry tree honey is rich in amino acids and have antioxidant properties (Rosa et al. 2011). Homogentisic acid has been identified as a possible marker for this honey (Tuberoso et al. 2010). Besides, other substances such as (±)-2-*cis*,4-*trans*-abscisic acid (*c,t*-ABA), (±)-2-*trans*,4-*trans*-abscisic acid (*t,t*-ABA) and unedone, can be used as complementary markers in the identification of this monofloral honey (Spano et al. 2006; Tuberoso et al. 2010).

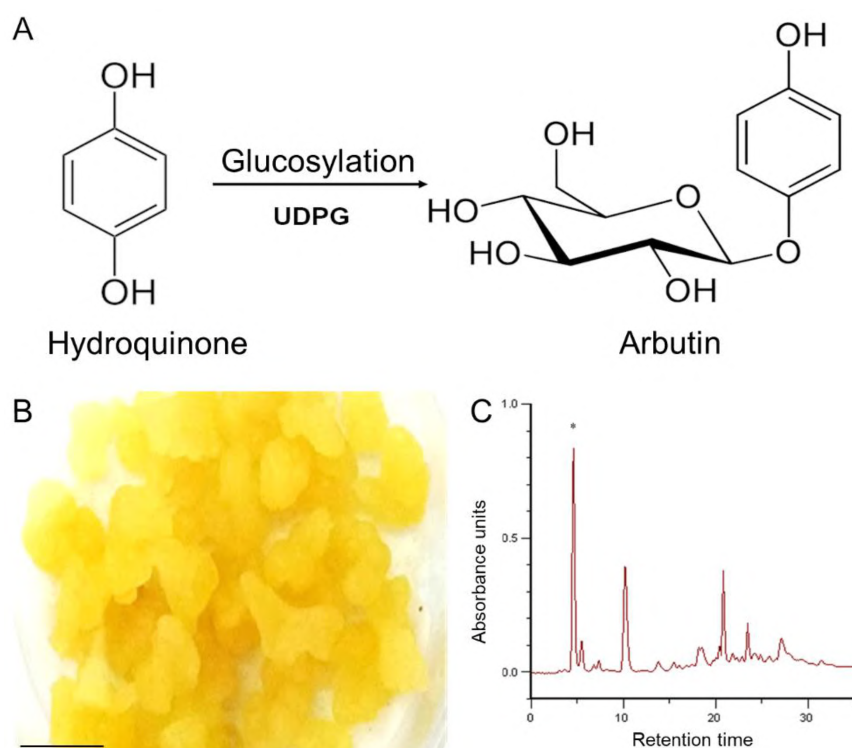

Figure 3. Glycosylation process of hydroquinone into arbutin (A), *calli* used in the biotransformation of hydroquinone into arbutin (B, bar = 50 mm) and chromatogram of the extract recovered from the biotransformation with the arbutin peak (*) (C).

2.3. Microbiome

Endophyte organisms live on a symbiotic relationship with plants without causing any symptoms of disease, and might be highly advantageous to their host by producing plant growth promoting hormones and antimicrobial compounds (Firáková et al. 2007; Waqas et al. 2012; Kusari et al. 2012). Several fungi have found to be strawberry tree endophytes, such as: *Allantophomopsis lycopodina*, *Alternaria alternata*, *Aureobasidium pullulans*, *Cladosporium* sp., *Cryptosporiopsis diversispora*, *Discostroma* sp., *Microsphaeropsis olivacea*, *Penicillium* spp., *Stemphylium globuliferum* and *Umbelopsis* spp. (Borges 2014), as well as *Talaromyces pinophilus* (Vinale et al. 2017). On other members of the Ericaceae family (*Rhododendron* spp., *Enkianthus perulatus* and *Pieris japonica*), *Alternaria* spp., *Aureobasidium pullulans*, *Cladosporium* sp. and *Penicillium* spp. were also found, as well as several other *taxa* such as: *Aspergillus*, *Colletotrichum*, *Fusarium*, *Glomerella*, *Phoma*, *Phomopsis*, *Septoria* and *Trichoderma*) (Okane et al. 1998; Purmale et al. 2012). Strawberry tree endophytes produce several volatile compounds and show antimicrobial activity (Borges 2014). Strawberry tree plants are likely to be affected by biotic stress, in particular plant pathogens. Several fungi have been identified to be causing foliar diseases on strawberry tree, such as: *Alternaria* sp. (Thomma 2003), *Glomerella* sp. (sexual stage of *Colletotricum* sp.) (Polizzi et al. 2011), *Mycosphaerella* sp. (sexual stage of *Septoria* sp.) (Romero-Martin and Trapero-Casas 2003) and *Hendersonula toruloidea* (Tsahouridou and Thanassoulopoulos 2000). *Phytophthora*

cinnamomi, a wide spread invasive oomycete that causes root rotting has also been found on strawberry tree (Moreira and Martins 2005; Moralejo et al. 2008).

2.4. Drought stress

As one of the most restrictive factors on plant growth (Guarnaschelli et al. 2012), water balance is strongly regulated by plants, which have developed diverse adaptive characteristics and resistance mechanisms both morphological and physiological, including a tight stomatal control and osmotic adjustment, as well as resistance to cavitation, deep rooting, leaf thickness and cuticular waxes (Gratani and Ghia 2002; Bussotti et al. 2014). As should be expected, *A. unedo* has developed some of these features being considered a drought tolerant species (Castell and Terradas 1994; Ogaya et al. 2003; Munné-Bosch and Peñuelas 2004; Raimondo et al. 2015). As part of its protection mechanism against drought strawberry tree has developed a conservative water use strategy. Under water scarcity conditions photosynthesis is compromised and plant stomata close leading to an accumulation of CO_2 (Martins et al. 2019), a typical behavior of an isohydric species (Raimondo et al. 2015).

3. Germplasm conservation and plant propagation

The multiplication of the selected and improved material obtained on breeding programs is essential to provide such genotypes for producers. This can be achieved using conventional methods and micropropagation techniques. So far, seed orchards constitute most of the production area, but the demand for cloned plants has considerably increase on recent years, and micropropagation techniques might be crucial to fulfill these requests. The conservation *ex situ* of this germplasm is also very important and can be accomplished through micropropagation and/or cryopreservation.

3.1. Conventional propagation methods

The propagation of strawberry tree can be achieved through conventional methods such as cuttings or by seeds. The propagation trough cuttings allow the cloning of specific genotypes. Although (Metaxas et al. 2004) obtained rooting rates higher than 90% using indole-3-butyric acid, potassium salt (K-IBA), this technique can be difficult to apply on strawberry tree. Among other limitations, it proved to be genotype-dependent, and rooting rates as low as 22.2% were obtained on one of the tested genotypes.

Alternatively, propagation by seed is usually a fast and easy method of plant propagation. However, *A. unedo* seeds are characterized by physiological dormancy, which impairs the propagation of strawberry tree through this method (Tilki 2004; Demirsoy et al. 2010; Ertekin and Kirdar 2010). Different methods can be applied to overcome seed dormancy, such as stratification, scarification and treatments with gibberellins (Tilki 2004; Demirsoy et al. 2010; Ertekin and Kirdar 2010), and several studies have been carried out in order to increase strawberry tree seed germination rates. (Smiris et al. 2006) achieved a germination percentage close to 86% with a

combined treatment of 24 h in gibberellic acid (GA_3, 500 mg L^{-1}) followed by 3 months of stratification at 2-4 °C. In a study carried out by (Tilki 2004) the highest percentage of germination was 89% following seed treated treatment with GA_3 (600 mg L^{-1}). In the same study, seeds subjected to a stratification period of 9 weeks, at 4 °C, showed a germination rate of 86%. (Ertekin and Kirdar 2010), obtained germination percentages of 85% when stratification was applied for 90 days at 4 °C or during a 60 days at 9 ºC. (Demirsoy et al. 2010) tested five different genotypes and obtained a maximum germination percentage of ca. 43% when seeds were subjected to stratification for 15 weeks at 4 °C, without significant differences among genotypes. Germination rates higher than 90% have also been obtained by our research group, when seeds were stratified at 4 °C for 4 weeks (Martins 2012).

Due to the simplicity of this propagation technique, the study of seed dormancy of strawberry tree has a great economic and practical interest (Smiris et al. 2006). Moreover, sexual propagation promotes genetic diversity, particular important for habitat restoration purposes. However, it does not allow the production of true-to-type plants and the multiplication of selected genotypes with specific characteristics, a drawback when the objective is to establish orchards.

3.2. *In vitro* propagation

To overcome the limitations of conventional propagation techniques, micropropagation appears to be the best alternative. Besides the production of cloned plants, micropropagation techniques have other advantages when compared to the conventional cloning: it is not dependent of the time of the year, a small quantity of initial plant material is required, all the process is carried out under aseptic conditions assuring the phytosanitary quality of the propagated materials, and a large number of cloned plants can be obtained (Chawla 2009). Micropropagation techniques can also be useful for germplasm conservation purposes. The most common micropropagation techniques on woody plants are: shoot proliferation (from shoot apices or nodal segments), organogenesis and somatic embryogenesis.

In vitro propagation protocols have already been developed for strawberry tree (Figure 4 A) trough axillary shoot propagation (Mereti et al. 2002; Gomes and Canhoto 2009; Martins et al. 2019) as well as organogenesis (Martins et al. 2019) and somatic embryogenesis (Martins et al. 2016a).

3.2.1. Axillary shoot proliferation

The propagation of strawberry tree trough axillary shoot proliferation can be achieved using Woody Plant Medium minerals (Lloyd and McCown 1980) supplemented with Murashige and Skoog vitamins (Murashige and Skoog 1962) and 5 mg L^{-1} 6-benzylaminopurine (BAP) (Mereti et al. 2002) or using De Fossard medium (De Fossard et al. 1974) supplemented with MS micronutrients and 2 mg L^{-1} BAP (Gomes and Canhoto 2009). Other cytokinins, such as kinetin and zeatin can also be used with similar results (Lopes et al. 2010). Axillary shoot proliferation of

strawberry tree can also be accomplished in liquid media (Figure 4 B, (Martins et al. 2019). Higher multiplication rates can be obtained using this method when compared to the propagation on solid media, which allows the reduction of costs. However, shoots obtained by this method show signs of hyperhydricity, resulting in poor lignification and several anatomical malformations (Marques et al. 2020). Nonetheless, plants recover their normal phenotype during acclimatization and have normal physiological functions under water stress (Martins et al. 2019). High rooting rates of the micropropagated shoots can be obtained either with indole-3-acetic acid (IAA) or IBA - indole-3-butyric acid (Mereti et al. 2002; Gomes and Canhoto 2009) and plants can be easily acclimatized (Figure 4 C). According to (Lopes et al. 2010), the genotype of the donor plants plays an important role in the multiplication rates and the conditions used in the multiplication phase, can also interfere in the rooting process. Several other members of the Ericaceae family have also been propagated trough axillary shoot proliferation: *Arbutus xalapensis* (Mackay 1996), *Calluna vulgaris* (Gebhardt and Friedrich 1987), *Gaultheria fragantissima* (Bantawa et al. 2011), *Elliottia racemosa* (Radcliffe et al. 2011), *Rhododendron* spp. (Anderson 1984; Douglas 1984; Almeida et al. 2005) and *Vaccinium* spp. (Pereira 2006; Biol et al. 2015).

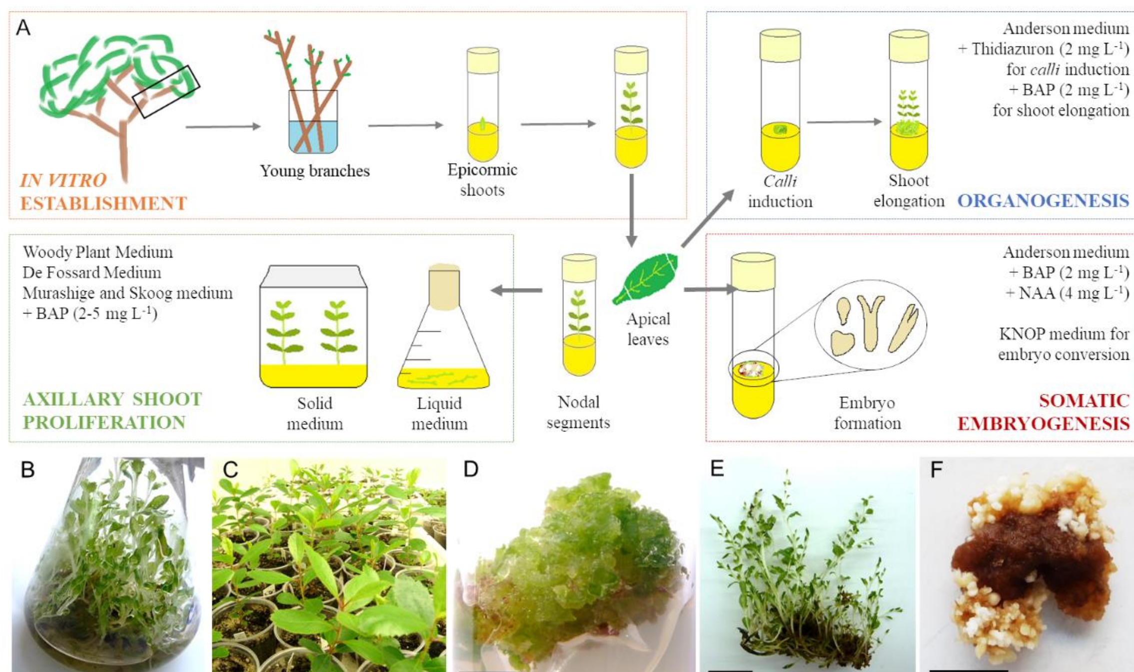

Figure 4. *In vitro* establishment and micropropagation of strawberry tree using different techniques, including axillary shoot proliferation, organogenesis and somatic embryogenesis (A), axillary shoot proliferation in liquid medium (B), acclimatized plants obtained by axillary shoot proliferation (C), *calli* with organogenic capacity (D), shoot obtained through organogenesis (E) and developing somatic embryos on a leaf explant (F). (bar = 50 mm)

3.2.2. Organogenesis

Organogenesis refers to the formation of adventitious shoots induced from shoot meristems usually from a pre-formed *callus*. This propagation method has a great potential for mass propagation, because a large number of shoots can be formed from a single explant. Strawberry tree can be successfully micropropagated through organogenesis (Martins et al. 2019). *Calli* with organogenic potential can be induced with thidiazuron on leaves of micropropagated shoots (Figure 4 D). The meristematic regions formed will produce new shoots when the *calli* is cultured on a medium with BAP (Figure 4 E). A similar method has been successfully applied on other members of the Ericaceae family, such as *Gaultheria fragrantissima* (Ranyaphia et al. 2011), *Rhododendron* (Harbage and Stimart 1987; Imel and Preece 1988; Iapichino et al. 1992; Mertens et al. 1996; Tomsone and Gertnere 2003) and *Vaccinium* spp. (Cao et al. 2002; Debnath 2003).

3.2.3. Somatic embryogenesis

Somatic embryogenesis is a useful tool for clonal propagation and for plant development studies, allowing a comparison with zygotic embryogenesis. Somatic embryos can be obtained in large amounts and can be germinated, stored or used for the production of artificial seeds. Different tissues can be used as explants, such as zygotic embryos, roots, stems and shoot tips, young leaves, sepals and petals. Somatic embryogenesis is usually affected by several variables, such as the genotype and physiological condition of the donor plant, type of explant, culture conditions and especially the composition of the medium.

A somatic embryogenesis induction protocol has been developed for strawberry tree from leaves of shoots propagated *in vitro* trough axillary shoot proliferation (Martins et al. 2016a, b). The embryos are obtained from a *callus* when segments of young leaves are cultured on Andersson medium (Anderson 1984) supplemented with 2 mg L^{-1} BAP and 4 mg L^{-1} of 1-naphthaleneacetic acid (NAA), through a one-step protocol. Somatic embryos start to form 1.5-2 months after the beginning of the culture (Figure 4 F) and their conversion into plantlets can be accomplished using the Knop medium (Knop 1865) without plant growth regulators, leading to the formation of plantlets with a well-developed root system. This technique has also been successfully applied on other Ericaceae, such as: *Conostephium pendulum* (Anthony et al. 2004a), *Elliotia racemosa* (Seong and Wetzstein 2008), *Leucopogon verticillatus* (Anthony et al. 2004b) and *Rhododendron catawbiense* (Vejsadová and Petrová 2003).

3.3. Cryopreservation

Cryopreservation may be a useful technique for germplasm maintenance *ex situ*, either for conservation and/or breeding purposes. It is cost effective and allows the preservation of large amounts of material for large periods of time. Unlike micropropagation, plant material do not undergo somaclonal variation and there is no risk of contamination (Panis and Lambardi 2006). (Damiano et al. 2007) were the first to developed a cryopreservation protocol for strawberry tree.

It consisted in the culture of shoot apices in WPM or MS solid media, with different concentrations of plant growth regulators. After this period, the apices were encapsulated in 3% alginate, and cultured in liquid MS media with different concentrations of sucrose during 1-7 days. After the desiccation period, which was performed in silica gel for 0-24 h, the beads were immersed in liquid nitrogen. The beads were then transferred to propagation medium and after two weeks the apices turned green and started to develop. The maximum regrowth rate (45%) was obtained when the encapsulated apices were precultured in medium containing 0.75M sucrose during 1 day, and the desiccation was done for 14 h.

4. Plant breeding

Some extensive work has been conducted in other members of the Ericaceae family, such as *Rhododendron* (Doorenbos 1955; Escaravage et al. 1997) and *Vaccinium* (Lyrene 1997; Usui et al. 2005; Drummond 2019) in order to characterize the pollination and breeding system of these species and to obtain new varieties with interesting traits. A characterization study of pollen morphology and germination has also been done on strawberry tree, as well as *in vitro* and *in vivo* controlled pollinations between selected plants, which produced several hybrid plants (Martins and Canhoto 2014). However, this method can be time consuming for trees with a long life-cycle, making it essential to develop a set of tools to speed-up the breeding process. The identification of QTL (Quantitative trait loci) associated to marker assisted selection (MAS) has been applied to other woody species and may also be applied to strawberry tree (Butcher and Southerton 2007). Several other techniques can be applied for this purpose, such as polyploidization, mutagenesis, mycorrhization and genetic transformation. Several of these techniques have already been applied on strawberry tree (Navarro and Morte 2009; Antunes 2010; Gomes et al. 2013b, 2016; Martins and Canhoto 2014).

4.1. Mycorrhization

Mycorrhization is often an advantage, in particular for woody plants, since it increases mineral and water uptake stimulating plant growth and crop production (Gomes et al. 2013b). Mycorrhization with edible species of fungi may also constitute an additional revenue for producers. *In vitro* synthesis of arbutoid mycorrhizae on strawberry tree plants was accomplished with two *Lactarius deliciosus* isolates, which persisted 9 months after the acclimatization of the plants (Gomes et al. 2016). Fungus-plant host compatibility has also been tested with *Pisolithus tinctorius* (Pers.) Coker and Couch (Navarro and Morte 2009; Gomes et al. 2013b). The association between strawberry tree and *P. tinctorius* proved to increase plant height and had a beneficial effect on root system development. Furthermore, higher stomatal conductance values were observed in the mycorrhizal plants which led to a higher photosynthetic activity (Navarro and Morte 2009). On other experiment, arbutoid mycorrhizae were observed one month after the *in vitro* inoculation, which seems to indicate the compatibility of the fungus with strawberry tree. However, in a field

trial the presence of the fungi previously inoculated were not observed on the roots of 20 months old plants, suggesting that fungal persistence can be a problem, once plants establish mycorrhizae with native fungi species present in the soil (Gomes et al. 2013b).

4.2. Polyploidization

Although most of the wild plant species are diploid, polyploidy is a common phenomenon in plants, not only in crop species but also in wild population, having been estimated to occur in approximately 70% of the Angiosperms. The duplication of chromosomes has been used in horticulture as a tool to obtain higher quality plants with improved yield as polyploidy can lead to thicker leaves, more intense colors, larger flowers and leaves, longer flowering period, disease resistance, fruit size increased and resistance to environmental stress conditions (Väinölä 2000). Additionally, this technique can also be used to restore fertility or prevent sterility of hybrids resulting from crosses between plants with different ploidy levels. Tetraploid plants can also be crossed with diploid plants to obtain sterile triploids (Väinölä 2000) which, in some cases, produce seedless fruits trough parthenocarpy, a feature highly appreciated by consumers (Picarella and Mazzucato 2019).

Attempts to obtain *A. unedo* tetraploid plants have been carried out by (Antunes 2010). Nodal segments micropropagated *in vitro* were treated with two c-mitotic agents (colchicine and oryzalin), on solid and liquid media. However, most of the treated plants remained diploid, some became mixoploid and only a tetraploid shoot was obtained on solid medium containing 125 µM oryzalin. A second attempt was carried out on similar conditions by (Martins and Canhoto 2014). Several mixoploid plants were obtained, but only three plants were found to be tetraploid after a treatment with oryzalin, a conversion rate of 1%. Besides the low conversion rates, the tetraploid plants died a few weeks after the treatment with the c-mitotic agent. Taken together, these findings suggest chromosome duplication are difficult to achieve on strawberry tree and a tetraploid genome is very unstable, which might be a major drawback on future attempts to obtain tetraploid plants. Despite the possible limitations of this method, tetraploid plants were successfully obtained on other woody Ericaceae, such as *Rhododendron* and *Vaccinium* (Väinölä 2000; Chavez and Lyrene 2009), which opens up good prospects to obtain strawberry tree tetraploid plants.

5. Thesis objectives

From a neglected species, strawberry tree has definitely caught the attention of local authorities, farmers and other stakeholders. Due to its resistance to abiotic and biotic stresses, the ability to colonize marginal lands and regenerate capacity after forest fires, strawberry tree has the potential to become one the most important species on the Mediterranean region. An extensive work has been done on recent years and several biotechnological tools have been developed for strawberry tree. Population genetics studies provided information essential for conservation and selection of individuals on wild populations while micropropagation techniques provide an efficient

way of *ex situ* conservation and multiplication. Some studies enlighten the tolerance mechanisms of strawberry tree against abiotic and biotic stress and a broad chemical fingerprint of the plant have been obtained along with its potential for pharmaceutical, cosmetic and food industries.

Nonetheless, some important pieces of this complex puzzle are still missing and need urgent attention. The chemical analysis carried out so far fail to evaluate the role of the plant genotype, as well as the effect of abiotic stimulus on the quantitative and qualitative fluctuation of the chemical compounds. The ecological relevance of such compounds is also generally neglected as studies tend to focus on pharmaceutical applications. Thus, the involvement of such compounds on plant development and defense mechanisms are poorly understood as well as its interaction with the plant microbiome. The study of endophyte communities has also been neglected and little is known about the interaction and relevance of these microorganisms with his host. Furthermore, endophytic bacteria have never been studied and non-culturable microorganisms are a grey field on this species. Moreover, the impact of pathogens on strawberry tree survival and production is not known and has never been quantified. Due to its relevance on plant selection and breeding, a better understanding of plant-pathogen interaction is necessary. Additionally, more efficient and eco-friendly plant protection strategies can also be developed based on this knowledge. A basic knowledge of the physiological response of strawberry tree plants under water deficit has also been provided, but the related biochemical and molecular mechanisms are still poorly understood and need to be studied in more detail. Finally, the first attempts of breeding using different approaches are surely a step forward but a long way has still to be done in order to obtain new strawberry tree varieties with improved characteristics.

In order to fill the knowledge gaps presented, and definitively boost the strawberry tree breeding program, this work aims to:

(1) optimize micropropagation protocols (somatic embryogenesis, axillary shoot proliferation and organogenesis in liquid medium), evaluate the ability of the micropropagated plants to cope with drought and study the latent microorganisms in the *in vitro* cultures of *A. unedo* and their prevalence after plant acclimatization (Chapter 1-3);

(2) characterize *A. unedo* reproductive system, obtain hybrid plants through controlled crosses, select genotypes more resistant to drought and provide insights on the physiological and biochemical resistance mechanism to water stress (Chapter 4-5);

(3) structural and functional characterization of *A. unedo* endophytic bacteria and fungi, leaves chemical profile, and antimicrobial activity of the plants extract and endophytes against phytopathogens (Chapter 6-8).

CHAPTER 1: Cloning adult trees of *Arbutus unedo* L. through somatic embryogenesis

(Martins J, Correia S, Pinto G, Canhoto J (2021) Cloning adult trees of *Arbutus unedo* L. through somatic embryogenesis. Submitted for publication)

Abstract

Arbutus unedo has an enormous economic potential and the demanding for plants by producers is increasing. In order to offer cloned material with assured quality, several micropropagation protocols have been developed. Because of the potential of somatic embryogenesis as a mass propagation tool, the main goal of this work is the development of an efficient somatic embryogenesis induction protocol for strawberry tree. Somatic embryogenesis was induced on apical expanded leaves, from shoots growing *in vitro* from several genotypes, in Andersson medium, with 3% sucrose and different concentrations of benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA). Embryogenesis induction rates ranged from 0 to 97.22% ± 4.81. Higher induction rates were achieved on the medium with 2 mg L^{-1} BAP and 2 mg L^{-1} NAA, and are genotype dependent. After the 3-month induction period, the maximum number of embryos per initial explant was 10.10 ± 2.78. Embryos on different developmental stages were found, as well as abnormal somatic embryos. SEM images showed different anomalies, being the most common embryos displaying more than two cotyledons or fused embryos. Embryo germination were not genotype dependent and the maximum embryo conversion rate achieved was 73.5% ± 13.3. However, only 39.21% ± 14.03 of the embryos were able to grow into fully developed plants. The developed somatic induction protocol proved to be very efficient, with high induction rates achieved, both from seedling and adult material, but its genotype dependent. However, embryo conversion must be improved, in order to fully seize the potential of this micropropagation technique.

Keywords: acclimatization, Ericaceae, germination, histology, plant growth regulators, strawberry tree

Introduction

In the last few years several research projects have been carried out in *A. unedo*, in order to develop efficient propagation and breeding protocols to obtain improved genotypes. The propagation of strawberry tree can be achieved through seeds, which do not assure true-to-type plants (Demirsoy et al. 2010; Ertekin and Kirdar 2010) or by cuttings, a conventional cloning method with low rooting rates in *A. unedo* (Metaxas et al. 2004). *In vitro* micropropagation protocols can overcome these limitations and have already been developed for strawberry tree by (Mereti et al. 2002) and (Gomes and Canhoto 2009). A propagation protocol through organogenesis in liquid medium was also developed (Martins et al. 2019). However, the potential of somatic embryogenesis is higher than other micropropagation techniques since large amounts of somatic embryos can be obtained and are usually easier to handle (Correia et al. 2011). Moreover, somatic embryos are bipolar units, which do not need to be rooted, thus avoiding a further step in the regeneration process, as occurs in other micropropagation methods such as axillary shoot proliferation or organogenesis. Somatic embryogenesis is also the ideal regeneration method following genetic transformation, and has a great potential for cryopreservation of selected genotypes (Park 2002; Von Arnold 2008; Graça et al. 2018). Last but not the least, somatic embryogenesis induction and further embryo development is an exceptional model system to analyse plant totipotency and the factors controlling embryo development, maturation and germination (Rose et al. 2010; Radoeva and Weijers 2014; Corredoira et al. 2019). Preliminary works have shown that somatic embryogenesis can be achieved in *A. unedo* (Gomes and Canhoto 2009; Martins et al. 2016a, b). However, a detailed study of plant regeneration through somatic embryogenesis in strawberry tree characterizing the induction, germination and acclimatization steps is missing. Besides, an effective protocol of somatic embryogenesis for plant cloning requires several assumptions, such as (1) the possibility of cloning different selected adult trees, allowing a phenotype-based selection approach, (2) the obtention of a considerable number of somatic embryos, and (3) the conversion of the somatic embryos into fully developed plants. In this context, the main goal of this work was the development of an efficient somatic embryogenesis induction protocol from adult plants, as well as the conversion and morphological characterization of the somatic embryos. For this purpose, different genotypes were tested as well as different combinations and concentrations of plant growth regulators (PGRs) in the induction medium. The morphology and anatomy of the obtained somatic embryos was characterized and compared with their zygotic counterparts. Finally, embryos were converted into plants and acclimatized. In this work, a reliable protocol for the induction of somatic embryogenesis from adult material of strawberry tree as well as embryo conversion and plant acclimatization is presented.

Material and Methods

Plant Material

Four genotypes with different origins were selected: AU1, AU2 and AU3 from adult trees, and AU4 from a single seedling. For genotypes AU1, AU2 and AU3, young branches were cut, dipped in 100 mg L^{-1} fungicide for 10 min (Ridomil Gold, Syngenta Crop Protection, Lisboa, Portugal) and rinsed with distilled water. After this treatment, the branches were kept on containers, covered with a plastic bag, watered with distilled water and placed in a walk-in chamber (FitoClima 10000 HP, Aralab, Rio de Mouro, Portugal), under 16-h photoperiod, 250 µmol m^{-2} s^{-1}, at 25 ºC. The developing epicormic shoots (2-3 cm) were removed and surface sterilized with 70 % ethanol (30 s) and calcium hypochlorite for 10 min (5 % w/v, Sigma-Aldrich, St. Louis, MO, USA). After 3 washes with sterile distilled water, the shoots were inoculated in Anderson Rhododendron medium (Anderson 1980) with 6-*benzylaminopurine* (BAP, 2 mg L^{-1}, Sigma), sucrose (3 %, w/v, Duchefa) and plant agar (0.6 %, w/v, Duchefa) in test tubes (25x150mm) with plastic caps (Duran, Mainz, Germany). The culture media were autoclaved at 121 ºC for 20 min (>1,100 g cm^{-2} gel strength after autoclaving) and the pH adjusted to 5.7 using KOH or HCl diluted solutions (0.01 M – 1M) before autoclaving and agar addition. Cultures were maintained under a 16 h photoperiod of 15–20 µmol m^{-2} s^{-1} (cool-white fluorescent lamps) at 25 ºC. Shoots were subcultured six times in a medium with the same composition within a 4-week interval.

For the *in vitro* establishment of genotype AU4, fruits were collected, and the seeds isolated and sterilized following the methodology used for shoots. Seeds were then sowed in Petri dishes with filter paper discs and sterile distilled water. After cold stratification (4 ºC) for a month, the seeds were transferred to a culture chamber (25 ºC and 16 h photoperiod with 15–20 µmol m^{-2} s^{-1}) for germination. After 1 month, a single plantlet was selected for shoot proliferation and subcultured six times in the medium previously described within a 4-week interval.

Somatic Embryogenesis Induction

For somatic embryogenesis induction, apical leaves (5-10 mm length) from proliferating shoots from the four genotypes were used (Figure 5 A-C). 4-6 transverse cuts were made in the abaxial side (Figure 5 C), and leaves were placed with this side down on Schott test tubes (25x150 mm, one leaf per tube) sealed with plastic caps, in a medium containing major salts from the Anderson medium (Anderson 1980), micronutrients from the Murashige and Skoog medium (Murashige and Skoog 1962) and organic compounds of the De Fossard medium (De Fossard et al. 1974). The medium was supplemented with sucrose (3%, w/v, Duchefa), plant agar (0.6%, w/v, Duchefa), and six combinations (IM1 – IM6) of BAP (2.0 mg L^{-1}) plus 1-naphthaleneacetic acid (NAA, 0.5, 1, 2, 5, 10, 15 and 20 mg L^{-1}). Media containing only BAP (IM8: 2.0 mg L^{-1}) and NAA (IM9: 2.0 mg L^{-1}) were also tested. A preliminary assay showed that media without PGRs were

unable to induce any morphogenic response in the explants, thus this control assay was not included in the data. The cultures were kept in a growth chamber, in the dark, at 25 ºC, for 90 days. After this period, somatic embryogenesis induction rate (%) was calculated as follows: IR = number of explants with embryos / total number of explants * 100.

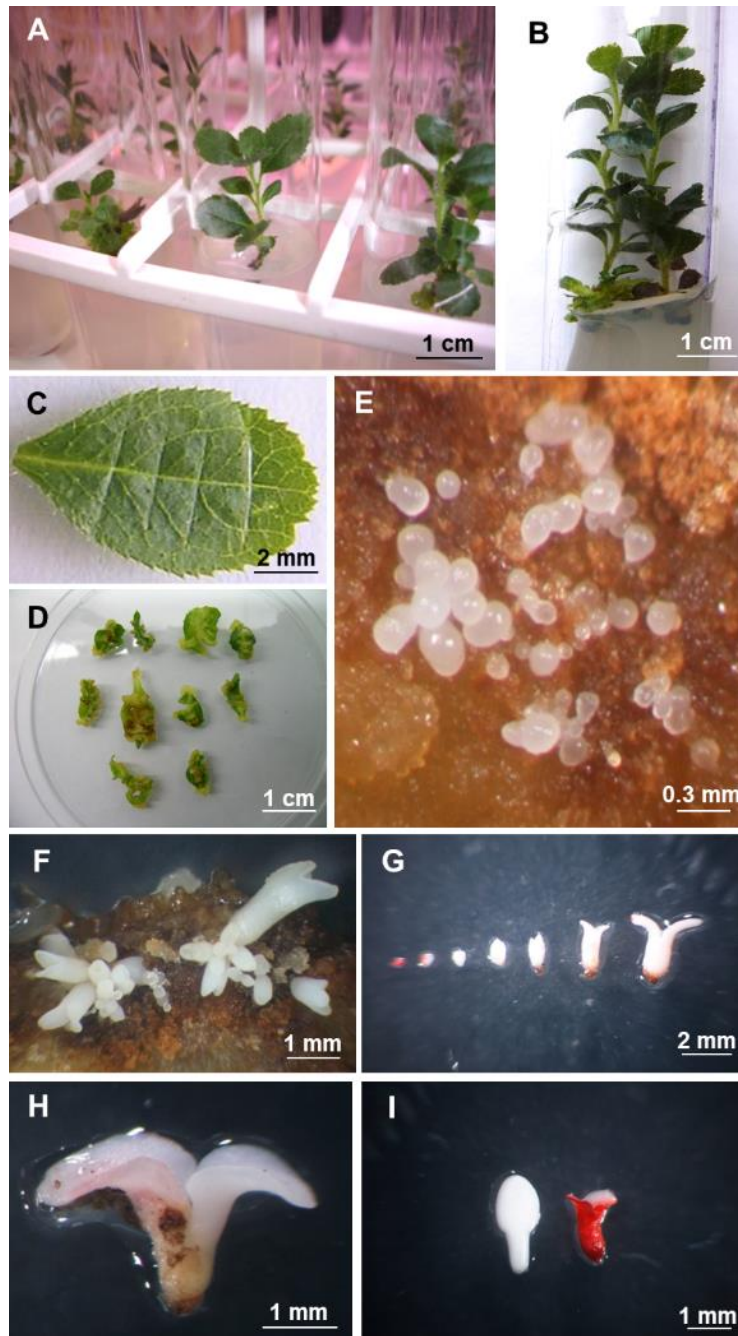

Figure 5. Somatic embryogenesis induction in *A. unedo*: established shoots from which leaves were collected for somatic embryogenesis induction (A-B); cut leave of strawberry tree before culture in the induction media (C); callus formation in cut leaf after 20 days of culture on a somatic induction medium (D); several somatic embryos developing in a callus after 60 days of culture (E); asynchronous somatic embryo development showing somatic embryos at different developmental stages (F); series of somatic embryos from globular (left) to cotyledonary (right) (G); isolated mature cotyledonary embryo (H); mature zygotic (left) and somatic (right) embryos (I).

Somatic embryo germination

For somatic embryo germination, mature embryos from the genotypes AU1, AU2 and AU4, obtained on four different induction media (IM2, IM3, IM4 and IM5) were transferred to a medium consisting of the Knop salts (Knop 1865), micronutrients of the MS (Murashige and Skoog, 1962) medium without potassium iodide, vitamins (without riboflavin) of the Fossard medium, sucrose (1.5%, w/v, Duchefa) and activated charcoal (1%, w/v, *Sigma*). Cultures were maintained for 2 months under a 16 h photoperiod of 15–20 µmol m^{-2} s^{-1} (cool-white fluorescent lamps) at 25 ºC. Genotype AU3 was not used in this assay due to the reduced number of somatic embryos obtained. After this period, somatic embryo germination rate (%) was calculated as follows: GR = number of embryos producing plantlets / total number of embryos in germination medium * 100.

Plant acclimatization

Plant roots were carefully washed on tap water to remove agar debris and placed on acclimatization containers, with perlite (Siro, Mira, Portugal), in a walk-in chamber (FitoClima 10000 HP, Aralab) under 16-h photoperiod with 40 µmol m^{-2} s^{-1}, 25 ºC and 70% humidity. The cover was gradually removed and after a month, the plants were transferred to individual containers with a substrate composed of peat and perlite (1:1, v/v, Siro). Two months after transfer to the containers, plant survival was recorded and acclimatization rate (%) was calculated as follows: AR = number of acclimatized plants / total number of plants * 100.

Histological and cytological analyses

For histological studies somatic embryos at different development stages (globular, earth, torpedo and cotyledonary) and mature cotyledonary zygotic embryos were fixed in glutaraldehyde (2.5%, v/v, Sigma) in cacodylate buffer (0.1M, pH 7.0, Sigma) for 4 h and post-fixed for 2 h, at room temperature, in osmium tetroxide (1%, w/v, Sigma) prepared in the same buffer. Dehydration was accomplished in an ethanol series (70, 80, 95 and 100%) and the samples were embedded in Spurr's resin (Spurr 1969). Ultrathin sections (1.5 µm) were made on a LKB Ultratome III and stained with toluidine blue (0.2%, w/v, Sigma) for general morphology (Roland 1978) and with Periodic acid-reactive Schiff (PAS) and bromophenol blue for specific compounds (starch and proteins, respectively). For carbohydrates staining, PAS was used according to (Mcmanus 1948). Sections were first immersed on Periodic acid (1%, v/v, Sigma), for 20 min, at room temperature. After a wash with 70% ethanol, the sections were washed twice on distilled water, and immersed on Schiff's reagent (Sigma), for 4 h, at room temperature in the dark. Finally, the samples were thoroughly washed on running water for 15 min and dried at 60 ºC. Proteins were stained with bromophenol blue, according to (Lison 1960). Briefly, samples were covered with bromophenol blue (0.1%, w/v), for 15 min at 60 ºC, immersed on acetic acid (0.5%, v/v) and finally washed on phosphate buffer (pH 7.0). Observations were carried out using a Nikon Eclipse Ci (Nikon

Instruments Europe BV, Amsterdam, Netherlands) coupled to a Nikon DS-Fi3 camera (Nikon Instruments) and processed with the software NIS-Elements D (version 4.60, Nikon Instruments). For cytological observations, isolated embryos or small pieces of callus with developing somatic embryos were treated for a few seconds in commercial bleach, washed in water, and then mounted in microscope slides in a drop of water or in a drop of a glycerol solution (40% v/v). To determine embryo viability, whole somatic or zygotic embryos were placed in a solution of 0.2% (w/v) of 2,3,5-triphenyltetrazolium chloride (TTC) for five hours, rinsed with distilled water and then checked for the appearance of a red color (Hartmann et al., 1999). Embryos evenly red stained were considered viable (Hartmann et al. 1997).

Chromosome counting

Chromosome counting of somatic-embryo derived plantlets was carried out after treatment of root tips with 0.05% (w/v) colchicine (Sigma) for 3 h at 25 °C, in the dark. The treated samples were then fixed in 3:1 (v/v) ethanol/ acetic acid overnight. The Feulgen technique (Darlington and La Cour 1976) was then applied. Accordingly, samples were hydrolysed in 1 N HCl at 60 °C for 10 min in a water bath, transferred to distilled water at room temperature for 5 min, stained in Schiff reagent for 1–3 h, in the dark, and squashed in 45% acetic acid. Slides were observed using a Nikon Eclipse E400 (Nikon Instruments Europe BV, Amsterdam, Netherlands) coupled to a Nikon DS-U1 camera (Nikon Instruments) and processed with the software NIS-Elements D (version 4.60, Nikon Instruments). As controls, the number of chromosomes was counted in roots of *in vitro* growing seedlings germinated in MS medium.

Scanning Electron Microscopy

Explant tissues with developing somatic embryos, somatic embryos at different development stages and mature zygotic embryos were fixed as described before. After dehydration on ethanol, samples were dried by the critical point method and sputter coated with a 20 nm layer of gold–palladium. Observations were carried out on a JEOL JSM-5400 (JEOL, Musashino, Japan) at 15 kV.

Statistical Analysis

Values are given as mean ± standard deviation of 3 replicates (N=30) for induction assays and a variable number of embryos (between 81 and 234) for embryo conversion assays. Data were analyzed and comparisons between treatments concerning the induction rates, number of embryos, and conversion rates were made using One Way ANOVA test on GraphPad Prism (v. 8.4.3 for Windows, San Diego, CA, USA), followed by a Tukey's multiple comparison test ($p<0.05$).

Results

Somatic embryogenesis induction and development

Strawberry tree was successfully propagated through somatic embryogenesis using the tested conditions, both from adult and juvenile genotypes with embryogenesis induction rates ranged from 0 to 94.5% ± 4.8 (Figure 6). On three genotypes (AU1, AU2 and AU4) higher induction rates were achieved on the medium with a combination of BAP and NAA on a 1:1 ratio (IM3: 2 mgL^{-1} BAP and 2 mgL^{-1} NAA) followed by the medium with a 1:2.5 ratio (IM4: 2 mgL^{-1} BAP and 5 mgL^{-1} NAA). Though, on genotype AU3, higher induction rates were obtained on IM5, with a significant amount of NAA (10 mgL^{-1}). Somatic embryogenesis induction on strawberry tree proved to be genotype dependent. Lower induction rates were obtained on genotype AU3 (young tree), when compared to the other genotypes tested. On some of the induction media tested, statistically significant differences were observed between genotypes in terms of induction rates (Figure 6 E-K). For instance, on the most efficient induction medium (IM3), an induction rate of 56.6 ± 13.2 were obtained on genotype AU1, 52.8 ± 9.6 on AU2, 19.5 ± 4.8 on AU3 and 94.5 ± 4.8 on AU4 (Figure 6 G).

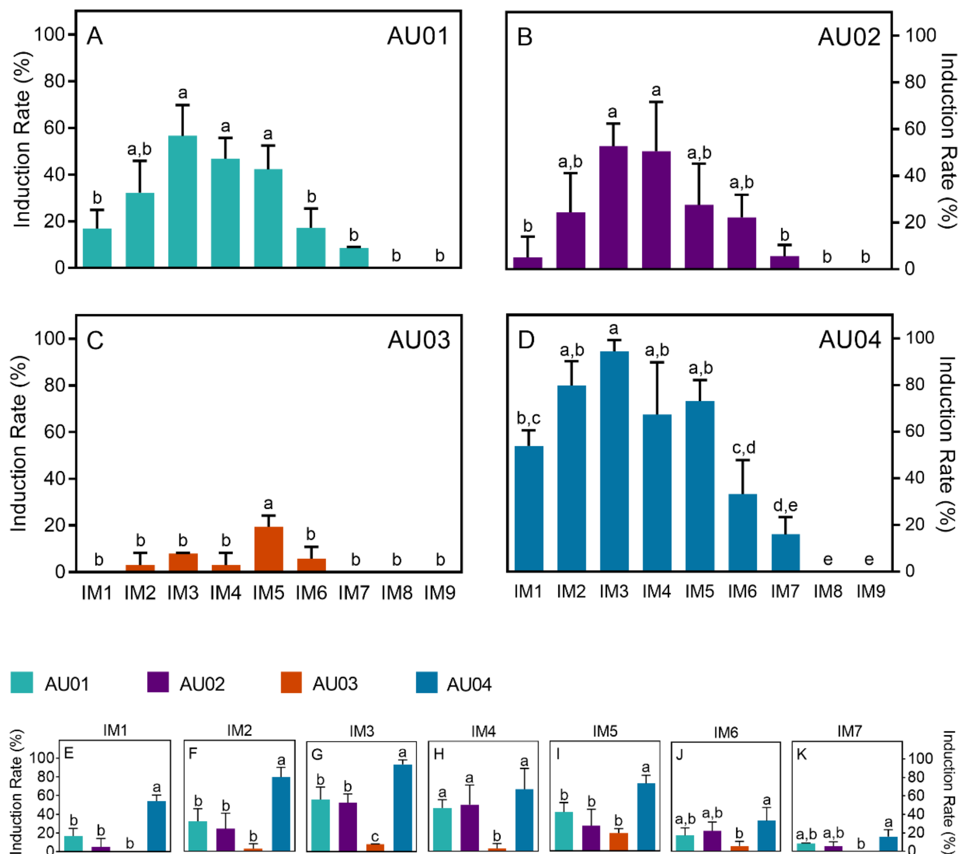

Figure 6. Somatic embryogenesis induction rates on strawberry tree on nine different induction media (IM 1-9) and four genotypes (AU 1-4): genotype AU1 (adult tree) on nine different induction media (A); genotype AU2 (adult tree) on nine different induction media (B); genotype AU3 (young tree) on nine different

induction media (C); genotype AU4 (seedling) on nine different induction media (D); four genotypes on induction medium 1 (E); four genotypes on induction medium 2 (F); four genotypes on induction medium 3 (G); four genotypes on induction medium 4 (H); four genotypes on induction medium 5 (I); four genotypes on induction medium 6 (J); four genotypes on induction medium 7 (K). Values are means ± SDs, n = 3. Each biological replicate consisted of 10 leaf explants induced on individual test tubes. Different letters indicate significant statistically differences between treatments at $P \leq 0.05$.

Leaf segments cultured on the different media started to produce *calli* in the wounded areas after 2-3 weeks of culture. Initially the *calli* were light green (Figure 5 D) but rapidly turned brownish and friable. In these *callus*, somatic embryos started to appear by the 6-8th week (Figure 5 E). Somatic embryo development was not synchronized and embryos at the different stage of development, from globular to cotyledonary, could be observed in a same explant (Figure 5 F-G). Like induction rates, the number of somatic embryos obtained varied according to the induction media composition, ranging from just a few (2-5) to more than 200 (Figure 7). Genotype AU4 gave the highest number of embryos per initial explant on medium IM2 in which an average of almost 200 embryos was obtained per explant (Figure 7 D), whereas genotype AU3 showed to be the less responsive (Figure 7 C). Statistically significant differences were observed between genotypes in the number of somatic embryos, on some of the induction media tested (Figure 7 E-K). In general, a higher number of embryos was obtained in genotype AU04 on most of the media used. Mature cotyledonary embryos (Figure 5 H) displayed two large cotyledons, a short hypocotyl and a radicle (Figure 5 H). The major difference when compared with mature zygotic embryos was the expanded cotyledons, which in zygotic embryos were in close contact with each other due to the mechanical constraints imposed by the seed coat (Figure 5 I). Tetrazolium staining also showed that somatic embryos were viable, as can be seen in figure 5 I by the intense red color they display.

Somatic embryo germination and acclimatization

When mature cotyledonary embryos (Figure 8 A) were transferred to the germination medium, the first signs of germination were detected during the first week of culture, namely elongation of the hypocotyl and the appearance of a green color in the cotyledons (Figure 8 B). Then the shoot apical meristem started to produce new phytomers and root elongation could be seen (Figure 8 C). By the 3rd week of plantlet development, a well-developed root system, showing many lateral roots, was established as well as several leaves (Figure 8 D). By the 6-8 weeks of culture, well-developed plantlets showing in general 5 to 9 phytomers (Figure 8 E) and an extensive root system were ready to be transferred to pots and acclimatized (Figure 8 F). In spite of the reduced dimensions of the chromosomes, the expected chromosome number of $2n=26$ was found in root tips of the plantlets (Figure 8 G), showing that no chromosomal changes occurred during regeneration through somatic embryogenesis. Embryo germination were not genotype dependent and the maximum embryo conversion rate achieved was 73.5% ± 13.3, on embryos obtained from genotype AU2. Furthermore, higher rates of embryo conversion were achieved on embryos obtained on IM3 when compared to IM2, IM4 and IM5 (Figure 9). In the tested conditions

acclimatization (Figure 8 H) was easily achieved and after 6 months the success rates were close to 100%.

Histological observations

Histological analyses carried out in embryogenic explants showed the different stages of somatic embryo differentiation. Few-celled somatic embryos (Figure 10 A) found at the periphery of the callus-forming embryos showed a strong polarity with an apical part formed by round, dense cytoplasmatic cells, the embryo proper, and a basal pole with more irregular cells, corresponding to a suspensor-like structure. Further development confirmed these observations and globular embryos clearly showed a suspensor embryo-like structure (Figure 10 B). The embryo proper also showed two distinct regions, one with more stained cells towards the apical region and more vacuolated cells near the root pole (Figure 10 C). More intense cell proliferation was responsible for the transition from globular to hearth-shaped embryos (Figure 10 D-E), stage at which a prominent protoderm was detected. After this stage the hypocotyl-root axis as well as the cotyledons elongated (Figure 10 F) and the radial differentiation of tissues took place. Thus, beyond the protoderm (Figure 10 G), easily distinguished from the subjacent tissues due to the lower density of the cytoplasm, the procambium and the shoot apical meristem could also be observed (Figure 10 H). At the basis of the embryos, a multicellular layer of phenolic-rich cells was usually found connecting the somatic embryos to the maternal tissues (Figure 10 F and I).

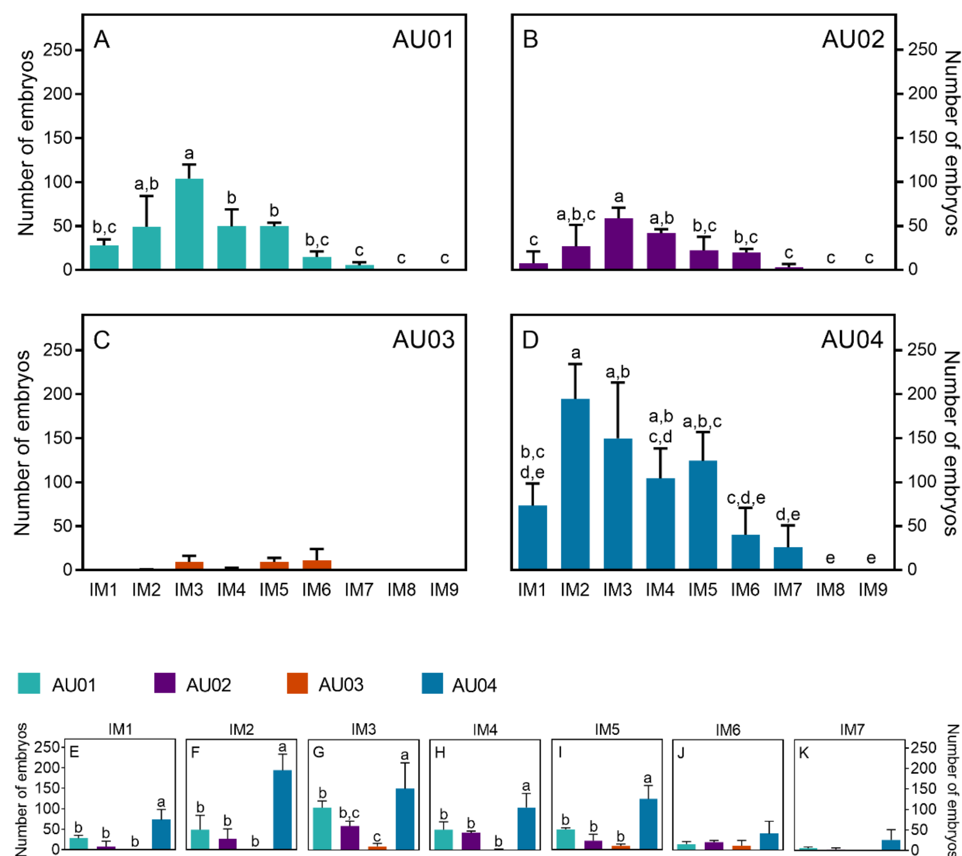

Figure 7. Number of somatic embryos obtained on strawberry tree on nine different induction media (IM 1-9) and four genotypes (AU 1-4): genotype AU1 (adult tree) on nine different induction media (A); genotype

AU2 (adult tree) on nine different induction media (B); genotype AU3 (young tree) on nine different induction media (C); genotype AU4 (seedling) on nine different induction media (D); four genotypes on induction medium 1 (E); four genotypes on induction medium 2 (F); four genotypes on induction medium 3 (G); four genotypes on induction medium 4 (H); four genotypes on induction medium 5 (I); four genotypes on induction medium 6 (J); four genotypes on induction medium 7 (K). Values are means ± SDs, n = 3. Each biological replicate consisted of 10 leaf explants induced on individual test tubes. Different letters indicate significant statistically differences between treatments at $P \leq 0.05$.

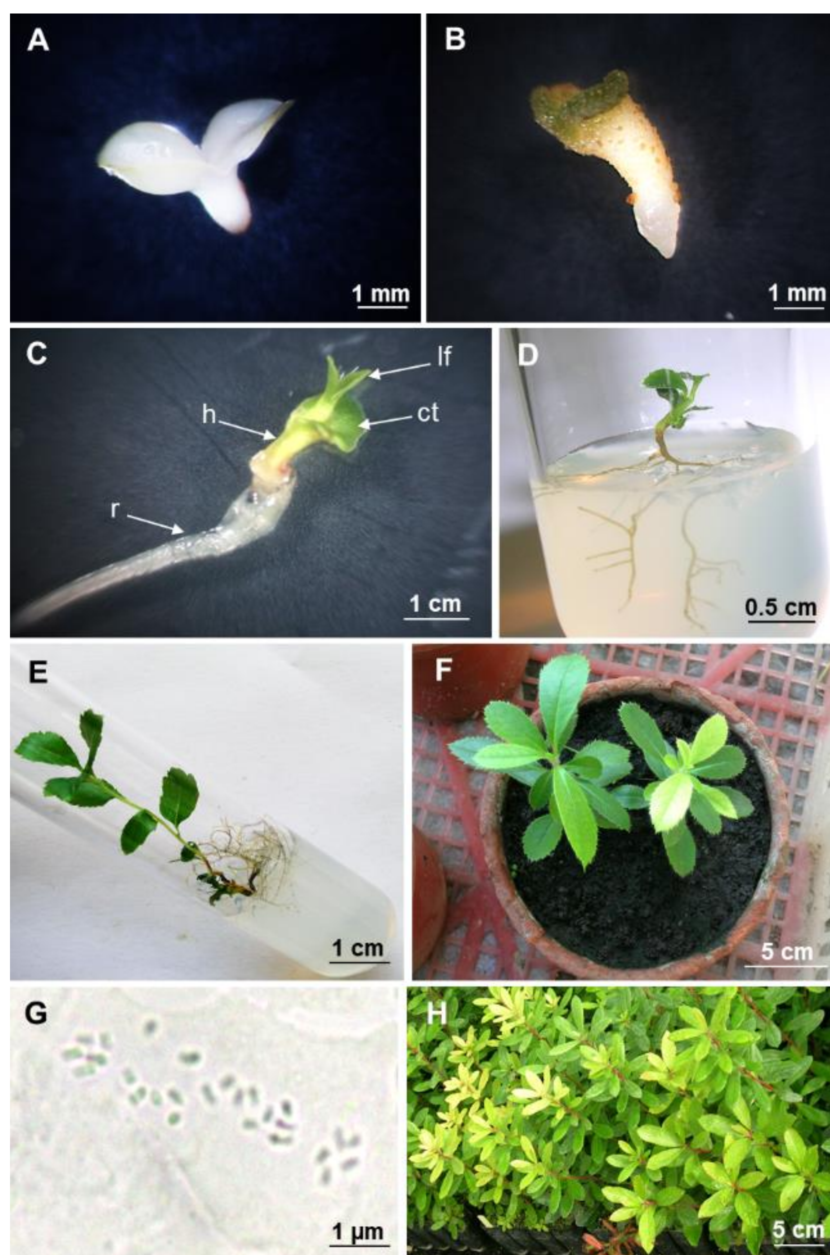

Figure 8. Somatic embryo germination and plant acclimatization: mature cotyledonary somatic embryo at the time of transfer to the germination medium; somatic embryo germination showing the green color of the cotyledons and hypocotyl elongation (B); young plantlet after 4 weeks of germination (C); developing plantlet after 8 weeks of culture in the germination medium (D); plantlet with several phytomers just before transferring to pots for acclimatization (E); somatic embryo derived plants growing in pots (F); c-metaphase

of a root shoot tip cell showing the normal chromosome number (2n=26) (G); *A. unedo* plants obtained through somatic embryogenesis (H). ct – cotyledon; h- hypocotyl; lf – leaf; r – root.

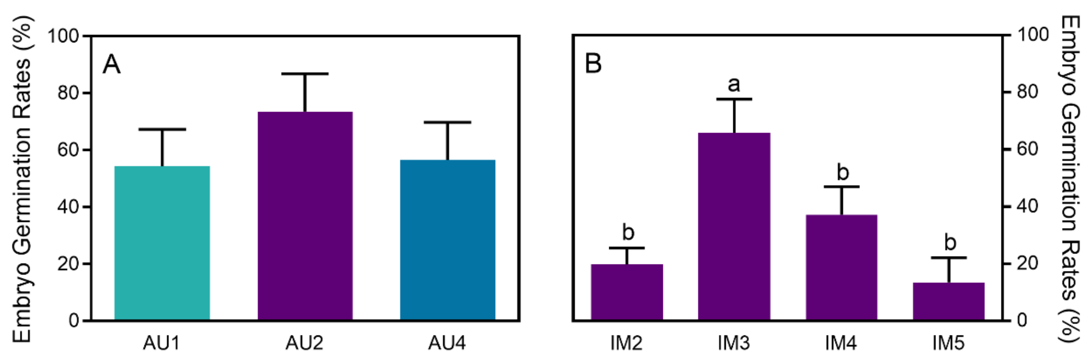

Figure 9. Somatic embryos germination rates: germination rates (%) of embryos obtained from 3 different genotypes, AU1 (adult tree), AU2 (adult tree) and AU4 (seedling) (A); germination rates of embryos based on the induction media they were obtained (B). Values are means ± SDs, n = 3. Each biological replicate consisted of a group with between 27 and 85 somatic embryos. Different letters indicate significant statistically differences between treatments at $P \leq 0.05$.

SEM observations

SEM observation confirmed most of the features of the embryogenic process already observed in the histological analysis. Thus, very young somatic embryos could be seen developing at the periphery of the embryogenic explants (Figure 11 A) usually showing a suspensor-like structure connecting the embryo proper to the subjacent maternal tissue (Figure 11 B). At these pre-globular stages, the three-dimensional organization of the embryo cells seems random since the protoderm was not yet formed. Continuous embryo growth ked to the appearance or somatic embryos at the periphery of the explants (Figure 11 C) mixed with callus cells not involved on somatic embryo differentiation. SEM analysis confirmed the presence of a suspensor-like structure in globular embryos (Figure 11 D). Globular somatic embryos involved into hearth-shaped (Figure 11 E), torpedo (Figure 11 F) and cotyledonary (Figure 11 G-H) morphologically normal somatic embryos. In the later stages of somatic embryo development, several anomalous embryos were found, usually showing an unusual number of cotyledons (1 or 3; Figure 11 I-J). SEM analysis also showed that mature zygotic and somatic embryos were very similar, the main difference being the expanded cotyledons found in somatic embryos as opposed to the face-to-face cotyledons of the zygotic embryos, which resemble a table tennis racket (Figure 11 K). The removal of a cotyledon showed also a similar organization in the apical regions of both somatic (Figure 11 H) and zygotic (Figure 11 L) embryos.

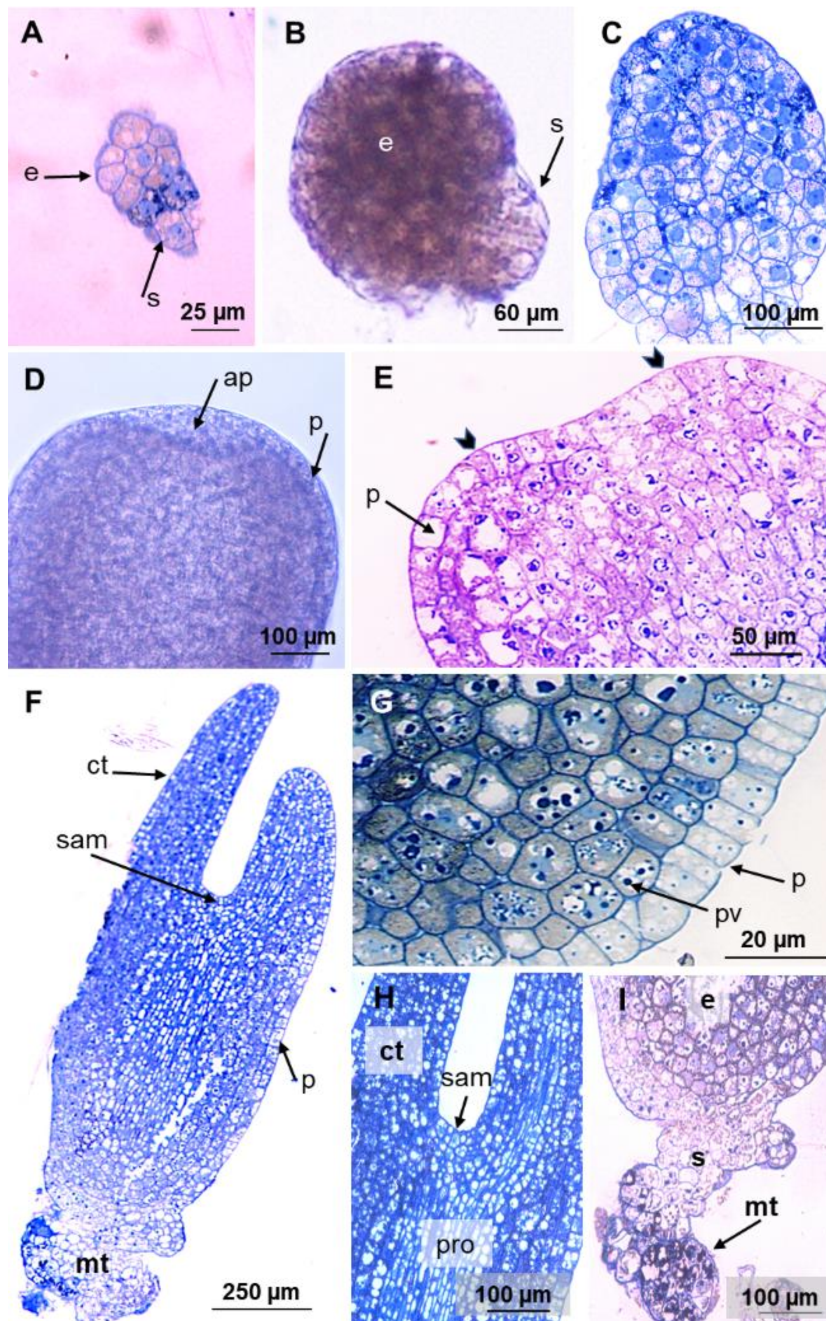

Figure 10. Histological observation during somatic embryogenesis induction: preglobular somatic embryo of *A. unedo* showing the apical part of the embryo (e) and a suspensor-like structure (s) (A); globular embryo showing the embryo proper (e) and the suspensor (s) (B); section of a globular embryo (C); apical part of a hearth-shaped somatic embryo were the apical region (ap) between the two cotyledons can be seen as well as the protoderm (p) (D); longitudinal section of a hearth-shaped somatic embryo showing the apical part where the two cotyledons have started to differentiate (arrows) and the protoderm is present (E); torpedo somatic embryo where the cotyledons (ct), shoot apical meristem (sam) the protoderm (p) and the mother tissue (mt) are indicated (F); section of a torpedo somatic embryo showing the protoderm and subjacent ground meristem cells (G); apical zone of a cotyledonary somatic embryo showing the shoot apical meristem (sam) between the two cotyledons (ct) and the procambium (pro) (H); basal part of a cotyledonary embryo (e) showing the suspensor (s) and the maternal tissue (mt) rich in phenolic compounds (I).

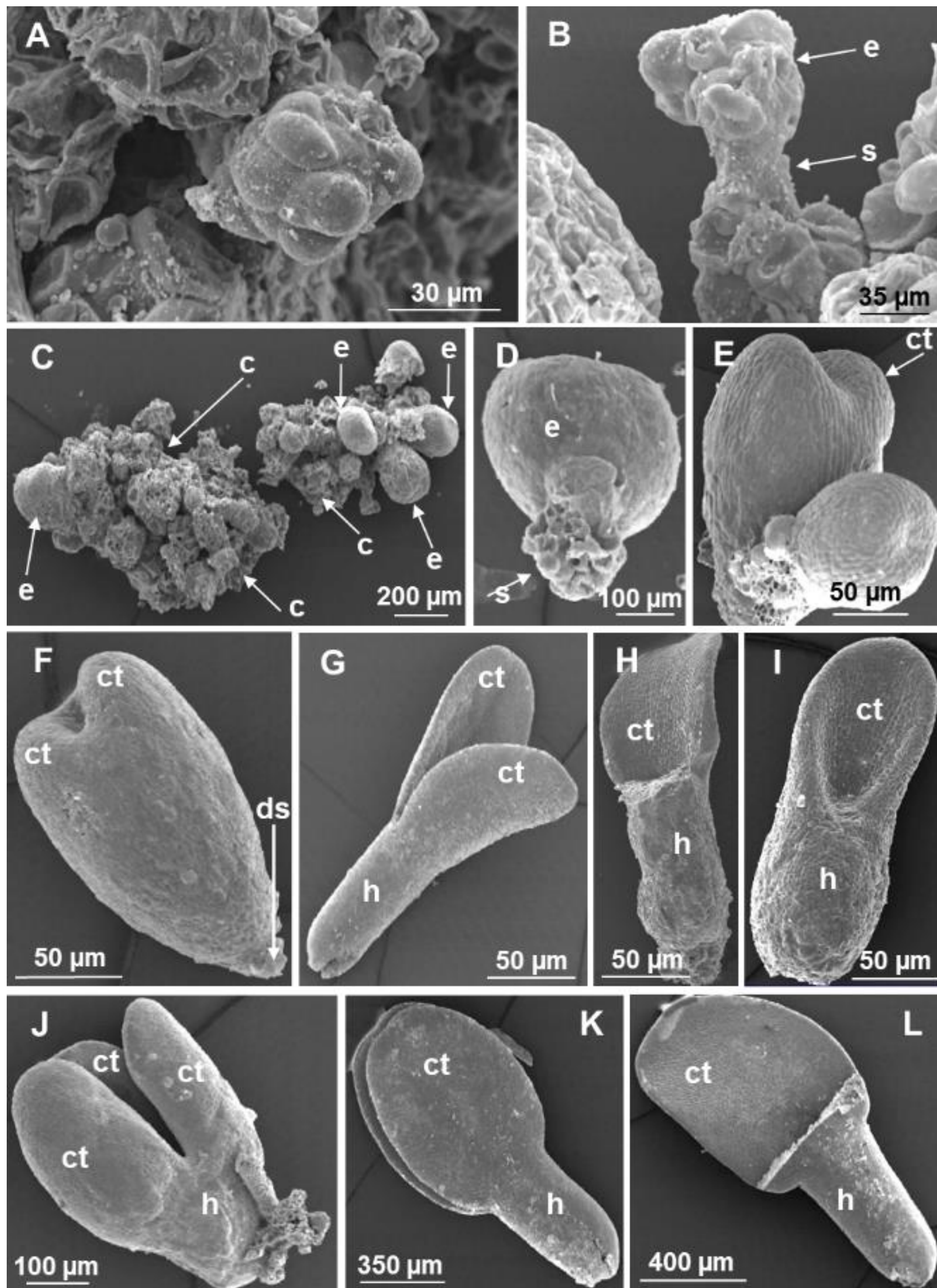

Figure 11. SEM observations during somatic embryogenesis induction in *A. unedo*: preglobular somatic embryo at callus surface. Note the absence of protoderm (A); preglobular embryo showing the embryo proper (e) and the suspensor (s) (B); callus (c) from which many somatic embryos (e) are developing (C); globular somatic embryo (e) showing a suspensor-like structure (s) (D); globular somatic embryos involved into hearth-shaped (E); torpedo somatic embryo with two cotyledons (ct) and a degenerated suspensor (ds) (F); cotyledonary somatic embryo with two cotyledons (ct) and hilum (h) (G); cotyledonary somatic embryo with one cotyledon removed (H); anomalous embryo with a single cotyledon (ct) (I); anomalous embryo with three cotyledons (ct) (J); zygotic embryo with face-to-face cotyledons (ct) (K); zygotic embryo with a cotyledon removed (L).

Discussion

Previous work carried out at our laboratory had already shown that strawberry tree could be propagated through somatic embryogenesis (Gomes et al. 2009; Martins et al. 2016a, b). In this work a more detailed study about the induction supported by histological and SEM analyses is presented. In this species, somatic embryogenesis is achieved by a one-step direct induction protocol, in which somatic embryos develop in the cultured explant, following callus formation. This protocol is faster than the two-step protocol described for other species, in which an embryogenic callus is first produced in the presence of an auxin and, following transfer to a second medium without or with lower auxin concentrations, proembryogenic masses develop into somatic embryos (Capuana et al. 2007; Correia et al. 2011; Corredoira et al. 2013; Lu et al. 2017).

Although a one-step direct induction protocol is faster than indirect embryogenesis, as there is no need for a *calli* induction medium and an embryo induction medium, in the long run, this kind of protocol might be less efficient, as no *calli* with embryogenic capacity is available. For this reason, new cultures are necessary for new inductions, in this case leaves from *in vitro* shoots.

Somatic embryogenesis induction from leaves of *in vitro* propagated shoots of selected adult trees has been already achieved in other species such as *Solanum betaceum* (Correia et al. 2011), *Castanea sativa* (Corredoira et al. 2003) and *Quercus alba* (Corredoira et al. 2012). The ideal protocol would be to induce somatic embryogenesis from leaves directly collected from field-growing selected trees. However, attempts to achieve this goal were unsuccessful (data not published) because the leaves hardly form callus and to the high levels of contamination observed. However, the induction of somatic embryogenesis from leaves of shoots or plantlets from selected genotypes kept *in vitro* has proved to be an interesting alternative to the direct culture of leaves of selected genotypes since high rates of somatic embryo formation and acclimatization can be achieved.

Embryogenesis induction rates of 97.22% were achieved, much higher than those of other tree species, such as *Eucalyptus sp.* (68.8%, (Corredoira et al. 2015), *Quercus alba* (50.7%, (Corredoira et al. 2012), *Alnus glutinosa* (16.6%, (Corredoira et al. 2013) and the Ericaceae *Leucopogon verticillatus* (50.8%, (Anthony et al. 2004b). Similar induction rates (97.3%) have been obtained by (Canhoto et al. 1999) on the shrub *Myrtus communis*.

Somatic embryogenesis is usually induced by the application of plant growth regulators or stress conditions to the explants (refs). The role of auxins is particularly known and most of the protocols for somatic embryogenesis induction are based on the application of a strong auxin, usually 2,4-D. In the case of *A. unedo*, cytokinins seem to have a crucial role since best results were obtained when the cytokinin BAP was also present in the culture medium. The role of cytokinins on somatic embryogenesis induction is poorly understood. Some works have shown that this type of hormones may increase the rates of somatic embryogenesis (Corredoira et al. 2003; Ramakrishna

and Shasthree 2016) but little is known about the mechanisms underlying their role. Due to the high levels of somatic embryogenesis induction achieved in *A. unedo* in the presence of BAP, this system may be interesting to analyse the role of cytokinins on promoting somatic embryogenesis.

Somatic embryo formation in strawberry tree was only achieved when the calli turned brown, due to oxidation. Similar observations were made in another species, such as *Acca sellowiana* (Reis et al. 2008) and *Coffea arabica* (Neuenschwander and Baumann 1992). Tissue browning is related with the oxidation of phenolic compounds that may have considerable impact in the endogenous levels of plant hormones thus modifying the balance between endogenous and exogenous growth regulators. The role of tissue browning and its eventual role on somatic embryogenesis induction deserves further analysis and the somatic embryogenesis induction system of *A. unedo* could be interesting to deeper analyse whether phenolic oxidation has a role on somatic embryogenesis.

Somatic embryogenesis induction proved to be genotype dependent on strawberry tree as lower induction rates were obtained on genotype AU3, when compared to the other genotypes tested. The importance of genotype on somatic embryogenesis induction has been extendedly referred by several authors (Sharma and Rajam 1995; Merkle et al. 1998; Fiuk and Rybczyński 2008; Corredoira et al. 2015). Embryos on different developmental stages were found, which shows embryos formation and development is asynchronous, as it has been referred to happen on *Myrtus communis* (Canhoto et al. 1999). Somatic embryos were also very diverse in shape and size especially when compared to the zygotic counterparts, probably due to not been confined to a limited space (Gray 2005).

Although the high induction rates obtained, only 39.21% ± 14.03 of the embryos were able to grow into fully developed plants, with a normal rooting system. The low efficiency on conversion has been appointed as one the most important limitations that restricts the application of this technique, especially on woody species (Merkle et al. 1998). This limitation was also observed on *L. verticillatus*, as root development did not occur unless specific treatments were used, and a pulse treatment with IBA significantly increased root production (Anthony et al. 2004b). The conversion of somatic embryos into plants are usually dependent on several factors such as water relations, desiccation tolerance and reserve compounds accumulation (Santa Catarina et al. 2003). Sucrose concentration on the conversion media can play an important role, as it influences water relations (Komatsuda et al. 1992).

The lower conversion rates obtained on this work are likely due to an insufficient reserve accumulation during the maturation phase since lower amounts of starch and protein are accumulated on somatic embryos, when compared to its zygotic counterparts. Low reserves were also observed on *Acrocomia aculeate* somatic embryos, as well as vacuolated cells in contrast with zygotic embryos (Moura et al. 2010). These reserves are essential for embryo germination as they are consumed on the initial stages of germination. The lower accumulation of reserves on somatic

embryos may be due to the fact the only connection between the embryo and these substances is the suspensor. Other reason may be the accumulation of phenolic compounds, that might limit the accumulation of reserves or inhibit the germination (Canhoto et al. 1999).

The precise origin of somatic embryos in *A. unedo* could not be determined by histological studies. However, the data (histological and SEM) allowed the identification of somatic embryos since the early stages, only when a few cells were present. Thus, it is plausible to speculate that somatic embryos have a unicellular origin from cells at the periphery of the callus, since no meristematic layers of cells were found from which somatic embryos of multicellular origin could have originated, as found in other systems such as *Acca sellowiana* (Canhoto and Cruz 1996; CANHOTO 1996) feijoa. Also interesting was the observation that a suspensor-like structure was present in somatic embryos of strawberry tree. Similar structures have been observed in other dicots species such as *A. sellowiana* (Correia and Canhoto 2010) and *Brassica napus* (Supena et al. 2008) and in conifers suspensors are always associated with the somatic embryos (Salaj et al. 2020). The role a suspensor may have during somatic embryogenesis is not clear, but it may function as a carrier through which nutrients and/or signal molecules are transported from the maternal tissues to the developing embryos. However, further studies are required to determine the precise role of this part of the somatic embryos.

Histological and SEM observations also showed that somatic embryos of *A. unedo* are morphologically normal, in spite of soma anomalies have been detected. The data also showed the both the radial (tissue formation) and apical-basal (organ differentiation) occurs as has been described in other species. However, the observation that the number of somatic embryos able to germinate is much lower than the total number of embryos formed seems to indicate that other types of anomalies, other than morphological, may affect the embryonic development.

The somatic induction protocol developed for *A. unedo* proved to be very efficient, when a medium with cytokinins and auxins on a 1:1 ratio is used. High induction rates were achieved, and a great number of somatic embryos can be obtained, both from seedling and adult material. However, like in other species, it is genotype dependent and embryo conversion seems to be a limitation, that must be surpassed in order to fully seize the potential of this micropropagation technique.

CHAPTER 2: A baseline of *Arbutus unedo* L. microbiome for future research: *in vitro* vs. *ex vitro*

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Abstract

As micropropagation techniques are quite popular for the production of strawberry tree clonal plants, the characterization of its microbiome is essential, due to the possible impact in micropropagation success, including acclimatization. Thus, this study aims to identify the endophytic communities in strawberry tree plant tissues *in vitro*, as well as their prevalence and/or alteration upon plant acclimatization. For this purpose, the microbiome of two genotypes under micropropagation and *ex vitro* was identified using a culture-independent method. Bacterial OTUs were assigned to 7 phyla and 79 genera, whereas only one Archaea genus was identified. The most abundant and diverse bacterial phylum was Actinobacteriota (48%) followed by Proteobacteria (43%), Firmicutes (6%) and Bacteroidota (3%). Noticeable differences in terms of composition and diversity were found when the microbiome of genotypes *in vitro* was compared, whereas the composition of *ex vitro* samples was similar. A significant higher diversity was found on both genotypes *ex vitro* when compared to the respective *in vitro* plants. This study presents the first approach in the identification and characterization of strawberry tree microbiome, including *in vitro* plants, using a culture-independent method. The genotype proved to be a determinant factor shaping microbiota structure on *in vitro* plants. The relevance of this results for micropropagation and breeding are discussed.

Keywords: Archaea, bacteria, diversity, genotypes, micropropagation

Introduction

Strawberry tree has received much attention from researchers over the last two decades and a great deal of work has been done to select superior genotypes. Due to the low level of differentiation that seems to exist between populations, caused by a high gene flow, the screening should be take into account intra- rather than inter-population diversity (Takrouni and Boussaid 2010; Lopes et al. 2012; Takrouni et al. 2012). In order to supply producers with true-to-type plants from high quality selected genotypes, micropropagation protocols have been developed (Martins et al. 2016a, 2019). To obtain axenic cultures, micropropagation techniques require a sterilization procedure, to eliminate microorganisms from plant meristematic tissues, before *in vitro* establishment. Nevertheless, the presence of bacteria in micropropagated plants is a common scenario. Although some of these bacteria are contaminants resulting from human handling, others are endophytes that survive the sterilization procedure and persist in the cultures (Quambusch and Winkelmann 2018). An intriguing area in this field that few researchers have addressed is the presence of latent microorganisms in *in vitro* plant cultures, that do not grow in the plant culture medium. Some studies were able to identify a considerable variety of these microorganisms in plant tissues under micropropagation from different plant species, such as *Atriplex* spp. (Lucero et al. 2011), *Pogonatherum paniceum* (Koskimäki et al. 2010) and *Prunus avium* (Quambusch et al. 2014). As micropropagation techniques are the main approach used for strawberry tree plant production due to their great advantages over traditional methods (Martins et al. 2019), and due to the impact of endophytes on its host, the identification and characterization of endophyte communities in the strawberry tree *in vitro* cultures is very important, in particular those that prevail after plant acclimatization and transference to field conditions.

With this in mind, the aim of this study was the identification of latent bacteria in *A. unedo in vitro* cultures and test the effect of plant genotype on microbiota structure. Moreover, the prevalence of these endophytes upon plant transfer to *ex vitro* conditions was evaluated. Insights about possible synergies between those microorganisms and host plant are provided and the implications of these results on *A. unedo* selection and micropropagation are discussed.

Material and Methods

Micropropagation

Two genotypes with different origins, genotype A (young tree) and genotype B (seedling) were selected for this study from a population growing in Coimbra region, Central Portugal (N 40°12′17.472″ W 8°23′40.929″, altitude 103 m). Plants were selected based on their physiological performance under drought (Martins et al. 2019). Both genotypes were established *in vitro* and micropropagated according to Martins et al. (2019). Briefly, young branches of genotype A were

cut, dipped in 100 mg L^{-1} fungicide (Aliette, Bayer CropScience, Germany) for 10 min and rinsed with distilled water. After this treatment, branches were kept on containers, covered with a plastic bag, watered with distilled water and placed on a culture chamber (FitoClima 10000 HP, Aralab, Portugal) for 30 days under 16-h photoperiod, an irradiance of 250 µmol m^{-2} s^{-1}, a temperature of 25 ºC, and air humidity of 70%. The epicormic shoots formed were removed and surface sterilized with 70% ethanol (for 30s) and calcium hypochlorite (5%, w/v, Sigma-Aldrich, St. Louis, MO, USA) with two drops of Tween 20for 10 min. After 3 washes with sterile distilled water, the epicormic shoots (0.5-1 cm) were placed in Anderson Rhododendron medium (Anderson 1980); DuchefaBiochemie B.V, Haarlem, The Netherlands) with 6-benzylaminopurine (2 mg L^{-1}, Sigma-Aldrich), sucrose (3%, w/v, Duchefa) and agar (0,6%, w/v, Duchefa), on test tubes (25x150mm) with plastic caps (Duran, Mainz, Germany). The pH was adjusted to 5.7 using KOH or HCl diluted solutions (0.01 M – 1 M), and the culture medium was autoclaved at 121 ºC for 20 min (800–1,100 g cm^{-2} gel strength after autoclaving). The culture was done in a growth chamber at a 16-h photoperiod, an irradiance of 15 - 20 µmol $m^{-2}s^{-1}$ (cool-white fluorescent lamps), and a temperature of 25 ºC. For genotype B, isolated seeds were disinfected following the same methodology used for shoots and germinated on Petri dishes with filter paper discs and sterile distilled water. After 1 month of cold stratification (4 ºC), the seeds were transferred to a culture chamber under 16-h photoperiod at 25 ºC. After germination, the roots of the plantlets obtained were removed and the shoots placed on the medium described before. For axillary shoot proliferation, shoots were cultured on plastic containers (O118/80+OD118 with white filter, Microbox, Deinze, Belgium) with 100 mL of the previously described medium. The cultures were kept under a 16-h photoperiod at 25 ºC, with culture intervals of 8 weeks for a total period of 2 years. After this period, the multiplication rate was 2.2 ± 0.4 shoots/explant in genotype A and 4.3 ± 1.4 in genotype B.

Rooting and acclimatization

For root induction, 3 cm long shoots were dipped on indole-3-butyric acid (IBA; 1 gr L^{-1}, Sigma-Aldrich) for 30 seconds and placed on containers with perlite (Siro, Mira, Portugal), under 16-h photoperiod at 25 ºC and 70% humidity. The cover was gradually removed and after a one-month plants were transferred to individual containers (1700 cm^3) with a substrate composed of peat (30-0, Siro) and perlite (3:1, v/v). After one year, plants were transferred to larger containers (100 dm^3) with peat (30-0, Siro), and were kept in a greenhouse for another year under uncontrolled light, temperature and humidity conditions. All the substrates used were autoclaved at 121 ºC for 20 min.

Sample preparation

Plant material was collected from two different development stages of the plants: *in vitro* shoots (A1 and B1) and two years old plants (A2 and B2). *Ex vitro* plants height was 150 ± 15 cm (A2) and 180 ± 13 cm (B2). Leaves and stems from A1 and B1 (950 ± 72 mg) were ground in liquid

nitrogen with a sterile mortar and pestle, in a laminar flow cabinet, to avoid contaminations. Each sample consisted of 5 different shoots. Healthy leaves from A2 and B2 (10 ± 0.74 g) were collected from different branches and thoroughly washed in sterile water, surface-disinfected in 70% ethanol for 30 s, sodium hypochlorite (5%, w/v) for 10 min and washed three times in sterile distilled water. Finally, leaves were ground in liquid nitrogen as described before and 250 mg were used for DNA extraction.

DNA extraction and sequencing

DNA was extracted using DNeasy Powersoil Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Samples were prepared for Illumina Sequencing by 16S rRNA gene amplification of the bacterial community. The DNA was amplified for the hypervariable V5-V6 region with specific primers and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes. First PCR reactions were performed for each sample using KAPA HiFi HotStart PCR Kit according to manufacturer suggestions, 0.3 µM of each PCR primer: forward primer 799F-mod3 5'–CMGGATTAGATACCCKGG-3' (Hanshew et al. 2013) and reverse primer 1115R 5'–AGGGTTGCGCTCGTTG-3' (Turner et al., 1999) and 50 ng of template DNA in a total volume of 25 µL. PCR conditions involved a 3 min denaturation at 95 ºC, followed by 30 cycles of 98 ºC for 20 s, 58 ºC for 30 s and 72 ºC for 30 s and a final extension at 72 ºC for 5 min. Second PCR reactions added indexes and sequencing adapters to both ends of the amplified target region according to the manufacturer's recommendations (Illumina, 2013). Negative PCR controls were included for all amplification procedures. PCR products were then one-step purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al. 2017), pooled and paired-end sequenced in the Illumina MiSeq® sequencer with the V3 chemistry, according to manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal).

Sequencing reads and statistical analysis

Raw reads were extracted from Illumina MiSeq® System in fastq format and the obtained sequences were analyzed using the Quantitative Insights into Microbial Ecology bioinformatics pipeline, QIIME2 (version 2020.2.0; https://docs.qiime2.org/2020.2/) (Bolyen et al. 2019). The sequences were demultiplexed using the "demux" plugin according to their sequence code. The cleaning and replication of the sequences was done using the DADA2 (Divisive Amplicon Denoising Algorithm) pipeline (Callahan et al. 2016) within the QIIME2 program to remove sequencing adapters and trim bases with an average quality lower than Q25. Chimeric sequences were removed and Operational Taxonomic Units (OTU) were obtained at 3% of dissimilarity and were taxonomically classified using vsearch classifier against SILVA (release 138). OTUs were selected at a 97% similarity threshold using the open reference strategy. Rarefaction curves were performed with phyloseq package (McMurdie and Holmes 2013) using R software (version 4.0.3,

R Foundation for Statistical Computing, Vienna, Austria,(R Core Team 2020). The normalization of the data was performed rarefying all samples to the minimum number of reads found. To analyze the endophytic microbiota structure, Venn diagram, heatmaps, correlations, Principal Component Analysis (PCA) and diversity analysis were carried out using R software. Venn diagrams were constructed using the package VennDiagram (Chen 2018). To evaluate the interaction and significance of the different endophyte genera on microbiota composition, correlations were calculated using the package GGally (Schloerke et al. 2018), heatmaps were constructed using the package ComplexHeatmap (Gu et al. 2016) and a PCA was performed using the package factoextra (Kassambara and Mundt 2019). Diversity analysis - Shannon index (H'), Simpson's Index (D), Simpson's Index of Diversity (1-D') and Pielou index (J') - were obtained using the package vegan (Oksanen et al. 2019), to compare the diversity between samples from different genotypes and different ages. The similarity percentages (SIMPER)(Clarke 1993) were performed using a Bray-Curtis dissimilarity with the PAST software (version 4.0.3). Raw Illumina sequencing data was deposited in the NCBI as SRA files within the bioproject with the accession number PRJNA703435.

Results

Endophytic microbiota associated with two genotypes of *Arbutus unedo* L.

A total of 113.546 good quality 16S rRNA reads and 160 Operational Taxonomic Units (OTUs) were recovered by Illumina sequencing: A1 (22.349 reads), A2 (32.348 reads), B1 (34.773 reads) and B2 (24.076 reads). No chloroplasts reads were detected, and chimaeras, unassigned or mitochondrial reads were removed. Unassigned reads were only found in sample B1, representing 0.25% of the total good quality reads. Each sample reached the plateau phase, indicating that much of the diversity of the microbial community could be captured (Figure S1, available at: http://phd.freecluster.eu/). The level of sequencing coverage was 99% in all samples. Bacterial and Archaea communities were found: Bacterial OTUs were assigned to 7 phyla, 12 classes, 37 orders, 58 families and 79 genera where as for the Archaea communities only one unique phyla, class, order, family and genus was identified (Table S1, available at: http://phd.freecluster.eu/). The most abundant and diverse bacterial phylum was Proteobacteria (44.5%, comprising 32 genera), followed by Actinobacteriota (44.1%, comprising 26 genera), Firmicutes (6.2%, comprising 10 genera) and Bacteroidota (3.0%, comprising 8 genera) (Figure 12). At the genus level, *Pseudomonas* was the most abundant (27.8%), followed by *Mycobacterium* (21.9%), *Cutibacterium* (16.3%), *Serratia* (7.7%), *Acinetobacter* (2.8%) and *Halococcus* (2.0), accounting for 78.5% of the total diversity (Figure 12). Altogether, minor genera accounted for 21.0% of the diversity. The most abundant genera by phylum were: *Mycobacterium* (49.7%), *Cutibacterium* (36.9%), *Corynebacterium* (3.5%), *Lawsonella* (1.8%), *Micrococcus* (1.7%) amd *Janibacter*

(1.4%) within Actinobacteriota; *Chryseobacterium* (67.2%), *Alloprevotella* (10.4%), *Sphingobacterium* (6.7%), *Capnocytophaga* (4.7%), *Empedobacter* (4.1%), *Prevotella* (2.9%), *Proteiniphilum* (2.3%) and *Hymenobacter* (1.7%) within Bacteroidota; *Staphylococcus* (29.4%), *Streptococcus* (28.6%), *Bacillus* (17.8%), *Lactobacillus* (9.9%), *Anaerococcus* (4.4%), *Carnobacterium* (4.3%) and *Gemella* (3.5%) within Firmicutes; *Pseudomonas* (62.4%), *Serratia* (17.2%), *Acinetobacter* (6.3%), *Candidatus* Portiera (3.8%), *Wolbachia* (1.6%), *Escherichia-Shigella* (1.3%) and *Sphingomonas* (1.1%) within Proteobacteria (Figure 12).

A total of 37 species were identified, such as: *Bacillus thuringiensis*, *Kocuria rhizophila*, *K. palustris*, *Pseudomonas alcaligenes*, *P. mendocina*, *P. umsongensis*, *Sphingomonas azotifigens*, *S. mali* and *S. yabuuchiae* (Table S2, available at: http://phd.freecluster.eu/). From the identified species, 10 were found *in vitro* in genotype A and 9 in genotype B, whereas *ex vitro*, 17 and 20 species were found in genotype A and B, respectively.

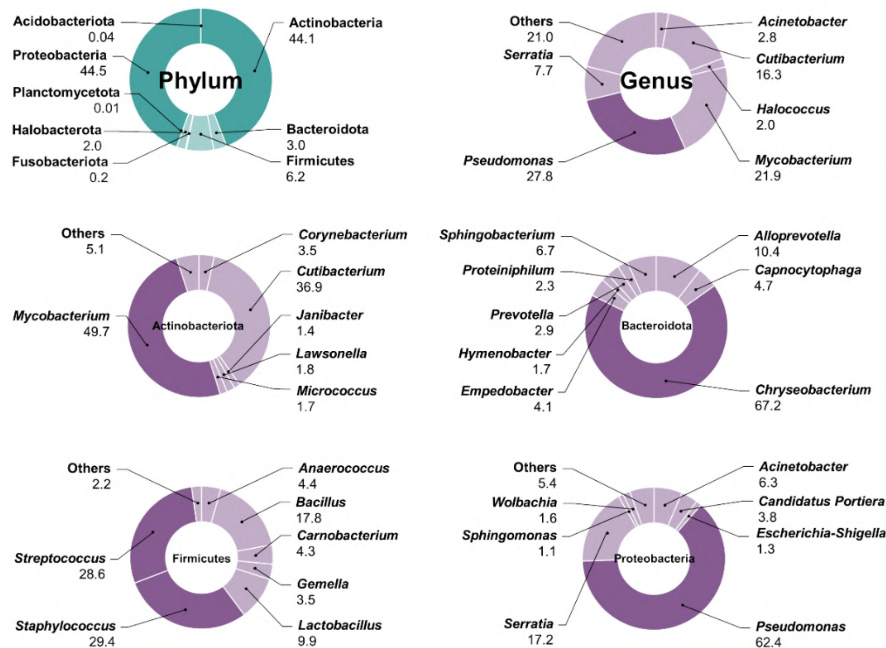

Figure 12. Overall endophytic microbiota relative abundance (%) at the phylum and genera level.

Differences in the composition of the endophytic microbiota between genotypes

Comparing the microbiota structure of the two strawberry tree genotypes there were noticeable differences. A substantial difference was that the domain Archaea was only identified in genotype A - *ex vitro*. At the phylum level, considerable differences in the relative abundance were observed between genotypes *in vitro*. While Proteobacteria was the most abundant phylum on genotype A, Actinobacteriota had a higher relative abundance on genotype B (Figure 13 A). This difference in relative abundance was also observed at the genus level (Figure 13 B). *In vitro*, a considerably higher number of unique genera was identified in genotype A (16) when compared to genotype B (6). At this development stage, 15 bacterial genera (40.5%) were found to be shared between genotypes (Figure 13 C, Table S3, available at: http://phd.freecluster.eu/). In the *ex vitro*

samples the number of unique genera was very similar between genotypes (21 in genotype A and 20 in genotype B). Furthermore, 22 genera (34,9%) were shared between genotypes (Figure 13 D, Table S3, available at: http://phd.freecluster.eu/). Only eight bacterial genera (10%) were shared between all the samples: *Bacillus, Chryseobacterium,* an unknown genus of the family *Comamonadaceae*, *Cutibacterium*, *Pseudomonas, Serratia, Staphylococcus* and *Streptococcus* (Figure 13 E).

Concerning the distribution of OTUs among genotypes, 15 were shared on the *in vitro* samples, most belonging to the Proteobacteria phylum (7; *Pseudomonas* 5, *Burkholderia-Caballeronia-Paraburkholderia* 1 and *Serratia* 1). Twice as much as unique OTUs were identified in genotype A than in genotype B, respectively 31 unique OTUs in genotype A, most belonged to the Actinobacteria (14) followed by Proteobacteria (7); and fifteen in genotype B, most belonged to the Proteobacteria (6) followed by Firmicutes (5). The number of unique OTUs in the *ex vitro* samples was very similar between genotypes: 47 and 49 in genotypes A and B respectively. In genotype A, most OTUs belonged to the Actinobacteria phylum (21) followed by Proteobacteria (16), whereas in genotype B most belonged to Proteobacteria (18), followed by Actinobacteria (15) and Firmicutes (14). 22 OTUs were found to be shared between strawberry tree genotypes in the *ex vitro* samples (Table S4, available at: http://phd.freecluster.eu/).

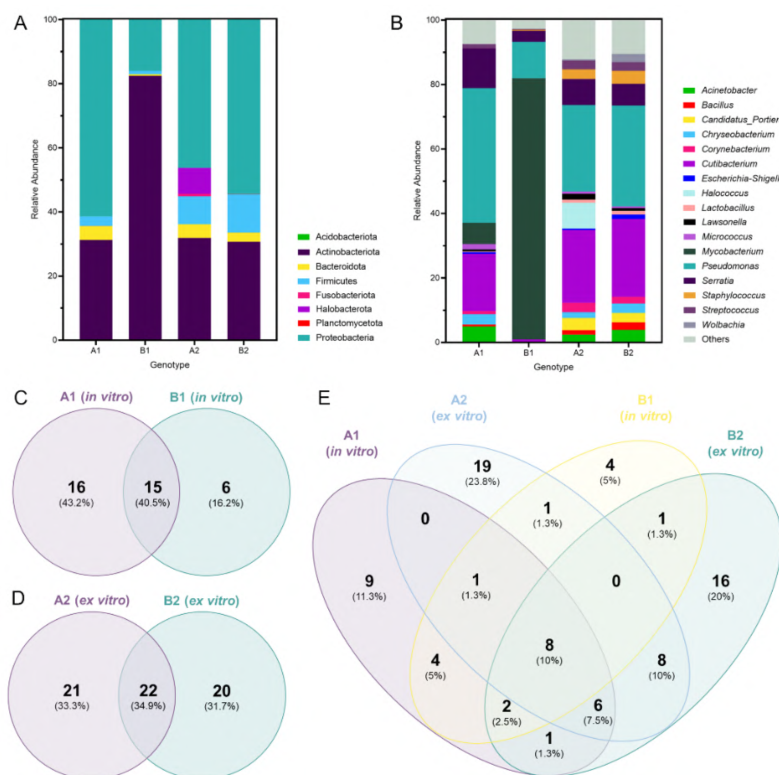

Figure 13. Relative abundance of endophytic microbiota at the phylum (A) and genus (B) level for samples A1 (genotype A - *in vitro*), B1 (genotype B - *in vitro*), A2 (genotype A – *ex vitro*), and B2 (genotype B – *ex vitro*); Venn diagram showing the common and exclusive genera and relative abundance of the endophytic microbiota of samples A1 and B1 (C), A2 and B2 (D), and between all samples (E).

Effect of plant development stage on the endophytic microbiota (*in vitro* vs. *ex vitro*)

Regarding the *in vitro* development stage of strawberry trees genotypes, Proteobacteria (61.4%) was found to be the most abundant phylum in genotype A, followed by Actinobacteria (31.3%), whereas Actinobacteria represented 82.5% of the endophytic microbiota in genotype B (Figure 14). In contrast, samples collected from *ex vitro* strawberry trees showed a similar composition between genotypes at the phylum level, most belonging to Proteobacteria (46.2-54.4%), followed by Actinobacteria (31.8-30.7%) and Firmicutes (8.8-11.9%), in genotype A and B respectively (Figure 14).

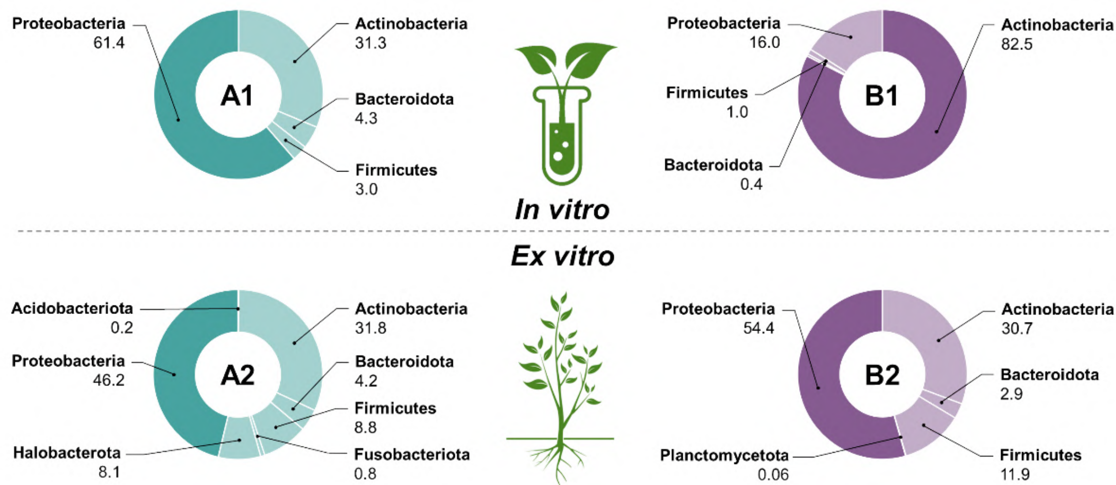

Figure 14. Relative abundance of the endophytic microbiota at the phylum level between genotype A and B at different development stages (*in vitro* and *in situ*).

At the genera level, 16 unique genera (27.1%) were identified *in vitro* in genotype A compared to 28 (47.5%) *ex vitro*, whereas in genotype B 10 unique genera (19.2%) were identified *in vitro* compared to 31 (59.6%) *ex vitro* (Figure 15 A-B). The difference between *in vitro* and *ex vitro* samples was also confirmed by the log2 fold change calculation of OTUs at a phylum and genera level (Figure 15 C-D). An increase in the number of OTUs was observed between *in vitro* and *ex vitro* samples in almost all the genera, except *Acinetobacter*, *Chryseobacterium*, *Escherichia-Shigella*, *Micrococcus*, *Pseudomonas* and *Serratia* in genotype A.

Similarity analysis between microbial communities

The dissimilarity value obtained (76.1%) for single genera between the *in vitro* samples of genotypes A (A1) and genotype B (B1) demonstrated that the microbiota at this stage of development was considerably different (Table S5, available at: http://phd.freecluster.eu/). *Mycobacterium*, *Pseudomonas* and *Cutibacterium* were the genera with the most contribution to the dissimilarity with 48.96%, 20.05% and 11.22% respectively. A lower dissimilarity value was determined between A2 and B2 (22.14%). In this case, *Halococcus, Pseudomonas* and *Wolbachia* were the genera with the most contribution to the dissimilarity with 18.24%, 10.08% and 4.89%

respectively. The relative abundance of endophytic microbiota found in the two developmental stages of strawberry trees was more similar in genotype A (A1/A2) with an average dissimilarity of 37.80%, than in genotype B (B1/B2) with an average dissimilarity of 83.11%. The genera with the most contribution to the dissimilarity in genotype A were *Pseudomonas* (19.76%), *Halococcus* (10.78%) and *Mycobacterium* (8.70%), whereas in genotype B were *Mycobacterium* (48.78%), *Cutibacterium* (14.12%) and *Pseudomonas* (12.06%).

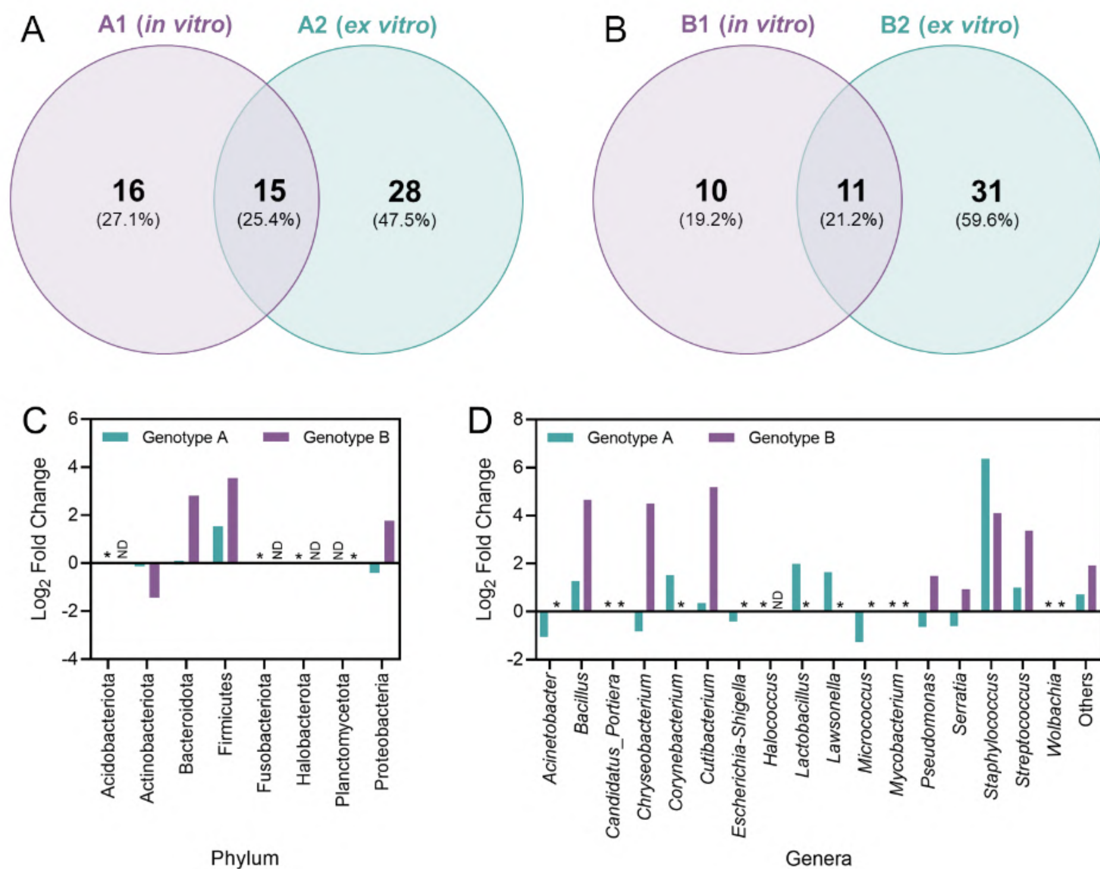

Figure 15. Venn diagram showing the common and exclusive genera and relative abundance of the endophytic microbiota of genotype A (A) and genotype B (B) *in vitro* and *in situ*; Log2 fold change calculation of OTUs between *in vitro* and *in situ* samples at a phylum (C) and genus (D) level for genotypes A and B.

Endophytic microbiota structure

A correlation analysis carried out at the genus level revealed a positive correlation between most genera in terms of OTUs relative abundance, whereas a negative correlation was observed between *Mycobacterium* and all other genera. Furthermore, no correlation was found between some genera (*e.g.*, *Micrococcus* and *Serratia* with *Bacillus*, *Candidatus* Portiera, *Halococcus*, *Lactobacillus*, *Staphylococcus* and *Wolbachia*) (Figure 16).

A PCA analysis using OTUs relative abundances at the genus level differentiated the endophytic microbiota according to the genotype (A vs B) and development stage (*in vitro* vs *ex*

vitro). Each axis reflects the percentage of the variation between the samples with the X-axis representing the highest dimension of variation (PC1-62.1%) and the Y-axis representing the second-highest dimension of variation (PC2-24.3%) (Figure 17 A). The analysis allowed clear differentiation among the development stage (*in vitro* vs *ex vitro*), especially in genotype B. The relative abundances of genera that contributed to the microbiotas divergence by development stage were mainly *Mycobacterium* for sample B1, and *Acinetobacter*, *Chrysobacterium*, *Micrococcus*, *Pseudomonas* and *Serratia* for sample A1. The differences observed between genotypes in the *in vitro* samples did not translate into *ex vitro*, which have a similar composition and relative abundance at the genus level. A high positive correlation was also obtained between samples A1, A2 and B2, whereas no correlation was found between those samples and B1 (Figure 17 B). These results were also confirmed in the heatmap, which shows the contribution of the bacteria genera mentioned before to the microbiota structure. The hierarchical clustering analysis identified the same relation between samples priorly revealed by the PCA analysis (Figure 17 C).

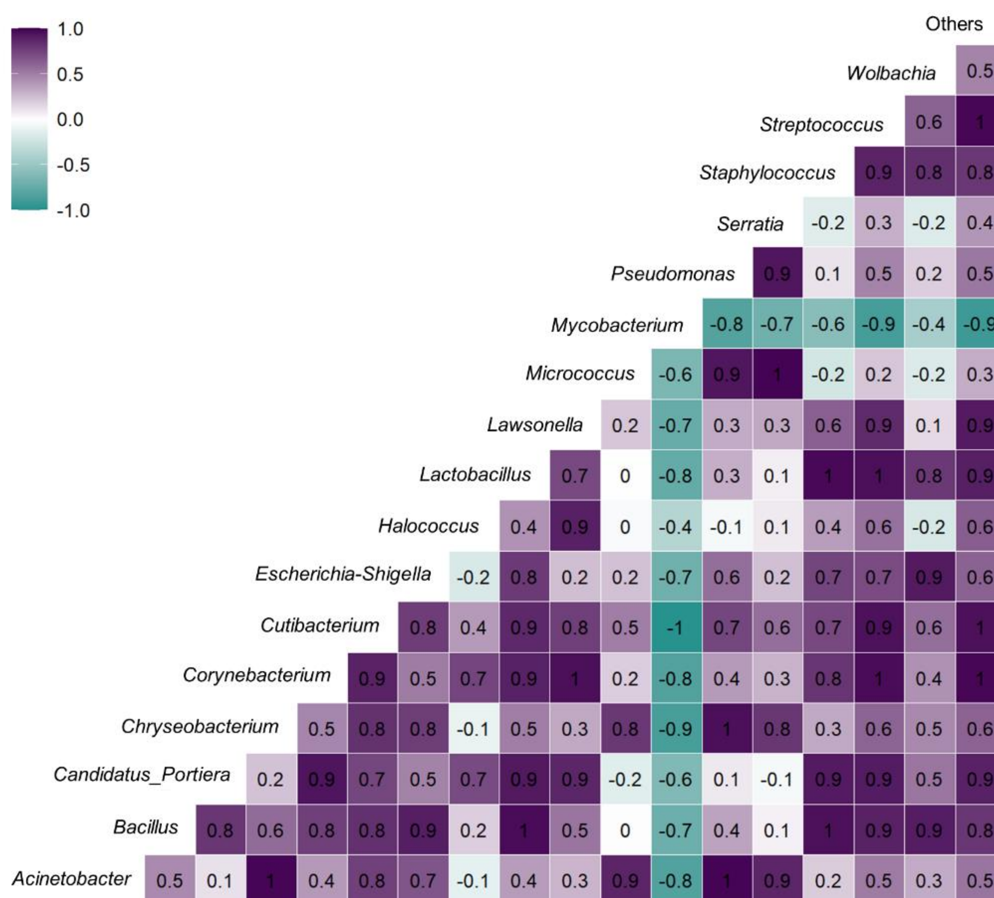

Figure 16. Pearson correlation analysis at the genus level for all the samples. Values close to 1 and -1 indicate a high positive or negative correlation respectively, and values close to 0 indicate no correlation.

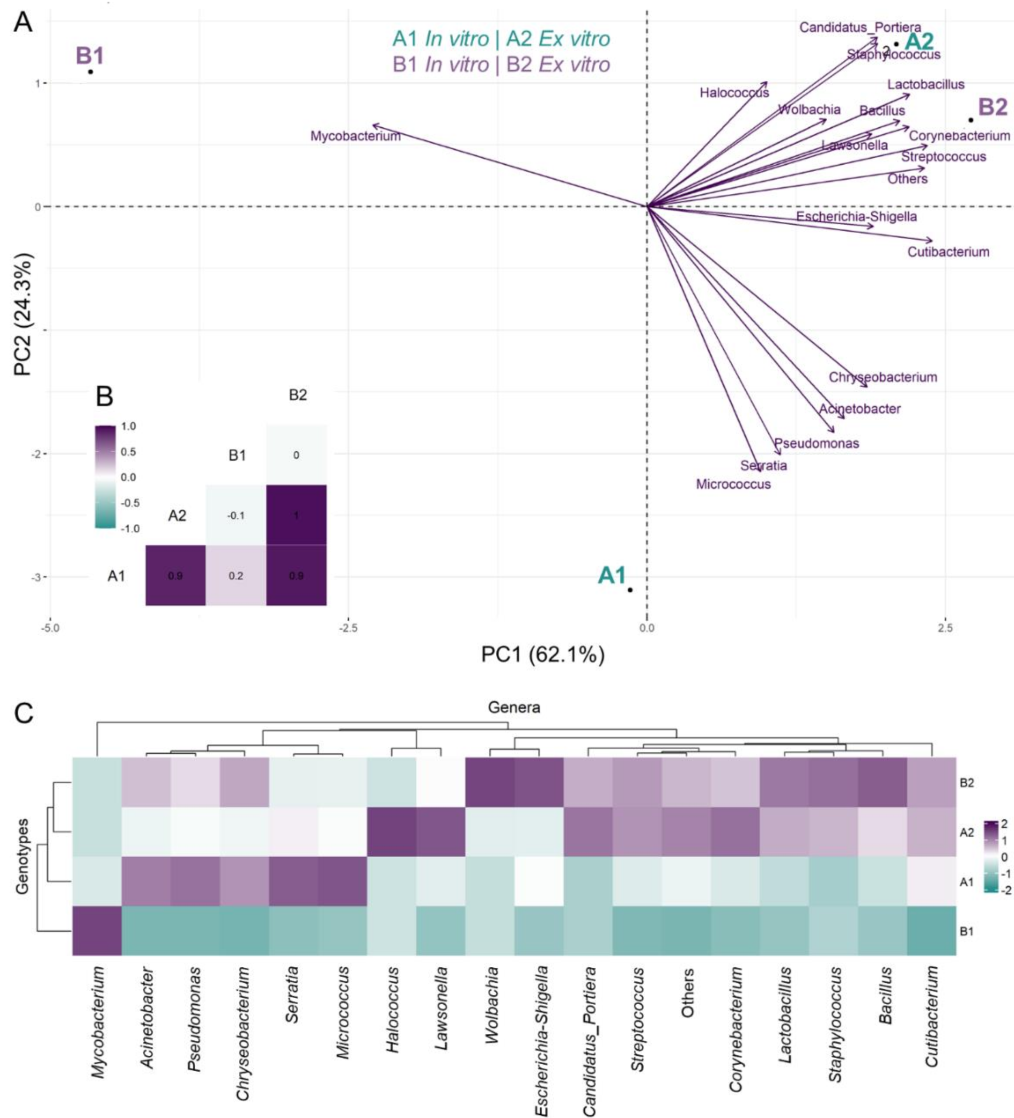

Figure 17. PCA analysis based on the OTUs relative abundance for genotypes A and B at different developmental stages (*in vitro* and *in situ*) (A); Correlation analysis (B) and Heatmap with a hierarchical clustering analysis using OTUs relative abundances for all samples (C).

Alpha diversity analysis

Diversity was estimated for each sample through the measurement of the Shannon index (H'), Simpson's Index, Simpson's Index of Diversity (1- D, sometimes called Dominance Index) and Pielou index (J'). All diversity indices support that the endophytic microbiota of strawberry trees varies according to genotype and/or developmental stage (Figure 18). The Shanon (H_{A1}= 2.04 and H_{B1}=0.79) and Simpson's (D_{A1}= 0.77 and D_{B1}=0.33) indexes from *in vitro* shoots supported that genotype was a structuring factor of the endophytic microbiota. On the other hand, this factor had no impact on the endophytic microbiota from the *ex vitro* samples (H_{A2}= 2.57 and H_{B2}=2.45; D_{A2}= 0.86 and D_{B2}=0.83). Simpson's Index of Diversity showed greater diversity on *ex vitro* samples when compared to *in vitro* in both genotypes (1-D_{A2} = 0.14 and 1-D_{B2} =0.17). This index also supports the low diversity found in the genotype B *in vitro* (1-D_{B1}= 0.67) as a consequence of the

high relative abundance found for the *Mycobacterium* genus. Pielou's evenness index (J'), which measures the distribution of genera by sample showed a very heterogeneous genus distribution on *in vitro* samples from genotype B (J'_{B1}= 0.26), once again supported by the high relative abundance of the genus *Mycobacterium* found, whereas *ex vitro*, the homogeneity found between genotypes is also supported by this index (J'_{A2}= 0.68 and J'_{B2}=0.66).

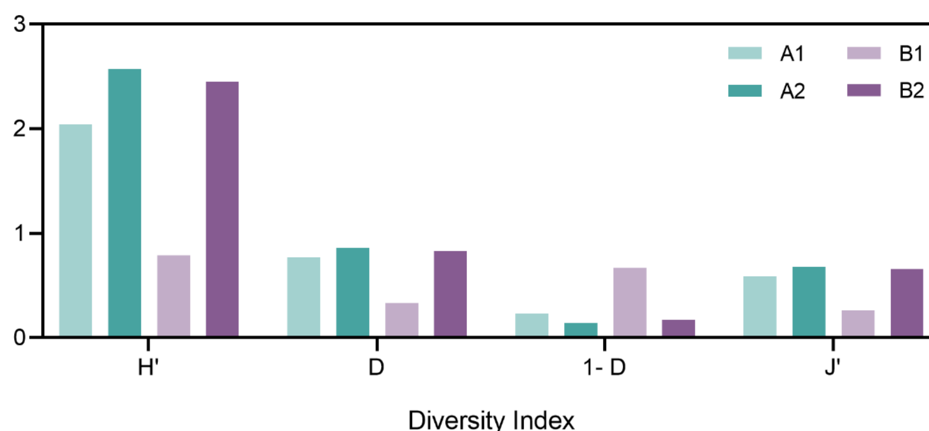

Figure 18. Alpha diversity indexes for each sample through the measurement of the Shannon index (H'), Simpson (D), Simpson's Index of Diversity (1- D) and Pielou index (J').

Discussion

For a long time, *in vitro* cultures were believed to be axenic, thus free of any microorganism contamination. However, this is far from the truth, and several archaea, bacteria and fungi species have been identified inside plant tissues growing *in vitro* using culturable methods. The fact that most of these microorganisms are latent and do not cause any symptoms or grow in the plant growth medium is undoubtedly the reason why they remain undetected. Nonetheless, culture-independent methods can bypass this limitation and usually reveal an unexpectedly high diversity. Strawberry tree *in vitro* cultures are not an exception and have been found to harbour a considerable diversity of bacteria. Most of the dominant genera identified in this study have already been found on other micropropagated species. *Acinetobacter*was identified in *Ipomoea batatas*(Izarra et al. 2020) and *Staphylea pinnata* (Szewczyk-Taranek et al. 2020), while *Cutibacterium*was found on *Triticum* spp. (Kuźniar et al. 2020). *Mycobacterium*was found in *Aglaonema* sp. (Fang and Hsu 2012), *Pogonatherumpaniceum* (Koskimäki et al. 2010)and *Prunus avium* (Quambusch et al. 2014), while *Pseudomonas*was detected in *Ipomoea batatas* (Izarra et al. 2020), *Aglaonema* sp. (Fang and Hsu 2012) and *Triticum* spp. (Kuźniar et al. 2020). *Staphylococcus*, another bacterial genus identified on strawberry tree is also commonly identified on *in vitro* growing plant tissues, such as *Atriplex* spp. (Lucero et al. 2011) and *Ipomoea batatas* (Izarra et al. 2020), as well as *Bacillus* that has been identified in plant material of poplar, larch and spruce, that had been micropropagated for at least 5 years (Ulrich et al. 2008).Other bacteria genera found on strawberry tree, such as

Chryseobacterium, *Pseudomonas* and *Serratia* are also commonly found in other species (Afzal et al. 2019).

Several authors hypothesized that the presence of endophytes might influence micropropagation success at different stages of the process (Laukkanen et al. 2000; Pirttilä et al. 2008). The endophyte composition, together with the plant genotype and tissue culture conditions are even pointed as the key elements in the process, influencing the regeneration capacity of the plant material (Pirttilä et al. 2008). The negative effects on plant micropropagation caused by different endophytes have been reported, such as those caused by *Methylobacterium* and *Mycobacterium* on *Pinus sylvestris*(Laukkanen et al. 2000; Pirttilä et al. 2008). *Mycobacterium* was once reported as a widespread and recurrent contaminant in plant tissue culture, due to its resistance to disinfection procedures(Taber et al. 1991),and recent studies indicated the presence of *Mycobacterium* on the rhizosphere of different plant species (Bouam et al. 2018). This bacterial genus was found on both strawberry tree genotypes sampled but only under *in vitro* conditions, which seems to indicate that this genus did not cope well with the *ex vitro* environment. Furthermore, as the relative abundance of *Mycobacterium* was the highest on genotype B, it would be expected that the micropropagation could be affected. However, this was not the case, and genotype B presented high micropropagation rates (data not shown).

Although these possible undesired effects, most plant endophytes seem to produce a positive effect on the host. Several mechanisms can be involved in endophyte-plant interaction, promoting plant growth and fitness, such as hormone production (IAA - indole-acetic acid and gibberellic acid), siderophores, cell-wall degrading enzymes and other metabolites, etc. Additionally, endophytes might also stimulate plant immunity, leading to metabolic changes in the host plant. All in all, these mechanisms often confer protection against abiotic and biotic stresses which translates into high crop yields (Radhakrishnan et al. 2017). Among the genera found on strawberry tree tissues, *Acinetobacter*, *Bacillus*, *Burkholderia*, *Paraburkholderia*, *Pseudomonas* and *Serratia* are some that might promote the mentioned positive effects. For example, *Acinetobacter* sp. strains isolated from the rhizosphere of *Pennisetum glaucum* were able to solubilize phosphates and produce IAA and siderophores. Additionally, *in vitro* assays showed they caused inhibition of *Fusarium oxysporum* and also enhanced the shoot height, root length, and root dry weights of inoculated *Pennisetum glaucum* plantlets (Rokhbakhsh-Zamin et al. 2011). Similarly, a *Serratia* sp. strain isolated from *Achyranthes aspera* was able to solubilize phosphate, produce ammonia, IAA and siderophores, greatly promoting plant growth (Devi et al. 2016). In this study, *Burkholderia* and *Paraburkholderia* were only found *in vitro*, with a very low relative abundance. *Acinetobacter* was found in all samples except B1, whereas *Bacillus*, *Pseudomonas* and *Serratia* were identified in all samples. In fact, *Pseudomonas* is the genera with the highest relative abundance in samples A1, A2 and B2.

Furthermore, strains of *Acinetobacter*, *Burkholderia*, *Serratia* and *Bacillus thuringiensis* (identified on sample A2), have been reported to have the ability to solubilize zinc, thus improving its content in plants and yield of several crops (Hakim et al. 2021). Additionally, the application of *B. thuringiensis*, an auxin producing bacteria, promoted growth of wheat plants under drought stress (Hakim et al. 2021). *Sphingomonas azotifigens* and *Pseudomonas mendocina* (both identified in sample B2) also proved their beneficial effects on plants, through nitrogen fixation and nutrient absorption (Kohler et al. 2006; Videira et al. 2009). *Kocuria palustris*, identified on *in vitro* samples, showed great resistance to arsenic (Zacaria Vital et al. 2019), whereas *Kocuria rhizophila*, identified on sample A2, greatly improved the absorption of nickel (Anum et al. 2019). *Streptomyces mirabilis* (identified in sample A1), also showed great resistant to heavy metals (Schütze et al. 2014), showing the potential of the aforementioned bacteria on heavy metal mobilization and consequent benefits on plants growing in contaminated soils. Finally, *Pseudomonas alcaligenes* (identified in B2), showed an inhibitory activity of more than 80% against *F. oxysporum* f.sp. *lycopersici* suppressing the disease when a bacterial suspension was applied on tomato (Widnyana et al. 2013). Likewise, *Sphingobacterium multivorum*, a chitosan-degrading bacteria (identified in A1), was also able to inhibit mycelial elongation of the same pathogen (Matsuda et al. 2001).

The difference observed in the microbiota composition between strawberry tree genotypes under *in vitro* conditions was expected. In fact, among the factors that govern plant microbiome structure, the genotype of the host plant and its age and developmental stage are pointed to play a crucial role (Whipps et al. 2008; Turner et al. 2013; Compant et al. 2019). Differences in the bacteria endophytic populations associated with different genotypes were also observed in shoots of *P. avium* growing *in vitro* (Quambusch et al. 2014).The fact that strawberry tree genotype A was established *in vitro* from an adult plant, whereas genotype B was obtained from a seed, might also be the reason for the difference observed in the microbiota structure between these genotypes under *in vitro* conditions. Because seed microbiome is believed to be recruited mainly from the mother plant (Nelson 2017), the aforementioned factors might also be decisive influencers on plant microbiome. Nevertheless, bacteria associated with seed are usually within the Actinobacteria, Bacteroidota, Firmicutes and Proteobacteria phyla (Nelson 2017), which is in accordance with our findings.

Changes in the structure of the microbiota can also be expected throughout the micropropagation process through successive colonization by bacterial communities as was observed in *Ananas comosus* (Abreu-Tarazi et al. 2010).

The transition between *in vitro* to *ex vitro* conditions translated into an increase in diversity. This was an expected result as endophytic populations are usually lower in plant tissues growing *in vitro* due to the small amount of tissue used to initiate the micropropagation as well as the specific conditions of these cultures (Quambusch et al. 2014). Other possible explanation is related to

horizontal transmission of these microorganisms. As the substrate used after plant acclimatization was sterilized, no microorganisms were transmitted into the plant by the soil. However, the surrounding environment is also an important source of organisms that might enter the plant through different paths integrating its microbiome (Wassermann et al., 2019). Moreover, a dramatic change was observed in genotype B microbiota composition when *in vitro* and *ex vitro* samples are compared. Furthermore, the composition of both genotypes *ex vitro* is quite similar, possible due to horizontal transmission, which in this particular case seems to suggest a minor role of the genotype on shaping endophytic microbiota. Finally, the diversity of archaea was considerably low on adult plants and were not detected on *in vitro* shoots. Although archaea species are commonly associated with plants, *e.g.*, in *Zea mays* roots (Chelius and Triplett 2001) and associated with *Eruca sativa* (Taffner et al. 2019), as far as we concern, there is hardly any report of their presence on plant tissues under micropropagation, possible because archaea might not be resilient enough to survive the sterilization procedures before *in vitro* establishment and/or struggle to thrive under the specific conditions of the *in vitro* cultures.

Due to the influence of endophytic bacteria in micropropagation, which is the major vegetative propagation technique used on *A. unedo*, microbiome studies are essential to improve the process. Furthermore, endophytes might mitigate the negative consequences of stress on plants and ameliorate their general fitness. Additionally, due to their biological control ability, some species might be ecological friendly alternatives to synthetic pesticides (Radhakrishnan et al. 2017). For example, bacteria from the genera *Bacillus* (Radhakrishnan et al. 2017; Fira et al. 2018), *Burkholderia* (Sandani et al. 2019), *Paraburkholderia* (Baccari et al. 2019) and *Pseudomonas* (Sandani et al. 2019) have been found on strawberry tree and according to several reports might have potential as biological control agents. Furthermore, several species identified on strawberry tree, such as *Bacillus thuringiensis*, *Kocuria palustris*, *K. rhizophila*, *Pseudomonas alcaligenes*, *P. mendocina*, *Sphingomonas azotifigens*, *S. multivorum* and *Streptomyces mirabilis*, might provide great benefits to their host plants as mentioned before (Matsuda et al. 2001; Kohler et al. 2006; Videira et al. 2009; Widnyana et al. 2013; Schütze et al. 2014; Anum et al. 2019; Zacaria Vital et al. 2019; Hakim et al. 2021). For this reason, the inoculation of microplants with bacterial formulations might be a great benefit not only during the multiplication phase but especially during *ex vitro* acclimatization which is a key step of the process but causes great stress to plants (Orlikowska et al. 2017). Our work provided the first insights on *A. unedo* microbiome composition, and both genotype and type of explant used for micropropagation seems to be a fundamental driver of the microbiome structure. However, more research is still required to fully characterize and understand the contribution of endophytic organisms on different aspects of strawberry tree micropropagation, including regeneration rates, rooting and acclimatization success, which might have great repercussions on plant selection, either for micropropagation or breeding purposes.

CHAPTER 3: Shoot proliferation and organogenesis on *Arbutus unedo*: physiological analysis under water stress

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Abstract

Micropropagated *A. unedo* clones are in high demand in the market. Although micropropagation have great advantages when compared to traditional plant production methods, *in vitro* cloning is considerably expensive. In order to reduce production costs, this work aims to improve strawberry tree micropropagation in liquid medium and test plant performance under water stress. For this purpose, three strawberry tree clones were established *in vitro* and propagated by axillary shoot proliferation on solid and liquid media (modified De Fossard medium with 2 mg L^{-1} benzylaminopurine). A *calli* with organogenic capacity was obtained from apical leaves of the *in vitro* grown shoots from the three genotypes, in the same basal liquid medium supplemented with 2 mg L^{-1} thidiazuron. Micropropagation through organogenesis in liquid medium proved to be more efficient than the other tested methods (considering the number of shoots produced), but the shoots showed signs of hyperhydricity. Shoots were successfully rooted on medium with indole-3-butyric acid and acclimatized *ex vitro* with rates higher than 90%. Six-month-old plants from the most proliferative genotype (AU1) and propagated *in vitro* by different methods were submitted to drought stress (no watering for 10 days) and several morphological and physiological parameters were evaluated and compared to a control group (watered to 70% field capacity). No significant differences were found in plant biomass, root length, and plant height; however, slight differences were observed in water potential, net photosynthetic rate, intercellular CO_2 concentration, and stomatal conductance between the plantlets propagated on solid or liquid medium. In general, the responses to drought stress imposed were similar in plants micropropagated by different propagation methods.

Keywords: chlorophyll fluorescence, micropropagation, photosynthesis, stomatal conductance, transpiration, water potential.

Introduction

The demand for *A. unedo* plants has been increasing on recent years due to its economic and ecological potential. Clonal plants are especially requested by producers who seek homogeneous productions among other advantages. In order to obtain true-to-type plants, *A. unedo* is essentially produced by micropropagation methods, due to low rooting rates of macro cuttings. Although these techniques have great advantages over traditional methods, the high cost usually involved is a drawback, and a great deal of work has been done to develop and improve alternative techniques that reduce production costs. One of the most popular is probably the use of bioreactors with liquid medium. However, as referred by (De Klerk and ter Brugge 2011), these protocols are only profitable, from the economic point of view, if the performance in field conditions of *in vitro* derived plants is adequate. Since strawberry tree orchards are usually established on dry and poor soils it is essential to assure drought tolerance of the micropropagated plants (Allen et al. 2010), especially because water is the most limiting factor for plant acclimatization and growth on field conditions (Guarnaschelli et al. 2012) even in species recognized as drought tolerant as *A. unedo* (Munné-Bosch and Peñuelas 2004). Work done with strawberry tree wild plants on field conditions under water stress has shown that plants follow a conservative water use strategy (Castell and Terradas 1994; Ogaya et al. 2003; Munné-Bosch and Peñuelas 2004). However, and as far as we know, the performance of these plants obtained through micropropagation under water deficit conditions has not been tested before. Thus, the objective of this work was: i) develop new protocols for strawberry tree shoot proliferation in liquid medium by organogenesis, ii) evaluate the potential of these protocols for mass scale propagation, including the potential use of bioreactors, and iii) analyze the effect of these protocols on plant performance under drought stress.

Material and Methods

In vitro establishment

Three phenotypes of *Arbutus unedo* were selected: AU1 (young tree less than 5-year-old and a shrub-like growing type), AU2 (adult tree more than 10-year-old), and AU3 (seedling). The AU1 and AU2 were propagated *in vitro* using young branches from two different trees that were cut, dipped in 100 mg L^{-1} fungicide Aliette (Bayer CropScience, Carnaxide, Portugal) for 10 min, and rinsed with distilled water. After this treatment, branches were kept in containers, covered with a plastic bag, watered with distilled water and placed in a culture chamber (FitoClima 10000 HP, Aralab, Portugal) at a 16-h photoperiod, an irradiance of 250 µmol m^{-2} s^{-1}, a temperature of 25 ºC, and an air humidity of 75% for 30 d. The epicormic shoots formed were removed and surface sterilized with 70% (v/v) ethanol (30 s) and 5% (m/v) calcium hypochlorite solution (Sigma-Aldrich, St. Louis, MO, USA) for 10 min with two drops of Tween 20. After 3 washes with sterile distilled water, the epicormic shoots (0.5 - 1 cm) were inoculated in a medium with De Fossard macronutrients and vitamins (De Fossard et al. 1974), MS microelements (Murashige and Skoog

1962), 2 mg L^{-1} benzylaminopurine (BAP; Sigma-Aldrrich), 3% (m/v) sucrose (Duchefa Biochemie, Haarlem, The Netherlands) and 0.6% (m/v) agar (Duchefa) and grown in test tubes (25 x 150 mm) with plastic caps (Duran, Mainz, Germany). The culture medium was autoclaved at 121 ºC for 20 min and pH was adjusted to 5.7 using KOH or HCl before autoclaving and agar addition. For micropropagation of AU3, seeds after 1 month of cold stratification (4 ºC) were disinfected following the same methodology as for shoots and germinated in Petri dishes on filter paper discs moistened with sterile distilled water in a growth chamber set to conditions mentioned above. The obtained seedlings were selected and multiplied by shoot proliferation in the medium described before.

Axillary shoot proliferation

For axillary shoot proliferation in solid medium (SM), AU1, AU2, and AU3 shoots were cultured in test tubes with 10 mL of the medium described before (one explant per tube and 10 shoots per replicate). Shoot proliferation in liquid medium (LM) was carried out in Erlenmeyer flasks (100 mL) with 25 mL of the same medium without the gelling agent (6 shoots per flask). The flasks were placed on an orbital shaker (80 rpm). The cultures were kept in a growth chamber, under a 16 h photoperiod with 15–20 µmol m^{-2} s^{-1} (cool-white fluorescent lamps) at 25 ºC. After 6 subcultures, micropropagation rates of the three genotypes were evaluated by the number of shoots longer than 1 cm obtained per initial explant.

Organogenesis

For micropropagation through organogenesis in liquid medium (LMO), *calli* with organogenic capacity were obtained from apical leaves from shoots obtained by shoot proliferation mentioned before. Leaves were cultured in the solid medium described before, but supplemented with 2 mg L^{-1} thidiazuron (TDZ; Sigma-Alrich), for a month, at 25 ºC in the dark (Martins and Canhoto 2014). Transversal cuts (4 - 6) were made in the central part of the leaves, before they were placed with the abaxial side down on the culture medium. After 2 months, the obtained *calli* were transferred to liquid medium with the same composition and under the same conditions as for the shoot proliferation on liquid media.

Rooting and acclimatization

For root induction, 3 cm long shoots were cultured in test tubes, in a medium with Knop salts (Knop 1865), De Fossard vitamins without riboflavin, MS microelements without KI, and 2 mg L^{-1} indole-3-butyric acid (IBA; Sigma-Aldrich) at 25 ºC under the dark for 10 d. Shoots were then transferred to a medium with the same composition but without IBA, 1% (m/v) charcoal (Sigma-Aldrich) was added and the shoots were grown in a growth chamber at a 16-h photoperiod, an irradiance of 15 - 20 µmol m^{-2} s^{-1} (cool-white fluorescent lamps), and a temperature of 25 ºC for 3 weeks. Rooting rates were calculated and the number of roots per shoot was counted (only roots longer than 1 mm), before plant acclimatization in covered containers with Perlite (Siro, Mira, Portugal) moistened with water in a culture chamber (FitoClima 10000 HP) under a 16-h photoperiod, an irradiance of 250 µmol m^{-2} s^{-1}, a temperature of 25 ºC, and 70% relative humidity.

The container cover was gradually removed and after a month, acclimatization rates were calculated and plants transferred to individual 200 cm^3 containers with peat and sand (1:1, v/v).

Drought stress assays

From the acclimatized six-month-old plants, obtained by the three methods of *in vitro* propagation, with at least 10 well developed leaves, 5 plants were randomly chosen and placed under two water regimes: WW - well watered (watered to 70% field capacity) or WS - water stressed (without watering). After 10 d under these conditions plant performance was evaluated based on growth and physiological parameters. Only genotype AU1 was used in this assay, since it presented the highest multiplication rates at all propagation methods tested.

Root length and plant height were measured at the end of the WS period. Total plant dry mass was determined after drying the samples at 70 °C until constant mass. Leaf area was determined using ImageJ (Schneider et al. 2012). Water potential (Ψ) was measured with a Scholander-type pressure chamber (PMS Instrument Co., Albany, OR, USA). Plant water status was further assessed by the determination of the relative water content (RWC) calculated as [(fresh mass - dry mass)/(water saturated mass - dry mass)] × 100. Water saturated mass was determined after 24 h on distilled water at 4 °C, and dry mass after drying at 70 °C for 48 h.

In situ leaf gas exchange measurements (net photosynthetic rate, PN, transpiration rate, E, intercellular CO2 concentration, ci, and stomatal conductance, gs) were performed on apical fully expanded leaves using a portable infrared gas analyzer (LCpro+, ADC, Hoddesdon, UK) operating in open mode under the following conditions: irradiance of 350 μmol m-2 s-1, air flow of 200 mol s-1; block temperature of 25 ºC, and atmospheric CO2 and H2O concentrations. Data were recorded when the measured parameters were stable (after 2 - 6 min).

Chlorophyll a fluorescence was determined *in situ* in fully expanded leaves, with a portable fluorimeter Mini-PAM (Walz, Effeltrich, Germany). Values of Fv/Fm= (Fm - F0)/Fm and ΦPSII = (F′m - F′0)/F′m were determined as described in this paper.

Statistical analysis

Values were given as means ± standard deviations of 3 replicates for micropropagation assays, and 5 replicates for drought stress. Micropropagation data were analyzed by one way ANOVA (GraphPad Prism for Windows v. 6.01), followed by a Tukey´s multiple comparison test ($P < 0.05$). Comparisons of eco-physiological parameters of the plants submitted to two water regimes was done by a Student t-test.

Results

Strawberry tree was successfully micropropagated on solid and in liquid medium (Figure 19 A-B) and no significant statistical differences were found in the number of shoots obtained in both treatments in the three genotypes tested (AU1, AU2, and AU3). However, the number of shoots obtained using organogenesis was substantially higher when compared to shoot proliferation. Although this difference was observed in all three tested genotypes, it was higher for

genotype AU2, especially when compared to the propagation on solid medium, as the number of shoots obtained by organogenesis was almost 8 times higher (15.57 ± 2.21) compared to axillary shoot proliferation (2.20 ± 0.40; Figure 20 A). Despite the highest multiplication rates achieved, hyperhydricity was observed on some of the shoots obtained on liquid medium, either by shoot proliferation or organogenesis, causing phenotypic differences, such as abnormal leaf shape and lower chlorophyll content (data not show).

The rooting rates obtained were mostly higher than 80% in most of the groups tested and no significant statistical differences were observed among propagation methods. Considering plants produced on solid medium the rooting rate was of 100% for genotype AU2. Moreover, the number of roots was similar on the plants produced on liquid and in solid medium, except in genotype AU2, where an average of 13.87 ± 5.8 roots was observed in shoots obtained on solid medium, compared to 5.2 ± 0.8 in liquid medium (Figure 20 C). Throughout the rooting process, most of the plants showing hyperhydricity and corresponding modifications recovered their normal phenotype.

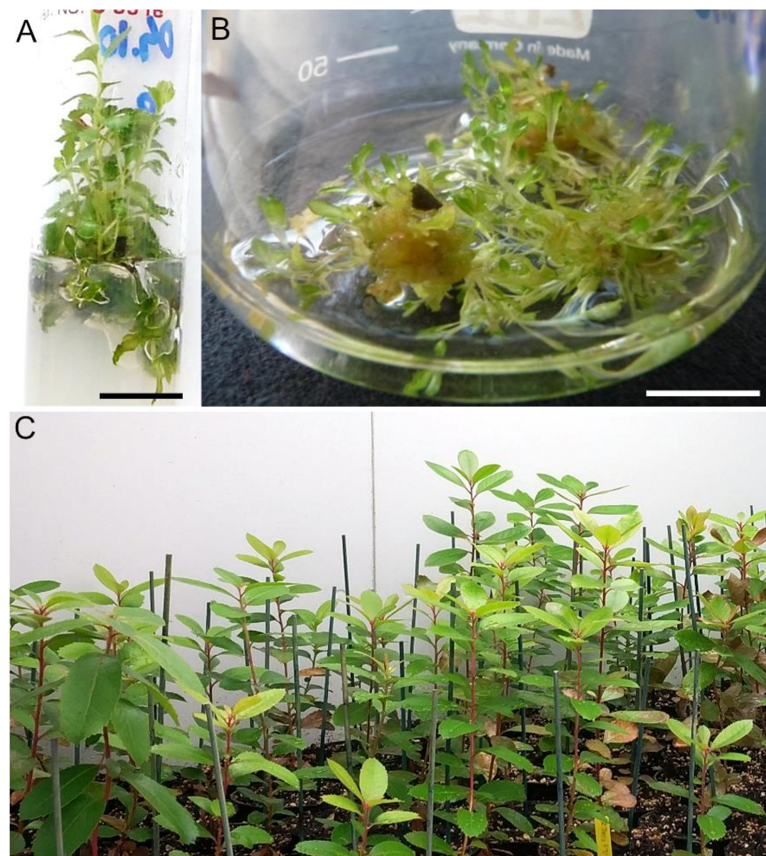

Figure 19. Micropropagation of strawberry tree by axillary shoot proliferation on solid medium (A); or by organogenesis in liquid medium (B); and *ex vitro* acclimatized plants after three months (C) (bar = 1 cm).

In all groups, most of the plants survived after the acclimatization (Figure 19 C), and no differences were found among propagation methods, with rates higher than 80%, except for plants produced through organogenesis from genotype AU3, established *in vitro* from a seedling (Figure 20 D). Three months after acclimatization, the plant height and leaf area of those obtained by shoot

proliferation, both in solid and on liquid medium, was very similar. However, the plants obtained through organogenesis showed lower height (Figure 21 A) and smaller leaf area (for genotypes AU2 and AU3, Figure 21 B). Mostly no significant differences were found in total plant biomass, height and root length under WW and WS regimes (Figure 22 D-F) in plants obtained by different micropropagation methods. Similar results were also obtained regarding leaf area, although slightly less values were found for plants propagated on solid medium (Figure 22 C). Water potential was lower on plants under drought stress conditions, when compared to well-watered plants. However, it was statistically different only for plants produced in liquid medium by axillary shoot proliferation (Figure 22 A). Very slight differences were found in RWC and only in plants propagated on solid medium (Figure 22 B). Gas exchange parameters, stomatal conductance, intercellular CO_2 concentration, and net photosynthetic rate (Figure 23 A-D) decreased under water deficit, especially when the plants were obtained by axillary shoot proliferation on solid and liquid medium. No marked differences were found in transpiration rates (Figure 23 B). The chlorophyll fluorescence parameters, the yield of photosystem II under steady-state conditions and maximum efficiency of photosystem II were very similar in all treatments (Figure 23 E-F). Only a slight increase in ᶲPS II was observed for plants from solid medium when submitted to water stress.

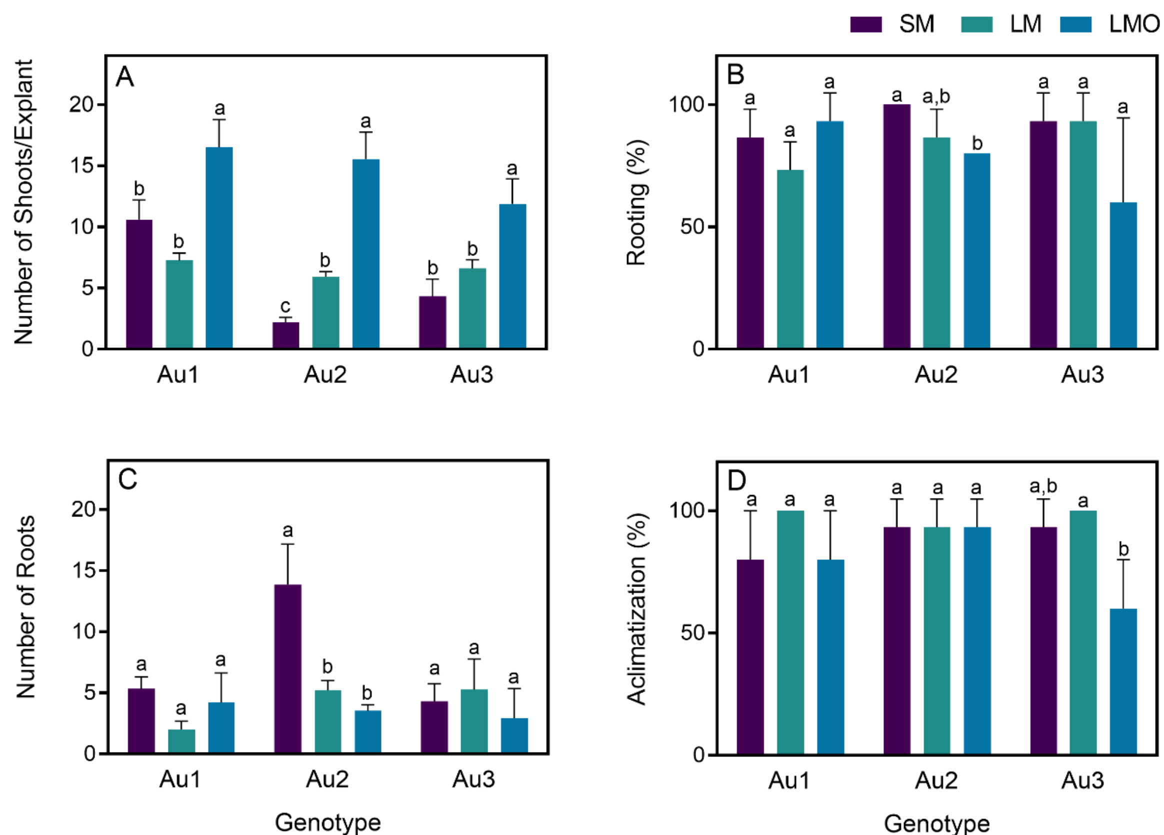

Figure 20. Number of shoots per explants (A); rooting rate (B); number of roots per plantlet (C); and acclimatization percentage (D) in genotypes AU1, AU2, and AU3 by using three micropropagation methods (SM - axillary shoot proliferation on solid medium, LM - axillary shoot proliferation on liquid medium, LMO - organogenesis on liquid medium). Means ± standard deviations, n = 3, on each genotype different letters indicate significant differences between treatments at $P \leq 0.05$.

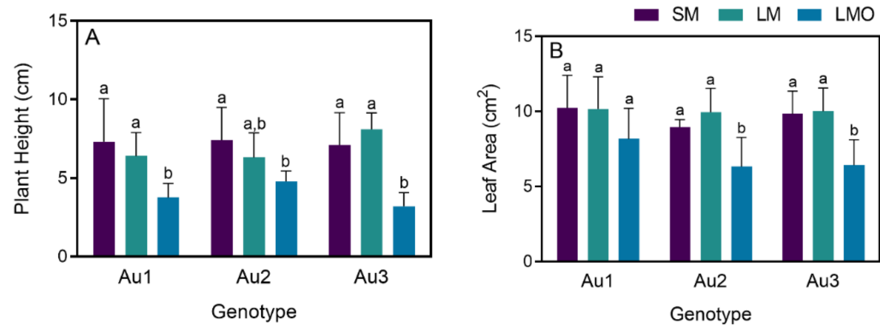

Figure 21. Effect of three micropropagation methods (SM - axillary shoot proliferation on solid medium, LM - axillary shoot proliferation on liquid medium, LMO - organogenesis on liquid medium) on height (A) and leaf area (B) of plants of different genotypes (AU1, AU2, and AU3) measured three months after acclimatization. Means ± standard deviations, n = 3, different letters indicate significant differences between treatments at P ≤ 0.05.

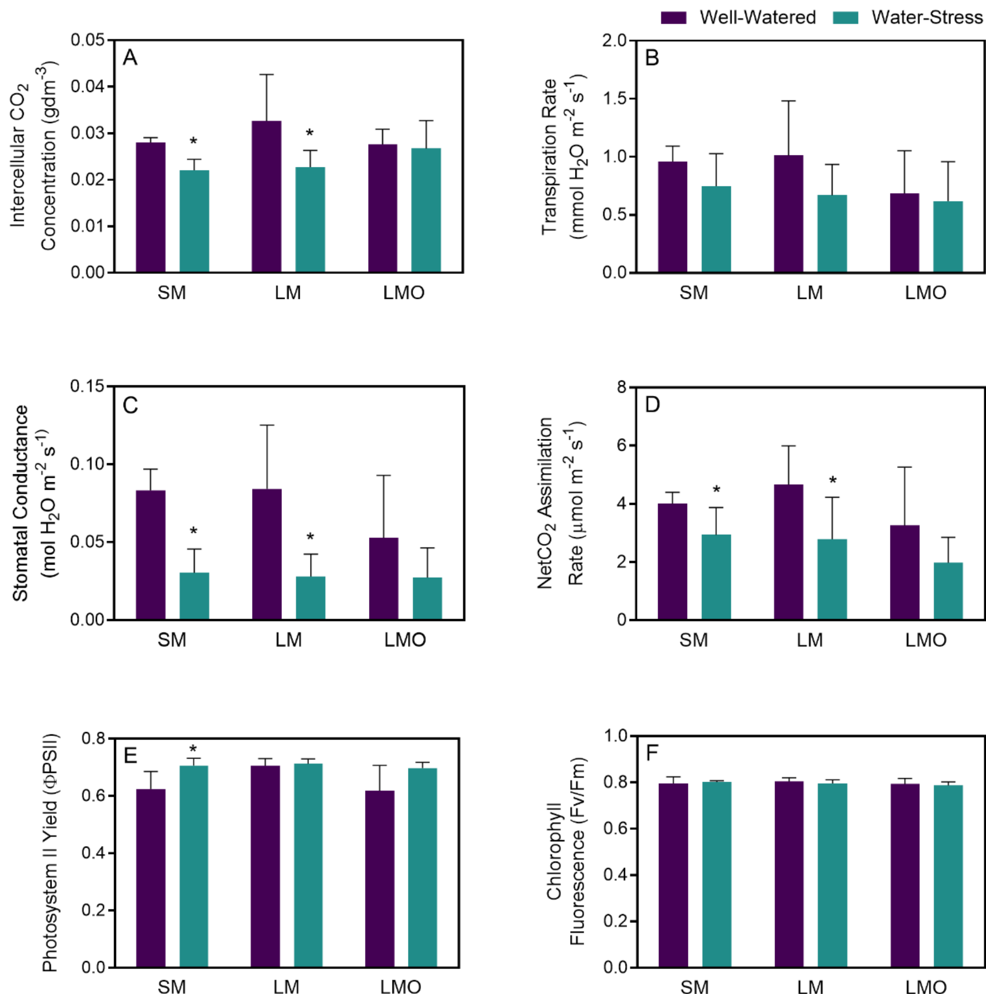

Figure 22. Effects of water stress (WS) imposed to AU1 plants propagated by three methods (SM - axillary shoot proliferation on solid medium, LM - axillary shoot proliferation on liquid medium, LMO - organogenesis on liquid medium) and acclimatized for 6 months on water potential (A); relative water content (B); leaf area (C); root length (D); total plant dry mass (E) and plant height (F) in comparison with well-watered plants (WW). Means ± standard deviations, n = 5, * indicate significant differences between treatments at P ≤ 0.05.

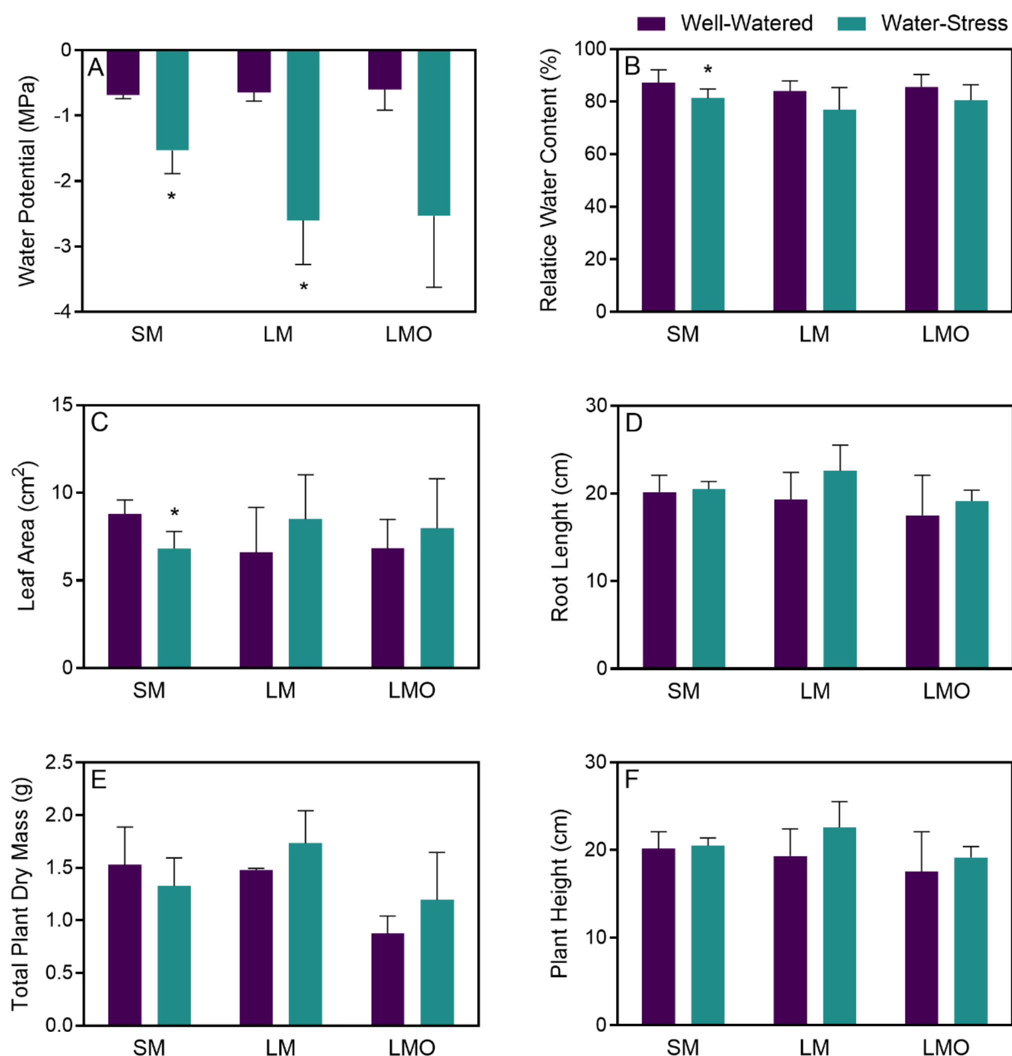

Figure 23. Effects of water stress (WS) imposed to AU1 plants propagated by three methods (SM - axillary shoot proliferation on solid medium, LM - axillary shoot proliferation on liquid medium, LMO - organogenesis on liquid medium) and acclimatized for 6 month on intercellular CO_2 concentration (A); transpiration rate (B); stomatal conductance (C); net photosynthetic rate (D); chlorophyll fluorescence parameters quantum yield of photosystem II (E) and photochemical efficiency of PS II (F) in comparison to well-watered plants (WW). Means ± standard deviations, n = 5, * indicate significant differences between treatments at $P \leq 0.05$.

Discussion

The protocol developed for the micropropagation of strawberry tree in liquid medium was proved to be very efficient. Similar results have been reported for some other species, such as *Camellia sinensis* (Sandal et al. 2001), *Dioscorea japonica* (Kadota and Niimi 2004), and *Catharanthus roseus* (Pati et al. 2011). These kinds of protocols tend to be more effective when compared to the ones on solid medium in terms of proliferation rates, shoot size, and biomass. This might be related to a more efficient uptake of nutrients due to the large area of contact with the medium, since nutrients can be uptake also by leaves via stomata and aqueous pores (De Klerk and

ter Brugge 2011) and even through the cuticule, which in leaves of *in vitro* growing shoots or plantlets is thin and may allow the change between the culture medium and plant tissues (Dias Ferreira et al. 2003). However, some of the drawback observed on strawberry tree, particularly the hyperhydricity of shoots, were also mentioned previously (Sandal et al. 2001; Kadota and Niimi 2004; Pati et al. 2011). Although some phenotypic differences were observed in strawberry tree plants produced in liquid medium either by shoot proliferation or organogenesis (Martins and Canhoto 2014), they were only a transitory and according to the parameters evaluated six-month after ex vitro transfer, they did not affect overall plant performance when submitted to drought stress.

The genotype plays an important role on micropropagation, as it has already been reported for strawberry tree (Lopes et al. 2010) as well as for other species such as *Paeonia suffruticosa* (Beruto et al. 2004) and *Hagenia abyssinica* (Feyissa et al. 2005). Our results show that organogenesis was the most effective method for the propagation of all strawberry tree genotypes. However, the results also show that different genotypes behaved differently accordingly to micropropagation method tested. For example, all the genotypes seemed to display no differences when the organogenesis was used whereas more clear differences were found when shoot proliferation was used. This might be due to inadequate culture conditions for specific genotypes, such as medium pH or nutrient composition as well as added plant growth regulators (Lopes et al. 2010). The age of the mother plant, as well as its physiological condition, may also be important factors for micropropagation efficiency (Chowdhury et al. 2004; Shukla et al. 2012). In fact, genotype AU1 (established *in vitro* from a young tree), has higher micropropagation rate by axillary shoot proliferation on solid medium when compared to genotype AU2, whose mother plant is an older tree.

In the present study, no significant morphological differences were observed among the plants acclimatized ex vitro for 6 months, which were originally obtained by the different micropropagation methods and also when they were submitted to different water regimes. A reduction in growth was reported in previous studies when strawberry tree plants and seedlings were submitted to a water deficit (Ogaya et al. 2003; Ogaya and Penuelas 2004; Vasques et al. 2013). This is a common response to dry environments, as plants tend to increase belowground biomass allocation in order to improve water uptake. However, no differences were observed in this assay in terms of biomass allocation, probably due to the short duration of the experiment because some time is required to activate the complex mechanisms induced by drought stress (Chaves et al. 2002; Chambel et al. 2005). Despite that, a slight reduction in leaf area was observed in plants produced by shoot proliferation on solid medium when submitted to water limitation. The reduced investment in foliar area is a relevant strategy to cope with water deficit conditions (Lopez et al. 1997).

In general, the physiological parameters evaluated in plants produced by different propagation methods were not significantly different after imposition of mild water stress and were in accordance with the values previously reported (Castell and Terradas 1994; Vasques et al. 2013).

Many of them were not significantly different between two water regimes. However, the water potential was significantly reduced in plants produced by shoot proliferation in the solid medium. Plants obtained by the other two methods were probably more dynamic in maintaining the physiological traits, which are particularly important for breeding drought-tolerant plants.

The stomatal conductance was lower in plants submitted to water stress, with a statistically significant difference for plants produced by shoot proliferation in the solid and liquid medium, which may indicate a better performance of plants produced by this technique, to be tested on future research. In fact, plants have evolved several mechanisms in order to adapt to unfavorable environmental conditions by reducing resources consumption and adjusting their growth (Osakabe et al. 2014). In water deficit conditions, changes in ion- and water-transport systems across membranes in guard cells stimulates stomatal closure, which directly affects photosynthetic rates and plant productivity due to lower levels of CO_2 available (Osakabe et al. 2014). According to previous reports (Castell and Terradas 1994; Gratani and Varone 2004; García et al. 2011), the reduction of stomatal conductance usually occurs under drought stress as part of the efficient water use strategy of strawberry tree, a typical drought resistant sclerophyllous Mediterranean species. Activation of anti-oxidative protection mechanisms has also been reported as a mechanism that might be used by this species to cope with water stress (Munné-Bosch and Peñuelas 2004).

The micropropagation in liquid medium was found very efficient for strawberry tree, especially by organogenesis, in the three genotypes tested. Nevertheless, strawberry trees micropropagated by different methods showed a very similar morphological and physiological performance and were not strongly affected under the imposed drought stress. However, plants produced by shoot proliferation on solid medium showed a slightly superior performance in some of the evaluated parameters, indicating different adaptation strategies to the imposed water stress, that should be considered when selecting a propagation technique. Overall, we can conclude that the tested propagation methods were efficient and did not considerably affect plant performance and productivity and can be used for strawberry tree micropropagation.

CHAPTER 4: Hybridization assays in Strawberry tree towards the identification of plants displaying increased drought tolerance

(Martins J, Monteiro P, Pinto G, Canhoto J (2021) Hybridization assays in Strawberry tree toward the identification of plants displaying increased drought tolerance. Forests 12:148 | DOI: 10.3390/f12020148)

Abstract

On the foreseen climate change context, breeding towards drought tolerance is necessary in order to ameliorate plant performance. The aim of this work was therefore to study the reproduction mechanisms of strawberry tree, obtain new genetic combinations by hybridization and select genotypes more tolerant to drought stress. A morphological analysis of flowers and pollen was carried out, and controlled pollinations performed both *in vitro* and *ex vitro*. The very first approach on strawberry tree breeding by means of hybridization is also presented. Several physiological parameters were evaluated on 26 genotypes submitted to a water deficit regime. Plant behavior under drought greatly varied among genotypes, which showed a high phenotype plasticity. Three genotypes that were able to cope with water restriction without compromising net CO_2 assimilation were identified as highly tolerant to drought stress. The results obtained elucidate the reproduction mechanisms of strawberry tree and open the way for a long-term breeding program based on the selection of drought tolerant plants.

Keywords: artificial Pollination, breeding, drought stress, microscopy, pollen, physiological performance

Introduction

Strawberry tree orchards are usually established on marginal dry areas where water is a scarce resource. Considering the expected increase on the frequency and severity of drought events in southern Europe in the near future (Bussotti et al. 2014), it is urgent to obtain new genotypes more tolerant to drought stress, in order to ameliorate their performance and increase productivity. Several studies have been carried out to study the effects of drought stress on water relations, growth rate and photosynthesis in *A. unedo* under field conditions (Munné-Bosch and Peñuelas 2004). Although crucial, data focusing on drought performance at early stages of plant development are missing. Thus, early selection decisions are currently only based on productivity/fruit quality traits. Considering the increasing demand of high quality plant stocks of *A. unedo*, *in vitro* propagation protocols were developed to cloning selected genotypes (Gomes and Canhoto 2009; Martins et al. 2016a, b) and studies to evaluate how these *in vitro* propagation systems change drought tolerance of regenerated plants have been carried out (Martins et al. 2019).

Some studies have been conducted on strawberry tree pollen and a morphological description have been provided (Villa 1982; Mateus 1989). However, as far as is known, no work has been done in order to improve strawberry tree throughout conventional breeding, although some extensive experiments have been carried out on other Ericaceae, such as *Rhododendron* (Doorenbos 1955; Escaravage et al. 1997) and *Vaccinium* (Lyrene 1997; Usui et al. 2005) species. Although conventional breeding is a lengthy process, particularly in tree species with long life-cycles, improved varieties of several tree species such as *Populus* spp., *Platanus* spp. and *Malus* x *domestica* have been produced through classical breeding (Aravanopoulos 2010; Igarashi et al. 2016). The first step to initiate the development of new cultivars based on conventional breeding is a deep knowledge of the mechanisms of sexual plant reproduction, in particular the compatibility between the male and female reproductive structures, as well as the time of their maturation and phenology (Fryxell 1957).

In order to set up the basis for a long-term breeding program on strawberry tree, the aim of this work was to study the reproduction system of *A. unedo*, from pollen morphology to pollen-stigma interactions and analyze the tolerance of the F1 plants towards drought. For this purpose, a morphological analysis of flowers and pollen was carried out, and controlled pollinations were made *in vitro* and *in situ* to obtain hybrid plants. Moreover, the plants obtained by the artificial crossings were tentatively selected based on its drought tolerance. For this purpose, they were submitted to a water deficit regime in order to identify individuals able to maintain higher photosynthetic levels under water deficit conditions, that might be used on future micropropagation and/or breeding programs.

Material and Methods

Plant material

Flowers from three different populations were used in this study: CH (N 41°42’31.868’’ W 7°26’32.506’’, altitude 579m), from Chaves (North Portugal) and populations C1 (N 40°12’17.472’’ W 8°23’40.929’’, altitude 103m) and C2 (N 40°11’33.604’’ W 8°23’37.163’’, altitude 123m) from Coimbra (Central Portugal). Plants were selected based on its fruit quality and production (data not shown). A tree from population CH was used as a pollen donor for morpho-histological analysis (section 2.2), germination studies (section 2.3) and *in vitro* and *in situ* pollination assays (section 2.4). For pollen release and gathering anthers were removed from the flowers and placed on a Petri dish coated with aluminum foil for 1-2 days at room temperature. *In vitro* and *in vivo* pollinations (section 2.4) were carried out using emasculated flowers from ten trees from C1 and C2 populations (5 from each population) and the collected pollen as described before (Figure 24).

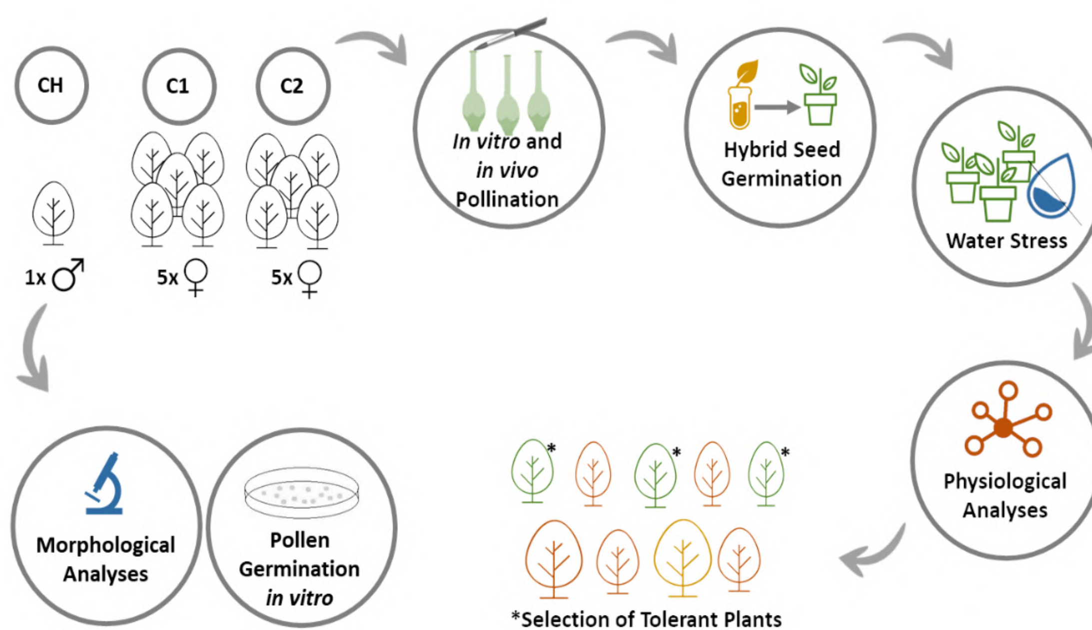

Figure 24. Methodology scheme applied in this work to study the phenology and reproductive anatomy of strawberry tree, as well as selection of drought tolerant plants.

Reproductive phenology and anatomy

Trees from populations C1 and C2 were monthly monitored throughout the year in order to characterize *A. unedo* reproductive phenology. Flowers and fruits were gathered and characterized, including anther position and fruit maturation stages. To analyse its morphology, pollen from CH was treated by the standard method of acetolysis (Erdtman 1960). Briefly, after being washed in water and acetic glacial acid (100%, v/v), pollen grains were treated with the classic acetolysis mixture (9:1, acetic anhydride:sulphuric acid), and heated in a water bath at 70 °C for 5

min. After being treated with acetone (100%, v/v), acetolysed pollen material was mounted in glycerin jelly (Sigma-Aldrich, St. Louis, MO, USA). Measurements (D: diameter of the tetrad; d: single grain diameter; and the ratio D/d) were taken under light microscopy (Nikon EclipseCi) with an ocular micrometer, from 30 randomly chosen pollen tetrads from 3 different slides (10 per slide). Terminology based on that of Punt et al. (Punt et al. 2007) was used for pollen morphology characterization. For scanning electron microscopy, pollen was placed on stubs and coated with gold on a JEOL JFC 1100 apparatus (JEOL, Musashino, Japan). Pollen observations were performed on a JEOL JSM 5400 microscope. For anther anatomy studies, whole anthers were fixed for 3h at room temperature in glutaraldehyde (1.5%, v/v, Sigma), prepared with phosphate buffer (0.1M) and postfixed in osmium tetroxide (1%, w/v, Sigma) prepared with the same buffer. Samples were further dehydrated with ethanol and embedded with Spurr resin (Spurr 1969). After the polymerization, ultrathin sections (1.5 µm) were obtained on an LKB Ultratome III and the cross sections stained with toluidine blue (Roland 1978) (Roland 1978) and observed on a light microscope (Nikon EclipseCi) and photographs were collected with a Nikon DS-Fi3 camera and processed with the software NIS-Elements D (version 4.60).

Pollen germination

Previously to the pollination assays the viability of the collected pollen was checked and the effect of sucrose concentration evaluated. Mature pollen was cultured on Petri dishes containing a basal germination medium (Jahier et al. 1992) composed of H_3BO_3 (5 mg L^{-1}), $CaCl_2$ (15 mg L^{-1}), KNO_3 (10 mg L^{-1}), agar (8%, w/v, Duchefa Biochemie B.V, Haarlem, The Netherlands) and different concentrations of sucrose (0, 3, 6, 9, 12, 15 and 18%, w/v, Duchefa), for 6 and 24 hours, at room temperature. Pollen grains were then stained with aceto-carmine and observed under a light microscope. As *A. unedo* pollen grains are dispersed as tetrad units (Mateus 1989), germination rates were determined by scoring 100 pollen tetrads from 5 replicates (a total of 500 tetrads and 2000 pollen grains). A pollen tetrad was considered germinated when the pollen tube length of at least a pollen grain surpassed the diameter of a pollen grain. On a second experiment, different carbon sources as well as the effect of some plant growth regulators on pollen germination were also tested. For this purpose, pollen was cultivated for 6 hours on the medium described before, with sucrose, glucose and fructose at three different concentrations (3%, 9% and 15%). The effect of 1-naphthaleneacetic acid (NAA, Sigma), indole-3-butyric acid (IBA, Sigma) and gibberellic acid (GA_3, Sigma) was also tested in three concentrations (10, 100 and 500 mg L^{-1}), on the same basal germination medium containing 15% sucrose.

***In vitro* and *in situ* pollination assays**

For *in vitro* pollination, flowers immediately before anthesis, from C1 and C2 populations were used. After emasculation, a total of 120 pistils from each population was placed on baby food jars (5 pistils per container) with a jellified medium for support (with water and 8 g L^{-1} agar). Pollen

from population CH collected as described in section 2.2. was then carefully placed at the stigma using a spatula. Open and closed non-pollinated flowers were used as controls and all treatments were done in triplicate. From each population, a total of 75 pistils were crosspollinated (15 pistils/tree from 5 different trees), 15 autopollinated (from a single tree), and 30 used as negative and positive controls (15 each from a single tree). Following artificial pollination, the pistils were kept in the dark at 25 °C, for 24 h, and the efficiency of the pollination was evaluated. For this purpose, pistils were fixed in FAA (formalin:acetic acid:ethanol, 5:5:90, v/v/v) at room temperature for 24 h, washed in water, softened on a NaOH solution (8N) and mounted with aniline blue (0.1%, w/v, Sigma) as described by Martin (Martin 1959). The observations were carried out in a fluorescence microscope (ex: 370 nm, Leica DM4000 B), and pollination was considered efficient when pollen germination was observed on the stigma, and pollen tubes grown along the style and reached the ovaries.

For *in situ* pollination assays pollen with a viability over 80% from a single tree (population CH) was used to hand pollinate flowers from the trees used for *in vitro* pollination. After the emasculation with forceps, the pollen was carefully placed on the stigma and the pollinated flowers covered with polypropylene pollination bags for 7 days, in order to avoid pollen contamination. 10 flowers from three different inflorescences (a total of 30 per tree) were pollinated on each of the 10 trees. All the immature and old flowers from the pollinated inflorescences were removed. During the assays the minimum absolute temperature was 7.1 °C and the maximum absolute temperature was 26.9 °C, while the total precipitation recorded was 145.5 mm, according to the data provided by the meteorological station of Coimbra/Cernache (www.ipma.pt).

Seed germination and plant development

Mature fruits resulting from hand-pollination were gathered and washed with tap water. Isolated seeds were washed with distilled water for 10 min. Following a 30s surface sterilization with ethanol (70%, v/v, Merck), the seeds were sterilized in a calcium hypochlorite solution (5%, w/v, Sigma) and 2-3 drops of Tween 20 for 10 min, washed 3 times with distilled sterilized water and sowed on sterilized Petri dishes (9 cm) with cotton wool imbibed with sterile distilled water and covered with filter paper. The seeds were kept at 4 °C for 30 days and then transferred to a culture chamber (25 °C) for another 60 days. After this period the germination rate was recorded and Relative Germinability (RG) was calculated: RG = (number of seeds produced * 100) / number of viable seeds germinated (Yang et al. 1999). Seedlings were then transferred to acclimatization containers with sterilized perlite, and kept in a growth chamber at 25 °C and 70% relative humidity, under a 16 h daily illumination regime of 15–20 µmol $m^{-2}s^{-1}$ photosynthetically active radiation (PAR, cool-white fluorescent lamps). After 15-30 days, the plants were transferred to individual containers (5 dm^3) with a substrate composed of peat and perlite (3:1, v/v, Siro, Mira, Portugal) for further growth on a greenhouse.

Drought stress assays

Three-year-old plants resulting from cross pollination (a tree from each population) were then submitted to water stress. A total of 26 plants (#1-13 from population C1 and #14-26 from population C2) were watered to full field capacity, and plant performance was evaluated after 24 hours (t0). After that period watering was interrupted and plants submitted to 3 weeks (t3) of water deficit. Leaf gas exchange was evaluated on t0 and t3 while due to the destructive nature of leaf water potential (Ψ_w) and leaf relative water content (RWC) measurements, sampling was performed in the end of the experiment (t3). The experiment was conducted during July, and the temperature ranged from 13 °C (15.8 ± 1.4) to 32 °C (24.1 ± 2.7). The average temperature at each sampling point was: 20.5 ± 4.5 °C (t0) and 17.5 ± 6.4 °C (t3).

In situ leaf gas exchange measurements (net CO_2 assimilation rate: *A*, transpiration rate: *E*, stomatal conductance: *gs* and intercellular CO_2 concentration: *ci*) were measured on a young and fully expanded leaf (normally the fifth leaf from the top) using a portable infrared gas analyzer coupled to a broad leaf chamber (*LCpro+*, *ADC*, Hoddesdon, UK), operating in open mode and under the following conditions: photosynthetic photon flux density - 650 µmol m^{-2} s^{-1} (based on a light curve: 0-1750 µmol m^{-2} s^{-1}); air flux - 200 mol s^{-1}; block temperature - 25 °C; and atmospheric CO_2 and H_2O concentration. Data were recorded when the measured parameters were stable (2–6 min). Water potential was measured with a Scholander-type pressure chamber (PMS Instrument Co., OR, USA). Relative water content (RWC) was calculated as: RWC (%) = (FW − DW)/(TW − DW)*100, where FW is the fresh weight of the leaf, TW the turgid weight (after 24 h on distilled water at 4 °C) and DW is the dry weight (after drying at 70 °C for 48 h).

Statistical analysis

Pollen germination and physiological data was analyzed using ANOVA (GraphPad Prism for Windows v. 6.01) followed by a Tukey´s multiple comparison test ($P < 0.05$). Data expressed as percentages were first submitted to arcsine transformation. A heatmap with dendrogram and a principal component analysis (PCA) were carried out using R software (R Core Team 2020) to evaluate the interaction and significance of all the physiological parameters measured on the analyzed trees. A heatmap with physiological data from all the samples was constructed using the Heatmap function and the package ComplexHeatmap (Gu et al. 2016). The dendrogram within the heatmap was calculated with Euclidean distance as dissimilarity measure. Finally, data was classified with a PCA, using the prcomp function and the package ggbiplot (Vu 2011).

Results

Reproductive phenology and anatomy

The reproductive cycle of strawberry tree is long, and lasts for almost two years (Figure 25 A). During this period three distinct stages can be identified: flower buds, flowers at anthesis and fruit development. During June, the inflorescences (panicles) start to appear from terminal meristems of young stems (Figure 25 B). Flower development proceeds trough summer months and flower anthesis usually begins on October (Figure 25 C).

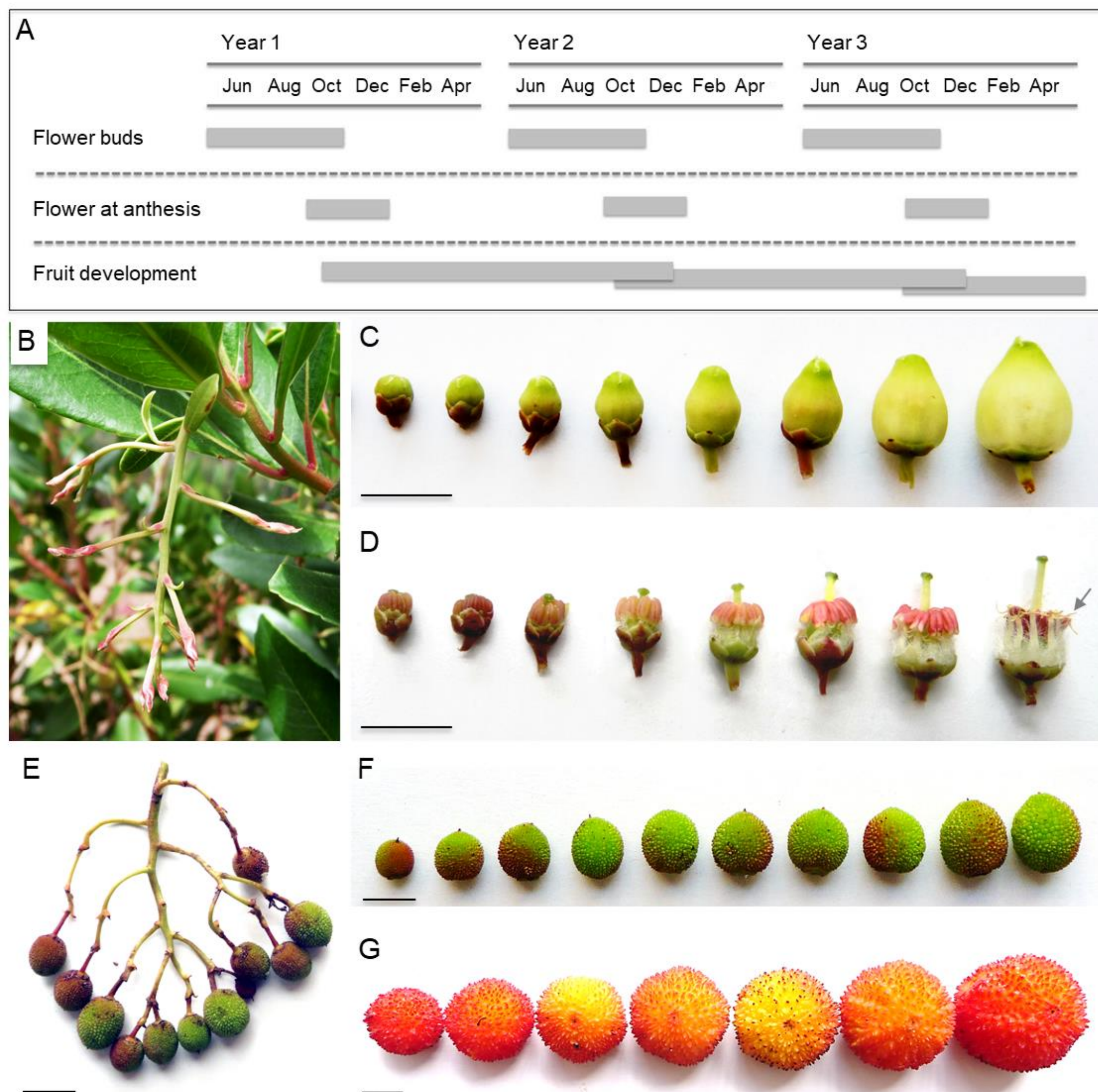

Figure 25. Different aspects of strawberry tree phenology and reproductive anatomy: phenological cycle of strawberry tree (A); terminal hanging panicle at an early development stage during June (B); general morphology of the bell-shaped flowers from early stages of development to flower anthesis (C); developmental process of the stamens (arrow indicates anther appendage) (D); infructescence at an early development stage (E); fruit developmental stages before ripening (F) and mature fruits with different sizes

and shapes (G). Feb - February, Apr - April, Jun - June, Aug - August, Oct - October, Dec - December. (bar: C and D = 50 mm, E-F = 1 cm).

The flowering period can be long, from early October to late January depending on the trees and location. The flower is complete, bell-shaped, sympetalous and white to slightly pink (Figure 25 C). Each pistil is formed by a pentalocular ovary, a style and a stigma who becomes receptive to pollen just before flower anthesis. Each stamen possesses a hairy filament and an anther with two pores located at the top. During flower development and just before flower anthesis, anthers suffer an inversion process from an extrorse to an introrse position and develop two appendages on the apical end (Figure 25 D). After pollination, the slow fruit development process begins. Each infructescence will usually bear between 1 to 20 fruits (Figure 25 E) that will develop along the year until fully ripped (Figure 25 F). Consequently, fruit ripening occurs simultaneously with the next flowering period, during autumn (Figure 25 A). Fruits at different developmental and ripening stages can be found at the same time on a tree. When fully ripped, fruits present a variable size and shape and a bright red color (Figure 25 G). Anthers of *A. unedo* have four microsporangia or pollen sacs arranged in pairs (Figure 26 A). Pollen in dispersed in groups of four and each anther contains on average 500 pollen units. On the earlier developmental stages some pollen grains were found to be aborted on the pollen tetrad (Figure 26 B).

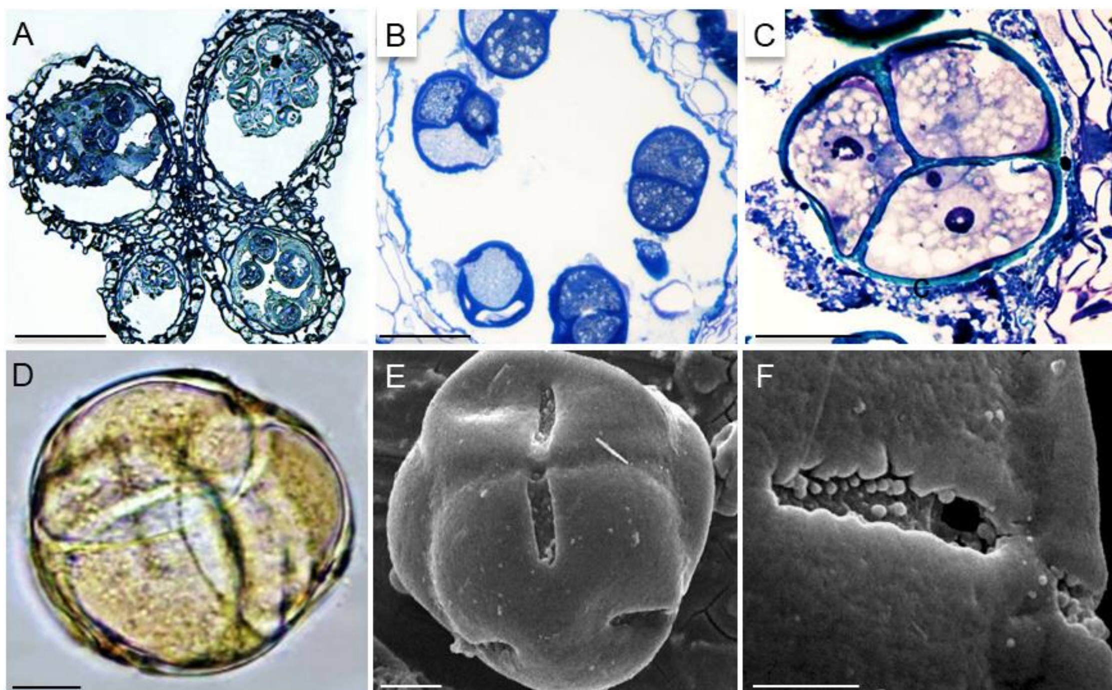

Figure 26. Anther and pollen morphology of strawberry tree: anther cross section stained with toluidine blue (A); pollen sac with aborted pollen grains (B); pollen tetrad section stained with toluidine blue showing a bi-nucleated pollen grain (C); non acetolised pollen tetrad (D); pollen tetrad on SEM (E) and aperture and ornamentation detail of the pollen tetrad on SEM (F) (bar: A = 100 μm, B = 50 μm, C = 20 μm, D -E = 10 μm, F = 5 μm).

Pollen tetrads became mature and are released just after flower anthesis. Single pollen grains are 3-zonocolporate and have a circular or slightly elliptic outline on optical slice (Figure 26 C-D). The ectoapertures are long colpus with a granulate membrane, and the endoapertures are pores with regular outline (Figure 26 E-F). The exine (≈ 1.5 µm) has a psilate surface and is tectate and slightly columellate. The size of the pollen tetrads (D) ranged from 42 µm to 67 µm (53.8 µm ± 3.6) whereas the size of single pollen grains (d) varied from 22 µm to 36 µm (29.5 µm ± 0.8). The relation D/d was between 1.6 and 2.2 (1.8 ± 0.1).

Pollen germination

Pollen germination was higher on media with higher sucrose concentrations for both periods analyzed (6 and 24 h). After 24 h, best germination rates were obtained on 15%, 18% and 21% sucrose, without statistical differences. However, a decrease on germination was observed on the medium with 18% sucrose after 6 h (Figure 27 A-B). The highest germination rates were obtained on a medium with 21% sucrose, after 24 hours (83.29% ± 10,85), and 15% sucrose after 6 hours (80.52% ± 12.55; Figure 27 A-B). In most of the pollen tetrads scored as germinated, only one of the pollen grains developed a pollen tube (70.98% ± 1.81), whereas the germination of more than two pollen grains was only observed occasionally (Figure 28 A).

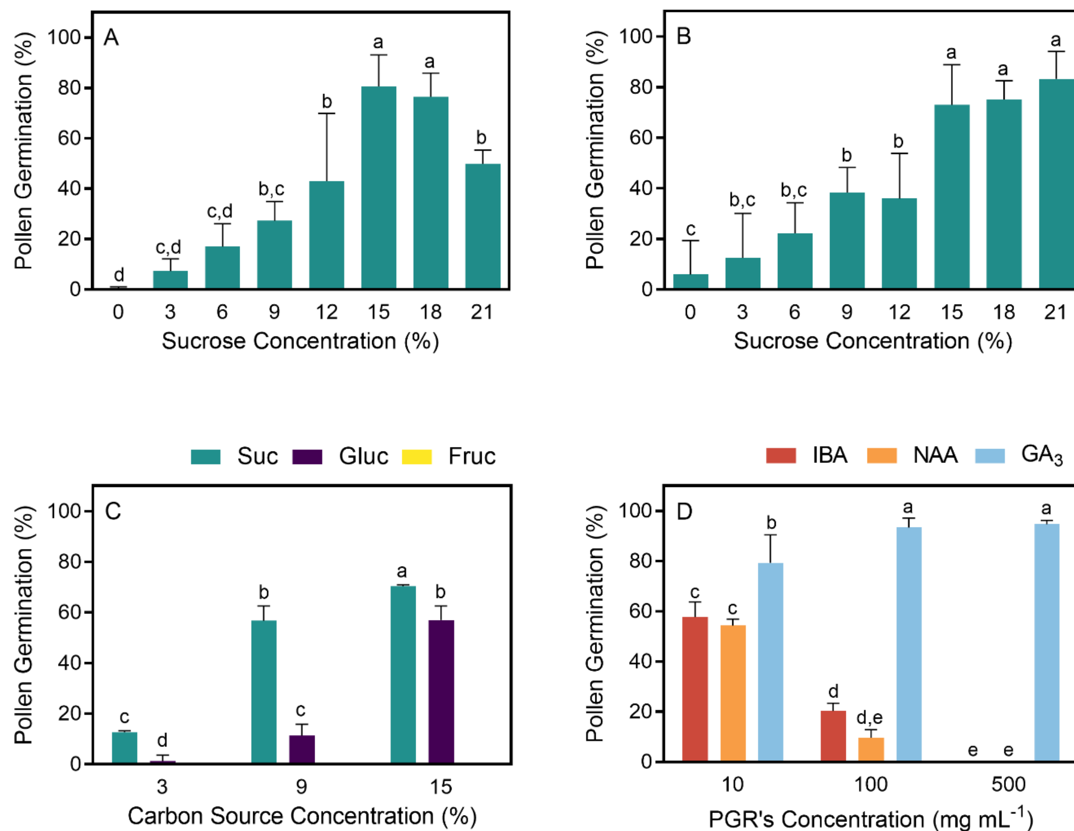

Figure 27. Effect of different sucrose concentrations (0, 3%, 6%, 9%, 12%, 15% and 18%) on pollen germination rates after 6 hours (A) and 24 hours (B); effect of different carbon sources (sucrose, glucose and fructose) at three different concentrations (3%, 9% and 15%) on pollen germination after 24 hours (C) and

effect of plant growth regulators (IBA, NAA and GA_3) on germination rates at different concentrations (10, 100 and 500 mg L^{-1}) after 24 hours (D). Values (%) are means ± standard deviations, n = 5, different letters indicate significant differences between treatments at $P \leq 0.05$.

When different carbon sources were tested, sucrose gave the best results on the three concentrations tested, with statistical differences when compared to glucose and fructose. The maximum germination rate was obtained with 15% sucrose (70.33% ± 1.89). Although the glucose was not as efficient as sucrose, a germination rate of 57.00% ± 4.55 was obtained with the maximum concentration tested (Figure 27 C). No pollen germination was observed when fructose was used as carbon source. Likewise, NAA and IBA had an inhibitory effect on pollen germination, even on the lowest concentration tested (10 mg L^{-1}). When these two auxins were applied at higher concentrations, pollen germination was completely inhibited with statistical difference between concentrations for both NAA and IBA (Figure 27 D). On the other hand, GA_3 highly promoted pollen germination and germination rates of 93.33 ± 3.09 and 94.67 ± 1.25 were obtained with 100 mg L^{-1} and 500 mg L^{-1} of GA_3 respectively (Figure 27 D). These values are higher than those obtained when a concentration of 10 mg L^{-1} was applied, with statistic significant differences. When the concentration of $CaCl_2$ was highly incremented on the germination medium (10 x increase), similar results were obtained (data not show).

***In vitro* and *in situ* pollination assays**

From the 12 combinations of crosses carried out *in vitro*, including 2 auto-pollinations, the average success rate obtained was 78.9% ± 22.7. Pollen germination was observed on stigma 1-2 hours after the pollination (Figure 28 B). The pollen tubes grow along the stile, reaching the ovary in 24 h (Figure 28 D), and the tips of pollen tubes enter the micropyle (Figure 28 C). The effectiveness of the cross-pollinations was 82.51% ± 19.81, while the effectiveness of the self-pollinations was 71.65 ± 29.50. It was observed the accumulation of callose along the pollen tubes as well as on the tips. In some cases, pollen showed no signs of germination, both on self- and cross-pollinations.

The growing pattern of pollen tubes seemed to be very similar on all the crosses made. In most of the flowers from the positive control (open flowers) pollen germination and pollen tube growth were observed, while all the flowers from the negative control (closed flowers) showed no signs of pollen in the stigma. Most of the pollinated flowers *in situ* were lost along the fruit developmental process. From the total of 300 pollinated flowers, after one year under development, only 3 fruits reached the mature stage which represents a very low success rate of only 1%.

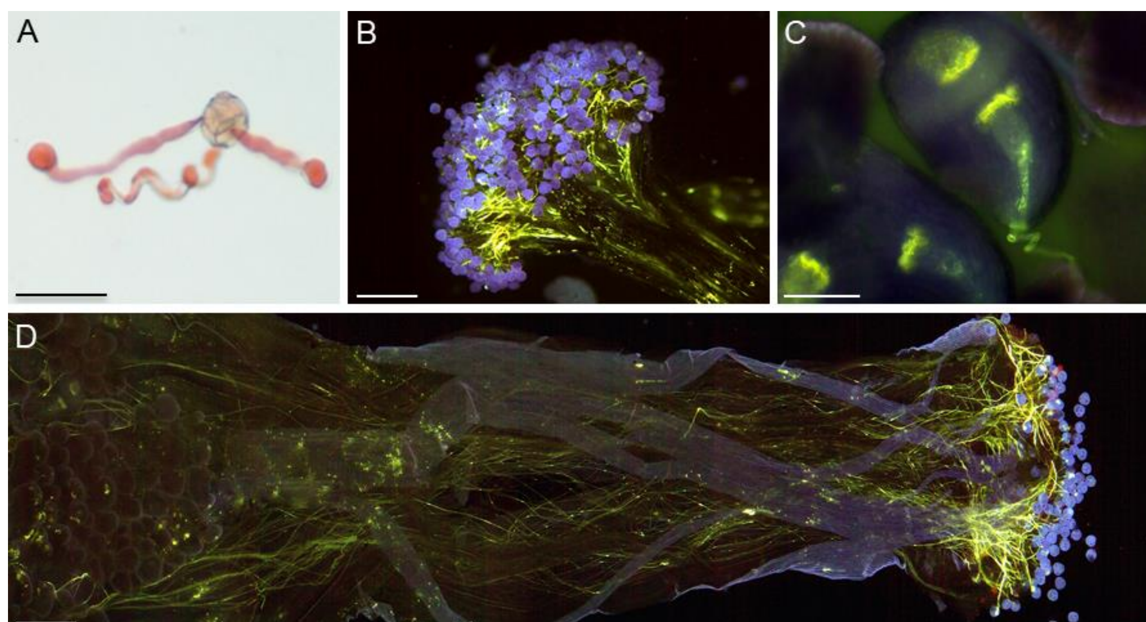

Figure 28. Strawberry tree pollen germination: germinated pollen tetrad stained with aceto-carmine (A); germination of pollen grains on stigma (B); pollen tube entering the ovule micropyle (C) and pollen germination on the stigma and pollen tube growth along the pistil (D) (bar: A = 100 µm, B = 200 µm, C = 1 mm, D = 200 µm).

Seed germination and plant development

From the three fruits retrieved from the field, a relative germination rate of 85.0% was obtained for group C1 and 86.7% for C2. After *in vitro* germination, seedling development proceeded rapidly, and after the acclimatization period the root system was well developed (Figure 29 A).

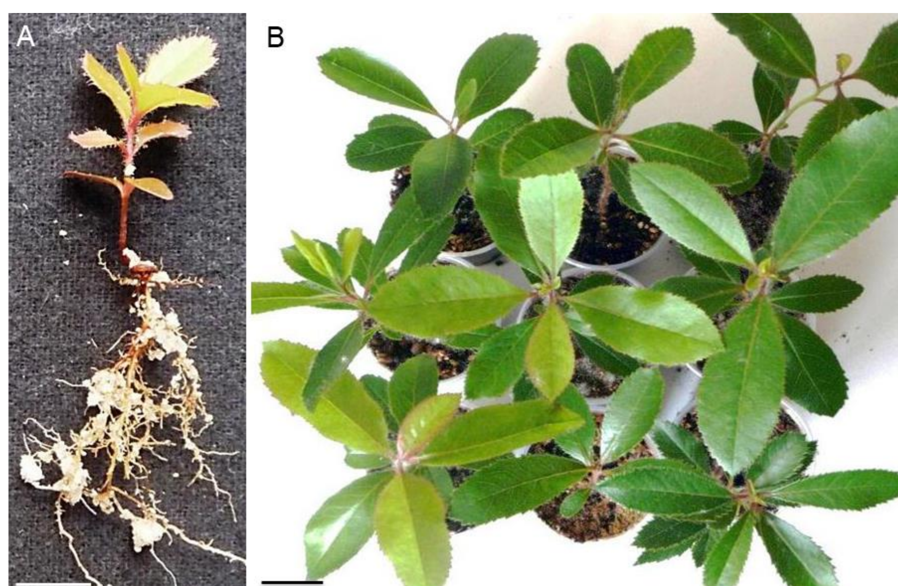

Figure 29. Strawberry tree hybrid plants development: two months-old hybrid seedlings (A) and six months-old hybrid plants with distinct morphological characteristics (B) (bar: A = 1 cm, B = 1 cm).

The hybrid plants were morphological diverse (Figure 29 B) in terms of height and leaf morphology (data not shown). A total of 30 plants were obtained, 17 from group C1 and 13 from C2. After 3 years under development, 13 plants from each group were submitted to drought stress.

Plant water status and gas exchange

Before the imposed water stress deficit regime (t0) a considerable variance was found among genotypes on all the physiological parameters measured. While some of the tested genotypes presented higher net CO_2 assimilation rates (*e.g.*, 3, 5, 13, 21 and 25), others had considerably lower values (*e.g.*, 2 and 17) (Table 1, Figure 30 A). After 3 weeks under water deficit, stomatal conductance, net CO_2 assimilation and transpiration rates decreased (Table 1, Figure 30 B). On the other hand, intercellular CO_2 concentration increased on most of the plants throughout the imposed water stress. Although a great reduction on stomatal conductance, transpiration and net CO_2 assimilation rates was observed on genotype 13 after drought stress, these parameters are still considerably higher than on most of the evaluated genotypes. A similar behavior was observed on genotypes 12 and 17, but with a less marked decreased on net CO_2 assimilation rate. Meanwhile, these parameters remained unchanged or slightly increased on genotypes 14, 15 and 18. Relative water content and water potential were in general higher on plants with a higher net CO_2 assimilation rate.

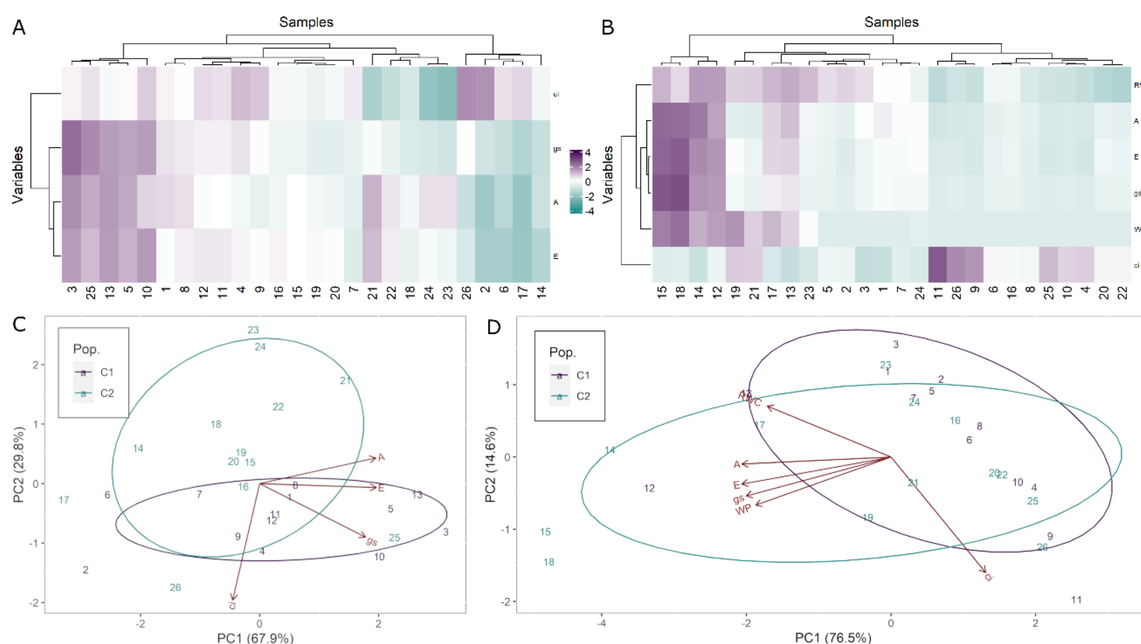

Figure 30. Physiological parameters measured on 26 hybrid plants from populations C1 and C2 on t0 and t3 (3 weeks under water deficit): heatmap with dendrogram on t0 and t3 (A-B) and principal component analysis (PCA) on t0 and t3 (C-D). ci - intercellular CO_2 concentration, E - transpiration rate, gs - stomatal conductance, A - net CO_2 assimilation rate, RWC - relative water content, WP - water potential.

Table 1. Gas exchange related parameters water status of 26 hybrid plants on t0 (control) and t3 (3 weeks under water deficit). ci - intercellular CO_2 concentration (ppm), E - transpiration rate (mmol H_2O m^{-2} s^{-1}), gs - stomatal conductance (mol H_2O m^{-2} s^{-1}), A - net CO_2 assimilation rate (µmol m^{-2} s^{-1}), RWC - relative water content (%), WP - water potential (MPa). + plants with an intermediate performance. ++ plants identified with the best performance. * equipment detection limit.

Genotype	**ci**		**E**		**gs**		**A**		**RWC**	**WP**
	t0	*t3*	*t0*	*t3*	*t0*	*t3*	*t0*	*t3*		
1	181	166	2.76	0.86	0.32	0.06	25.87	7.67	63.4	-50*
2	243	220	1.98	0.34	0.18	0.02	11.81	2.31	72.8	-50*
3	181	159	3.5	0.64	0.51	0.04	32.36	5.49	70.5	-50*
4	215	302	2.88	0.14	0.32	0.01	21.2	0.45	47.5	-50*
5	176	223	3.34	0.55	0.42	0.03	29.84	3.07	70.9	-50*
6	193	258	1.98	0.53	0.16	0.03	14.7	2.21	59.4	-50*
7	186	204	2.48	0.84	0.23	0.05	19.59	5.18	63.9	-50*
8	172	230	2.91	0.45	0.31	0.02	25.23	2.32	53.0	-50*
9	209	364	2.82	0.19	0.28	0.01	19.85	0.15	48.6	-50*
10	205	296	3.52	0.29	0.43	0.02	26.22	0.82	50.0	-50*
11	192	435	2.96	0.27	0.3	0.01	22.51	-0.46	45.7	-50*
12+	196	220	2.96	2.52	0.3	0.22	22.1	15.61	84.6	-21
13+	165	165	3.5	1.63	0.43	0.11	31.25	12.51	81.2	-32
14++	160	171	2.11	2.7	0.16	0.23	16.92	20.27	84.7	-24
15++	163	201	2.8	3.27	0.24	0.33	22.5	22.19	79.4	-14
16	179	232	2.89	0.63	0.24	0.03	20.86	2.89	59.6	-50*
17+	199	190	1.87	1.59	0.12	0.1	11.24	10.47	78.8	-26
18++	139	208	2.58	3.41	0.2	0.34	20.96	21.7	71.7	-10.5
19	157	305	2.85	0.91	0.22	0.05	21.26	2.01	73.3	-18.5
20	163	260	2.82	0.68	0.22	0.03	20.68	2.45	44.0	-50*
21	105	302	3.28	0.63	0.26	0.03	29.13	1.24	74.0	-31.25
22	125	260	2.93	0.6	0.23	0.03	24.77	2.16	42.3	-50*
23	76	188	2.64	0.64	0.18	0.03	25.25	3.73	77.6	-42.5
24	87	191	2.72	0.92	0.19	0.05	25.27	5.3	58.2	-48.5
25	191	325	3.28	0.19	0.46	0.01	29.66	0.55	50.6	-50*
26	246	377	2.42	0.33	0.27	0.02	16.36	0.02	50.5	-50*

This result is confirmed by the dendrogram within the heatmap (Figure 30 A-B) as well as the PCA biplot (Figure 30 C-D), that revealed a high positive correlation between E, gs and A with relative water content and water potential (Figure 30 B). Moreover, a negative correlation was found between these parameters and ci. On most of the plants, water potential was below the detection limit (-50 MPa). The PCA analysis have also revealed a very diverse behavior of plants, regardless of their provenience either on t0 or t3 (Figure 30 C-D). Thus, plants with best and worst performance under water stress are from both proveniences. On t0, principal component 1 (PC1) contributes with 67.8% to the total variance and A, gs and E are the parameters with a higher weight on this component, whereas PC2 contributes with 29.8% to the total variance and ci is the variable that most contributes to this variance. The genotypes identified due to a better performance (3, 5, 13, 21 and 25) are grouped (Figure 30 C). On t3, principal component 1 (PC1) contributes with 76.5% to the total variance. A, gs, E, relative water content and water potential are the parameters with a higher weight on this component. PC2 contributes with 14.6% to the total variance. As mentioned before, some of the tested plants showed a better overall performance in terms of net CO_2 assimilation under drought. Thus, genotypes 12, 13, 14, 15, 17 and 18 are grouped together, by the influence of some gas exchange parameters (gs, E and A), relative water content and water

potential. Genotype 19 is also on this cluster as it was able to maintain relatively high values of relative water content and water content, in spite of its low performance in terms of net CO_2 assimilation. Plants with a worst overall performance are grouped together by the influence of ci (Figure 30 D).

When comparing the two groups (C1 and C2), although no statistical differences were found between groups (Figure 31 A), transpiration rates greatly decreased after 3 weeks on both groups (Figure 31 B), as well as stomatal conductance (Figure 31 C) and net CO_2 assimilation rate (Figure 31 D). No statistical difference was found for relative water content as well, with values of 62.4 ± 13.0% on C1 and 65.0 ± 14.7% on group C2.

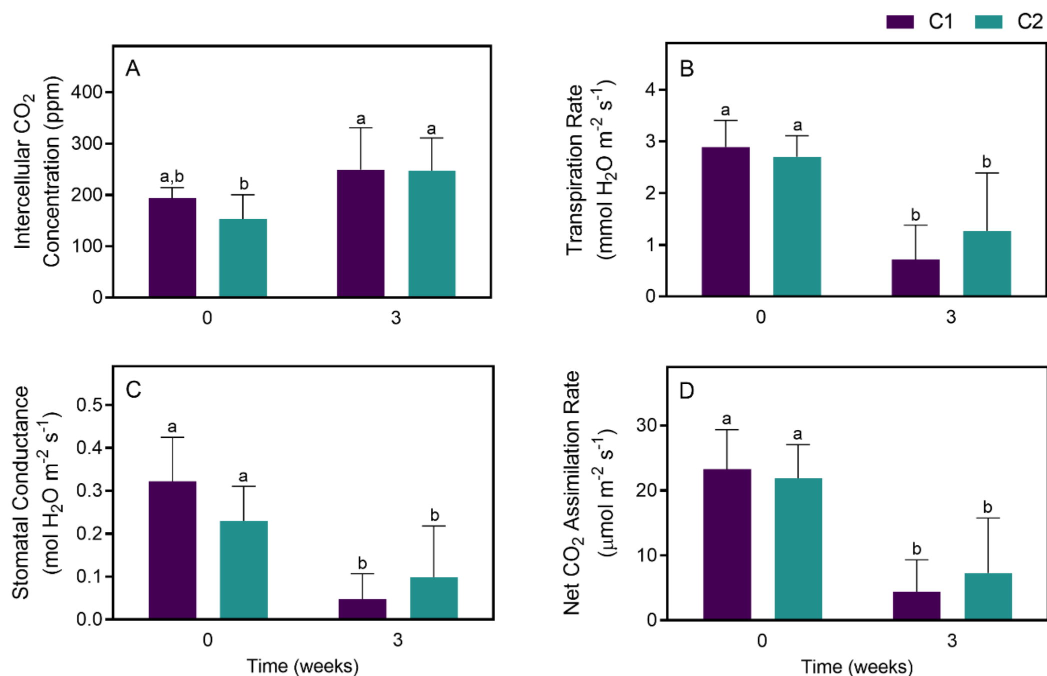

Figure 31. Gas exchange related parameters of hybrid plants from populations C1 and C2 on t0 (plants watered to full field capacity) and t3 (3 weeks under water deficit): intercellular CO_2 concentration (A); transpiration rate (B); stomatal conductance (C) and net CO_2 assimilation rate (D). Values are means ± standard deviations, n = 13, different letters indicate significant differences between treatments at $P \leq 0.05$.

Discussion

The long phenological cycle observed on the trees analyzed on this work, is similar to the one that has been reported by (Villa 1982)(Villa 1982). The inversion process of the anthers observed, as well as the development of the two appendages, has been described as a characteristic feature of the Ericaceae family (Hermann and Palser 2000). The pollen morphology observed is similar to data reported by (Villa 1982)(Villa 1982), but slightly differs from that described by Mateus (Mateus 1989). According to this author, the endoapertures of strawberry tree pollen are endocolpus. In contrast, on this study the endoapertures observed were endopores. The size of the pollen tetrads is

slightly different as well: the diameter of the tetrads (D) determined on this work range from 42 µm to 67 µm, compared to 49-66 µm obtained by Mateus (Mateus 1989), while the size of single pollen grains (d) range from 22 µm to 36 µm compared to 33-41 µm.

The pollen germination rates obtained on the germination medium with 12% sucrose (89.40%) are similar to the ones obtained on other species, such as *Prunus domestica* (Ćalić et al. 2013) and *Pistacia* spp. (Acar and Gopal 2010), and much higher than the germination rates obtained on *Annona charimola* (Rosell et al. 1999) and *Olea europaea* (Vuletin Selak et al. 2013). Therefore, the germination medium used is adequate for strawberry tree and should be used for pollen viability tests. Although it has been reported by (Cane 2009)(Cane 2009) that 90% of the tetrads generated 3-4 pollen tubes on other member of the Ericaceae (*Vaccinium macrocarpon*), we found that most of the strawberry tree pollen tetrads analyzed had only one pollen grain germinated. This can be due to the fact that some of the grains on the pollen tetrads were found aborted, even in the initial developmental stages. The inhibition of pollen germination caused by fructose has been reported on the literature by (Okusaka and Hiratsuka 2009) (Okusaka and Hiratsuka 2009). According to these authors, fructose completely inhibited pear pollen germination, but without pollen viability loss. Thus, fructose is not an adequate sugar for strawberry tree pollen germination, and other carbon sources should be used instead, preferably sucrose. Plant growth regulators (PGRs) can be an extremely useful tool for plant breeders, either as gametocides or on the contrary by promoting pollen germination and eventually increasing fruit seed-set. Different PGRs have been tested on diverse crop species including rye (Nátrová and Hlaváč 1975), barley (Verma and Kumar 1978), onion, tomato, eggplant, pepper, watermelon (Saimbhi and Brar 1978) and wheat (Chakraborty and Devakumar 2006), on most cases in concentrations similar to the ones tested on this study (10-500 mgL^{-1}). Our results showed an inhibitory effect of IBA and NAA on strawberry tree pollen germination in similar concentrations to those reported on the literature: the application of NAA (50 mg L^{-1}) on eggplant and IBA or NAA (10-100 mg L^{-1}) on onion, proved to have an efficient gametocide effect (Saimbhi and Brar 1978). On the other hand, the applications of at least 100 mg L^{-1} of GA_3 greatly promoted pollen germination, an effect that has also been observed on strawberry with a similar concentration (50 mg L^{-1}) (Voyiatzis and Paraskevopoulou-Paroussi 2002) and blueberry where the application of GA_3 on flowers (on concentrations approximately between 30 and 500 mg L^{-1}) lead to an increased fruit set (Cano-Medrano and Darnell 1998). For this reason, the effect of GA_3 on strawberry tree pollen germination and fruit set should be further tested due to its potential as a breeding aiding tool.

Although a previous study had suggested that pollen tubes growth speed is slower on self-pollination due to higher rates of attrition (Horsley and Johnson 2007), this was not the case in strawberry tree and similar pollen tube growth patterns on self- and cross-pollinations was observed. Moreover, no difference was found between the effectiveness of self- and cross-

pollinations. The observed accumulation of callose on pollen tubes of strawberry tree has also been observed in *Chaenomeles japonica* (Kaufmane and Rumpunen 2002). Only a small portion of the hand-pollinated flowers on the field were able to complete the long development process and bear fruits. Due to the long development process that takes a year to be completed, strawberry tree pollinated flowers and fruits under development are subjected to a wide range of environmental conditions and interferences and only a small portion of fruits is able to complete its development. This might help to explain the low success rate of hand-pollinations along with the high manipulation required to carry the pollination procedure. In fact, it has been reported that fruit production on *Vaccinium* spp. is lower when plants were cross- or self-pollinated by hand than when natural pollination occur (Usui et al. 2005). Thus, the improvement of pollinations conditions is something to be pursued in the near future in order to increase success rates of hand-pollinations. The increase of the amount of pollen placed on the stigma and/or the use of PGRs (*e.g.*, GA_3) should be considered, as well as the implementation of open pollinated seed orchards. Size and seediness of fruits may also be affected when hand-pollination is carried out, as referred by (Usui et al. 2005). On this work, the average of viable seeds obtained on hand-pollinated fruits was 50%, much lower than the 77% obtained on open pollinated trees (data not shown). Nonetheless, due to the low amount of hybrid fruits obtained, these results are not significative and further analyses should be carried out in the future. The high germination rates obtained are similar to other works (Tilki 2004; Demirsoy et al. 2010; Ertekin and Kirdar 2010) indicating that germination ability of the hybrid seeds is not compromised. However, such rates were obtained after cold stratification, a procedure that should be followed in order to break seed dormancy.

When the hybrid plants were submitted to drought stress (t3), a decrease on transpiration rates, stomatal conductance as well as net CO_2 assimilation rate was observed, has a consequence of the efficient conservative water use strategy adopted by strawberry tree (Castell and Terradas 1994). As mentioned before, strawberry tree is an isohydric species, with a tight stomatal control. When under water deficit conditions, plant will adopt a conservative water use strategy by closing stomata, thus keeping a low gas exchange rate (Raimondo et al. 2015; Forner et al. 2018). The increase of intercellular CO_2 concentration throughout the imposed water stress is caused by a reduction of the photosynthetic machinery due to stomatal limitations and probably oxidative stress (Munné-Bosch and Peñuelas 2004). The high positive correlation found between water availability (relative water content and water potential) and gas exchange parameters evaluated is not surprising, as water is one of the most limiting factors on the entire photosynthetic process. Thus, plants with a higher ability to maintain their water status have as expected a better overall performance.

Overall, the measured values of physiological parameters are in accordance with those obtained on a previous study (Martins et al. 2019). However, net CO_2 assimilation rates measured

were higher than those obtained on previous reports (Vasques et al. 2013; Martins et al. 2019). This difference can be related to the older age of the plants evaluated on this study, that hypothetically might have a higher photosynthetic ability. Considerably lower values of water potential were also measured on this work, which is probably related with the period under water stress as well as the age of the plants that might have more lignified tissues and a higher resistance to cavitation and low water potential. Still, this hypothesis should be further tested and confirmed on future analysis.

Although most of the tested plants showed a poor performance under drought stress, we successfully identified two groups of plants that followed a different strategy to cope with water deficit and were able to maintain a high stomatal conductance and consequently higher net CO_2 assimilation rates. Genotypes 14, 15 and 18 were able to maintain their basal levels of photosynthesis, which was accomplished by maintaining stomata open as these plants were able to maintain relatively high levels of water (relative water content and water potential). Genotypes 12, 13 and 17 showed to have an intermediate performance under drought stress. Although these plants were able to maintain the photosynthetic mechanisms active after 3 weeks under water deficit conditions, they were already probably close to their resistance limits, and a significant drop on net CO_2 assimilation rates was expected after a few more days under stress. Finally, some genotypes (*e.g.*, 19) were able to maintain relatively high levels of water (relative water content and water potential), but were unable to maintain satisfactory levels of net CO_2 assimilation which might be due to biochemical limitation of photosynthesis rather than stomatal constraints. From the genotypes identified on t0 has to have higher net CO_2 assimilation rates, only genotype 13 was able to maintain a similar performance under stress. On the other hand, genotypes with lower net CO_2 assimilation rates on t0 (*e.g.*, 17) were able to cope with drought stress and maintain the levels measured at t0. The genotypes identified on t0 for its high net CO_2 assimilation rates (3, 5, 13, 21 and 25) might have high potential and their productivity should be evaluated. Nonetheless, on the water restriction scenario we hypothesize on this work, with the exception of genotype 13, they generally fail to cope with water stress.

Besides revealing the importance of genotype on strawberry tree physiological performance and response to drought, these results show that strawberry tree plants have a high phenotypic plasticity and are able to adjust differential strategies to cope with stress. In order to facilitate and considerably reduce the necessary required time for selection, the identification of other adequate selection parameters should be pursued. In particular, metabolites like phenols, proline, chlorophyll, anthocyanins and several hormones (*e.g.*, abscisic acid, jasmonic acid and salicylic acid), that are known to be essential on plant response mechanisms to drought stress, might be used as markers to identify plants with a better appetence to undergo extreme drought events.

Although a great variance was observed between individuals from the same population, no differences were observed between populations on all the tested parameters. These results suggest that intra-population variation should be take into account and prioritized over inter-population on

future selection endeavors, and a large number of individuals from within a population must be sampled. In contrast, results obtained by Vasques et al. (Vasques et al. 2013) showed that seedlings provenience might influence the tolerance of plants under water stress, thus suggesting local adaptations of plant populations, which reinforces the importance of inter-population variance on plant behavior. Due to its implication on plant selection, this hypothesis should be further investigated. Overall, the obtained results will have important repercussions on strawberry tree phenotyping and early plant selection as well as breeding towards the obtention of drought stress resistant genotypes.

As a basis for any breeding program that includes plant hybridization, a deep knowledge of the plant reproduction system is necessary. This work provides the firsts insights of strawberry tree reproduction system which will be crucial on future breeding attempts. As a tool for plant selection, the physiological parameters used on this study proved to be adequate. However, the analysis of biochemical parameters could not only elucidate the tolerance mechanism of *A. unedo* but also identify key metabolites (*e.g.*, phenols, hormones and pigments) that could be used as markers for early plant selection. Three genotypes (14, 15 and 18) showed a particular appetence to cope with water stress and may be the basis for a future breeding program. However, due to the influence of genotype on plant response to water stress and the observed phenotypic plasticity, the analysis of a large number of individuals should be carried out in order to develop a long-term breeding program. The selection and breeding of strawberry tree genotypes more tolerant to drought stress is essential in order to maintain species sustainability and our promising results are a step forward in order to ameliorate strawberry tree adaptation while preserving productivity on drought prone areas.

CHAPTER 5: Genotype determines *Arbutus unedo* L. physiological and metabolomic responses to drought and recovery

(Martins J, Pétriacq P, Flandin A, Gómez-Cadenas A, Monteiro P, Pinto G, Canhoto J (2021) Genotype determines *Arbutus unedo* L. physiological and metabolomic responses to drought and recovery. Submitted for publication)

Abstract

Most *A. unedo* orchards are particularly exposed to drought as they are usually installed on marginal lands where water is a scarce resource. Thus, selection of plants that can better cope with water restriction is essential, and a better comprehension of the resistance mechanism is necessary. For this reason, the present study aims to shed light on the metabolic pathways relevant to drought resistance mechanisms. Furthermore, the contribution of genotype and its bio-geographic origin on plant performance was also assessed. For this purpose, two different water regimes plus a recover assay were simulated on 2-years-old micropropagated plants. Several eco-physiological and biochemical parameters were evaluated, the metabolomic profile of the plants was studied and important stress related plant hormones were quantified. Drought resistance showed to be genotype dependent, and a relation between metabolomic response with drought tolerance seems to exist. In fact, the genotype with a better performance under drought presented a different metabolomic profile, with 382 down-regulated and 188 up-regulated metabolites, including hormones like abscisic and salicylic acids. This genotype was also able to recover faster when the imposed stress was interrupted, which seems to indicate the relevance of metabolism adaptation under water deficit conditions. We expect these results will contribute to plant selection and breeding with improvements in plant production.

Keywords: phenolic compounds, micropropagation, plant hormones, proline, Strawberry tree

Introduction

Strawberry tree production area has been constantly growing on recent years, especially on marginal lands with poor soils and water scarcity where most species hardly thrive, being particularly exposed to drought conditions. Water availability is probably the most limiting factor for plant growth and development (Guarnaschelli et al. 2012) with impact in all physiological and biochemical processes. Water status is tightly regulated by several mechanisms, such as stomatal control, osmotic adjustment, accumulation of cuticular waxes and leaf morphological adaptations (Polle et al. 2019). *A. unedo* is recognized as a drought tolerant species, being able to thrive in conditions where water availability is scarce (Castell and Terradas 1994; Munné-Bosch and Peñuelas 2004). However, on a changing global climate context, that foreseen harsh conditions to southern Europe and the Mediterranean area, even the most tolerant species towards water stress may exceed their acclimation and drought resistance ability (Nardini et al. 2014). Plants are able to cope with water reduction at different levels (morphological, physiological and molecular), and have developed a wide range of adaptive traits, including leaf thickness and shedding, deep rooting and resistance to cavitation, according to an avoidance or tolerance strategy (Bussotti et al. 2014; Nardini et al. 2014; Forner et al. 2018; Polle et al. 2019). Nonetheless, sclerophyllous Mediterranean species are considered slow adapters (Bussotti et al. 2014). Independently of the species tolerance and resistance strategy, irreversible damages may be caused by prolonged or successive drought events compromising plant normal functions, and eventually leading to plant dead (Forner et al. 2018), which aggravates drought and desertification (Polle et al. 2019) and causes significant ecological and economic impacts (Nardini et al. 2014).

Due to the large distribution of *A. unedo* on the Mediterranean region, as well as its economic and ecological relevance, the knowledge of its natural tolerance mechanisms to stress, in particular its resistance to drought, is of the utmost importance to assure the survival and productivity of the species. Furthermore, the information obtained has potential for easier transfer to other sclerophyllous fruit trees, but also other species in regions with similar environmental pressures and a Mediterranean like climate, such as California, Chile, South Africa and South-Western Australia, and even subtropical regions where harsh conditions are expected in the near future. Additionally, such knowledge can be useful for forest and agricultural management and of great advantage for future breeding programs of different tree species (Polle et al. 2019). The works done so far, have highlighted the conservative water use strategy of strawberry tree, an isohydric resprouter with a tight stomatal control and able to maintain a low gas exchange rate, useful during long drought stress periods (Martins et al. 2019, 2021b). However, the resistance mechanism is not yet fully understood, and a more detailed analysis is aimed. Drought stress triggers downstream pathways that involves plant hormones like abscisic, jasmonic and salicylic acids (Khan et al. 2015; Vishwakarma et al. 2017; Yang et al. 2019) and initiates the biosynthesis of different types of protective secondary metabolites like phenols, flavonoids and proline (Hayat et al. 2012; Sarker and Oba 2018). These responses minimize adverse effects of drought by reducing water loss and

oxidative stress (Fàbregas and Fernie 2019; Yadav et al. 2021). Thus, the comprehension of metabolic pathways leading to the synthesis of water stress-related compounds is crucial for helping to understand the physiological mechanisms underlying drought resistance. Moreover, metabolomic data can also be used as a phenotype predictor of plant performance under drought stress, which would considerably reduce the time required in breeding programmes (Sprenger et al. 2018). Furthermore, the recover ability of evergreen plants after water stress is extremely important, in order to compensate the lower carbon assimilation during the dry season (Baldocchi et al. 2010). Thus, in this work, metabolic profiles of *A. unedo* plants under water deficit conditions and during plant recovery, originated from different geographic regions were sampled to study the influence of plant provenance on their predisposition to cope with drought and ability to recover. Furthermore, the contribution of the genotype on plant performance was also assessed since a previous work showed that a component of the high phenotypic plasticity of *A. unedo* under water is in part under the control of genetic traits (Martins et al. 2021b).

Material and Methods

Plant material

Four genotypes from different provenances were selected: A1 and A3 from the centre region of Portugal, from an area with a high (>1000 mm) and a medium (500-1000 mm) average rainfall, respectively, and A2 and A4 from the south region, both from an area with a low (<500 mm) average rainfall (Figure 32 A). Genotype A1 was established *in vitro* from an adult tree, whereas genotypes A2, A3 and A4 from seedlings, as described by (Martins et al. 2019). For axillary shoot proliferation shoots were inoculated in Anderson Rhododendron medium (Anderson 1980) with 6-benzylaminopurine (2 mg L^{-1}, Sigma-Aldrich, St. Louis, MO, USA), sucrose (3%, w/v, Duchefa) and agar (0.6%, w/v, Duchefa). The pH was adjusted to 5.7 using KOH or HCl diluted solutions (0.01 M – 1 M), and the culture medium was autoclaved at 121 ºC for 20 min (800–1100 g cm^{-2} gel strength after autoclaving). Culture was done on plastic containers (O118/80+OD118 with white filter, Microbox, Deinze, Belgium) with 100 mL of media, on a growth chamber at a 16-h photoperiod, an irradiance of 15 - 20 µmol m^{-2} s^{-1} (cool-white fluorescent lamps), and a temperature of 25 ºC, with culture intervals of 8 weeks.

For rooting, 3 cm long shoots were dipped on indole-3-butyric acid (IBA; 1 gr L^{-1}, Sigma-Aldrich) for 30 seconds and placed on covered containers with perlite (Siro, Mira, Portugal), on a walk-in growth chamber (FitoClima 10000 HP, Aralab, Rio de Mouro, Portugal) under 16-h photoperiod at 25 ºC, 70% humidity and 250 µmol m^{-2} s^{-1} irradiance). The cover was gradually removed and after a month, plants were transferred to individual containers (1700 cm^3) with a substrate composed of peat (30-0; Siro) and perlite (3:1; v/v). Plants were kept under these conditions for two years, watered to 70-80% field capacity. A fertilizer (NPK, 12-12-17; Siro) was applied after one year.

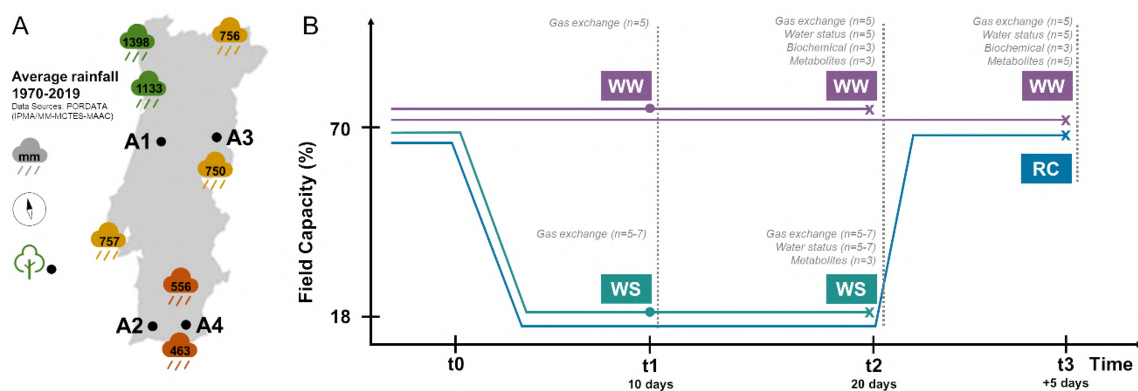

Figure 32. Average rainfall (mm) from 1970 to 2019 in seven meteorological stations across continental Portugal and location of the four mother plants used for micropropagation (A) and water irrigation conditions used on the experiment (B).

Experimental design: Drought stress and recovery assay

Two-year-old plants kept on the conditions described before were placed under three water regimes using the gravimetric method: WW – well watered (watered to 70% field capacity), WS – water stress (watered to 18% field capacity) and RC – recover (watered to 18% and then to 70% field capacity) (Figure 32 B). Three sampling points were setup: t1 (10 days) and t2 (20 days) for WS groups and t3 (20 days with 18% field capacity plus 5 days with 70%) for RC groups. A WW group (control) was also sampled at each time point. Plant performance was evaluated on the four genotypes based on physiological (gas exchange and water status), biochemical (phenols, ortho-phenols, flavonoids, proline, chlorophyll a and b, carotenoids and anthocyanins) and metabolomic parameters (untargeted and targeted). Five plants from each of the four genotypes were sampled for WW and RC groups and 6-7 plants for WS group at each sampling point (t1, t2 and t3). For biochemical and metabolomic analysis, apical leaves were collected and flash-frozen in liquid nitrogen to rapidly arrest metabolic activity, ground in liquid nitrogen with a mortar and pestle and transferred to 2 mL microtubes. Samples for metabolomic analysis were freeze-dried for 48 h (Coolsafe, Labogene, Lynge, Denmark). Biochemical analysis was carried out at t2 (n=3) and t3 (n=3). Untargeted metabolomic analysis was carried out at t2 (n=3) and t3 (n=5). Targeted metabolomic analysis (abscisic, jasmonic and salicylic acids quantification) was also carried out at t2 (n=3) and t3 (n=3) but only with genotypes A1 and A4, that were selected based on the physiological and untargeted metabolomic data due an antagonic performance.

Gas exchange and water status

In situ leaf gas exchange measurements (net CO_2 assimilation rate: *A*, transpiration rate: *E*, stomatal conductance: gs, and the intercellular CO_2 concentration: *ci*) were performed using a portable infrared gas analyser (*LCpro+, ADC*, Hoddesdon, UK), operating in open mode and under the following conditions: photosynthetic photon flux density: 350 µmol m^{-2} s^{-1}; air flux: 200 mol s^{-1}; block temperature: 25ºC; and atmospheric CO_2 and H_2O concentration. Data were recorded when

the measured parameters were stable (2–6 min). Water potential (Ψ) was measured in the stems with a Scholander-type pressure chamber (PMS Instrument Co., Albany, OR, USA).

Biochemical analysis

Phenols

Total phenols in the extracts was estimated according to (Attard 2013). Briefly, 1.5 mL methanol (70%, v/v) was added to 40 mg of frozen plant material. Samples were kept on an orbital shaker at 700 rpm, 25 °C for 1 hour and centrifuged for 15 min, 10000 *g,* 4 °C. The supernatant was collected and the extraction was repeated thrice. The final volume was adjusted to 10 mL with methanol (70%, v/v). For quantification, 20 µL of sample was mixed with 90 µL distilled water, 10 µL Folin-Ciocalteau reagent solution and 80 µL sodium carbonate (7%, w/v). After 2-hour incubation in the dark, the absorbance was read at 520 nm in a microplate reader. The concentration of phenols was determined as gallic acid equivalents from a standard curve (0-250 µg, y = 5.2429x + 0.0541, R^2 = 0.9998).

Ortho-Phenols

Ortho-phenols were quantified according to (Maestro Durán et al. 1991). Methanolic extracts were prepared as described before. Quantification was done by mixing 160 µL of sample with 40 µL sodium molybdate (5%, w/v). After 15 min incubating in the dark the absorbance was read at 370 nm in a microplate reader. Ortho-phenols concentration was determined as equivalents of gallic acid from a standard curve (0-250 µg, y = 5.6032x + 0.0503, R^2 = 0.9999).

Flavonoids

Total flavonoids were estimated according to (Zhishen et al. 1999). Methanolic extracts were prepared as described before and quantification was done by mixing 60 µL of sample with 28 µL sodium nitrite (5%, w/v). After 6 min incubating in the dark, 28 µL aluminium chloride (10%, w/v) was added and samples were incubated in the dark for 6 min. Finally, 120 µL sodium hydroxide (4%, w/v) was added to the mixture and absorbance was read at 510 nm in a microplate reader. The concentration of flavonoids was determined as equivalents of catechin from a standard curve (0-125 µg, y = 8.8685x + 0.0545, R^2 = 0.9997).

Proline

Total proline in the extracts was estimated according to (Bates et al. 1973). Briefly, 750 µL of sulfosalicylic acid (3%, v/v) was added to 50 mg of frozen plant material and centrifuged for 10 min, 10000 *g,* 4 °C. 500 µL of supernatant was collected and 500 µL of ninhydrin and glacial acetic acid were added. Samples were then incubated at 100 °C for 1 hour and cooled on ice. Finally, the reaction mixture was extracted with toluene and the absorbance of the chromophore-containing toluene was read at 520 nm in a microplate reader. Proline concentration was determined from a L-Proline standard curve (0-15 µg, y = 0.0799x + 0.0056, R^2 = 0.9998).

Chlorophyll and carotenoids

Total chlorophyll and carotenoids in the extracts was estimated according to (Sims and Gamon 2002). 50 mg of frozen plant material was ground in 2 mL of Acetone:Tris 50mM (80:20) buffer pH 7.8 and centrifuged for 5 min (10000 *g*, 4 °C). Supernatant was collected and extraction was repeated with 3 mL Acetone:Tris. Finally, Acetone:Tris was added to the supernatants to obtain a final volume of 6 mL. Samples were kept on ice and protected from light during the entire process. The absorbance of supernatants was read at 470nm, 537nm, 647nm and 663nm on a UV-Vis spectrophotometer. Acetone:Tris 50mM (80:20) buffer pH 7.8 was used as blank. Chlorophyll *a* (Chla), Chlorophyll *b* (Chlb) and Carotenoids (Car) contents were calculated according to the following equations: Chla = 0,01373 * A663 – 0,000897 * A537 – 0,003046 * A647; Chlb = 0,02405 * A647 – 0,004305 * A537 –0,005507 * A663; Car = ((A470 – (17,1 * (Chla + Chlb) – 9,479 * Anthocyanins)) / 119,26 and Anthocyanins = 0,08173 * A537 – 0,00697 * A647 – 0,002228 * A663.

Anthocyanins

Total anthocyanins was estimated following the protocol of (Close et al. 2004), with minor adaptations. 50 mg of frozen plant material was ground in 2 mL of acidified ethanol (ethanol:HCl, 99:1, v/v). The homogenate was immersed in boiling water for 90 seconds, and kept in the dark for 24 hours at 4 °C. After centrifugation (10000 *g*, 10 min, at 4 °C), absorbance was measured using a UV-Vis spectrophotometer at 530 and 657 nm. The formula A530 – 0.25 * A657 was used to calculate anthocyanins content.

Metabolomic analysis

Untargeted metabolomic profiling

Ten milligrams of each replicated sample were weighed into 1.1 mL-micronic tubes (MP32033L, Micronic, Lelystad, Netherlands), randomised onto a 96-micronic rack (MPW51001BC6, Micronic) then capped using a robotised capper–decapper (Decapper 193000/00, Hamilton, Bienne, Switzerland). Each rack also contained an empty tube corresponding to the extraction blank. The resulting micronics were then stored at −80 °C. Metabolites extraction was conducted on three or four biologically replicated leaf samples (n = 3-4) using a robotized extraction method developed at Bordeaux Metabolome Facility (https://metabolome.cgfb.u-bordeaux.fr/en, Villenave d’Ornon, France) as described by (Luna et al. 2020).

Untargeted metabolic profiling by UHPLC-LTQ-Orbitrap mass spectrometry (LCMS) was conducted using an Ultimate 3000 ultra-high-pressure liquid chromatography (UHPLC) system coupled to an LTQ-Orbitrap Elite mass spectrometer interfaced with an electrospray (ESI) ionisation source (ThermoScientific, Bremen, Germany) operating in negative ion mode as described previously (Luna et al. 2020). MS1 full scan detection of ions was performed by FTMS (50 - 1500 Da) at a resolution of 240k at 200 *m/z*. Ten QC samples and five blank extracts were

injected to correct for mass spectrometer signal drift, and to filter out variables detected in blanks, respectively. MS2 Data Dependent Analysis (DDA) was also performed on QC sample to generate fragmentation information for further annotation with the following parameters: FTMS (50 - 1500 Da) at a resolution of 60k at 200 *m/z*; activation type, CID; isolation width, 1 Da; normalized collision energy, 35 eV; activation Q, 0.250; activation time, 10 ms). Phytohormone standards were also injected along with sample extracts for annotation purposes.

Raw LCMS data were processed using MS-DIAL v 4.60 (Tsugawa et al. 2015), yielding 12 282 RT-*m/z* features. MS-DIAL parameters were as follows: MS1, tolerance, 0.01 Da; MS2 tolerance, 0.025 Da; retention time begin, 0 min; retention time end, 18 min; minimum peak height, 10000; mass slice width, 0.1 Da; smoothing level, 3 scans; minimum peak width, 5 scans; sigma window value, 0.5. After data-cleaning (blank check, SN > 10, CV QC < 30%), 3953 variables were retained for further chemometrics. MS-DIAL annotation of metabolic features was performed using the online library MSMS-Public-Neg-VS15.msp (36,848 records) with the following parameters: retention time tolerance, 100 min; accurate mass tolerance (MS1), 0.01 Da; accurate mass tolerance (MS2), 0.05 Da; identification score cut off, 80%. Putative annotation of differentially expressed metabolites resulted from MS-DIAL screening of the MS1 detected exact HR *m/z* and MS2 fragmentation patterns against multiple online databases (http://prime.psc.riken.jp/compms/msdial/main.html#MSP) (Tsugawa et al. 2015).

Targeted metabolomic analysis (hormone quantification)

Leaf content of abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) from Au01 and Au04 genotypes (WW, WS and RC) were analysed by UPLC MS/MS (ultra-performance liquid chromatography, tandem mass spectrometry) as described by (Durgbanshi et al. 2005) with slight modifications. For extraction, 2 mL of ultrapure water were added to 0.1 g of lyophilized leaf material and blended in a ball mill (MillMix20, Domel, Železniki, Slovenija). Water was spiked with 50 ng of [$^{2}H_{6}$]-ABA, [$^{13}C_{6}$]-SA and dihydrojasmonic acid. After centrifugation at 4000 g at 4 °C for 10 min, supernatants were recovered and pH adjusted to 3 with acetic acid (30%, v/v). All water extracts were partitioned twice against 2 mL of diethyl-ether and the organic layer was recovered and evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France). The dried residue was resuspended in water: methanol (9:1, v/v) by gentle sonication. The resulting solution was filtered through a 0.22 µm polytetrafluoroethylene membrane syringe filter (Albet S.A., Barcelona, Spain) and injected into an ultra-performance liquid chromatography system (Acquity SDS, Waters Corp., Milford, MA, USA). Chromatographic separations were carried out on a reversed-phase C18 column (Luna, Omega, 50 × 2.1 mm, 1.6 µm particle size, Phenomenex, Madrid, Spain) using a methanol:water gradient (both supplemented with acetic acid (0.1%, v/v)) at a flow rate of 300 µL min^{-1}. Hormones were quantified with a triple quadrupole mass spectrometer (Micromass, Manchester, UK) connected online to the output of the column though an orthogonal Z-spray electrospray ion source.

Statistical analysis

To compare genotypes under different water conditions, data were analysed by two-way ANOVA using GraphPad Prism (v. 8.4.3 for Windows, San Diego, CA, USA), followed by a Tukey´s multiple comparison test ($P < 0.05$). Values are given as means ± standard deviations. Principal Component Analysis (PCA) and heatmaps were constructed using R software (version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria, (R Core Team 2020), to evaluate the interaction and significance of the biochemical and physiological parameters on the analysed genotypes. The PCA was performed using the prcomp function and the package ggbiplot (Vu 2011). The heatmap was constructed with the Heatmap function and the package ComplexHeatmap (Gu et al. 2016). The dendrogram within the heatmap was calculated with Euclidean distance as dissimilarity measure. Metabolomic data was normalized by median normalization, cube-root transformation and Pareto scaling using MetaboAnalyst v. 5.0 (Pang et al. 2021), before statistical analyses. PCA and PLS-DA were performed with MetaboAnalyst providing satisfactory validation parameters of the multivariate models ($R2 > 0.89$ and $Q2 > 0.28$). Heatmaps were constructed with the most significant different metabolites (one-way Anova, $P < 0.01$). Volcano plots were used to identify statistically significant variation on metabolites between control (WW) and treatment (WS and RC) groups ($FC > 2$, $P < 0.05$). To check for shared metabolites between genotypes, Venn diagrams were constructed with R software using the package VennDiagram (Chen 2018). Finally, linear regression models were calculated using R software, to predict net CO_2 assimilation rates with metabolites concentration. Cook's distance was calculated to remove outliers, and Jarque-Bera and Durbin-Watson tests were performed to test the normality and independence of residuals, respectively. A multi regression analysis was done with the three metabolites with higher R^2: quercetin, galloyl hyperin and guajavarin (quercetin-3-O-arabinoside).

Results

T1 (water stress)

At this point, apart from a slight wilting, no other symptoms caused by drought were observed on plants. The wilting signs were more evident on plants from genotype A1, and barely visible on genotype A4.

Physiological analysis

Net CO_2 assimilation rate decreased on plants under water stress after 10 days (t1), with statistically significant differences on genotypes A1 and A2 (Figure 33 A). Although a considerable reduction was observed on genotype A3, no statistical differences between the control and stress groups were found. Finally, only a slightly decrease was observed on genotype A4, without statistical differences. Moreover, plants under stress from genotype A4 had higher rates of net CO_2 assimilation than those from genotypes A1 and A2 under stress (Figure 33 A). A decreased on

stomatal conductance and transpiration rate was also observed with statistical differences on genotypes A1, A2 and A3 (Figure 33 B-C). Nevertheless, no statistical differences were found on intercellular CO_2 concentration between well-watered (WW) and water stress (WS) groups, on the four genotypes tested (Figure 33 D).

This result can be observed on a heatmap, where group A4WS has been clustered with control groups (Figure 33 E). A PCA analysis also revealed a well-defined cluster with the control groups and a heterogenous distribution of the water stress groups (Figure 33 F), according to the genotype. Whereas samples from genotype A4 under stress (A4WS) are close to the respective control group, some individuals under water stress are distant from the control groups. This difference is more evident on genotype A1 under water stress (A1WS). Principal component 1 (PC1) contributes with 90.1% to the total variance, and A, gs and E are the parameters with a higher contribution on this component.

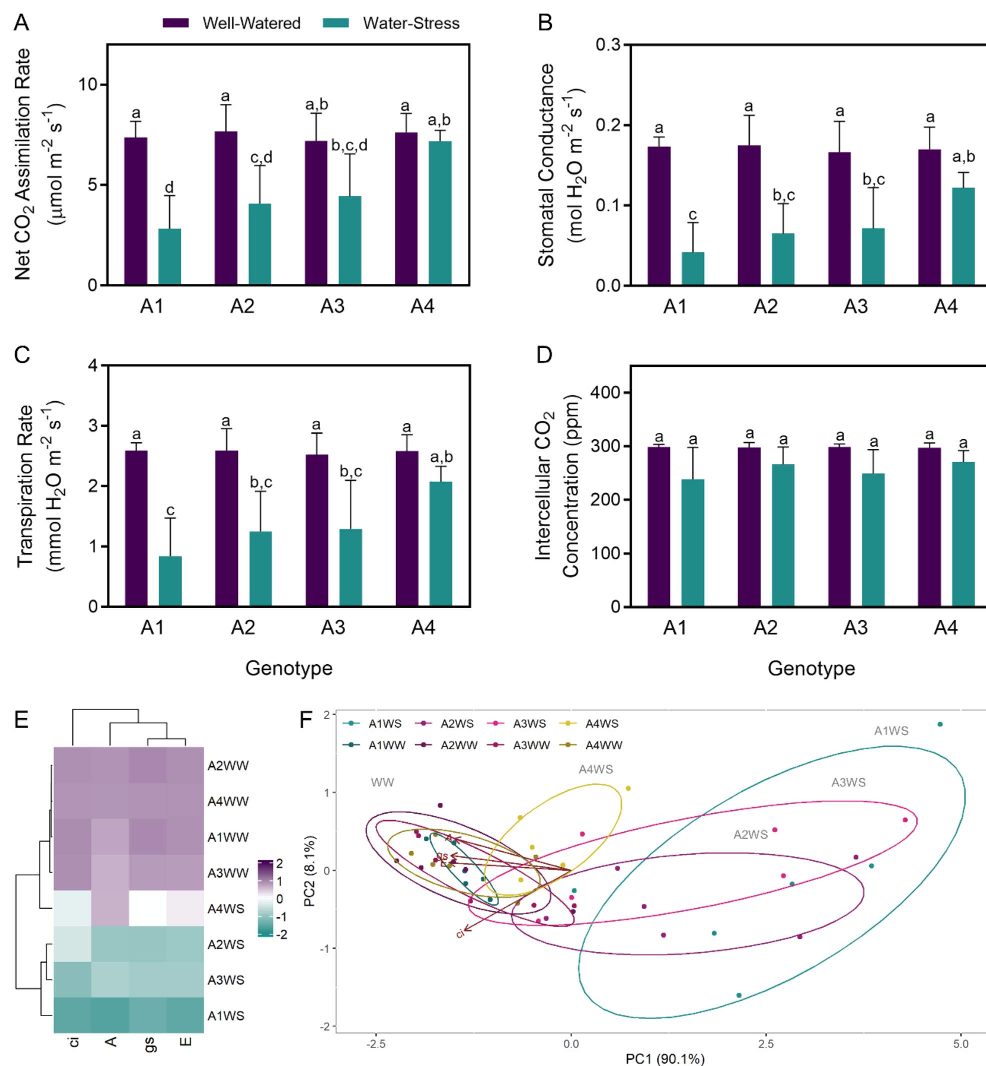

Figure 33. Physiological parameters measured on t1 (10 days) on four genotypes (A1, A2, A3 and A4) and two water regimes (well-watered and water-stress): Net CO_2 assimilation rate (A), stomatal conductance (B), transpiration rate (C), intercellular CO_2 concentration (D), heatmap (E) and principal component analysis (F). Means ± SDs, n = 7, different letters indicate significant differences between treatments at $P \leq 0.05$.

T2 (water stress)

On t2, wilting symptoms intensified on plants under drought from all genotypes, especially on genotype A1. Changes in the leaf pigmentation were also observed, in particular on genotype A1, as leaves turn from deep green to orange-red. No change in colour was observed on genotype A4. Bud development ceased on all genotypes.

Physiological and biochemical analysis

As it has been observed after t1, net CO_2 assimilation rate was lower on plants under stress after 20 days (t2). Similarly, no statistical differences were observed on genotypes A3 and A4 between control and stress groups (Figure 34 A). Moreover, stomatal conductance considerably decreased in plants under water restrictions, with statistical difference between WW and WS groups, in the four genotypes (Figure 34 B), as well as transpiration rates (Figure 34 C). The only exception was intercellular CO_2 concentration and water potential on genotype A2 (Figure 34 D-E). Additionally, no differences were found on plant height (Figure 34 F).

In general, no differences were found on phenols, ortho-phenols, flavonoids and proline between WW and WS groups (Figure S2 A-D, available at: http://phd.freecluster.eu/). A statistical difference was only found on genotype A4, with lower amounts of phenols on plants under stress (Figure S2 A, available at: http://phd.freecluster.eu/). Although a slight reduction was observed on the levels of chlorophyll a and b and carotenoids on plants under water stress, no statistical differences were found on all the samples (Figure S2 E-G, available at: http://phd.freecluster.eu/). On the other hand, the levels of anthocyanins increased in plants under water stress from genotypes A1 and A2, whereas a decrease was observed on genotype A4 (Figure S2 H, available at: http://phd.freecluster.eu/). Nonetheless, no statistical differences were obtained between WW and WS groups on all genotypes. This pattern can be cleared observed on a heatmap, especially due to physiological parameters (Figure 34 G). Moreover, samples are clustered according to treatments. The scores plot between PC1 and PC2 also confirm this result by revealing two distinct groups: control samples (WW) and drought samples (WS) from the four genotypes (Figure 34 H).

Metabolomics

The untargeted metabolomic analysis detected a total of 3953 peaks, of which 74 were identified as known metabolites, whereas the remaining peaks were unknown metabolites. The identified metabolites include abscisic acid, lipids, terpenes, methoxyphenols, flavonoids and flavonoid glycosides (Table S6, available at: http://phd.freecluster.eu/). A heatmap with the 1590 most significant metabolites ($p < 0.01$) revealed a specific metabolic profile of plants from genotype A4 under water stress (Figure 35 A). The scores plot of PC1 and PC2 also confirm this result by revealing two distinct groups: (i) A4WS samples and (ii) drought samples from the other four genotypes and all the control samples (Figure 35 B). Nonetheless, when analysed individually, samples from genotype are still clustered according to the water regime applied (Figure 35 C), and

a volcano plot (FC>2.0 and p-value<0.05) revealed the concentration of 334 metabolites were changed between WW and WS treatments (130 reduced and 204 increased) (Figure 35 D). As expected, the scores plot between PC1 and PC2 also showed two distinct groups for genotype A4 (A4WW and A4WS) (Figure 35 E). For this genotype, the concentration of 570 metabolites were changed between WW and WS treatments (382 reduced and 188 increased) (Figure 35 F, Table S7, available at: http://phd.freecluster.eu/). When the metabolites that were found to have statistically significant differences between treatments were compared between genotypes (A1 and A4), only a few were found to be common: 14 metabolites were reduced on both genotypes (A1WS and A4WS) and only 2 were increased on both (Figure 35 G-H). On the other hand, 116 and 368 metabolites were exclusively reduced on genotype A1 and A4 respectively, whereas 202 and 186 were found to be exclusively increased (Figure 35 G-H). On genotype A2, samples from control and stress groups were clustered relatively close together, and only two metabolites were found to be increased in the stress group (Figure S3 A-B, available at: http://phd.freecluster.eu/). A similar result was obtained for genotype A3, but no metabolites were found to be up- and/or down-regulated (Figure S3 C-D, available at: http://phd.freecluster.eu/).

The metabolites found to be differently expressed that were putatively identified, are mainly phenols and fatty acids. In genotype A1, 15 up-regulated metabolites were identified (abscisic acid 1, flavonoid glycosides 9 and flavonoids 5), whereas in genotype A4, only three up-regulated metabolites (flavonoids 1, lipids 1, methoxyphenols 1) and nine down-regulated (flavonoids 2, fatty acids 6, terpenes 1) were identified. Finally, two oxidized fatty acids that were down-regulated on both genotypes were identified (Figure 36 A). Some of the up-regulated metabolites in genotype A1 under stress are myricetin, quercetin and quercitrin (Fig. 5 B). In genotype A4, luteolin was also up-regulated, whereas a decrease α-linolenic acid and epicatechin was observed on plants under water deficit (Figure 36 B).

Although no statistical differences were detected, the amount of ABA increased on plants under stress on both genotypes tested (Figure 36 C). The amount of abscisic acid ranged from 4.5 ± 0.7 to 13.0 ± 0.7 on genotype A1 between WW and WS groups, and from 15.8 ± 3.7 to 28.3 ± 11.3 on genotype A4. Although the amount of ABA increases on both genotypes, no statistically significant differences was found on genotype A4. The total amount of jasmonic acid increased from 2.1 ± 0.2 to 3.4 ± 0.3 on genotype A4 between tested groups, and no differences were observed on genotype A1 (Fig. 5 C). Finally, although no statistical difference was obtained, the variation of salicylic acid on plants under stress was similar to those of ABA, with an increase on plants under water deficit (Figure 36 C).

From the up-regulated and/or down-regulated and identified metabolites, 14 were tested for the construction of general linear models. 10 of these metabolites provided a general linear model with a p value below 0.05 (Figure S4 A, available at: http://phd.freecluster.eu/). However, a R^2 above 0.7 ($p < 0.0001$) was only obtained with three metabolites quercetin, galloyl hyperin and

guajavarin (quercetin-3-O-arabinoside). A model with a satisfactory adjusted R^2 was also obtained when a multiple regression was calculated with the three metabolites mentioned previously. Nonetheless, the p-value of the three variables was higher than 0.05 (0.381, 0.621 and 0.422). The normality and independence of the residuals was confirmed in the four models by a Q-Q plot, a distribution histogram and by the Jarque-Bera and Durbin-Watson tests (Figure S4 B-E, available at: http://phd.freecluster.eu/).

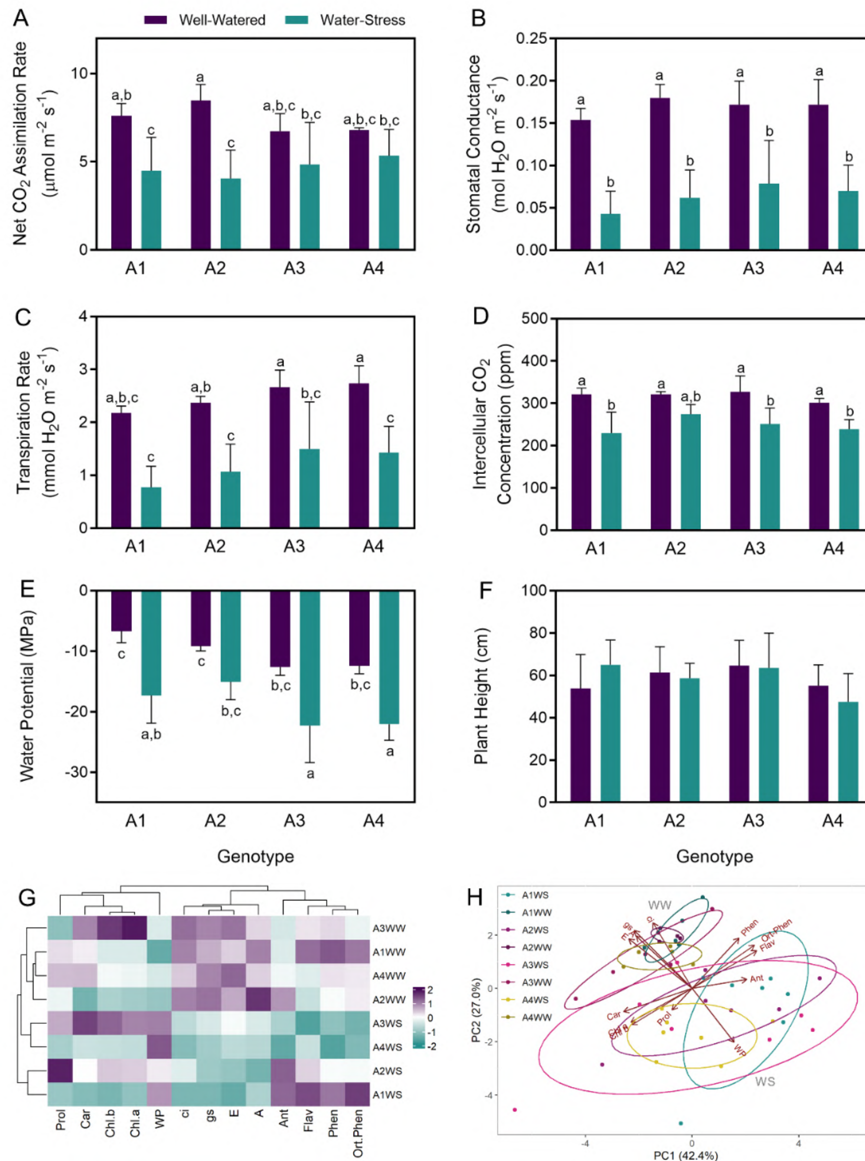

Figure 34. Physiological parameters measured on t2 (20 days) on four genotypes (A1, A2, A3 and A4) and two water regimes (well-watered and water-stress): Net CO_2 assimilation rate (A), stomatal conductance (B), transpiration rate (C), intercellular CO_2 concentration (D), water potential (E), plant height (F), heatmap with physiological and biochemical parameters (G) and principal component analysis with physiological and biochemical parameters (H). Means ± SDs, n = 7, different letters indicate significant differences between treatments at $P \leq 0.05$. A - net CO_2 assimilation rate, Ant - anthocyanins, Car - carotenoids, Chl.a - chlorophyll a, Chl.b - chlorophyll b, ci - intercellular CO_2 concentration, E - transpiration rate, Flav - flavonoids, gs - stomatal conductance, Phen - phenols, Prol - proline, Ortho.Phen - ortho-phenols, WP - water potential.

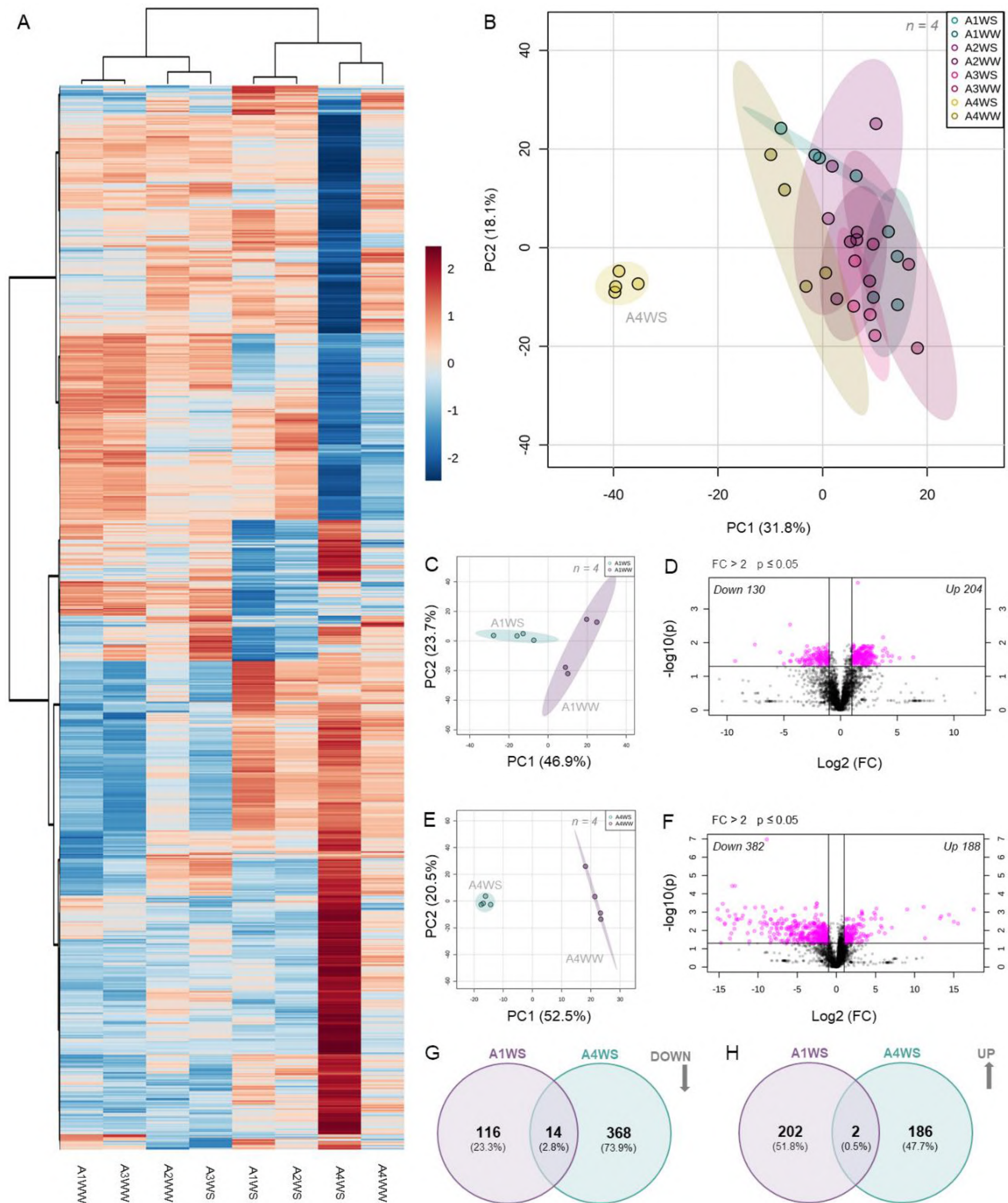

Figure 35. Untargeted metabolomic analysis on t2 (20 days under stress): heat map representing top 500 metabolites significantly different ($p < 0.01$) between groups (group is indicated at the bottom of the figure) (A), principal component analysis with all the metabolites (B), principal component analysis for genotype A1 (C), volcano plot for genotype A1 indicating significantly ($p < 0.05$ and FC > 2) up- and down-regulated metabolites on water stress group when compared to well-water group (D), principal component analysis for genotype A4 (E), volcano plot for genotype A4 indicating significantly ($p < 0.05$ and FC > 2) up- and down-regulated metabolites on water stress group when compared to well-water group (F), Venn diagram with the down-regulated metabolites shared between A1 and A4 genotypes under water stress (G), Venn diagram with the up-regulated metabolites shared between A1 and A4 genotypes under water stress (H). Data was normalized by median, cube root transformed and Pareto-scaled (n = 3).

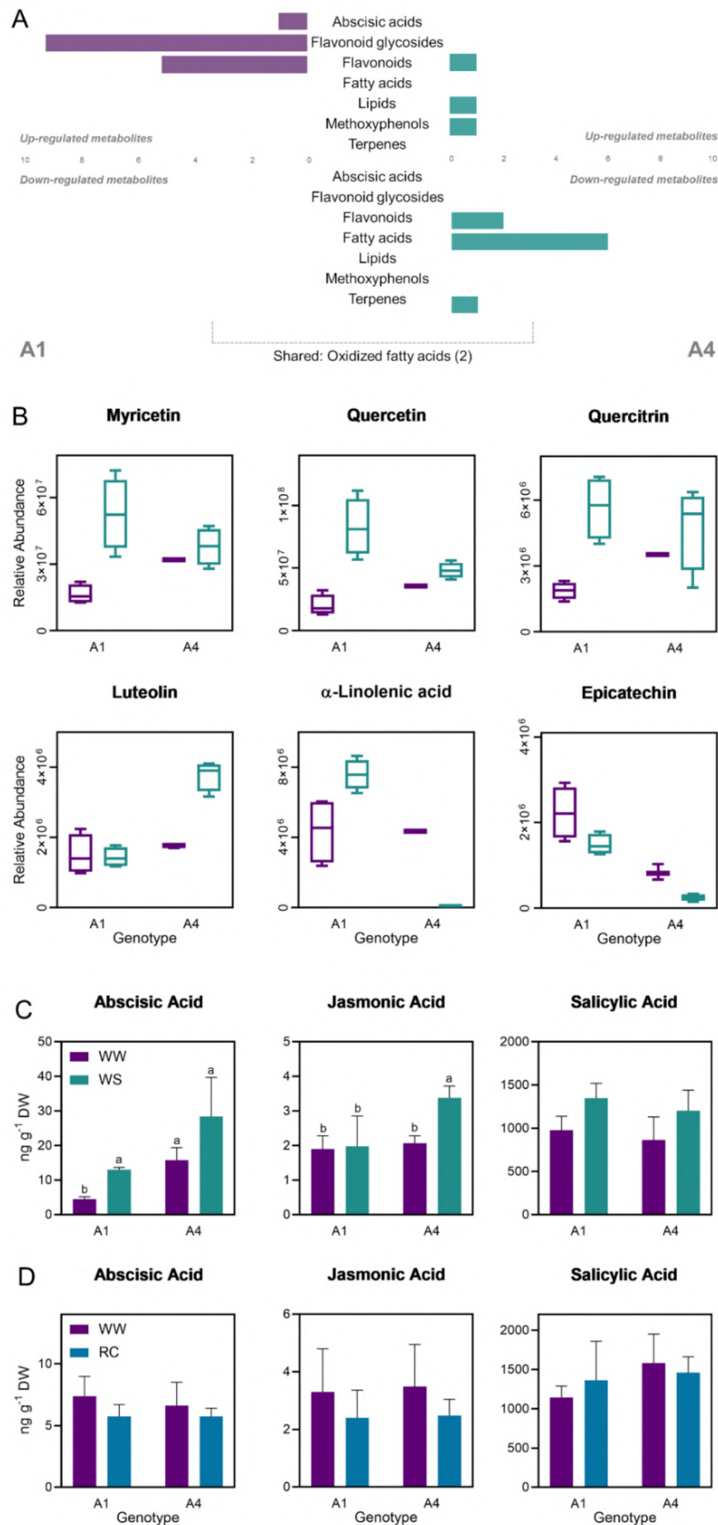

Figure 36. Up- and down-regulated metabolites on water stress groups from A1 and A4 genotypes on t2 (20 days) (A), relative abundance of myricetin, quercetin, quercitrin, luteolin, α-linolenic acid and epicatechin on well-watered and water stress groups from A1 and A4 genotypes on t2 (20 days) (B), abscisic acid, jasmonic acid and salicylic acid on well-watered and water stress groups from A1 and A4 genotypes on t2 (20 days) (C), abscisic acid, jasmonic acid and salicylic acid on well-watered and recover groups from A1 and A4 genotypes on t3 (20 days plus 5 days under recover) (D). Means ± SDs, n = 4, different letters indicate significant differences between treatments at $P \leq 0.05$.

T3 (recover)

When normal irrigation was resumed (*i.e.*, to 70% field capacity), wilting symptoms completely disappeared on all genotypes after a few hours. The orange-red colour has also intensified on plants from genotype A1 that have been exposed to drought.

Physiological and biochemical analysis

On the recovered plants, all the physiological and biochemical parameters were very similar when compared to the control group on the four genotypes tested (Figure 37 A-F and Figure S5 A-G, available at: http://phd.freecluster.eu/). The only exception was the higher amounts of anthocyanins on recovered plants from genotype A1 and lower on genotype A3, with statistical differences (Figure S5 H, available at: http://phd.freecluster.eu/). Although no statistical differences were observed, the levels of proline, chlorophyll a and b and carotenoids were lower on recovered plants from genotype A1. These results were confirmed by a heatmap (Figure 37 G) where genotype A1 is grouped on a separate cluster. The PCA (Figure 37 H) shows three distinct groups: (1) control and recovered plants from genotypes A2, A3 and A4, (2) control plants from genotype A1, and (3) recovered plants from genotype A1, revealing a distinct behaviour of recovered plants from genotype A1.

Metabolomics

Although differences were found between WW and RC groups in the concentration of some metabolites, as revealed by the heatmap (Figure 38 A, Table S8, available at: http://phd.freecluster.eu/), samples were clustered relatively close together on a PCA (Figure 38 B). Nonetheless, individual analysis by genotype showed a significant between groups in genotype A1, with a contribution of 48% and 16.3% of PC1 and PC2, respectively, to the variance (Figure 38 C). A volcano plot (FC > 2 and p-value ≤ 0.05) revealed the concentration of 350 metabolites were changed between WW and RC treatments (141 reduced and 209 increased) (Figure 38 D). On the other hand, samples from genotype A4 were clustered together on a PCA (Figure 38 E) and the volcano showed only one metabolite were up-regulated with statistical significance (Figure 38 F). A similar result was obtained for genotypes A2 and A3, with no metabolites found to have different concentrations (Fig. S3 E-H, available at: http://phd.freecluster.eu/). When the metabolites from genotype A1, that were found to have statistically significant differences between WW and RC treatments, were compared with WS treatment, only a few were found to be commonly down-regulated (22). Nevertheless, the common up-regulated metabolite was considerably higher (89) (Figure 38 G-H, Table S9, available at: http://phd.freecluster.eu/). Although a slight reduction of ABA and JA was observed on the recover plants (RC) from both genotypes A1 and A4, no statistical differences were obtained (Figure 36 D) and no differences were observed as well in the amount of salicylic acid in the recover group (Figure 36 D).

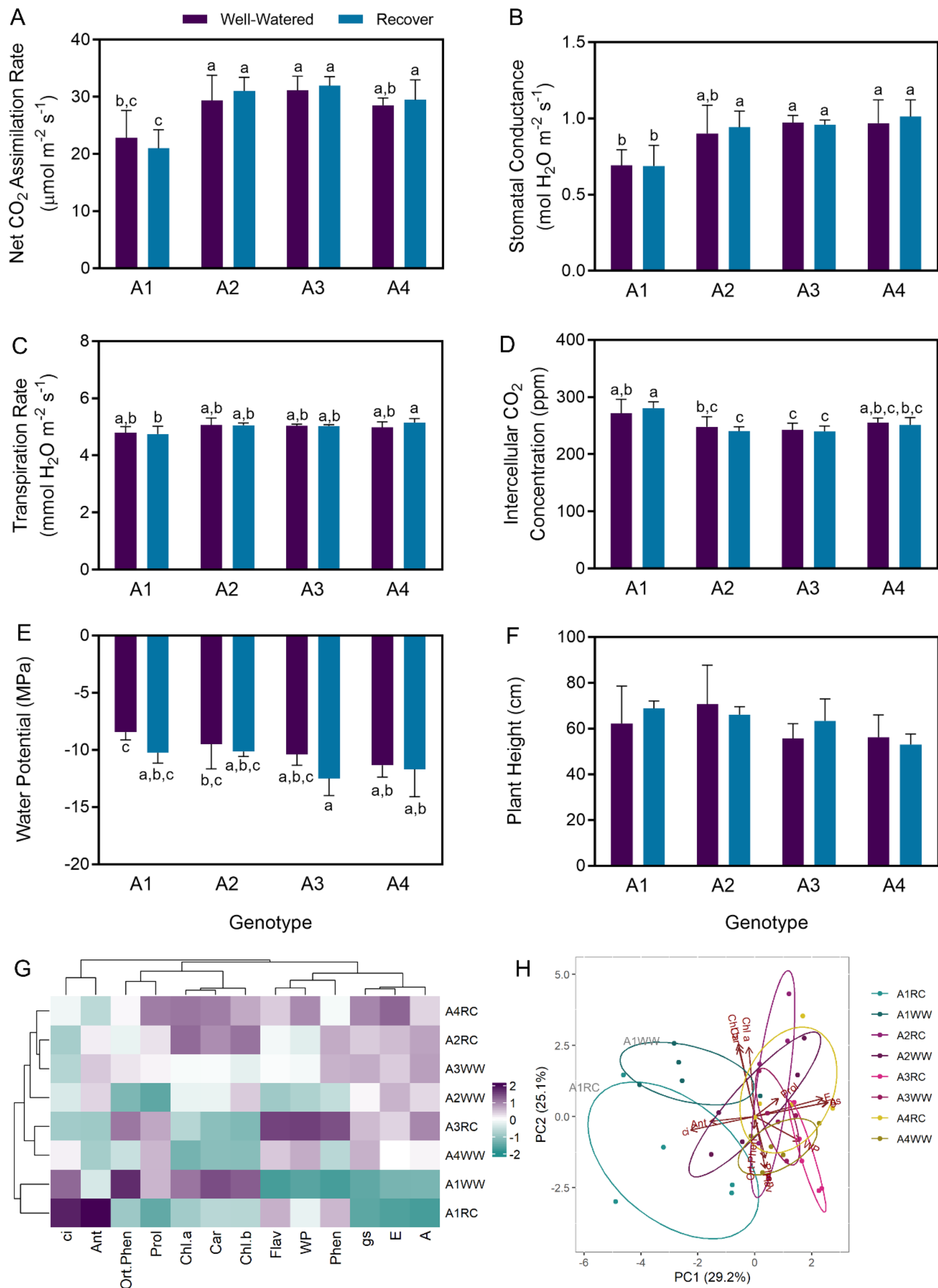

Figure 37. Physiological parameters measured on t3 (20 days stress plus five days under recover) on four genotypes (A1, A2, A3 and A4) and two water regimes (well-watered and recover): Net CO_2 assimilation rate (A), stomatal conductance (B), transpiration rate (C), intercellular CO_2 concentration (D), water potential (E), plant height (F), heatmap with physiological and biochemical parameters (G) and principal component analysis with physiological and biochemical parameters (H). Means ± SDs, n = 7, different letters indicate significant differences between treatments at $P \leq 0.05$. A - net CO_2 assimilation rate, Ant - anthocyanins, Car - carotenoids, Chl.a - chlorophyll a, Chl.b - chlorophyll b, ci - intercellular CO_2 concentration, E - transpiration rate, Flav - flavonoids, gs - stomatal conductance, Phen - phenols, Prol - proline, Ortho.Phen - ortho-phenols, WP - water potential.

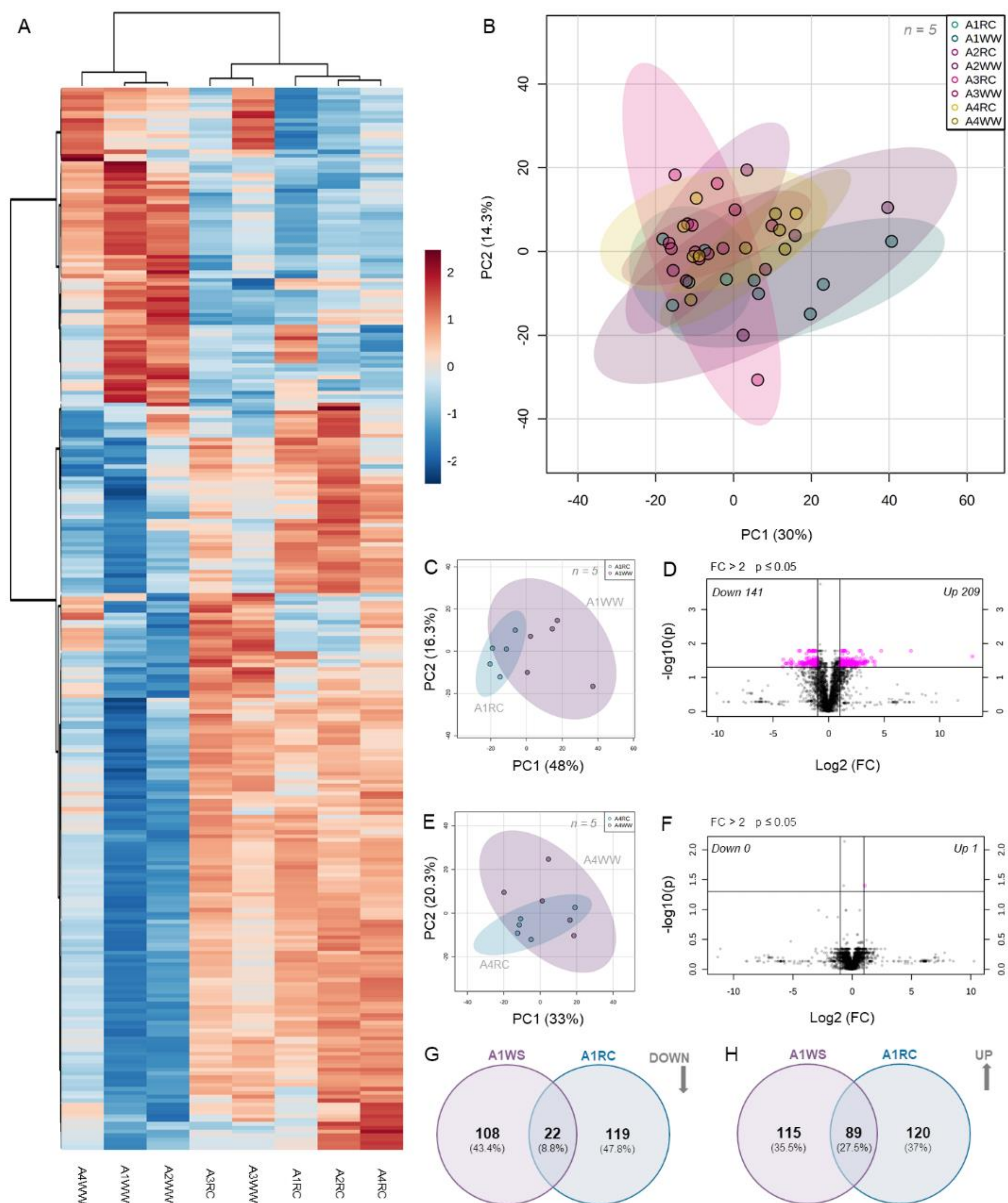

Figure 38. Untargeted metabolomic analysis on t3 (20 days stress plus 5 days recover): heat map representing top 500 metabolites significantly different ($p < 0.01$) between groups (group is indicated at the bottom of the figure) (A), principal component analysis with all the metabolites (B), principal component analysis for genotype A1 (C), volcano plot for genotype A1 indicating significantly ($p < 0.05$ and FC > 2) up- and down-regulated metabolites on recover group when compared to well-water group (D), principal component analysis for genotype A4 (E), volcano plot for genotype A4 indicating significantly ($p < 0.05$ and FC > 2) up- and down-regulated metabolites on recover group when compared to well-water group (F), Venn diagram with the down-regulated metabolites shared between recover and water-stress treatments on genotype A1 (G), Venn diagram with the up-regulated metabolites shared between recover and water-stress treatments on genotype A1 (H). Data was normalized by median, cube root transformed and Pareto-scaled (n = 5).

Discussion

In this study, the effects of water stress in plant performance were analysed at different levels, including photosynthetic and plant water status, as well as hormonal and metabolomic dynamics. Our results pinpoint a differential plant response related with sampling time and water regime depending on the genotype tested.

General response to water stress

In order to prevent embolism and hydraulic damage, resprouter species such as *A. unedo*, normally exhibit a conservative water use strategy accomplished by stomatal closure (Vilagrosa et al. 2014), which results in lower photosynthetic rates and CO_2 accumulation. The results presented in this analysis are in accordance with our previous studies (Martins et al. 2019, 2021b), and confirm this response pattern, *i.e.* lower stomatal conductance, transpiration and net CO_2 assimilation rates with higher intercellular CO_2 concentrations under water limitation scenarios (Figure 39).

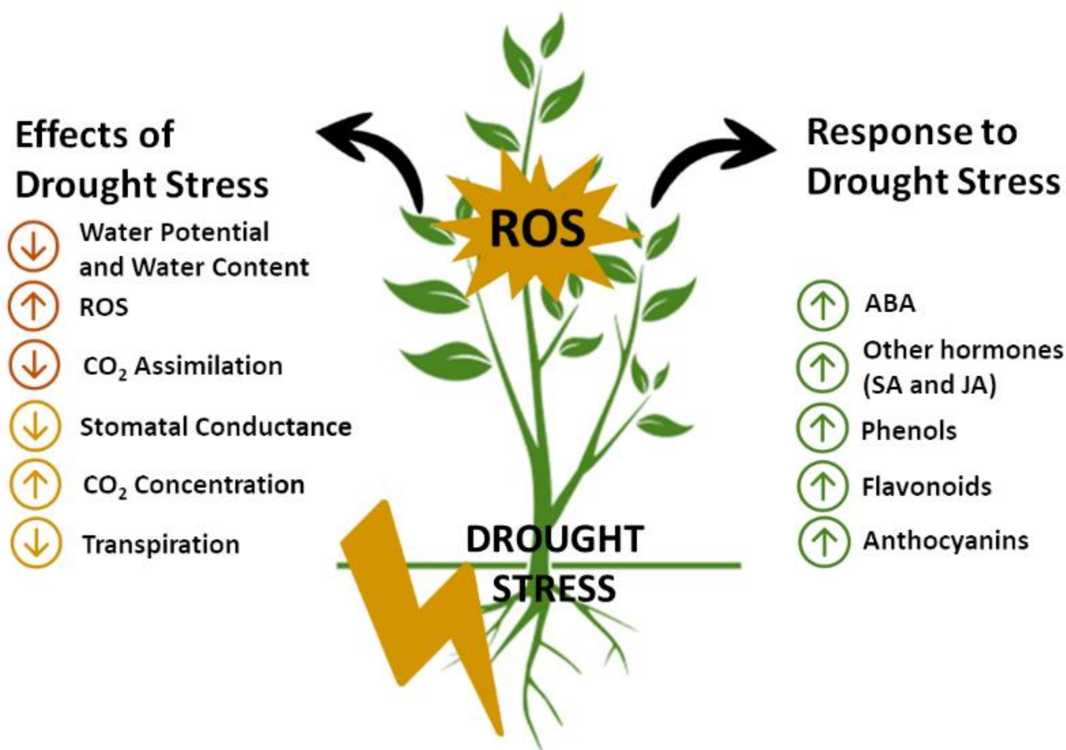

Figure 39. General response of *A. unedo* plants under drought.

Other mechanisms are usually involved on plant response to drought stress, including changes in several metabolites such as phenols and proline as well as plant hormones like abscisic, jasmonic and salicylic acids (Hayat et al. 2012; Khan et al. 2015; Vishwakarma et al. 2017; Sarker and Oba 2018; Yang et al. 2019), that might play an important role, among others, on ROS scavenging (Agati et al. 2012; Mittler 2017). However, the biochemical analyses carried out did

not seem to confirm this idea, as hardly any differences were observed in terms of phenols and proline concentrations between treatments. Though, a reduction in the level of chlorophyll a and b was observed in plants under water stress, which might be due to a lower biosynthesis and/or higher degradation rates. The reduction of chlorophyll levels on plants under drought have already been reported as a consequence of water limitation (Fahad et al. 2017). In contrast, an increase of chlorophyll and carotenoid levels in recovered plants from genotype A4 seems to indicate that its biosynthesis is up-regulated and that this genotype has a better recover ability. An increase was also observed in anthocyanins levels in genotype A1, but only in the recovering plants. This increase was expected, because anthocyanins have been related to an adaption mechanism of plants to stressful environments, through a reduction in leaf light perception, causing a stress-resistant phenotype (Cirillo et al. 2021). However, as the increase was only observed in the recover group, this may indicate a delay in this response mechanism and/or the involvement of such compounds in repairing rather than in tolerance mechanisms.

Although the preliminary biochemical analysis did not corroborate the involvement of metabolites such as phenols in the drought resistance mechanism of *A. unedo*, a more detailed analysis proved otherwise. In fact, several metabolites were up- and down-regulated especially in genotypes with a more contrast performance (*i.e.,* A1 and A4). In particular, genotype A4 presented a specific metabolic profile, which seems to correlate with its better ability to perform under water stress, at least at short-term, as shown by the physiological parameters. Alter metabolic levels have also been linked to different genotypes of *Cicer arietinum* (Khan et al. 2019) and *Sesamum indicum* (You et al. 2019).

Although only a few metabolites were putatively identified, they are essentially phenols (flavonoids, flavonoid glycosides and methoxyphenols), but also lipids and terpenes. Phenols such as myricetin, quercetin and quercitrin were up-regulated in genotype A1 under water stress. Interestingly, the basal levels (*i.e.*, in the control group) of these phenols in genotype A4 was considerably higher when compared to basal levels on genotype A1. These phenols have already been identified on strawberry tree (Martins et al. 2021a), and a variation of their concentrations along the year was observed, probably as a response to specific environmental conditions, which seems to support the involvement of these compounds on plant tolerance mechanisms to stress. Furthermore, on this previous study, chemotypes were identified, confirming the relevance of genotype on metabolomic plant response. Although not as well studied as primary metabolites, secondary metabolites such as phenols play an essential role on plant resistance to stress, an idea that is shared by several authors (Fàbregas and Fernie 2019; Yadav et al. 2021). Other important metabolites that were found to increase in concentration in response to drought conditions are hormones. As mentioned before, stomatal control in strawberry tree is highly regulated and essential for plant stress tolerance/avoidance under water limitation. Stomatal closure can be

accomplished through a hydraulic effect or via receptors activation in the guard cells by ABA. Although even small changes in ABA concentration in the apoplast are known to be enough for the stomata to close due to the high sensitivity of the guard cells (Gallé et al. 2013), a considerable increase in the ABA levels was observed in plants under stress from both genotypes. While a more marked increased occurred in genotype A1, which had the worst performance under water deficit, the basal levels of ABA in genotype A4 were considerably higher when compared to basal levels on genotype A1, as it has been observed in some phenols, and seems to indicate a predisposition of this genotype to cope with drought. Because this hormone is also involved on other important pathways, higher levels of ABA is a common response on plants under water deficit conditions. Its biosynthesis and allocation is usually promoted by ROS, or through other hormones like jasmonic acid (Kuromori et al. 2018; Yang et al. 2019), that also increased on genotype A4. Salicylic acid also plays an important role on several signal pathways, and is amount usually increases as part of the response of plants to stress. Although a slight increase was observed in our experiment, this difference was not statistical significant, possibly because on prolonged stress conditions there is usually a return to basal levels (Ali and Baek 2020). Furthermore, although the increase of salicylic acid proved to be essential on plant defence mechanisms under drought on several species, it has also been shown the accumulation of high amounts of this hormone might promote programmed cell death (Borsani et al. 2001; Miura and Tada 2014).

Genotype dependent response to water stress

Our data clearly shows plant performance under water stress is genotype dependent, which is in accordance with our previous study (Martins et al. 2021b). This was also observed in other species, such as *Eucalyptus globulus* (Correia et al. 2014) and *Populus* × *euramericana* (Monclus et al. 2006). Genotype A4 showed a better performance not only under water restriction but also recovering, which indicates a high phenotypic plasticity. Furthermore, as genotype with the worst performance (A1) is from a region with a high average rainfall, whereas genotype with the best performance (A4) is from an area with a low average rainfall, a correlation between provenience and performance seems to exist. In fact, a previous study was also able to correlate *A. unedo* plant provenience with performance under drought (Vasques et al. 2013), as seedlings from a driest region coped better with drought. A similar observation was made by (Matías et al. 2016) when *Abies alba* from different geographic locations was under controlled stress conditions. (Meijón et al. 2016) was even able to establish a connection between the metabolomic profile of *Pinus pinaster* genotypes and their region of origin. Nevertheless, in our study, this correlation was not found on all genotypes. Actually, genotype A2, which is from the region with a lower average rainfall, had a poor performance similar to genotype A1. This data, seems to indicate antioxidative response and resistance to drought is not always correlated with the strawberry tree plants biogeographic history, something that might be related to the high intra-specific diversity found by several authors on strawberry tree wild populations (Lopes et al. 2012; Gomes et al. 2013a). A similar observation has

been made by (Arend et al. 2011) on *Quercus* spp. populations. Thus, although provenience is certainly one of the aspects influencing plant performance under water deficit and should be take into account for plant selection, genotype seems to be the key determining factor. Interestingly, (Polle et al. 2019) have suggested that a more flexibly response to stress could be expected from trees rarely exposed to such conditions, which is something to take into account when plant selection is pursued.

On this work, although all genotypes recover their normal physiological performance when the irrigation was resumed, this may not be the case if a more prolonged stress occurs. In fact, under chronic stress, plants with an isohydric strategy usually suffer from cavitation and general degradation of the hydraulic system. Even tough strawberry tree has developed functional strategies, such as a deep root system, leaf shedding and even resprout ability, nefarious consequences can arise from prolonged stress events. On a natural environment, the genetic diversity might buffer the negative impacts on sensitive populations caused by extreme climate events, whereas the selection of the less sensitive genotypes would definitely minimize productions losses on orchards. On large natural populations such as those of *A. unedo*, whose characteristics are mostly unknown, plant selection usually represents a great deal of work. Thus, the development of prediction tools, like general linear models, are essential to facilitate this task. In this work, satisfactory general linear models were obtained with quercetin, galloyl hyperin and guajavarin (quercetin-3-O-arabinoside), that could be used as predictors of net CO_2 assimilation rates. Nonetheless, no satisfactory multiple regression models were obtained so far, which are essential when dealing with such complex data. Although the model obtained has a satisfactory adjusted R^2, the high p values of the variables indicate low model precision.

This study has shown that sensitivity to water stress is highly genotype dependent. The research has also shown that physiological control seems to be the primary response mechanism of *A. unedo* under water stress, with a trade-off between transpiration water loss and CO_2 assimilation. Furthermore, the recover ability is also genotype dependent, and in general, genotypes with a better performance under stress also have a great recover ability. The results of this investigation also show that plant provenience is an important factor, but genotype is factually the key factor governing plant ability to cope with drought. Finally, one of the more significant findings to emerge from this study is the relevance of metabolomic changes to tackle drought stress, which is clearly supported by the current findings. Overall, although a drought avoidance strategy is generally followed by strawberry tree plants, sensitivity to drought is highly genotype dependent, which may be related to downstream pathways that are activated as repairing mechanisms. In fact, two distinct mechanisms were observed: (1) highly drought sensitive and water conservative and (2) drought tolerant and less conservative. On the first case, plants close the stomata earlier to avoid water loses as they are not able to activate efficient repairing mechanism, whereas on the second mechanism, plants successfully activate a response by up-regulating signalling hormones that will activate a response cascade that includes the production of secondary metabolites essential for ROS scavenging and repairing mechanisms. Thus, plants are able to maintain stomata open for longer

periods maintaining normal CO_2 assimilation rates. Furthermore, the fast recover ability of plants following the second mechanism, also reveals a quick response mechanism that results in a high phenotypic plasticity. Our data seems to indicate that plants following this efficient strategy are predisposed to drought, as several metabolites were found in high concentrations on control groups. Besides the genotype influence, this might be putatively related to the plant provenance and a pre-conditioning.

The insights gained from this study are of particular interest to plant selection and breeding as they confirm previous data that pointed to a selection based on a single population, thus focused on the intra- rather than inter-specific diversity. Although these findings shed new light on strawberry tree drought tolerance mechanisms, some questions still remain to be answered. Plant geographic provenance and the possible benign effect of previous exposure to stress is still not clear. Thus, the exploration of epigenetics for drought improvement would be a fruitful area for further work. Other natural progression of this work would be to perform a targeted analysis on a broad range of metabolites to better comprehend the metabolic pathways involved on the response to drought and also because some metabolites might emerge as reliable predictors of plant performance under drought and be used as selection markers.

CHAPTER 6: Fungi endophytes isolation and identification of *Arbutus unedo* L. fungi endophytes and biological control of *Phytophthora cinnamomi in vitro*

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Abstract

This study aims to characterize the fungi microbiome of strawberry tree in order to develop biological control strategies in the increasing orchard production area. For this purpose, fungi endophytes were isolated from wild strawberry tree plants and a molecular identification was carried out. *In vitro* assays were carried out to evaluated and characterize the antagonism of some endophytes. Among the several fungi endophytes isolated from strawberry tree (a total of 53 from 20 genera), a *Trichoderma atroviride* strain proved to have antagonism effect against several phytopathogens, including *Alternaria alternata*, *Botrytis cinerea*, *Glomerella cingulata* and *Mycosphaerella aurantia*. This antagonism was particularly effective against *Phytophthora cinnamomi*, causing a reduction in growth of about 80% on this invasive oomycete. An enzymatic assay revealed the production of several enzymes by *T. atroviride*, such as cellulases, chitinases, glucosidases, alkaline phosphatases and proteases, which is one of the several mechanisms known to be involved on *Trichoderma* biological control ability. The enzymatic activity, in particular that of cell-wall degrading enzymes, was accentuated when in a dual culture with *P. cinnamomi*. The production of serine proteases, aspartyl proteases, metalloproteases and cysteine proteases was also detected in an experiment carried out in liquid medium, suggesting the involvement of this proteases on *Trichoderma* mycoparasitism mechanisms. Finally, in a 3-way interaction with *in vitro* strawberry tree plants, the *T. atroviride* strain identified on this study (Au50) was able to protect the plants against *P. cinnamomi*, thus proving its potential as a biological control agent.

Keywords: cellulases, chitinases, enzymatic activity, proteases, Strawberry tree, *Trichoderma atroviride*

Introduction

The negative impact on plant productivity caused by plant pathogens is a chronic problem on agriculture worldwide. A higher chemical input, *i.e.*, the use of higher amounts of pesticides is usually the response on intensive farming systems (Aktar et al. 2009), which is an undesirable practice due to the adverse effect of some of these molecules on ecosystems and human health. Additionally, chemical pesticides are in some cases no longer effective due to acquired resistance of plant pathogens (Bardin et al. 2015). For this reason, the reduction of chemical pesticide usage is widely recognized as a desirable goal for agriculture and forestry, which can be accomplished using biological control methods (Bardin et al. 2015). A wide range of endophytic fungi have shown potential as biological control agents for phytopathogens and can be efficiently used as alternatives to chemical control (Knudsen and Dandurand 2014). Endophytes are widely spread and diverse microorganisms that can thrive asymptomatically in different tissues of living plants and have great impact on their host by promoting their growth and increasing their fitness and resistance to biotic and abiotic stresses (Faeth and Fagan 2002). Some bioactive compounds produced by endophytic fungi have shown antimicrobial activity against pathogens (Firáková et al. 2007; Waqas et al. 2012; Kusari et al. 2012). They can also act as a biological trigger of plant defense systems, by increasing hormonal levels in plant tissues (*e.g.*, jasmonic and salicylic acid), through nutritional elements uptake and by activation of specific genes (Zhang et al. 2006; Rodriguez et al. 2009; Hermosa et al. 2012; Morelli et al. 2020). The production of enzymes like chitinases and β-1,3-glucanases, either by plants is a frequent mechanism involved on fungi antagonism by degrading chitin and β-1,3-glucan, the most common polymers in fungi cell wall (Downer et al. 2001). Such enzymes might also be produced by plants as a defense mechanism in response to pathogens (Gupta et al. 2012).

A better understanding of the interaction between these pathogens and strawberry tree is necessary to develop efficient strategies for the control and mitigation of such organisms. In particular, the characterization of endophyte communities might be a step forward, as such organisms play a crucial role on the resistance of their host against adverse conditions including pathogen attacks, either by direct competition and antagonism or by promoting plant defense mechanisms (Morelli et al. 2020). However, very little is known about the strawberry tree microbiome and its role on plant defense. Thus, the objectives of this study were: (1) the isolation and identification of fungi endophytes from different parts of *A. unedo* wild plants, (2) to test the interaction and antagonism effect using the direct opposition method and (3) the identification of enzymatic classes that might be involved on biological control. To the best of our knowledge, some endophytes have been identified in strawberry tree for the first time in this work and their antagonism ability evaluated. A very detailed enzymatic profile of a *T. atroviride* strain in dual

culture with *P. cinnamomi* is also provided. The role of the endophytic community on strawberry tree resistance to pathogens and possible future biotechnological applications are discussed.

Material and Methods

Isolation and Identification of Endophytic Fungi

Samples were collected from three adult *A. unedo* trees growing in the wild, in Chaves (N 41º42'31.868'' W 7º26'32.506'', altitude 579 m), in the Northern region of Portugal. Roots, stems and leaves were excised and stored on sterile plastic bags, at 4 ºC, and processed within 24h. Only disease-free parts of the plant, *i.e.*, without any visible symptoms of disease. Roots were obtained at three different points for each tree by soil excavation (30 cm depth and 0.5 m^2) at 0.5 m from the tree trunk.

Plant material was thoroughly washed in sterile water, surface-disinfected in ethanol (70%, v/v) for 30 sec, sodium hypochlorite (3%, v/v) for 3 min and washed three times in sterile demineralized water. Small pieces of plant tissues were placed on PDA medium (Difco™ Potato Dextrose Agar: 4 g L^{-1} potato starch, 20 g L^{-1} dextrose, 15 g L^{-1} agar, Becton, Dickinson and Company, NJ, USA) in Petri plates (90 mm) and incubated at 25 ºC for 10 days. Colony growth was evaluated daily and different species, identified based on time of growth and morphology, were isolated in PDA medium. Aliquots of the water used on the last wash were also plated on PDA medium to confirm the efficiency of the disinfection protocol.

For DNA extraction, 100 mg of mycelia from each of the isolated fungi were used, and genomic DNA was isolated using the NucleoSpin® Plant II kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions and stored at -20 ºC. PCR was performed using the primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990; Gardes and Bruns 1993). The amplification of rDNA was carried out on a 25 µL final volume reaction containing 1 µL of total DNA, 0.5 µL of each primer and 12 µL (30U) of JumpStart™ Taq DNA Polymerase with $MgCl_2$ (Sigma, St. Louis, MO, USA). Primers were synthesized by Eurofins (Ebersberg, Germany). The PCR reaction was carried out on a Arktik™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) under the following conditions: 94 ºC for 5 min, followed by 35 cycles at 94 ºC for 1 min, annealing at 57 ºC for 30 s and extension at 72 ºC for 45 s, before a final extension at 72 ºC for 10 min. Detection of PCR-amplified products was performed by electrophoresis in a 1.5% (w/v) agarose gel stained with GelRed™ (Biotium, Fremont, CA, USA). In each lane, 4 µL of PCR-amplified product plus 1 µL of DNA loading buffer (Bioline, London, UK) were loaded, and 4 µL of HyperLadder II (50 – 2000 bp, Bioline) was also run in parallel. The PCR products were sequenced by StabVida sequence services (Oeiras, Portugal) using ITS1-F and ITS4 primers. As an underlying basis to identify the fungi, the rDNA-ITS sequences were manually edited using

Sequence Scanner Software 2 (Applied Biosystems, Foster City, California, USA), submitted to GenBank (accession numbers are listed in Table 1), and compared with available data from GenBank databases (closest identified relatives based on a BLAST search, National Centre for Biotechnology Information website; http://www.ncbi.nlm.nih.gov/). The close related sequences obtained from Genbank were used for a phylogenetic analysis to identify endophytes. Sequences were aligned using ClustalW and the best DNA substitution model was assessed using the "find best DNA/Protein Models (ML)" function on MEGA (version 10.0 for Windows) by implementing the maximum likelihood (ML) statistical method to test the goodness of fit of several models of evolution. A phylogenetic reconstruction was calculated using the maximum Likelihood method with bootstrap values calculated from 1000 replicate runs using Kimura 2 parameter model (K2) and gamma distributed (+G) model (=K2+G). For the identification of *Trichoderma* strains, a phylogenetic reconstruction was calculated using Kimura 2 parameter model (=K2), with close related sequences obtained from Genbank.

Antagonism assays

Antagonism in solid medium

Fungal isolates were tested *in vitro* for their antagonistic activity using the direct opposition method (Dennis and Webster 1971). Briefly, two mycelia plugs (5 mm in diameter) were placed on opposite sides of a Petri dish (90 mm) with PDA medium, at 1 cm from the edge. *Aureobasidium pullulans* (Au04)*, Epicoccum nigrum* (Au23), *Trichoderma atroviride* (Au50), *Umbelopsis* sp. (Au53) were tested against *Alternaria alternata* (Au01), *Botrytis cinerea* (Au09), *Glomerella cingulata* (Au26) and *Mycosphaerella aurantia* (Au28). Additionally, *T. atroviride* was tested against *Phytophthora cinnamomi*. Plates were incubated at 25 ± 1 ºC, and each experiment was carried out in triplicate. Growth reduction of *Alternaria* sp., *Botrytis cinerea*, *Glomerella cingulata*, *Mycosphaerella* sp. and *P. cinnamomi* was calculated after 3, 7 and 14 days using the following formula: RG = (TG - CG) / CG *100 (Lahlali et al. 2007), where TG is the growth of the colony in dual cultures, and CG is the growth of a control group. Additionally, the mycellium of *T. atroviride* growing with *G. cingulata*, *Mycosphaerella* sp. and *P. cinnamomi* was collected and protein extracted as described in the next section (*Protein extraction and quantification*).

Antagonism in liquid medium

Due to the effect of *T. atroviride* on *P. cinnamomi* found during the antagonism tests carried out on solid medium, further analyses were conducted to test their interaction. Both microorganisms were grown on PDB medium (Difco^{TM} Potato Dextrose Broth: 4 g L^{-1} potato starch, 20 g L^{-1} dextrose, Becton, Dickinson and Company) and the resulting medium used for subsequent cultures. For this purpose, 5 mm plugs of mycelium from both organism growing on PDA medium, were placed on 100 mL Erlenmeyer's with 25 mL of PDB medium. After a month, the medium was

filtered through a PES membrane filter (0.22 μm, Ø33 mm), and the culture was repeated using this medium. A 5 mm plug of *T. atroviride* was inoculated on the medium were *P. cinnamomi* was growing and on its own medium as a control. The same was performed with *P. cinnamomi*. The mycelium was collected and protein extracted as described in the next section.

Proteomics

Protein extraction and quantification

Protein from *T. atroviride* and *Phytophthora cinnamomi* was extracted twice with phosphate buffer (50 mM, pH 7.0). Briefly, mycelium was homogenized for 5 min, at 10000 rpm, on a T25 digital ULTRA-TURRAX® disperser (IKA, Staufen, Germany) and centrifuged at 5000 rpm, 10 min, 4 °C. Total protein was assessed using the Bio-Rad Protein Assay based on Bradford's reaction (Bradford 1976) in 96-well microplates. A calibration curve was constructed using concentrations of BSA between 8 to 70 μg mL^{-1} for PDB medium and 0.05 to 0.5 mg mL^{-1} for the mycelium protein extracts. All measurements were made simultaneously and in triplicate at 595 nm in a SPECTRAmax PLUS 384 spectrophotometer (Molecular Devices, San Jose, CA, USA).

Enzymatic Activity

The production of extracellular enzymes by *T. atroviride* and *P. cinnamomi* was assessed by growing a 5 mm mycelium plug on GYP medium (Glucose Yeast Extract Peptone Agar: 1 g L^{-1} glucose, 0.1 g L^{-1} yeast extract, 0.5 g L^{-1} peptone, 16 g L^{-1} agar) with specific substrates. After incubation for 5 days, enzymatic activity was detected by the clear zones observed around the colony. Amylolytic activity was assessed by growing the mycelium on GYP medium (pH 6.0) with soluble starch (0.2%, w/v). After incubation, the plates were flooded with iodine (1%, w/v) in potassium iodide (2%, w/v). For cellulase activity, carboxy-methylcellulose (0.5%, w/v) was added to the GYP medium and the plates were flooded with aqueous Congo red (0.2%, w/v) and distained with 1 M NaCl for 15minutes. Finally, proteolytic activity was tested on GYP medium (pH 6.0) with gelatin (0.4% w/v) prepared separately. The plates were then flooded with saturated aqueous ammonium sulfate.

Hydrolytic Activity

The proteolytic specificity was tested against several amino-fluorogenic synthetic substrates: (Arg, Lys, Leu, Met, Phe)-AMC, Bz-Arg-AMC, Gly-Pro-AMC, Gly-Pro-Arg-AMC, Boc-Val-Pro-Arg-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Boc-Ala-Gly-Pro-Arg-AMC, Boc-Phe-Ser-Arg-AMC and Suc-Leu-Leu-Val-Tyr-AMC (PeptaNova, Sandhausen, Germany). The proteolytic activity of protein extracts was measured on a fluorescence spectrophotometer for multiplate (SpectraMax®GeminiTMEM Microplate Spectrofluorometer, Molecular Devices). Reactions were initiated with the addition of 2 μl of substrate (dissolved in DMSO, with a final concentration of 0.1 mM) to 200 μL of sample. Fluorescence intensity was recorded for 60 min, with a premixing time of 3 sec, at 37 ºC (λem: 380 nm and λex: 460 nm). A control without enzyme was performed.

The amount of released AMC was calculated from a standard AMC curve using a 1 mM AMC stock solution in DMSO. One unit of specific proteolytic activity was defined as the quantity of enzyme that hydrolyzes 1 pmol AMC/min/mg protein.

Additionally, four enzyme substrates based on 4-methylum-belliferone (MU) were tested, namely 4-Methylumbelliferyl-β-D-galactopyranoside (MU-G for the detection of β-glucosidases activity), 4-Methylumbelliferyl N-acettyl-β-D-glucosaminide (MU-NAG for chitinase activity), 4-Methylumbelliferyl β-D-cellobioside (MU-C for cellulase acticity) and 4-Methylumbelliferyl Phosphate (MU-P for alkaline phosphatase activity, Sigma-Aldrich), in a final concentration of 0.5 mM. The conditions were similar to those described before but with different wavelengths (λex = 365 nm and λem = 460 nm). The results were obtained as before and converted to pmol MU/min/µg of protein.

Proteolytic inhibition assays

Protein extracts obtained from the mycelium (growing on PDA and PDB medium) were incubated with enzymatic inhibitors cocktails. Six different cocktails were prepared, each containing five inhibitors, and lacking a different one from the following list: pepstatin A - aspartyl protease inhibitor, EDTA - metalloprotease inhibitor, TPCK (Tosyl phenylalanyl chloromethyl ketone) - chymotrypsin-like serine and cysteine protease inhibitor, TLCK (Tosyl-L-lysyl-chloromethane hydrochloride) - trypsin-like serine protease inhibitor, E-64 ((1*S*,2*S*)-2-(((*S*)-1-((4-Guanidinobutyl)amino)-4-methyl-1-oxopentan-2-yl)carbamoyl) cyclopropane carboxylic acid) - cysteine protease inhibitor and Pefabloc (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride) - serine proteases inhibitor. Inhibitors cocktails were prepared in Tris-HCl buffer (50 mM, pH 7.4), with each inhibitor at a final concentration of 1 mM, and were incubated with 200 µl of extract for 10 min at room temperature. The reaction was initiated with the addition of 2 µl (0.1 mM) of the substrate with the highest enzymatic activity on the proteolytic activity assay (Gly-Pro-Arg-AMC) and enzyme activity was measured as described before. A negative control, without inhibitors, and a positive control, with all the six inhibitors, were also carried out. Results are expressed as relative proteolytic activity compared to a control without inhibitors. Enzymatic activity was considered positive for a 10% threshold.

Antagonism tests with plants

Two genotypes with different origins were selected, genotype A1 (young tree) and genotype A2 (seedling) and were established *in vitro* following the method described by (Gomes and Canhoto 2009) with slightly modifications. Briefly, genotype A1 was established *in vitro* using epicormic shoots that were surface sterilized with ethanol (70%, v/v) for 30 s and calcium hypochlorite (5%, w/v, Sigma-Aldrich, St. Louis, MO, USA) for 10 min with two drops of Tween 20. After 3 washes with sterile distilled water, the epicormic shoots (0.5-1 cm) were inoculated in Anderson Rhododendron medium (Anderson 1980) with 6-benzylaminopurine (2 mg L^{-1}, Sigma-

Aldrich), sucrose (3%, w/v, Duchefa) and agar (0,6% w/v, Duchefa), on test tubes (25x150 mm) with plastic caps (Duran, Mainz, Germany). The culture medium was autoclaved at 121 ºC for 20 min (800–1.100 g cm^{-2} gel strength after autoclaving), the pH was adjusted to 5.7 using KOH or HCl diluted solutions (0.01M – 1M) before autoclaving and agar addition. The culture was done on a growth chamber at a 16-h photoperiod, an irradiance of 15 - 20 µmol m^{-2} s^{-1} (cool-white fluorescent lamps), and a temperature of 25 ºC. For genotype A2, isolated seeds were disinfected following a similar methodology used for shoots and sowed on Petri dishes with filter paper discs and sterile distilled water. After 1 month of cold stratification (4 ºC), the seeds were transferred to a culture chamber under 16-h photoperiod at 25 ºC. The roots of the plantlets obtained were removed and the shoots inoculated on the medium described before. For axillary shoot proliferation shoots were cultured on plastic containers (O118/80+OD118 with white filter, Microbox, Deinze, Belgium) with 100 mL of the medium described before. The cultures were kept under a 16-h photoperiod at 25 ºC, with culture intervals of 8 weeks. For root induction, 3 cm long shoots were cultured on test tubes, on a medium with KNOP salts (Knop 1865), De Fossard vitamins without riboflavin (De Fossard et al. 1974), MS microelements without KI (Murashige and Skoog 1962) and IBA (2 mg L^{-1}, Sigma-Aldrich), under dark conditions, 25 ºC, for 10 days. Shoots were then transferred to a medium with the same composition but without IBA, and added charcoal (1%, w/v, Sigma-Aldrich), and kept on a culture chamber, 16-h photoperiod at 15–20 µmol m-2 s-1 (cool-white fluorescent lamps at 25 ºC), for 3 weeks.

In order to test the three-way interaction, the protocol developed by (Macías-Rodríguez et al. 2018) was used with some adaptations. Plants were placed in Petri dishes (100 mm x 25 mm, Corning, NY, USA) with 10 mL Knop salts (Knop 1865) and 7 g L^{-1} plant agar (Duchefa). Two plants were placed on each petri dish with their roots on media surface and two 5 mm plugs of *T. atroviride* and *Phytophthora cinnamomi* (Tr+Pc) were placed at 1 cm from the roots and 1 cm apart from each other (see annotation in Fig. 9 D). Three control groups were also carried out: without microorganisms, with *T. atroviride* (Tr) and with *Phytophthora cinnamomi* (Pc). Plates were shaded in the root area and placed vertically in a growth chamber at a 16-h photoperiod, an irradiance of 15 - 20 µmol m^{-2} s^{-1} (cool-white fluorescent lamps), and a temperature of 25 ºC for 30 days. Plates were examined daily and the development of disease symptoms recorded. 30 days after inoculation, plants without any visible symptoms were considered as non-infected. Root and stem necrosis as well as apical leaves senescence were considered symptoms caused by *P. cinnamomi*. The oomycete was also re-isolated from the symptomatic plants to confirm the infection. 10 plants per treatment were analyzed in five independent inoculation assays.

Statistical analysis

Values are given as means ± standard deviation. Reduction in growth in the antagonism assays was analyzed by one-way ANOVA (GraphPad Prism v. 8.4.3 for Windows, San Diego, CA, USA), followed by a Tukey´s multiple comparison test ($P < 0.05$). Specific enzymatic activity data

(Table S10-S13, available at: http://phd.freecluster.eu/) was analyzed using the same method. The Venn diagram, Upset plot, heatmaps and principal component analysis (PCA) were carried out using R software (version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria,(R Core Team 2020). Venn diagram of fungi species in different plant organs was constructed using the package VennDiagram (Chen 2018). To evaluate the interaction and significance of different enzymatic substrates on *T. atroviride* and *P. cinnamomi* extracts, heatmaps were constructed using the package ComplexHeatmap (Gu et al. 2016) and PCA were performed using the package ggbiplot (Vu 2011). Finally, an Upset plot was constructed with enzymatic inhibitors data using UpSetR package (Gehlenborg 2019).

Results

A total of 53 endophytic fungi strains were isolated from strawberry tree tissues (Figure 40, Table 2). Most isolates belong to the Ascomycota phylum, whereas only one Zygomicota (*Umbelopsis* sp. – Au53) and one Basidiomycota was identified (*Coprinopsis gonophylla* – Au17).

The isolates were identified belonging to 20 genera, including *Alternaria*, *Aureobasidium*, *Botrytis*, *Diaporthe*, *Epicoccum*, *Glomerella*, *Mycosphaerella*, *Trichoderma* and *Umbelopsis*. Only a *Trichoderma* strain (Au50) was identified to the species level as *Trichoderma atroviride*, based on a phylogenetic analysis (Figure S6, available at: http://phd.freecluster.eu/). The amplification of genes encoding exo-β-1,3-glucanase, alkaline protease, α-1,3 glucanase and aorsin serine protease using primers specific to *T. atroviride* (Morán-Diez et al. 2019) also confirmed this identification as well as the production of volatile organic compounds characteristic of this fungi such as 6-penyl-α-pyrone (Morath et al. 2012).

Table 2. Maximum nucleotide identity match for 50 fungal isolates based on ITS sequences using BLAST analysis.

Isolate ID (GenBank Accession)	Plant organ	Species	Match identity (%)	E value	GenBank Accession	Phylum, Class, Family
Au01[a] (MW560214)	Leaf	*Alternaria alternata*	99.46	0.0	MH521178.1	Ascomycota, Dothideomycetes, Pleosporaceae
Au02 (MW560215)	Leaf	*Alternaria* sp.	99.30	0.0	JF694748.1	Ascomycota, Dothideomycetes, Pleosporaceae
Au03 (MW560216)	Leaf	*Alternaria* sp.	99.83	0.0	MN636331.1	Ascomycota, Dothideomycetes, Pleosporaceae
Au04[a] (MW560217)	Root	*Aureobasidium pullulans*	98.07	0.0	MN922121.1	Ascomycota, Dothideomycetes, Saccotheciaceae
Au05 (MW560218)	Twig	*Aureobasidium pullulans*	99.65	0.0	KX067792.1	Ascomycota, Dothideomycetes, Saccotheciaceae
Au06 (MW560219)	Twig	*Aureobasidium pullulans*	99.65	0.0	KC897669.1	Ascomycota, Dothideomycetes, Saccotheciaceae
Au07 (MW560220)	Leaf	*Aureobasidium pullulans*	99.47	0.0	KX664386.1	Ascomycota, Dothideomycetes, Saccotheciaceae

Isolate	Tissue	Closest match	Identity (%)	E-value	Accession	Taxonomy
Au08 (MW560221)	Leaf	*Aureobasidium pullulans*	99.82	0.0	KX664386.1	Ascomycota, Dothideomycetes, Saccotheciaceae
Au09[a] (MW560222)	Twig	*Botrytis cinerea*	99.22	0.0	MF996364.1	Ascomycota, Leotiomycetes, Sclerotiniaceae
Au10 (MW560223)	Twig	*Botrytis cinerea*	99.43	0.0	KT723007.1	Ascomycota, Leotiomycetes, Sclerotiniaceae
Au11 (MW560224)	Leaf	*Botrytis cinerea*	99.08	0.0	MF442632.1	Ascomycota, Leotiomycetes, Sclerotiniaceae
Au12 (MW560225)	Leaf	*Botrytis cinerea*	99.42	0.0	MN689856.1	Ascomycota, Leotiomycetes, Sclerotiniaceae
Au13 (MW560226)	Leaf	*Cladosporium cladosporioides*	99.26	0.0	LT603044.1	Ascomycota, Dothideomycetes, Cladosporiaceae
Au14 (MW560227)	Leaf	*Cladosporium* sp.	99.63	0.0	MN486549.1	Ascomycota, Dothideomycetes, Cladosporiaceae
Au15 (MW560228)	Leaf	*Cladosporium* sp.	99.81	0.0	KJ361484.1	Ascomycota, Dothideomycetes, Cladosporiaceae
Au16 (MW560229)	Leaf	*Cladosporium* sp.	98.81	0.0	MG548567.1	Ascomycota, Dothideomycetes, Cladosporiaceae
Au17 (MW560230)	Root	*Coprinopsis gonophylla*	99.71	0.0	MH856190.1	Basidiomycota, Agaricomycetes, Psathyrellaceae
Au18 (MW560231)	Twig	*Cytospora* sp.	99.63	0.0	MK912130.1	Ascomycota, Sordariomycetes, Valsaceae
Au19 (MW560232)	Leaf	*Diaporthe* sp.	98.96	0.0	KU712217.1	Ascomycota, Sordariomycetes, Diaporthaceae
Au20 (MW560233)	Leaf	*Diaporthe* sp.	99.82	0.0	MG719626.1	Ascomycota, Sordariomycetes, Diaporthaceae
Au21 (MW560234)	Twig	*Diaporthe viticola*	99.65	0.0	KC145906.1	Ascomycota, Sordariomycetes, Diaporthaceae
Au22 (MW560235)	Twig	*Diaporthe viticola*	99.83	0.0	KC145904.1	Ascomycota, Sordariomycetes, Diaporthaceae
Au23[a] (MW560236)	Twig	*Epicoccum nigrum*	99.62	0.0	KR909153.1	Ascomycota, Dothideomycetes, Didymellaceae
Au24 (MW560237)	Twig	*Epicoccum nigrum*	100.00	0.0	KR023621.1	Ascomycota, Dothideomycetes, Didymellaceae
Au25 (MW560238)	Twig	*Epicoccum nigrum*	99.81	0.0	KX664321.1	Ascomycota, Dothideomycetes, Didymellaceae
Au26[a,b] (MW560239)	Leaf	*Glomerella cingulata*	99.66	0.0	AJ301952.1	Ascomycota, Sordariomycetes, Glomerellaceae
Au27 (MW560240)	Twig	*Microsphaeropsis olivacea*	99.05	0.0	MH627286.1	Ascomycota, Dothideomycetes, Coniothyriaceae
Au28[a,b] (MW560241)	Leaf	*Mycosphaerella aurantia*	99.62	0.0	EU853472.1	Ascomycota, Dothideomycetes, Mycosphaerellaceae
Au29 (MW560242)	Leaf	*Mycosphaerella aurantia*	99.81	0.0	AY509742.1	Ascomycota, Dothideomycetes, Mycosphaerellaceae
Au30 (MW560243)	Leaf	*Mycosphaerella aurantia*	99.81	0.0	EU853472.1	Ascomycota, Dothideomycetes, Mycosphaerellaceae
Au31 (MW560244)	Leaf	*Mycosphaerella punctiformis*	98.50	0.0	EU343240.1	Ascomycota, Dothideomycetes, Mycosphaerellaceae
Au32 (MW560245)	Leaf	*Mycosphaerella punctiformis*	99.25	0.0	EU343240.1	Ascomycota, Dothideomycetes, Mycosphaerellaceae
Au33 (MW560246)	Leaf	Fungal sp. NLR-2013 strain E48 (*Rhododendron*) *	99.61	0.0	KC867914.1	Ascomycota, Dothideomycetes, Mycosphaerellaceae
Au34 (MW560247)	Leaf	Fungal sp. NLR-2013 strain E20 (*Rhododendron*) *	99.81	0.0	KC867830.1	Ascomycota, Dothideomycetes, Mycosphaerellaceae
Au35 (MW560248)	Leaf	Fungal sp. NLR-2013 strain E18 (*Rhododendron*) *	99.61	0.0	KC867813.1	Ascomycota, Dothideomycetes, Mycosphaerellaceae

Au36 (MW560249)	Leaf	Fungal sp. NLR-2013 strain E211 (*Rhododendron*) *	99.62	0.0	KC867839.1	Ascomycota, Dothideomycetes, Mycosphaerellaceae
Au37 (MW560250)	Root	*Penicillium* sp.	98.90	0.0	MT035966.1	Ascomycota, Eurotiomycetes, Trichocomaceae
Au38 (MW560251)	Root	*Penicillium* sp.	99.47	0.0	KF367528.1	Ascomycota, Eurotiomycetes, Trichocomaceae
Au39 (MW560252)	Root	*Penicillium* sp.	98.58	0.0	KT354994.1	Ascomycota, Eurotiomycetes, Trichocomaceae
Au40 (MW560253)	Root	*Phialocephala fortinii*	99.82	0.0	HM190137.1	Ascomycota, Leotiomycetes, Vibrisseaceae
Au41 (MW560254)	Root	*Phialocephala fortinii*	99.46	0.0	HM190137.1	Ascomycota, Leotiomycetes, Vibrisseaceae
Au42 (MW560255)	Twig	*Pyronema* sp.	99.48	0.0	KF128839.1	Ascomycota, Pezizomycetes, Pyronemataceae
Au43 (MW560256)	Leaf	*Saccothecium sepincola*	96.92	0.0	MH860330.1	Ascomycota, Dothideomycetes, Saccotheciaceae
Au44 (MW560257)	Twig	*Seimatosporium lichenicola*	97.76	0.0	MH860239.1	Ascomycota, Sordariomycetes, Sporocadaceae
Au45 (MW560258)	Twig	*Seimatosporium lichenicola*	97.71	0.0	MH860239.1	Ascomycota, Sordariomycetes, Sporocadaceae
Au46 (MW560259)	Leaf	*Septoria* sp.	99.81	0.0	JN215415.1	Ascomycota, Dothideomycetes, Mycosphaerellaceae
Au47 (MW560260)	Twig	*Sordaria* sp.	99.46	0.0	KP401930.1	Ascomycota, Sordariomycetes, Sordariaceae
Au48 (MW560261)	Root	*Trichoderma* sp.	99.34	0.0	MK870621.1	Ascomycota, Sordariomycetes, Hypocreaceae
Au49 (MW560262)	Root	*Trichoderma* sp.	99.67	0.0	MH170872.1	Ascomycota, Sordariomycetes, Hypocreaceae
Au50[a,b] (MW560263)	Root	*Trichoderma atroviride*	99.50	0.0	KU942400.1	Ascomycota, Sordariomycetes, Hypocreaceae
Au51 (MW560264)	Root	*Trichoderma* sp.	99.83	0.0	MH170872.1	Ascomycota, Sordariomycetes, Hypocreaceae
Au52 (MW560265)	Root	*Trichoderma* sp	100.00	0.0	JF311965.1	Ascomycota, Sordariomycetes, Hypocreaceae
Au53[a] (MW560266)	Root	*Umbelopsis* sp	99.67	0.0	JQ912671.1	Zygomycota, Umbelopsidaceae

[a]Isolates used on the antagonism assay. [b]Isolates used on the proteomics assay. * Identified as *Mycosphaerella* sp. based on morphological characteristics.

Five genera (*Coprinopsis*, *Penicillium*, *Phialocephala*, *Trichoderma* and *Umbelopsis*) were found exclusively on root tissues, six on twigs (*Cytospora*, *Epicoccum*, *Microsphaeropsis*, *Seimatosporium*, *Sordaria* and *Pyronema*), and a total of six fungi genera were identified on leaves (*Alternaria*, *Cladosporium*, *Glomerella*, *Mycosphaerella*, *Septoria*, *Saccothecium*). Although *Diaporthe viticola* was identified only in twigs, some unidentified *Diaporthe* species were also isolated from leaves. *Botrytis cinerea* was also identified in both twigs and leaves, whereas *Aureobasidium pullulans* was isolated from all the plant tissues sampled (Figure 41).

The antagonism assays showed that *A. pullulans* was only slightly effective against *Alternaria alternata* and *Botrytis cinerea* with a reduction in growth around 20% and 30%, which was more accentuated after 7 days in the case of *B. cinerea*, with statistically significant differences (Figure 42 A-B). Although a promotion in growth was observed against *Glomerella cingulata*, a slightly reduction in growth was observed after 14 days (Figure 42 C). No effect was observed on the growth of *Mycosphaerella aurantia* (Figure 42 D). A very similar result was obtained for

Epicoccum nigrum, that was slightly effective against *A. alternata* and *B. cinerea*, with a more accentuated antagonist effect after 7 days in the latter, with statically significant differences (Figure 42 E-F). A slightly reduction in the growth of *G. cingulata* was observed after 14 days with statistical differences (Figure 42 G), and no effect was observed on *M. aurantia* (Figure 42 H). *T. atroviride* caused a considerable reduction in growth on all the four fungi tested that was closed to 80% in some cases (Figure 42 I-L). Although there are no statistical differences between 7 and 14 days (except for *Mycosphaerella aurantia*), an increasing reduction in growth over time was observed (Figure 42 I-L). When *Umbelopsis* sp. was tested, no effect on the growth of *A. alternata* was detected (Figure 42 M), whereas *B. cinerea* was slightly inhibited (Figure 42 N), as it has been observed with *A. pullulans* and *E. nigrum*. Likewise, *Umbelopsis* sp. slightly promoted *G. cingulata* growth (Figure 42 O) and no effect was observed on *M. aurantia* (Figure 42 P). In general, *T. atroviride* showed higher efficiency as a biological control agent against the four fungi tested after 7 days in the dual culture (Figure 42 Q-T), with statistical differences after 7 and 14 days against *A. alternata* and *M. aurantia*, and differences on the three time points tested against *B. cinerea*. No statistical differences were found on the effect of *A. pullulans* and *T. atroviride* after 7 days, as well as between *A. pullulans*, *E. nigrum* and *T. atroviride* after 14 days, against *G. cingulata*.

When *T. atroviride* was tested against *Phytophthora cinnamomi*, it was able to overgrown the pathogen, without the formation of a deadlock (Figure 43 A). A reduction in growth of 69.16% (± 0.83) was found after 3 days, 75.33% (± 0.67) after 7 days and 79.44% (± 0.56) after 14 days in the dual culture (Figure 43 B). Enzymatic activity plate assays of both microorganisms revealed the production of enzymes, including amylases, cellulases and proteases (Figure 43 C-H). However, by the size of the halos, *T. atroviride* produced a considerably large amount of enzymes, especially proteases, when compared to *P. cinnamomi* (Figure 43 E and H).

In the dual culture enzymatic assay in solid medium, *T. atroviride* control (Tr) showed higher enzymatic activity on most proteases substrates (except Arg-AMC and Gly-Pro-AMC) and also chitinase substrate (MU-NAG) after 3 days (Figure 44 A and Table S10, available at: http://phd.freecluster.eu/) when compared to other samples in a dual culture with pathogens. In fact, when *T. atroviride* was tested against *G. cingulata* (Tr/Gc) less enzymatic activity on almost all substrates was recorded. However, in the antagonism assays with *M. aurantia* (Tr/Ma) higher values for cellulases, chitinases, glucosidases and phosphatases substrates were obtained. The dendrogram revealed a similarity between Tr/Ma and Tr/Pc groups, something that has also been confirmed by a PCA (Figure 44 B). This multivariate analysis perfectly grouped the samples on four different clusters, according to the treatments. The principal component 1 (PC1) contributed with 69.8% to the total variance while PC2 with 20.1%. After 6 days, a different result was obtained, and a higher enzymatic activity was obtained on all the substrates, except MU-C, when *T. atroviride* was in dual culture with *P. cinnamomi* (Tr/Pc; Figure 44 C and Table S11, available at: http://phd.freecluster.eu/). This difference was confirmed by a dendrogram and also a PCA

(Figure 44 D), as these samples (Tr/Pc) are grouped together by the influence of all vectors except 17, which corresponds to MU-C. In this case, the principal component 1 (PC1) contributed with 86.6% to the total variance while PC2 with only 7.2%.

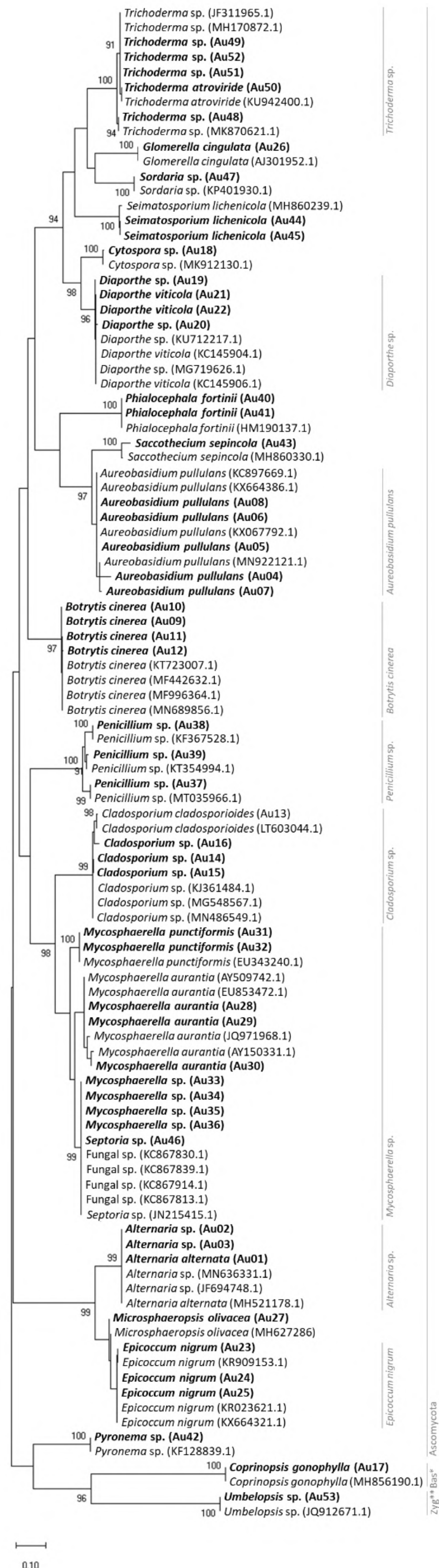

Figure 40. Overall endophytic microbiota relative abundance (%) at the phylum and genera level. Maximum Likelihood (ML) phylogenetic tree based on rDNA ITS sequences of endophytic fungal isolates and fungal ITS sequences from GenBank database. ML tree was constructed using the Kimura 2 parameter model (K2) and gamma distributed (+G) (=K2+G). All positions containing gaps and missing data were included for analysis. Clade supports were calculated based on 500 replicate runs. Accession numbers (GeneBank) of the sequences belonging to the endophytic fungi isolated in this study and the reference sequences from GeneBank are listed in Table 1. a - isolates used on antagonism assays. b - isolates used on enzymatic assays. * Basidiomycota, ** Zygomycota

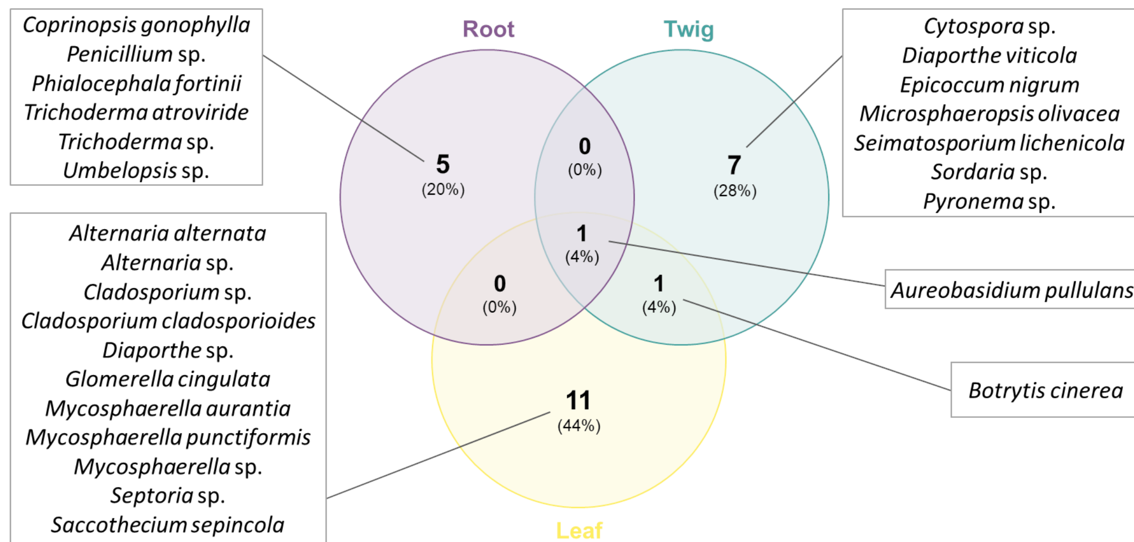

Figure 41. Venn diagram with the endophytic fungi species isolated from roots, twigs and leaves of Arbutus unedo

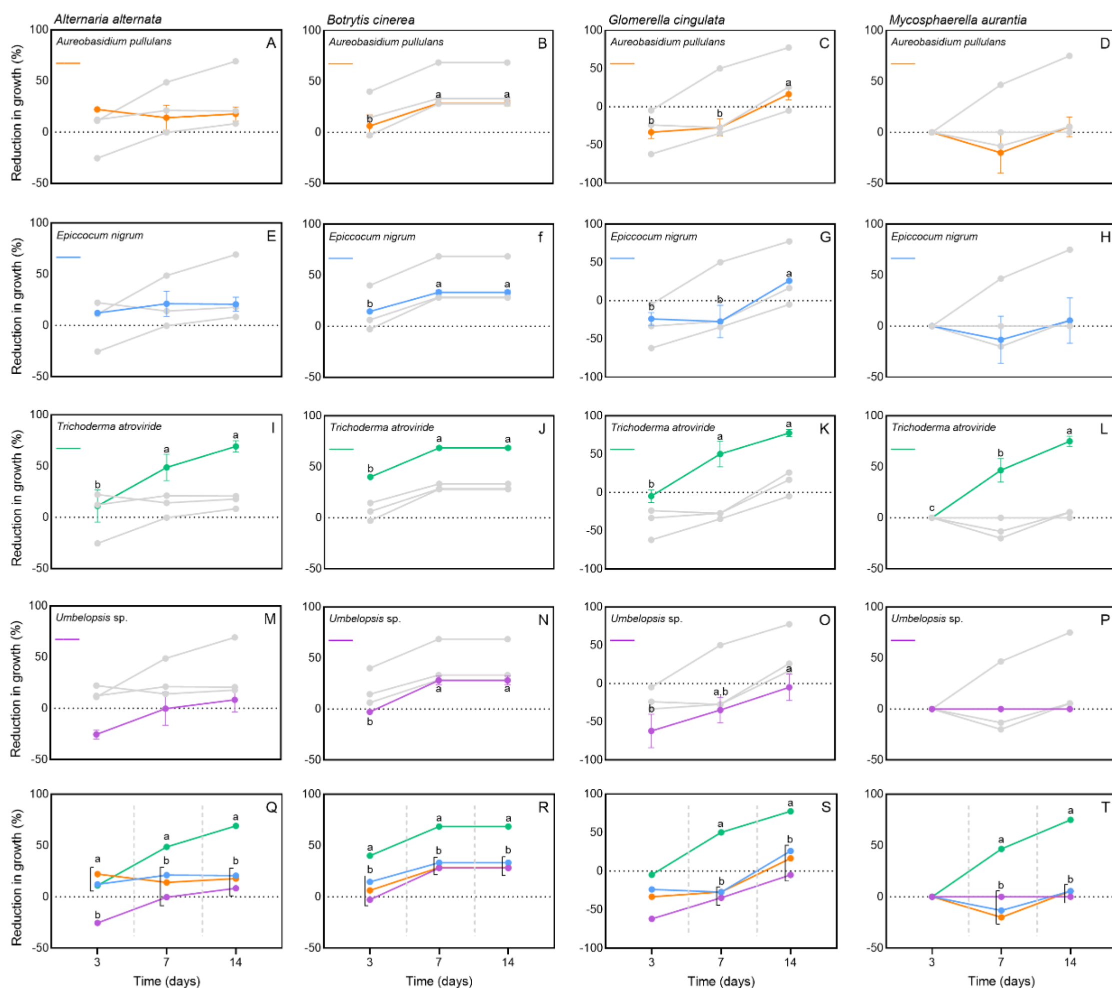

Figure 42. Antagonism assay between some of the endophytic fungi isolated from *Arbutus unedo*. Reduction in growth of *Alternaria alternata* (Au01), *Botrytis cinerea* (Au09), *Glomerella cingulata* (Au26) and *Mycosphaerella aurantia* (Au28) caused by *Aureobasidium pullulans* (Au04), *Epiccocum nigrum* (Au23),

Trichoderma atroviride (Au50) and *Umbelopsis* sp. (Au53): *A. pullulans* and *A. alternata* (A); *A. pullulans* and *B. cinerea* (B); *A. pullulans* and *G. cingulata* (C); *A. pullulans* and *M. aurantia* (D); E. *nigrum* and *A. alternata* (E); *E. nigrum* and *B. cinerea* (F); *E. nigrum* and *G. cingulata* (G); *E. nigrum* and *M. aurantia* (H); *T. atroviride* and *A. alternata* (I); *T. atroviride* and *B. cinerea* (J); *T. atroviride* and *G. cingulata* (K); *T. atroviride* and *M. aurantia* (L); Umbelopsis sp. and A. alternata (M); Umbelopsis sp. and B. cinerea (N); Umbelopsis sp. and G. cingulate (O); Umbelopsis sp. and M. aurantia (P); A. pullulans, E. nigrum, T. atroviride, Umbelopsis sp. and A. alternata (Q); A. pullulans, E. nigrum, T. atroviride, Umbelopsis sp. and B. cinerea (R); A. pullulans, E. nigrum, T. atroviride, Umbelopsis sp. and G. cingulate (S); A. pullulans, E. nigrum, T. atroviride, Umbelopsis sp. and M. aurantia (T). The reduction in growth was calculated after 3, 7 and 14 days (%, means ± SDs, n = 3). A-P: different letters indicate differences between times ($P \leq 0.05$). Q-T: different letters indicate differences between microorganisms at each time point ($P \leq 0.05$)

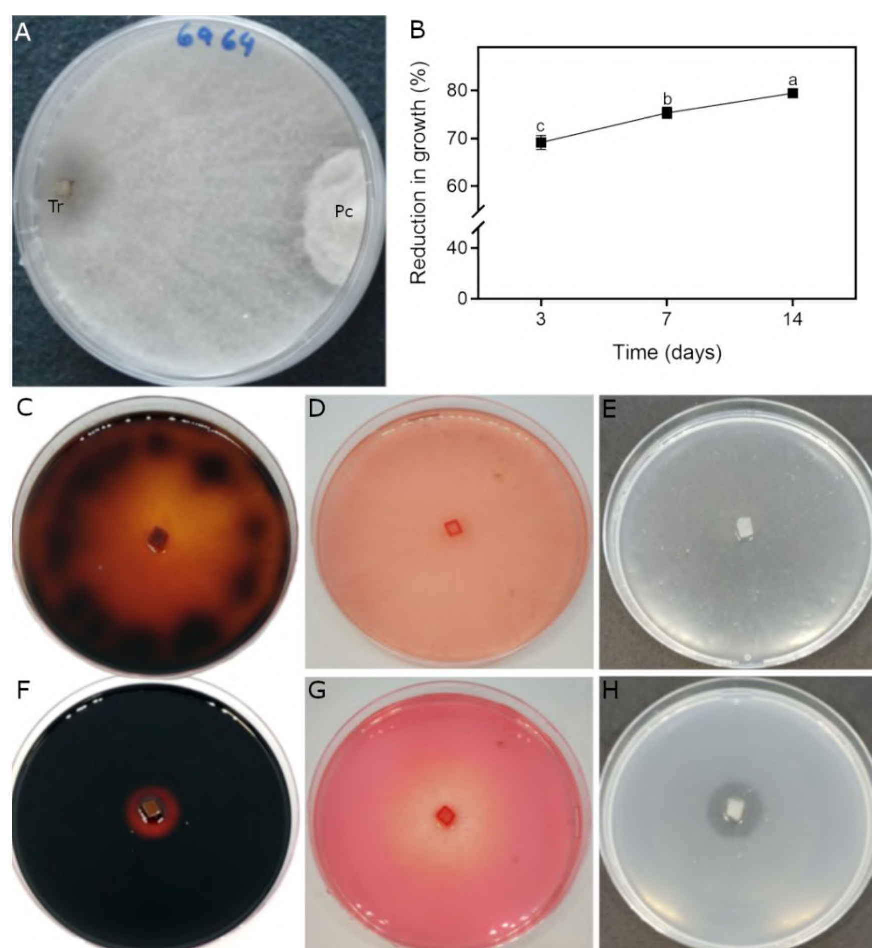

Figure 43. Antagonism assay between *T. atroviride* (Au50) and *P. cinnamomi* and enzymatic activity plate assay of *P. cinnamomi* and *T. atroviride* (Au50): dual culture between *T. atroviride* (Tr on the left) and *P. cinnamomi* (Pc on the right) after 7 days in culture (A); reduction in growth of *P. cinnamomi* caused by *T. atroviride* calculated after 3, 7 and 14 days (%, means ± standard deviations, n = 3) (B); amylolytic activity of *T. atroviride* (C); cellulolytic activity of *T. atroviride* (D); proteolytic activity of *T. atroviride* (E); amylolytic activity of *P. cinnamomi* (F); cellulolytic activity of *P. cinnamomi* (G); proteolytic activity of *P. cinnamomi* (H).

When the antagonism assay was carried out in liquid medium (PDB), a reduction in growth of *T. atroviride* was found when sub-cultured on the same medium (Tr/Tr) and in the medium where *P. cinnamomi* was growing (Tr/Pc) when compared to fresh medium (Tr), with statistical differences (Figure 45 A). However, no differences were observed between the two sub-cultures (Tr/Tr and Tr/Pc) in terms of total biomass. In the case of *P. cinnamomi*, a decrease in biomass was also observed when the microorganism was sub-cultured on the same medium (Pc/Pc; Figure 45 A-C). This decrease was even more accentuated when the culture was carried out on the medium where *T. atroviride* was firstly growing (Pc/Tr; Figure 45 A-D). Besides producing lower biomass, the growth of *P. cinnamomi* on *T. atroviride* medium was impaired and the mycelia presented morphological abnormalities and had an errant growth.

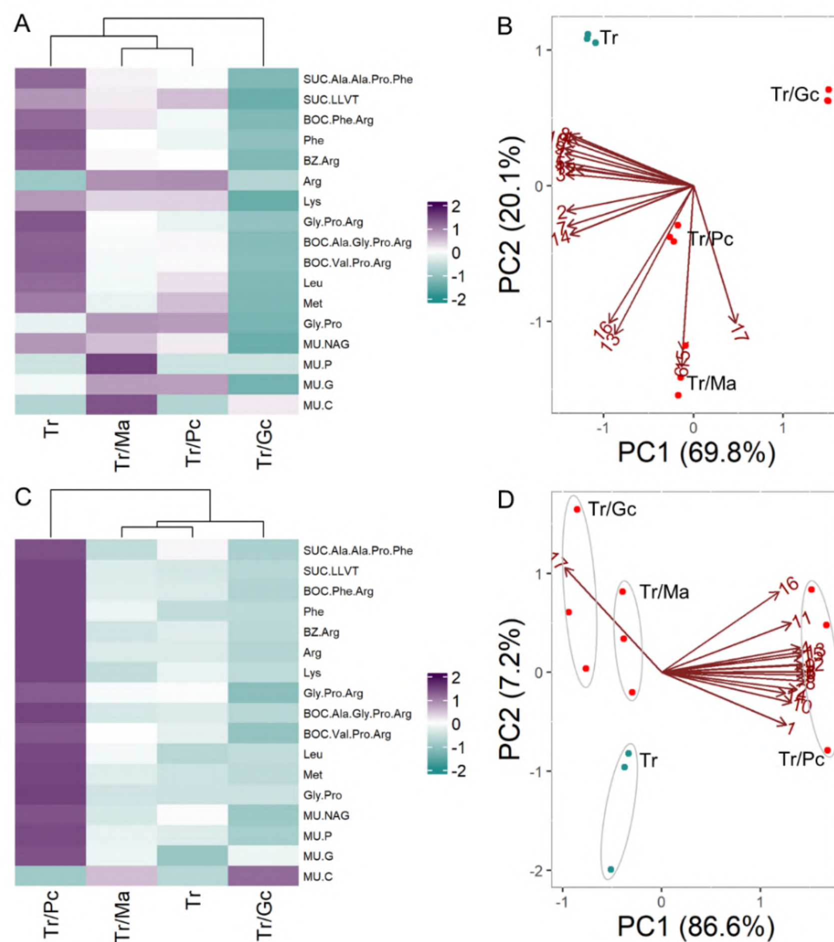

Figure 44. Specific enzymatic activity (pmol AMC/MU min^{-1} × mg protein) of *T. atroviride* mycelium growing on PDA medium on the control group (Tr), and in a dual culture with *G. cingulata* (Tr/Gc), *M. aurantia* (Tr/Ma) and *P. cinnamomi* (Tr/Pc) after t1 (3 days) and t2 (6 days): heatmap (A) and principal component analysis (PCA) biplot (B) of enzymatic activity after 3 days (t1); heatmap (C) and PCA biplot of enzymatic activity after 6 days (t2) (D). PCA vectors: (1) Suc-Ala-Ala-Pro-Phe-AMC, (2) Suc-Leu-Leu-Val-Tyr-AMC, (3) Boc-Phe-Ser-Arg-AMC, (4) Phe-AMC, (5) Bz-Arg-AMC, (6) Arg-AMC, (7) Lys-AMC, (8) Gly-Pro-Arg-AMC, (9) Boc-Ala-Gly-Pro-Arg-AMC, (10) Boc-Val-Pro-Arg-AMC, (11) Leu-AMC, (12) Met-AMC, (13) Gly-Pro-AMC, (14) MU-NAG, (15) MU-P, (16) MU-G and (17) MU-C

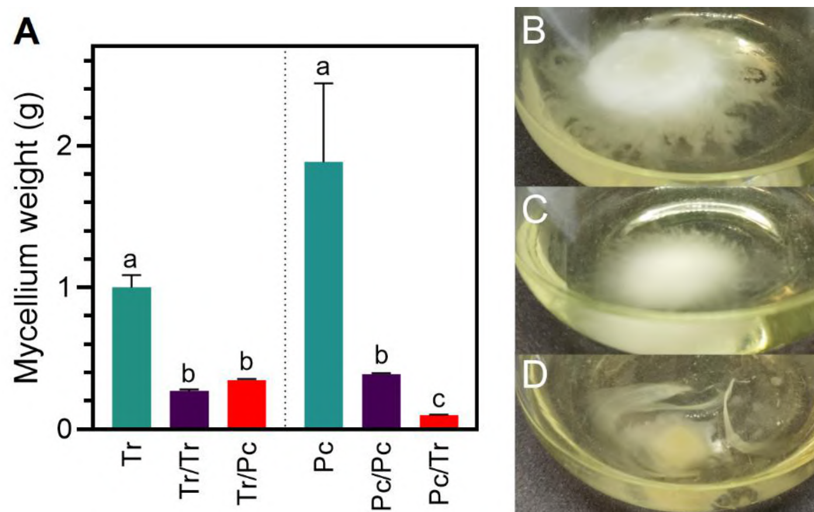

Figure 45. Antagonism assay between *T. atroviride* and *P. cinnamomi* on PDB medium: Mycelium weight (g) after growing for 30 days on different interactions (A); *P. cinnamomi* control group (Pc) (B); *P. cinnamomi* sub-cultured on the same medium (Pc/Pc) (C); *P. cinnamomi* sub-cultured on *T. atroviride* medium (Pc/Tr) (D). Tr – *T. atroviride* control group, Tr/Tr – *T. atroviride* sub-cultured on the same medium, Tr/Pc – *T. atroviride* sub-cultured on *P. cinnamomi* medium, Pc – *P. cinnamomi* control group, Pc/Pc – *P. cinnamomi* sub-cultured on the same medium, Pc/Tr – *P. cinnamomi* sub-cultured on *T. atroviride* medium

The enzymatic assay showed that *T. atroviride* (Tr) mycelium had higher enzymatic activity for some amino acids proteases substrates (Phe-AMC, Arg-AMC, Lys-AMC, Leu-AMC, Met-AMC) and phosphatases (Mu-P) (Figure 46 A and Table S12, available at: http://phd.freecluster.eu/). Pc had a different enzymatic profile with higher enzymatic activity on peptide protease substrates (BOC-Phe-Arg, BZ-Arg-AMC, Gly-Pro-Arg-AMC, BOC-Ala-Gly-Pro-Arg-AMC, BOC-Val-Pro-Arg-AMC, Gly-Pro-AMC), and also glucosidases (Mu-G) and cellulases (Mu-C). Moreover, *T. atroviride* growing in its own medium showed higher enzymatic activity for proteases (Suc-Ala-Ala-Pro-Phe-AMC, Suc-LLVT-AMC) and chitinases (Mu-NAG). However, when growing in the medium were *P. cinnamomi* had grown before, higher enzymatic activity on chitinases (Mu-NAG), glucosidases (Mu-G) and cellulases (Mu-C) were obtained. *P. cinnamomi* growing in its own medium (Pc/Pc) and also on *T. atroviride* medium (Pc/Tr) had lower enzymatic activity. The dendrogram showed a similarity between Pc/Tr and Pc/Pc groups, a result also confirmed by PCA analysis (Figure 46 B), whereas the other samples are also grouped together on the PCA according to the treatments. In this case, the principal component 1 (PC1) contributed with 41.7% to the total variance while PC2 with 29.0%. The analysis of the culture medium revealed different enzymatic activities.

The analysis performed on the culture medium revealed a different profile from that of mycelium and no enzymatic activity was detected in some of the protease substrates (Figure 46 C and Table S13, available at: http://phd.freecluster.eu/). Furthermore, Tr/Tr was the medium where higher enzymatic activity was measured for all protease substrates (except Gly-Pro-AMC) and also

cellulases (Mu-C). Tr and Pc/Tr medium had a similar result with higher chitinase (Mu-Nag), phosphatase (Mu-P) and glucosidase (Mu-G) activities, although higher protease activity was detected in the former. Finally, Pc, Pc/Pc and Tr/Pc medium had lower and similar activities on all the substrates, except Gly-Pro-AMC on Pc medium. In this case, the dendrogram revealed a similarity between Pc/Pc and Tr/Pc groups, and also Pc, while Tr/Tr is the more distant group. Again, these results were confirmed by a PCA (Figure 46 D), where samples were grouped according to treatments, and Pc/Pc were clustered with Tr/Pc and also Pc. The principal component 1 (PC1) contributed with 55.2% to the total variance while PC2 with 21.2%.

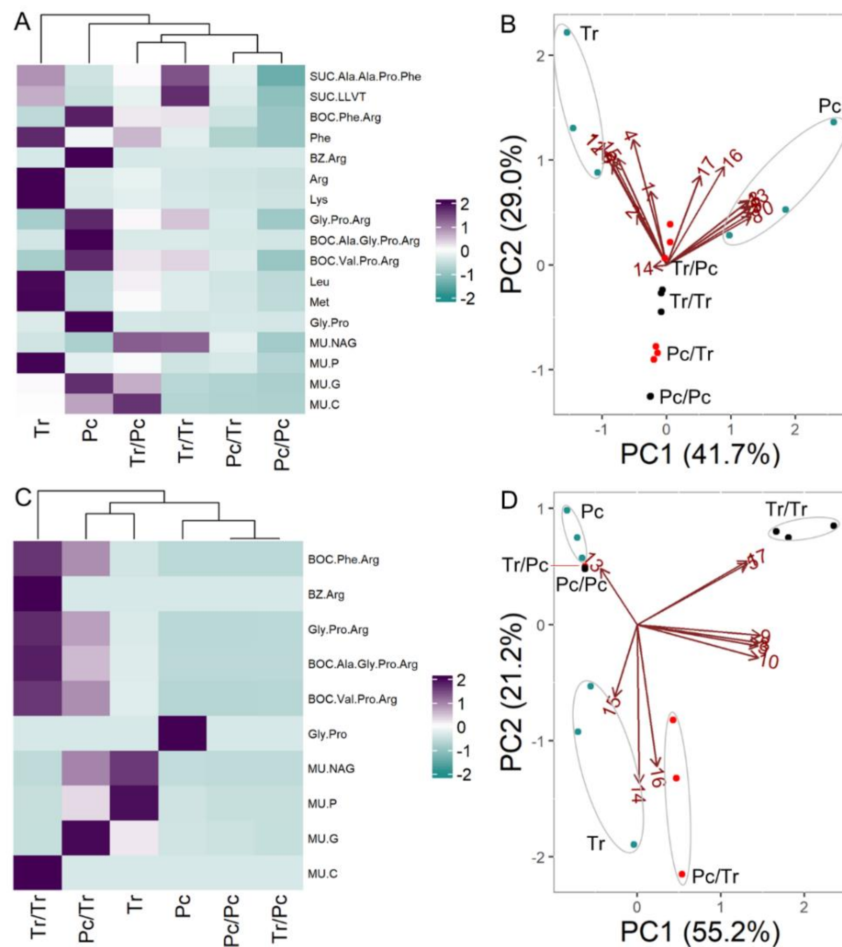

Figure 46. Specific enzymatic activity (pmol AMC/MU min^{-1} × mg protein) of *T. atroviride* and *P. cinnamomi* mycelium and medium on PDB medium after 30 days: heatmap (A) and principal component analysis (PCA) biplot (B) of enzymatic activity from the mycelium; heatmap (C) and PCA biplot (D) of enzymatic activity from the medium. Tr – *T. atroviride* control group, Tr/Tr – *T. atroviride* sub-cultured on the same medium, Tr/Pc – *T. atroviride* sub-cultured on *P. cinnamomi* medium, Pc – *P. cinnamomi* control group, Pc/Pc – *P. cinnamomi* sub-cultured on the same medium, Pc/Tr – *P. cinnamomi* sub-cultured on *T. atroviride* medium. PCA vectors: (1) Suc-Ala-Ala-Pro-Phe-AMC, (2) Suc-Leu-Leu-Val-Tyr-AMC, (3) Boc-Phe-Ser-Arg-AMC, (4) Phe-AMC, (5) Bz-Arg-AMC, (6) Arg-AMC, (7) Lys-AMC, (8) Gly-Pro-Arg-AMC, (9) Boc-Ala-Gly-Pro-Arg-AMC, (10) Boc-Val-Pro-Arg-AMC, (11) Leu-AMC, (12) Met-AMC, (13) Gly-Pro-AMC, (14) MU-NAG, (15) MU-P, (16) MU-G and (17) MU-C

When proteases inhibitors were used to test the mycelium samples growing on PDA medium, enzymatic activity was only observed when the cocktail of inhibitors without TLCK was used (Figure 47, Figure S7, available at: http://phd.freecluster.eu/), indicating the presence of trypsin-like serine proteases on all the samples with no differences between the control group (Tr) and the dual cultures (Tr/Gc, Tr/Ma and Tr/Pc). When a similar test was carried out on the mycelium growing in liquid medium (PDB medium), a more diverse result was obtained revealing a complex enzymatic cocktail produced by *T. atroviride* (Figure 47, Figure S7, available at: http://phd.freecluster.eu/). In the control (Tr) a wide variety of proteases, including aspartyl proteases, metalloproteases, chymotrypsin-like serine proteases, trypsin-like serine proteases and cysteine proteases were detected. A similar result was obtained on Tr/Tr when compared to Tr, except no metalloprotease and cysteine proteases activity was detected, as well as on Tr/Pc, but no cysteine proteases were detected.

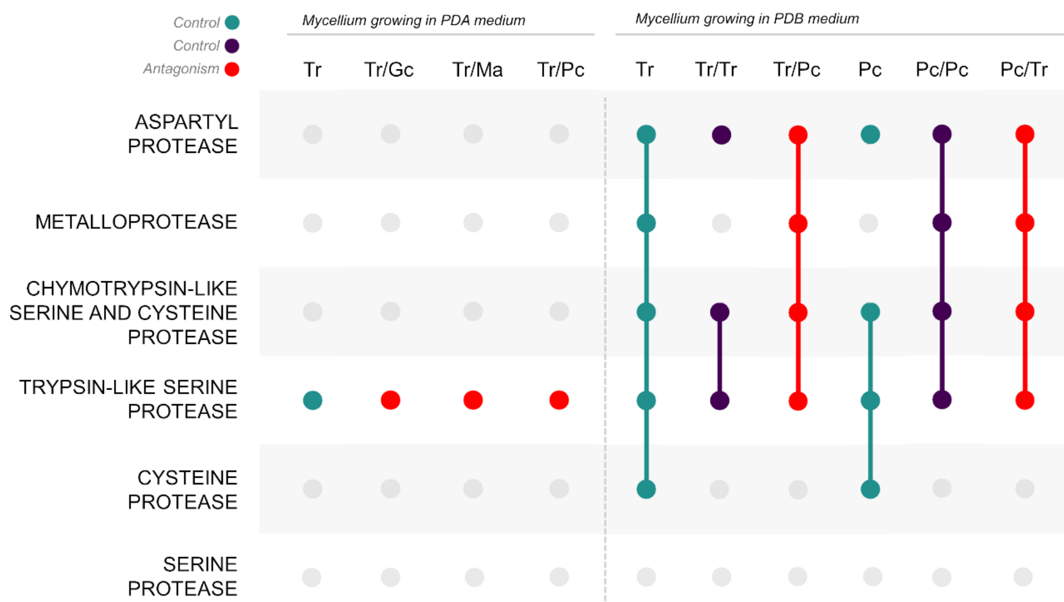

Figure 47. Upset diagram with relative enzymatic activity obtained with inhibitors cocktails for substrate Gly-Pro-AMC, with protein extracted from mycelium growing in PDA and PDB medium. On PDA medium: *T. atroviride* on the control group (Tr), and in a dual culture with *G. cingulata* (Tr/Gc), *M. aurantia* (Tr/Ma) and *P. cinnamomi* (Tr/Pc). On PDB medium: *T. atroviride* control group (Tr), *T. atroviride* sub-cultured on the same medium (Tr/Tr), *T. atroviride* sub-cultured on *P. cinnamomi* medium (Tr/Pc), *P. cinnamomi* control group (Pc), *P. cinnamomi* sub-cultured on the same medium (Pc/Pc), *P. cinnamomi* sub-cultured on *T. atroviride* medium (Pc/Tr). Colored dots indicate relative positive activity when compared to the control group with a 10% threshold: green dots - control group, black dots - sub-cultures on its own medium, red dots - antagonism

In the case of Pc, aspartyl protease, chymotrypsin-like serine proteases and cysteine protease, trypsin-like serine protease activities were detected. Similar results were obtained on Pc/Pc and Pc/Tr, with aspartyl protease, metalloprotease, chymotrypsin-like serine proteases and

cysteine protease as well as trypsin-like serine protease activities. No enzymatic activity was detected when the cocktail without the inhibitor pefabloc was applied (serine proteases).

When a 2-way and 3-way interaction culture was tested, *T. atroviride* was able to protect plants against *P. cinnamomi* (Figure 48 A-D). Plants inoculated with *P. cinnamomi*, showed the first disease symptoms after a few days (Figure 48 C), with root necrosis that continuously spread to the top of the plant, leading to plant death after 3 weeks. The oomycete was successfully re-isolated from dead plants, which confirms the infection. However, in cultures inoculated with both *P. cinnamomi* and *T. atroviride* on a 3-way interaction (Figure 48 D), *P. cinnamomi* growth was almost completely inhibited by *T. atroviride*, and all the plantlets tested from both genotypes remained asymptomatic without any visible necrosis (Figure 48 A-B).

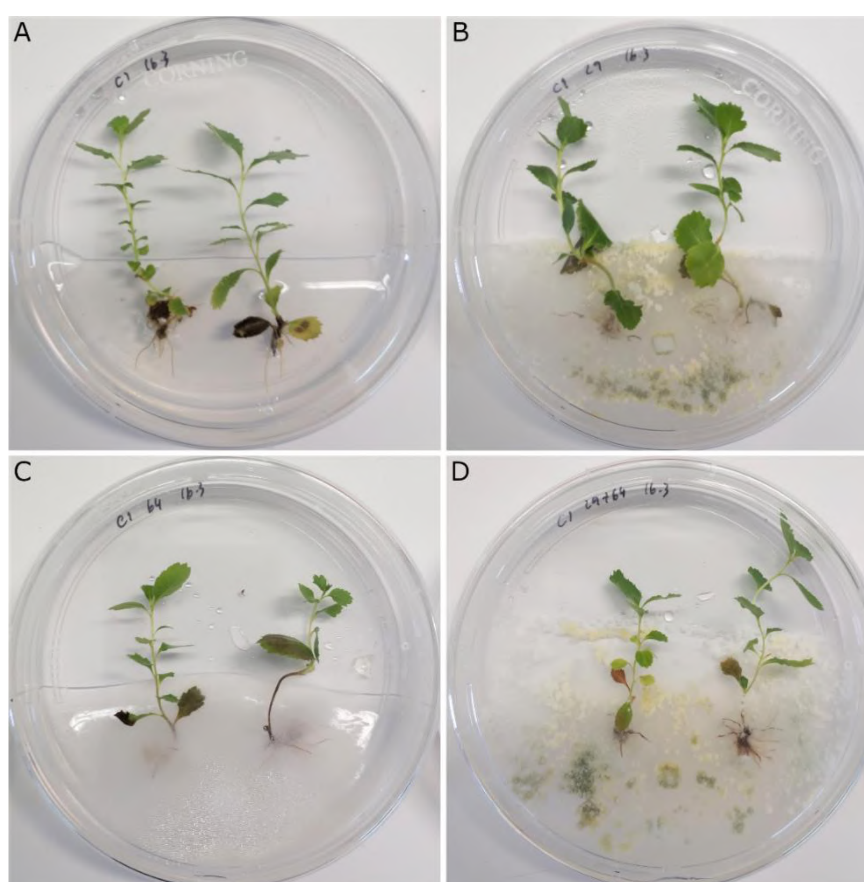

Figure 48. *In vitro* inoculation of strawberry tree plants with *T. atroviride* and *P. cinnamomi*: non-inoculated plants (control group) (A); plants inoculated with *T. atroviride* (B); plants inoculated with *P. cinnamomi* (C); plants inoculated with *T. atroviride* and *P. cinnamomi* (D).

Discussion

Although a relatively high number of fungi taxa have been isolated from strawberry tree, this number is certainly underestimated due to the dependent culture method used, as most of the microbiome species are non-culturable (Sun and Guo 2012). Furthermore, the medium used on this

study might not be appropriate for some species. Thus, in order to obtain a more detailed profile of the microbiota, new generation sequence methodologies as well as different media and growth conditions should be tested on future studies, focusing also on the influence of plant genotype and environment on microbiome composition. Although barely no work has been carried out to identify *A. unedo* endophytes, some of the fungi identified on this work have already been found to be strawberry tree endophytes, such as: *Alternaria alternata*, *Aureobasidium pullulans*, *Cladosporium* sp., *Penicillium* spp. and *Umbelopsis* spp. (Borges 2014). In these assays, several other fungi were identified as strawberry tree endophytes for the first time: *Botrytis cinerea*, *Coprinopsis gonophylla*, *Cytospora* sp., *Diaporthe* spp., *Epiccocum nigrum*, *Phialocephala fortinii*, *Pyronema* sp., *Saccothecium sepincola*, *Seimatosporium lichenicola*, *Sordaria* sp. and *Trichoderma* spp. In other members of the Ericaceae family (*Rhododendron* spp., *Enkianthus perulatus* and *Pieris japonica*) the fungi *Alternaria* spp., *Aureobasidium pullulans*, *Cladosporium* sp., *Glomerella* sp., *Penicillium* spp., *Septoria* sp. and *Trichoderma* sp. have also been found (Okane et al. 1998; Purmale et al. 2012). Not surprisingly, the isolated fungi consisted mainly of Ascomycota and only two isolates from Basidiomycota and Zygomycota were found. Endophytic fungi are known to be mainly Ascomycota and only a few taxa of Basidiomycota and Zygomycota are usually found in plant tissues (Sun and Guo 2012; Martin et al. 2015). Other interesting result was the fact most of the isolated species were only identified in a specific tissue (roots, twigs or leaves). This spatial distribution suggests these species are adapted to specific internal environments, something expected due to the great differences between these micro-ecosystems in terms of temperature, radiation and chemical composition. On the other hand, *B. cinerea* was found on twigs and leaves while *A. pullulans* was found on all the tissues sampled, which indicates a great adaptation of these fungi to different microenvironments.

Even though its rusticity nature, strawberry tree is also affected by foliar diseases caused by *Alternaria* sp. (Woudenberg et al. 2015), *Glomerella* sp. (Polizzi et al. 2011) and *Mycosphaerella* sp. (Romero-Martin and Trapero-Casas 2003), that have also been identified in the strawberry tree plants sampled in this study. For this reason, endophytes isolated from strawberry tree were screened for its potential biological control activity against these pathogens. *A. pullulans* showed antagonism against *A. alternata*, something that has been reported before (Yalage Don et al. 2020). The antagonism ability of *Trichoderma* spp. against *A. alternata* and *G. cingulata* has also been referred before by (Ramírez-Cariño et al. 2020) and (Ferreira et al. 2020). In the present study, *B. cinerea* was isolated from leaves and twigs of strawberry tree. Although this fungus has not been yet reported as a strawberry tree pathogen, it is the causing agent of blossom blight on other Ericaceae such as blueberry (Hildebrand et al. 2001). The fact that all the four endophytes tested showed to be effective antagonists of *B. cinerea*, might be the reason this fungus has not yet been reported as a problematic pathogen on strawberry tree, as their prejudicial effects might be suppressed by the endophytes. The antagonism effect on *B. cinerea* has been referred before in the

literature, caused by *A. pullulans* (Yalage Don et al. 2020), *E. nigrum* (Alcock et al. 2015) and also *T. atroviride* (Li et al. 2020).

T. atroviride showed to be the most effective biocontrol agent as it was able to cause a reduction in growth of all the four pathogenic fungi tested. Besides, this result is highly relevant for biotechnological applications and from an ecological perspective since *Trichoderma* spp. are usually found in the soil and as plant endophytes. Furthermore, the *T. atroviride* strain tested in this study also showed anti-oomycete activity against *P. cinnamomi*, a devastating wide spread invasive oomycete that causes root rotting, leading to plant death, and that infects a high diversity of crops (Sena et al. 2018), including strawberry tree (Jung et al. 2018). This is not a surprising result as the biological control ability of several *Trichoderma* species is well known and has been reported in the literature, including against *P. cinnamomi* (Kelley and Rodriguez Kabana 1976; Andrade-Hoyos et al. 2020). Moreover, *Trichoderma* spp. have been reported as biological control agents with relevant application in agricultural systems and also biotechnological applications (Hermosa et al. 2010). Some species such as *T. virens* and *T. atroviride* are cosmopolitan opportunists with a parasitism strategy (Atanasova et al. 2013). These species have developed a wide array of mycoparasitism mechanisms such as the production of cell wall degrading enzymes such as amylases, chitinases, glucanases and proteases (Hermosa et al. 2010; Xie et al. 2014; Sharma et al. 2016, 2017). Other biological control mechanisms include antibiosis and niche exclusion, through the production of secondary metabolites including VOCs - volatile organic compounds and siderophores (Qi and Zhao 2013; Bae et al. 2016). Some of these compounds may also work as elicitors, promoting plant growth and resistance to stress (Hermosa et al. 2010).

Our data showed inhibition of *B. cinerea* by *T. atroviride* after three days, even before direct contact, which seems to indicate VOCs might play an important role in this contactless interaction. In fact, several volatiles produced by the *T. atroviride* used in this study were identified (data not shown). For example, 6-pentyl-α-pyrone, a compound that has been previously identified in *Trichoderma* spp., in particular on *Trichoderma atroviride* (Morath et al. 2012), is largely produced by this strain (Au50). Besides its antibiotic properties, this compound is also responsible for the characteristic coconut odor of the fungi and seems to play an important role on fungi-plant cross-talk (Morath et al. 2012). *P. cinnamomi* was also affected after 3 days in a dual culture with *T. atroviride* which seems to indicate this pathogen is sensitive to VOCs produced by the endophyte.

On the other cases, the main inhibition mechanism seems to be related to the production of lytic enzymes and/or other inhibitory compounds as the inhibition was accentuated when *T. atroviride* was closer to the pathogen. Due to their importance in biological control as part of the mycoparasitism mechanism, but also their relevance for biotechnological applications, the enzymatic profile of the *T. atroviride* strain isolated from strawberry tree was studied in more detail. *T. atroviride* was able to produce all the tested enzymes on a plate assay, including amylases,

cellulases and proteases. As mentioned before, these enzymes are known to be produced by *Trichoderma* spp. and are essential for the mycoparasitism lifestyle and biocontrol. According to the halos, a large quantity of enzymes was produced by *T. atroviride* when compared to *P. cinnamomi*, even though this pathogen is known for the production of a large quantity of enzymes, in particular cellulases, that play a role on nutrient acquisition and are also involved on the pathogenic process allowing intracellular fungal penetration on plant tissues (McIntyre and Hankin 1978; Ferraris et al. 1996).

The enzymatic activity assay carried out in solid medium, revealed a specific enzymatic profile according to the pathogen *T. atroviride* was facing. Previous studies have already demonstrated the ability of *Trichoderma* spp. to distinguish different fungi species and/or strains and adjust their responses in accordance (Guzmán-Guzmán et al. 2017). Several studies have also demonstrated that *Trichoderma* spp. overexpresses several genes and produce different proteins when in contact with plants (Hermosa et al. 2010; Guzmán-Guzmán et al. 2017; Brotman et al. 2020). Some of these proteins, such as hydrophobins, are essential for the interaction between the fungi mycelium and plant roots, thus affecting the colonization of the root system and tissues penetration (Ruocco et al. 2015; Sharma et al. 2017).

When *T. atroviride* was cultured with *P. cinnamomi*, a great increase of the enzymatic activity was observed in all the substrates tested, indicating a high production of cellulases, chitinases, glucosidases, phosphatases and proteases. As mentioned before, cell-wall degrading enzymes like cellulases (cellobiohydrolase, endoglucanase), hemicellulases (glucan 1,3-b-glucosidase and arabinofuranosidases) and endochitinases have been identified and are essential on *T. atroviride* mycoparasitism lifestyle and biocontrol (Grinyer et al. 2005; Brotman et al. 2020). All together, these enzymes are able to hydrolase β-1,3 glucan and chitin, which are the most common cell wall-forming polymers in fungi and also *P. cinnamomi* cell walls that have a considerable amount of cellulose (β-1,4-linked glucans). Thus, the production of this enzymatic cocktail is particularly important against *P. cinnamomi* and other oomycetes with a similar cell wall composition. Besides being essential on fungal physiology, morphogenesis and metabolism (Yike 2011), proteases might also be involved on cell wall degradation, through the lysis of lipids and proteins that are part of the cell wall skeleton (Viterbo et al. 2002). According to the inhibitors assay, the majority of the proteases produced by *T. atroviride* in both the control group and dual cultures in PDA medium are trypsin-like serine protease. It has been reported that most proteases involved on *Trichoderma* spp. biological control process are serine proteases (Suárez et al. 2005). These enzymes play an important in fungal biology and are highly conserved in these microorganism (Pozo et al. 2004). Additionally, a trypsin-like protease has been identified on the biocontrol agent *Trichoderma harzianum* (Suarez et al. 2004), and it has also been reported this kind of protease might be correlated with pathogenicity of filamentous fungi (Yike 2011).

At a transcriptomic level, several genes encoding glucanases and proteinases were found to be up-regulated when *T. atroviride* is co-cultured with pathogenic microorganisms (Salas-Marina et al. 2015; Morán-Diez et al. 2019). In the interaction between *T. atroviride* and *P. cinnamomi* tested in this work, an increase in the expression of genes related to exo-β-1,3-glucanase, alkaline protease, α-1,3 glucanase and aorsin serine protease was verified (data not shown). The aorsin putative gene was particularly up-regulated, which seems to indicate the importance of this serine protease enzyme on the biocontrol mechanisms carried out by *T. atroviride* against *P. cinnamomi*. This result corroborates the previous finding regarding enzymatic activity.

A reduction in the growth of mycelial mass when both microorganisms were sub-cultured (*i.e.,* Tr/Tr, Tr/Pc, Pc/Pc and Pc/Tr) was observed when compared to the respective control group (Tr and Pc). As a consequence, lower enzymatic activities were also detected. This result was expected, due to nutrient depletion. However, while Tr/Pc had a similar growth to Tr/Tr, in the case of Pc/Tr the growth of *P. cinnamomi* was greatly reduced and the mycelium had abnormal characteristics. This situation may be caused by the cell wall degrading enzymes present in the culture medium secreted by *T. atroviride*. The change of enzymatic profile of Tr/Pc when compared to Tr (more cellulases, chitinases and glucosidases), is a possible response to *P. cinnamomi* enzymes and other molecules present in the medium. The presence of cell wall fragments might also trigger the release of higher amounts of enzymes by *T. atroviride* and work as an elicitor, something that has been demonstrated before (Grinyer et al. 2005). In fact, under carbon depletion situations, which might be the case, ß-1,3-glucanases have an important role on energy mobilization from glucan polymers (Viterbo et al. 2002).

The analysis carried out in the liquid medium, showed no enzymatic activity for some proteolytic substrates possible due to low concentrations of such enzymes in the culture medium, below the detection limit of the technique used in this study. These enzymes might also have been degraded by other proteases or they were not released into the medium as only a small portion of the fungi proteome is secreted (Krijger et al. 2014). An increase in the enzymatic activity with proteolytic substrates was observed on Tr/Tr probable due to a cumulative effect. On the other hand, in the case of Tr/Pc, a similar result to Pc/Pc and Pc was obtained. As mentioned before, *P. cinnamomi* lead to a higher enzymatic production by *T. atroviride*, but apparently these enzymes haven't been released to the medium.

The assay with inhibitors carried out in the mycelium growth in liquid medium, revealed a more complex matrix when compared to solid medium, with a wide variety of proteases detected. Some of the proteases detected are aspartyl proteases, cysteine proteases and metalloproteases. An aspartyl protease has been detected on a Trichoderma-plant coculture medium (Brotman et al. 2020). These acidic proteases, seem to be responsible for the degradation of enzymes that are overexpressed, like cellulases, chitinases and glucanases (Viterbo et al. 2002). Thus, the detection of this enzyme in the mycelium might be related to this feature. Likewise, cysteines are also

responsible for other proteins degradation, and might have a similar activity than that of aspartyl proteases. Moreover, several metalloproteases have been implicated in fungi virulence mechanisms (Pan et al. 2020), which may explain their detection in the medium. Finally, *T. atroviride* was able to control *P. cinnamomi* on a 3-way interaction with the plant under *in vitro* conditions. Although preliminary, this result is very promising and additional experiments should be carried out in field conditions to confirm the ability of this *T. atroviride* strain as a biological control agent of *P. cinnamomi*.

In conclusion, *A. unedo* harbours several endophytic fungi and provides a fungal resource for the study of ecological interactions between these microorganisms and also the production of metabolites. In fact, endophytes produce a large amount of compound with interesting properties that can be used for several purposes, widening their spectrum of possible applications. Their use as biological control agents has great potential on modern green agriculture, especially against *Phytophthora* species where sterols, the main target of many fungicides, are not present. Moreover, due to its high genetic flexibility, they can easily overcome chemical control (Bae et al. 2016). In particular, *Trichoderma* spp. are antagonists of several plant pathogens and due to their ability to survive under extreme competition conditions have the potential to be used under different environments, conferring protection to plant hosts and promoting their yield (Benítez et al. 2004). For these reasons, the characterization of strawberry tree microbiome and their role on stress tolerance mechanisms might be essential to ensure plant survival and productivity. Hence, future studies of mutual relationships, and the integration of beneficial endophytes (such as *T. atroviride* - Au50) on strawberry tree breeding program and orchard management are warranted.

CHAPTER 7: Identification and characterization of *Arbutus unedo* L. endophytic bacteria isolated from wild and cultivated trees for the biological control of *Phytophthora cinnamomi*

(Martins J, Ares A, Casais V, Costa J, Canhoto J (2021) Identification and characterization of *Arbutus unedo* L. endophytic bacteria isolated from wild and cultivated trees for the biological control of *Phytophthora cinnamomi*. Plants 10:1569 | DOI: https://doi.org/10.3390/plants10081569)

Abstract

Plant pathogens are a chronic problem in agriculture worldwide and several microorganisms are responsible for strawberry tree diseases leading to production constrictions. Thus, the development of alternative plant protection strategies is necessary and bacteria endophytes may confer great advantages to the host plant, increasing their overall fitness and productivity. This paper aimed to isolate, identify and characterize endophytic bacteria from strawberry tree leaves from plants growing spontaneously in a natural environment as well as from plants growing on orchards to identify naturally occurring bacteria with biocontrol activity against the most common *A. unedo* diseases. A total of 62 endophytes of strawberry tree leaves were isolated and identified as belonging to the genera *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Sphingomonas* and *Staphylococcus*. Although a slightly higher number of species was found in wild plants, no differences in terms of diversity indexes were found when compared to cultivated plants. Sixteen isolates were tested *in vitro* for their antagonistic effect against the most important *A. unedo* mycopathogens. *B. cereus* (Au06) was the most effective antagonist causing a growth reduction of 20% in *Glomerella cingulata* and 40% in *Phytophthora cinnamomi* and *Mycosphaerella aurantia*. Also, several endophytic isolates exhibited plant growth-promoting potential. This study provides insights into the diversity of endophytic bacteria in *A. unedo* leaves and their potential role as growth-promoters and pathogen antagonists. The implications of these results on the microbial structure, biological control strategies and plant phenotyping are discussed.

Keywords: *Bacillus cereus*, bacteria endophytes, biological control, *Phytophthora cinnamomi*

Introduction

Due to an increasing demand for Strawberry tree products and a consequent market value growth, the production area has been rising considerably in recent years, facing constraints due to phytosanitary problems. Several microorganisms such as *Glomerella cingulata* (Polizzi et al. 2011), *Mycosphaerella* sp. (Romero-Martin and Trapero-Casas 2003) and *Phytophthora cinnamomi* (Jung et al. 2018) are known to be strawberry tree pathogens causing several diseases and leading to a reduction of plant fitness and constraints on crop production. The most aggressive is *P. cinnamomi*, an invasive and widespread oomycete, responsible for huge losses on agriculture and forestry worldwide, as it leads to tree decay (Sena et al. 2018), calling for adequate responses to improve agricultural practices and upkeep production.

To achieve this goal, a deep understanding of plant defence mechanisms is required as well as the selection and breeding of improved genotypes. Still, a change of paradigm is needed, and an integrative approach must be followed taking into consideration all the different aspects influencing plant performance and productivity. In this sense, the microbiota should be taken into account, as they have great significance on natural and agricultural ecosystems (Whipps et al. 2008; Compant et al. 2019). In particular, endophytic bacteria may confer advantages to host plants, promoting their overall fitness and productivity by enhancing plant resistance to biotic and abiotic stress conditions, like pathogen attacks and drought (Compant et al. 2019). These microorganisms are known to be the dominant group in these communities and have been isolated from numerous plant hosts where they live in symbiosis (Zinniel et al. 2002; Yang et al. 2017). Such beneficial effects are accomplished through the production of phytohormones, secondary metabolites with important bioactive activities (*e.g.*, antifungal and antiviral) and by promoting an increase of mineral uptake or nitrogen fixation (Zinniel et al. 2002; Yang et al. 2017). Such features might be important in plant breeding and can be accomplished through the integration of specific endophytic bacteria genes on plants or by the inoculation of bacterial strains into the plant (Compant et al. 2019). In addition, to enhance crop output, this approach can be a sustainable alternative for conventional pesticides and fertilizers, with economic and environmental benefits (Rabiey et al. 2019). The structural characterization of plant microbiota, from their composition to the intricate interaction with the host plant, is essential for this knowledge to be applied by breeders and farmers (Toju et al. 2019). Although the endophyte population dynamics have not yet been fully understood, several factors have been reported to cause variations among endophyte populations (Zinniel et al. 2002).

Because the surrounding environment and agricultural practices are considered the most crucial driving factors shaping the microbiota composition and functionality (Wassermann et al. 2019), the aim of this work was the isolation and molecular identification of endophytic bacteria from strawberry tree leaves from plants growing spontaneously on a natural environment as well as from plants growing on a production orchard. Moreover, to assess the function of these bacteria

endophytes in plant defence, its antagonism effect was tested against the most important strawberry tree pathogens, namely *Glomerella cingulata*, *Mycosphaerella aurantia* and *Phytophthora cinnamomi*. Insights about the bacteria endophytic diversity and their plant growth-promoting features and antagonism effects are provided and the implications of these results on biological control strategies, plant phenotyping and breeding are discussed.

Material and Methods

Collection of Plant Material

Plant material was collected during May 2018 from 60 *A. unedo* trees growing in the wild (40.043334, -7.904996) and in an orchard (40.029581, -7.924739), in Pampilhosa da Serra, Coimbra district, central Portugal. Three replicates from each location were used, each replicate consisting of 10 healthy adult trees. Five leaves were randomly collected from each tree (a total of 50 leaves per replicate) and pooled together (Figure 49).

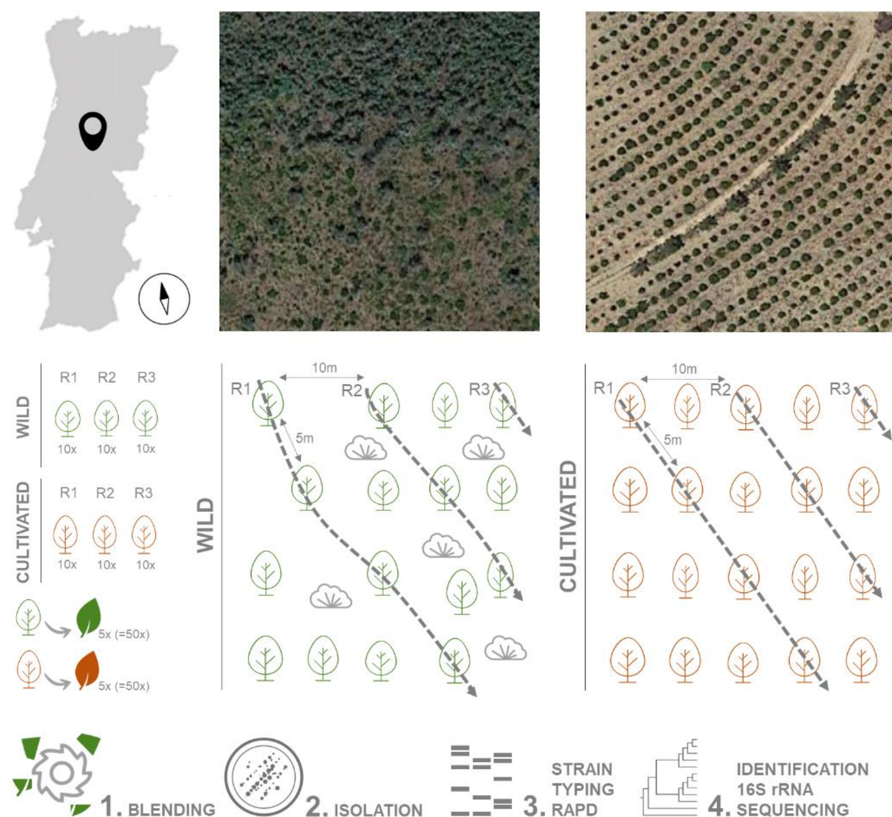

Figure 49. Strategies for sampling, isolation and identification of bacteria endophytes from strawberry tree leaves.

Leaves were stored in sterile plastic bags at 4°C and processed within 24h. Plants from the wild location were growing under uncontrolled conditions, on a mixed forest with *Pinus pinaster* and shrubs (*Erica arborea*, *Calluna vulgaris* and *Ulex* sp.) as well as several weeds. In the orchard

an irrigation system was installed, plants were regularly pruned, fertilized and the soil had been mobilized to remove weed species.

Isolation of Endophytic Bacteria

Leaves were sterilized as previously described by (Eevers et al. 2015). Briefly, leaves were thoroughly washed in sterile ultra-pure water, surface-disinfected in ethanol (70%, v/v) for 90 sec, sodium hypochlorite (1%, w/v) for 3 min and finally rinsed five times in sterile ultra-pure water. The last rinsing water was inoculated in alkaline buffered *medium* 2 (ABM2, (Tiago et al. 2004) and incubated at 25ºC for 72 h to confirm the efficiency of the process. Approximately 10 g of sterilized leaves were shredded in a blender with 10 mL PBS buffer (10 mM, pH 7.2), and filtered through a sterile gauze cloth to remove plant debris. Several serial dilutions were prepared (0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) and 100 µL of each dilution was inoculated in Petri dishes containing ABM2 and 1/10 869 medium (Mergeay et al. 1985), at pH 5.5, 7.0 and 8.5, and cultured at three different temperatures: 20, 25 and 30 ºC. Petri dishes were examined daily, and bacteria colonies were isolated in the same medium with the same pH and kept at 25 ºC.

Identification of Endophytic Bacteria

DNA extraction and RAPD fingerprinting

For DNA extraction, a crude lysate was prepared by resuspending a bacteria colony on 50 µL of 0.5M NaOH, heated at 95 ºC for 4 min and immediately chilled on ice for 10 min. After centrifugation at 10000 rpm for 5 min to remove debris, the supernatant was collected and stored at -20 ºC (adapted from (Wiedmann-Al-Ahmad et al. 1994). RAPD-PCR was performed as previously described (Costa et al. 2005) to assess the genetic diversity of bacterial strains using the primer OPA-03 (5'-AGTCAGCCAC-3'). PCR products were visualized on a 2% (w/v) agarose gel stained with GreenSafe Premium (NZYTech, Oeiras, Portugal), under UV light. Clusters were formed by visual inspection based on the similarity and intensity of the fluorescence of each band observed in RAPD profiles, namely the number and weight of the bands when compared with each other and with the molecular weight marker.

Phylogenetic analysis

Phylogenetic analyses were performed in the representative bacterial strains selected from the previously established clusters based on the RAPD-PCR fingerprinting analysis (1-3 strains per cluster). Isolates were identified based on the complete sequence of the 16S rRNA gene (approximately 1500 bp) amplified by polymerase chain reaction (PCR) using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-GGTTACCTTGTTACGACTT-3') (Rainey et al. 1996). PCR reactions contained 1x NZYTaq II 2x Colorless Master Mix (NZYTech), 10 mM primer, 50 ng DNA in a final reaction volume of 50µl. The reactions were performed in a T100™ Thermal Cycler (BioRad) under the following conditions: 95 ºC for 5 min, followed by 30 cycles at 95 ºC for 1 min, annealing at 55 ºC for 1 min and extension at 72 ºC for 2 min, before a final

extension at 72 ºC for 10 min. PCR products were visualized on a 1.5% (w/v) agarose gel stained with GreenSafe Premium (NZYTech), under UV light. PCR products were purified and sequenced by StabVida, Portugal, using the reverse 519R primer (5'-GWATTACCGCGGCKGCTG-3') (Turner et al. 1999) and the forward primers 357F (5'-CCTACGGGAGGCAGCAG-3') (Muyzer et al. 1993) and 803F (*5'-ATTAGATACCCTGGTAGTC-3'*) (Turner et al. 1999). Sequences were manually edited using Sequence Scanner Software 2 (Applied Biosystems, Foster City, California, USA) and compared with available data from EZBioCloud databases (Yoon et al. 2017). Those obtained from EZBioCloud with more than 97% similarity with our isolates were used for phylogenetic analysis and aligned using ClustalW. This operation was done for each bacterial genus separately. Best suitable DNA substitution models were assessed using the "find best DNA/Protein Models (ML)" function on MEGA (version 10.0 for Windows; (Kumar et al. 2018) by implementing the maximum likelihood (ML) statistical method to test the goodness of fit to several models of evolution. According to the estimated values of all parameters for each model, the best fitting model to the dataset from the 16S rRNA gene sequences was Kimura 2-parameter (K2) and gamma-distributed (+G) with invariant sites (+I) (=K2+G+I) (Kimura 1980) for *Bacillus* sp. and *Paenibacillus* sp., Hasegawa-Kishino-Yano (HKY+G+I) (Hasegawa et al. 1985) for *Pseudomonas* sp., Tamura 3-parameter (T92+G+I) (Tamura 1992) for *Sphingomonas* sp. and Tamura-Nei (TN93+G+I) (Tamura and Nei 1993) for *Staphylococcus* sp. Phylogenetic reconstruction was calculated using the Maximum Composite Likelihood method with bootstrap values calculated from 1000 replicate runs. Finally, a phylogenetic reconstruction was similarly calculated using the Kimura 2-parameter model (K2+G+I) with the 16S rRNA sequences obtained from the *Arbutus unedo* bacteria isolates along with the most similar sequences retrieved from EZBioCloud database (Table 1).

Antagonism ability of bacterial endophytic against *A. unedo* fungi pathogens

Bacteria isolates were tested *in vitro* for their antagonistic effect against microorganisms (two fungi and one oomycete) known to be pathogenic to *A. unedo*, using the direct opposition method (Dennis and Webster 1971). A strain randomly selected from each bacterial species from the genera *Bacillus*, *Paenibacillus*, *Pseudomonas* and *Sphingomonas* was used (a total of 16). Briefly, mycelia plugs with 5 mm taken from the outside ring of a 7 days old actively growing colony of *Glomerella cingulata*, *Mycosphaerella aurantia* and *Phytophthora cinnamomi*, from the Plant Biotechnology Laboratory collection (isolated from *A. unedo*), were placed at 1 cm from the wall of a petri dish (90 mm) with PDA medium (Difco™ Potato Dextrose Agar: 4 g L^{-1} potato starch, 20 g L^{-1} dextrose, 15 g L^{-1} agar). On the opposite side, 10 µL of a bacteria inoculum ($1x10^8$ CFU mL^{-1}) was placed. Plates were done in triplicate and incubated at room temperature. The reduction in the growth of *G. cingulata* and *P. cinnamomi* was calculated after 7, 14 and 21 days using the following formula: RG = (TG - CG) / CG *100 (Lahlali et al. 2007), where TG is the

growth of the colony in dual cultures and CG is the growth of a control group. The reduction in the growth of *M. aurantia* was calculated after 3, 6 and 9 weeks, as it is a slow grower fungus.

Plant growth-promoting potential of endophytic bacteria

Endophytic bacteria plant growth-promoting potential was tested on the same 16 isolates used on the antagonism assays. For this purpose, the siderophores production, phosphate solubilization, ammonia and indol-3-acetic-acid (IAA) production was tested.

Siderophores production

Siderophore production was determined according to (Almoneafy et al. 2012). Bacteria isolates were grown on LB broth (10 g L^{-1} of peptone, 5 g L^{-1} of yeast extract and 5 g L^{-1} of NaCl) for 24 h. A 10 µL drop of each culture was plated on LB medium (LB broth supplemented with 12 g L^{-1} agar) with chrome azurol S (CAS) complex (Schwyn and Neilands 1987) and incubated for 3 days at 28 ºC. The siderophore production was determined by the presence of an orange halo around the colony. Plates were done in triplicate.

Phosphate solubilization

The ability of bacterial strains to solubilize phosphate was determined as previously described (Almoneafy et al. 2012). Briefly, isolates were grown for 24 h in GY medium (10 g L^{-1} glucose and 2 g L^{-1} yeast extract, supplemented after autoclaving with 50 mL of a 10% K_2HPO_4 solution and 100 mL of a 10% $CaCl_2$ solution). A 10 µL drop of the bacterial suspension was then plated in GYA medium (GY medium with 15 g L^{-1} agar) and incubated for 7 days at 28 ºC. The presence of a clear visible halo around colonies was indicative of phosphate solubilization by the bacterial isolate. Plates were done in triplicate.

Ammonia production

The production of ammonia was tested as previously described ((Singh and Kumar Yadav 2016). Briefly, bacterial isolates were inoculated in peptone water medium (10 g L^{-1} of peptone and 5 g L^{-1} of NaCl) and incubated for 4 days at 30 ºC. Nessler's reagent was added to the tubes and colour development from brown to yellow indicated ammonia production. The production level of ammonia was classified as 0 when no colour change was observed, 1 when a faint yellow colour was observed, and 2 for high levels of ammonia production, and clear development of the solution colour to yellow. Tubes were done in triplicate.

Indole-3-acetic acid production

The production of IAA was tested following a procedure previously described (Almoneafy et al. 2012). Briefly, isolates were cultured on LB medium supplemented with L-tryptophan (40 µg mL^{-1}) and incubated for 48 h at 30 ºC and 160 rpm. After centrifugation at 10000 rpm for 15 min, 1 mL of the filtrate culture and 1 mL of Salkowski's reagent (1.5 ml of $FeCl_3.6H_2O$ 0.5 M solution, in 80 ml of 60% (v/v) H_2SO_4) were mixed and incubated at room temperature for 30 min. The

presence of pink colour indicates the presence of IAA. The concentration of IAA produced by each bacterial isolate was calorimetrically quantified at 530 nm using an IAA standard curve (0 - 25 µg mL^{-1}, $y = 0.0359x – 0.0349$, $R^2 = 0.995$). Tests were done in triplicate.

Statistical analysis

Venn diagrams of RAPD profiles and bacteria species from cropped and wild plants were constructed on R software (version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria, (R Core Team 2020), using the package VennDiagram (Chen 2018). Shannon index (H'), Simpson (D), Simpson reciprocal index (1-D') and Pielou index (J') were obtained to compare the diversity between populations. Diversity indexes were compared by a t-student test, whereas antagonism and IAA production data were analysed by one-way ANOVA using GraphPad Prism (v. 8.4.3 for Windows, San Diego, CA, USA), followed by a Tukey´s multiple comparison test ($P < 0.05$).

Results

Isolation of endophytic bacteria from cultivated and wild strawberry trees

A total of 62 endophytic strains were isolated from the leaves of strawberry tree. A similar number of isolates were obtained on the two media and three temperatures tested. In contrast, the number of isolates obtained greatly varied according to the medium pH. A considerably higher number of isolates was obtained for pH 7 on both mediums and no bacteria growth was observed on ABM2 medium with pH 5.5 (Table S14, available at: http://phd.freecluster.eu/).

From the 62 isolates, 32 were obtained from wild plants, whereas the other 29 from the cultivated ones. RAPD profiles were obtained for all the 62 isolates and grouped in 50 different clusters. Most of the RAPD profiles (39) were unique. Eight clusters were formed by two isolates while the other three by three or more isolates.

Isolates selected for 16S rRNA gene amplification and sequencing were identified belonging to five genera (*Bacillus*, *Paenibacillus*, *Pseudomonas*, *Sphingomonas* and *Staphylococcus*) and 17 bacteria species (Figure 50, Table 3).

Table 3. Identification of endophyte bacteria isolates by 16S rRNA gene sequencing.

RAPD profile	Isolate	Species (NCBI accession number)	EZBioCloud database match (partial 16S sequence)	Similarity	EZBioCloud database match (complete 16S sequence)	Similarity
1	Au01	*Pseudomonas avellanae* (MW534840)	*Pseudomonas avellanae* (AKBS01001374)	100	*Pseudomonas avellanae* (AKBS01001374)	99.93
1	Au10					
1	Au18	*Pseudomonas avellanae* (MW534847)	*Pseudomonas avellanae* (AKBS01001374)	100	*Pseudomonas avellanae* (AKBS01001374)	99.93
1	Au19					

2	Au02	*Bacillus toyonensis* (MW534841)	*Bacillus toyonensis* (CP006863)	99.57	*Bacillus toyonensis* (CP006863)	99.86
3	Au03	*Paenibacillus humicus* (MW534842)	*Paenibacillus humicus* (BIMD01000104)	100	*Paenibacillus humicus* (BIMD01000104)	100
4	Au04	*Bacillus megaterium* (MW534843)	*Bacillus megaterium* (JJHM01000057)	98.93	*Bacillus megaterium* (JJHM01000057)	99.66
5	Au05					
5	Au54	*Bacillus toyonensis* (MW534862)	*Bacillus toyonensis* (CP006863)	99.35	*Bacillus toyonensis* (CP006863)	100
6	Au06	*Bacillus cereus*	*Bacillus cereus* (AE016877)	100		
6	Au07					
7	Au08	*Bacillus toyonensis*	*Bacillus toyonensis* (CP006863)	100		
8	Au09	*Paenibacillus humicus* (MW534844)	*Paenibacillus humicus* (BIMD01000104)	99.33	*Paenibacillus humicus* (BIMD01000104)	99.8
8	Au35	*Paenibacillus humicus*	*Paenibacillus humicus* (BIMD01000104)	100		
8	Au71	*Paenibacillus humicus*	*Paenibacillus humicus* (BIMD01000104)	100		
9	Au11	*Pseudomonas avellanae*	*Pseudomonas avellanae* (AKBS01001374)	100		
10	Au12	*Bacillus toyonensis*	*Bacillus toyonensis* (CP006863)	100		
11	Au14	*Bacillus safensis* subsp. safensis (MW534845)	*Bacillus safensis* subsp. *safensis* (ASJD01000027)	99.35	*Bacillus safensis* subsp. *safensis* (ASJD01000027)	100
12	Au15	*Paenibacillus pabuli* (MW534846)	*Paenibacillus pabuli* (BCNM01000057)	100	*Paenibacillus pabuli* (BCNM01000057)	98.78
13	Au16	*Paenibacillus humicus*	*Paenibacillus humicus* (BIMD01000104)	99.33		
13	Au30					
14	Au20	*Paenibacillus humicus*	*Paenibacillus humicus* (BIMD01000104)	99.33		
14	Au27					
15	Au21	*Bacillus simplex* (MW534848)	*Bacillus simplex* (BCVO01000086)	100	*Bacillus simplex* (BCVO01000086)	99.83
16	Au22	*Paenibacillus humicus* (MW534849)	*Paenibacillus humicus* (BIMD01000104)	100	*Paenibacillus humicus* (BIMD01000104)	100
17	Au23	*Bacillus toyonensis* (MW534850)	*Bacillus toyonensis* (CP006863)	100	*Bacillus toyonensis* (CP006863)	100
18	Au24	*Paenibacillus taichungensis*	*Paenibacillus taichungensis* (EU179327)	99.13		
18	Au40					
18	Au49					
19	Au26	*Paenibacillus humicus* (MW534851)	*Paenibacillus humicus* (BIMD01000104)	100	*Paenibacillus humicus* (BIMD01000104)	99.18

20	Au28	*Paenibacillus pabuli*	*Paenibacillus pabuli* (BCNM01000057)	100		
21	Au29	*Paenibacillus taichungensis* (MW534852)	*Paenibacillus taichungensis* (EU179327)	99.78	*Paenibacillus taichungensis* (EU179327)	99.3
22	Au31	*Paenibacillus pabuli*	*Paenibacillus pabuli* (BCNM01000057)	100		
23	Au32	*Paenibacillus marchantiophytorum* (MW534853)	*Paenibacillus marchantiophytorum* (KP056549)	98.99	*Paenibacillus marchantiophytorum* (KP056549)	98.31
24	Au33	*Paenibacillus humicus*	*Paenibacillus humicus* (BIMD01000104)	100		
25	Au34	*Paenibacillus humicus* (MW534854)	*Paenibacillus humicus* (BIMD01000104)	99.56	*Paenibacillus humicus* (BIMD01000104)	99.18
26	Au37	*Bacillus cereus* (MW534855)	*Bacillus cereus* (AE016877)	100	*Bacillus cereus* (AE016877)	100
27	Au38	*Bacillus cereus* (MW534856)	*Bacillus cereus* (AE016877)	100	*Bacillus cereus* (AE016877)	100
28	Au39	*Pseudomonas poae* (MW534857)	*Pseudomonas poae* (JYLI01000039)	99.78	*Pseudomonas poae* (JYLI01000039)	99.86
29	Au43	*Sphingomonas panni* (MW534858)	*Sphingomonas panni* (AJ575818)	98.48	*Sphingomonas panni* (AJ575818)	98.93
30	Au45	*Paenibacillus taichungensis*	*Paenibacillus taichungensis* (EU179327)	99.79		
31	Au46	*Bacillus cereus*	*Bacillus cereus* (AE016877)	100		
31	Au51	*Bacillus cereus*	*Bacillus cereus* (AE016877)	100		
32	Au47	*Paenibacillus etheri*	*Paenibacillus etheri* (KC625558)	100		
33	Au48	*Paenibacillus humicus* (MW534859)	*Paenibacillus humicus* (BIMD01000104)	100	*Paenibacillus humicus* (BIMD01000104)	99.93
34	Au50	*Paenibacillus taichungensis* (MW534860)	*Paenibacillus taichungensis* (EU179327)	99.79	*Paenibacillus taichungensis* (EU179327)	99.73
35	Au53	*Paenibacillus taichungensis* (MW534861)	*Paenibacillus taichungensis* (EU179327)	99.32	*Paenibacillus taichungensis* (EU179327)	99.3
36	Au55	*Paenibacillus taichungensis*	*Paenibacillus taichungensis* (EU179327)	99.32		
37	Au57	*Staphylococcus capitis* subsp. *capitis* (MW534863)	*Staphylooccus capitis* subsp. *capitis* (L37599)	100	*Staphylococcus capitis* subsp. *capitis* (L37599)	99.93
38	Au59	*Paenibacillus pabuli*	*Paenibacillus pabuli* (BCNM01000057)	100		
39	Au61	*Bacillus taxi*	*Bacillus taxi* (MK355518)	100		
40	Au62	*Staphylococcus epidermidis* (MW534864)	*Staphylococcus epidermidis* (UHDF01000003)	100	*Staphylococcus epidermidis* (UHDF01000003)	99.86
41	Au65	*Bacillus taxi* (MW534865)	*Bacillus taxi* (MK355518)	99.57	*Bacillus taxi* (MK355518)	99.73
42	Au67	*Sphingomonas panni* (MW534866)	*Sphingomonas panni* (AJ575818)	98.49	*Sphingomonas panni* (AJ575818)	98.93

43	Au69	*Paenibacillus humicus*	*Paenibacillus humicus* (BIMD01000104)	100		
44	Au70	*Paenibacillus etheri* (MW534867)	*Paenibacillus etheri* (KC625558)	100	*Paenibacillus etheri* (KC625558)	99.62
45	Au72	*Bacillus cereus*	*Bacillus cereus* (AE016877)	100		
46	Au73	*Staphylococcus epidermidis* (MW534868)	*Staphylococcus epidermidis* (UHDF01000003)	99.35	*Staphylococcus epidermidis* (UHDF01000003)	99.93
47	Au74	*Paenibacillus pabuli*	*Paenibacillus pabuli* (BCNM01000057)	100		
48	Au75	*Bacillus cereus* (MW534869)	*Bacillus cereus* (AE016877)	99.35	*Bacillus cereus* (AE016877)	99.93
49	Au76	*Paenibacillus marchantiophytorum*	*Paenibacillus marchantiophytorum* (KP056549)	98.32		
50	Au77	*Paenibacillus humicus*	*Paenibacillus humicus* (BIMD01000104)	100		

A total of 21 distinct RAPD profiles were obtained from the bacterial isolates from cultivated plants, while 27 were obtained from strains isolated in wild plants (Figure 51 A, Figure 52). Only two RAPD profiles were common between isolates from cultivated and wild plants, identified as *B. toyonensis* and *P. humicus*. This difference was more marked in the number of species since seven different species were restricted to wild plants, while only four species were exclusively found in cultivated plants (Figure 51 B, Figure 52). Additionally, five bacterial species were shared between wild and cultivated plants, to know *B. cereus*, *B. toyonensis*, *P. humicus*, *P. taichungensis* and *S. epidermidis*. In terms of distribution between replicates, *P. taichungensis* was found on all the three replicates, from cultivated and isolated plants, whereas *B. cereus* was identified in all replicates from cultivated plants and *P. humicus* in wild plants (Figure 51 C-D).

Bacillus and *Paenibacillus* were the genera more often recovered comprising 17 and 26 RAPD profiles, corresponding to 19 and 32 isolates, respectively. On the contrary, only two *Pseudomonas* species (*P. avellanae* in cultivated and *P. poae* in wild trees) were identified corresponding to three RAPD profiles with six isolates (Table 3). The same number of *Staphylococcus* species were identified (*S. epidermidis* and *S. capitis*), with three RAPD profiles and three isolates. Two isolates obtained from cultivated plants, with the same RAPD profile, were identified as *Sphingomonas hankookensis*, the only species found from this genus. Although seven species had only one RAPD profile, others had a considerably higher number of RAPD profiles: *B. cereus* (8), *P. taichungensis* (12) and *P. humicus* (15). As far as we know, this is the first report of *Paenibacillus etheri* as a plant endophyte. However, the other endophytes isolated from strawberry tree leaves have already been identified in other plant species (Table 4).

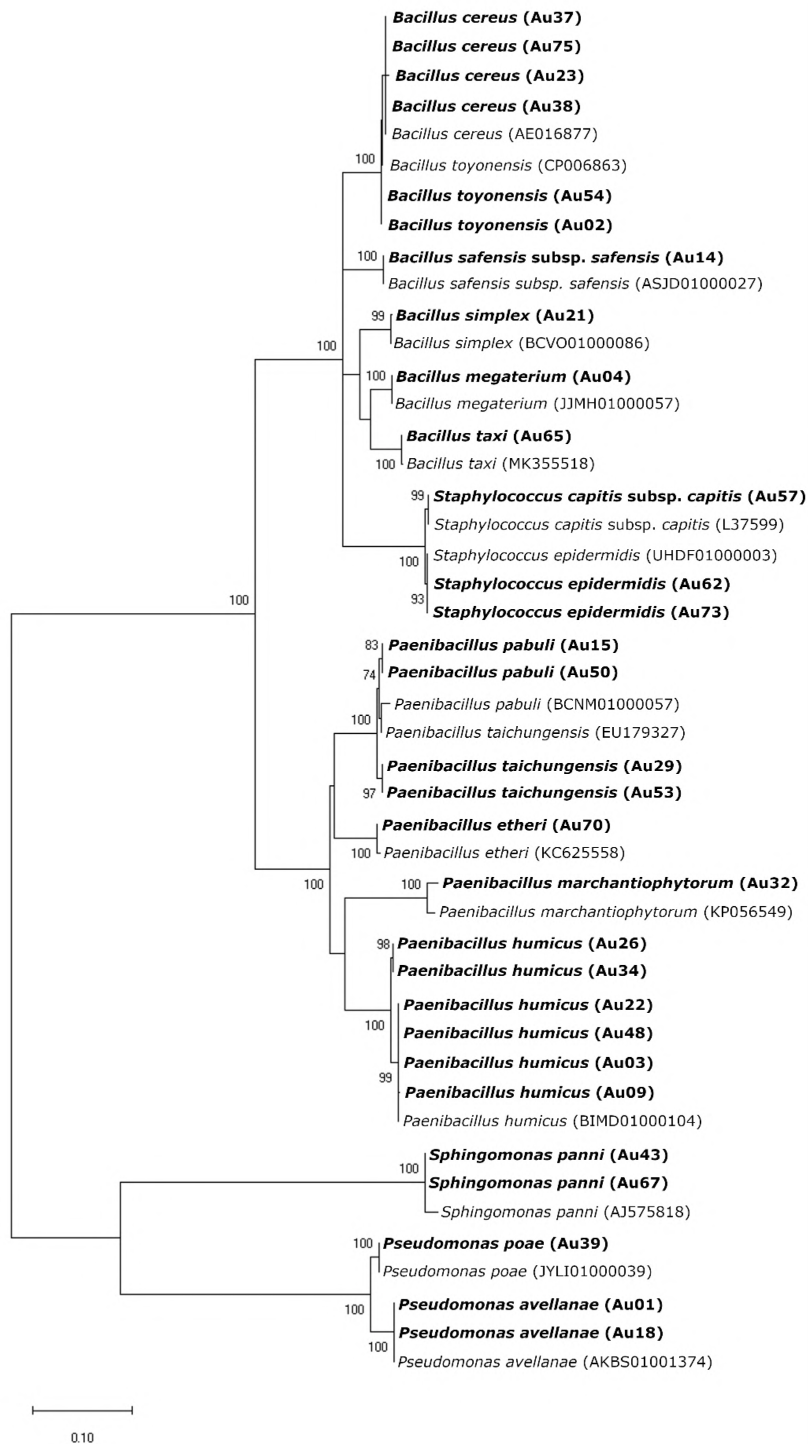

Figure 50. Maximum Likelihood (ML) phylogenetic tree based on 16S rRNA sequences of endophytic bacteria and sequences obtained from the EZBioCloud database**.** ML tree was constructed using the Kimura 2 parameter model (K2) and gamma-distributed (+G) with invariant sites (+I) (=K2+G+I). All positions containing gaps and missing data were included for analysis. Clade supports were calculated based on 1000 replicate runs. Accession numbers (GeneBank) of the sequences belonging to the endophytic bacteria isolated in this study and the reference sequences from EZBioCloud are listed in Table 3.

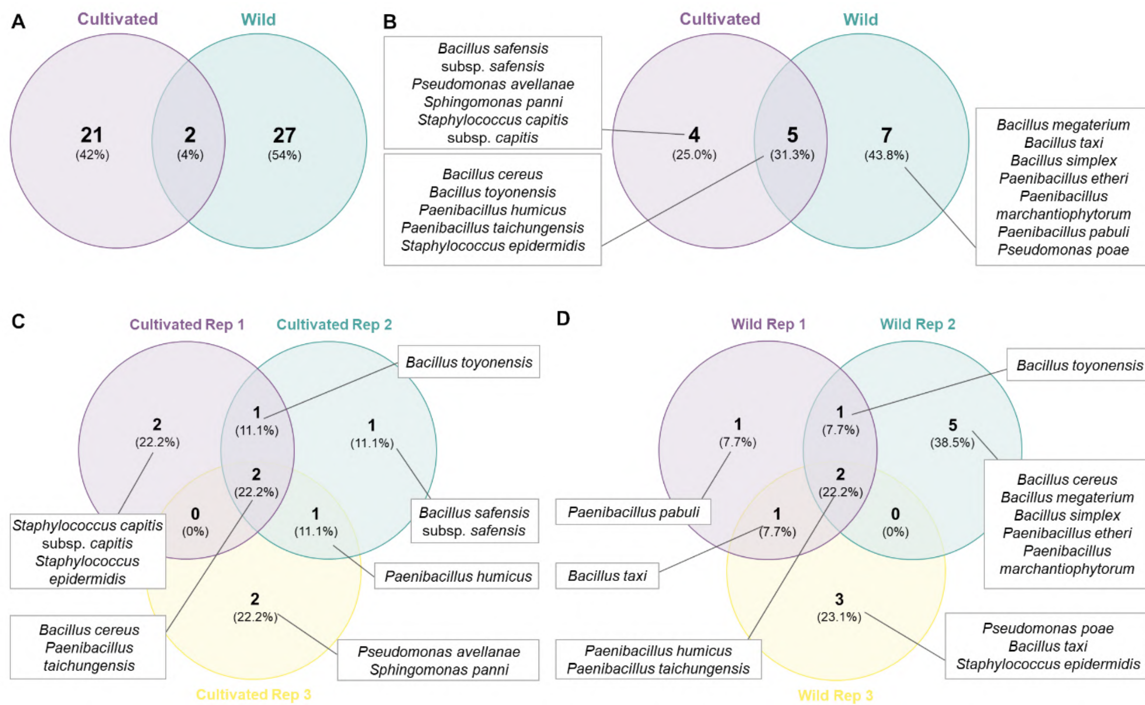

Figure 51. Venn diagrams with the number of RAPD profiles and bacteria species: RAPD profiles from orchard and wild plants (A); endophytic bacterial species from orchard and wild plants (B); endophytic bacterial species from cultivated plants replicates (C); endophytic bacterial species from wild plants replicates (D).

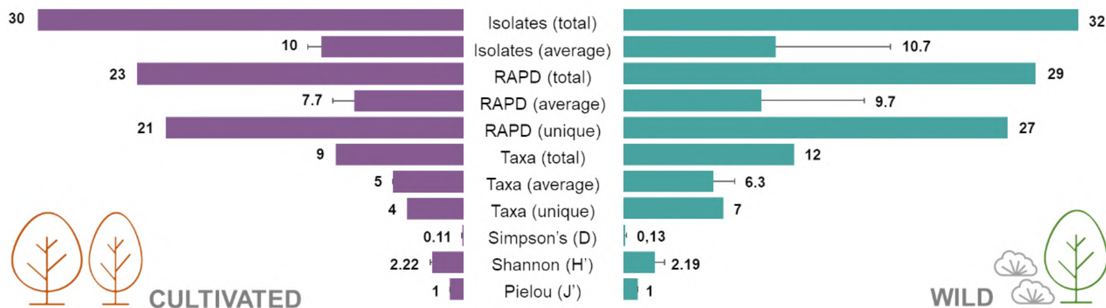

Figure 52. Number of isolates, RAPD profiles, taxa, and diversity indexes (Simpson, Shannon and Pielou) of the endophytic bacterial isolates from cultivated and wild strawberry tree populations.

Table 4. Bacteria endophytes isolated from *A. unedo*, and some plant species where these bacteria have been identified before.

Bacteria endophyte	Plant species
Bacillus cereus	*Teucrium polium* and *Sophora alopecuroides* (Lopes et al. 2018), *Solanum lycopersicum* (Rocha et al. 2017), *Polygonum cuspidatum* (Sun et al. 2013), *Saccharum officinarum* (Varma et al. 2017), *Clitoria ternatea* (Khaksar et al. 2016), *Fragraria ananassa* and *Dyospiros kaki* (Hu et al. 2017)

B. megaterium	*Retama monosperma* (Dahmani et al. 2020), *Eucalyptus* spp. (Ferreira et al. 2008), *Gossypium hirsutum* and *Zea mays* (McInroy and Kloepper 1995), *S. lycopersicum* (Rocha et al. 2017), *S. officinarum* (Varma et al. 2017) and *V. radiata* (Pandya et al. 2015)
B. nealsonii	*Nicotiana attenuate* (Long et al. 2010)
B. safensis	*Osmanthus fragrans* (Rong et al. 2020), *Musa* sp. (Sekhar and Thomas 2015), *P. cuspidatum* (Sun et al. 2013), *S. officinarum* (Varma et al. 2017), *Chloris virgata* (Wu et al. 2019) and *V. radiata* (Pandya et al. 2015)
B. simplex	*P. cuspidatum* (Sun et al. 2013)
B. taxi	*Taxus chinensis* (Tuo et al. 2020)
B. toyonensis	*S. lycopersicum* (Rocha et al. 2017)
Paenibacillus etheri	-
P. humicus	*Acacia* sp. (Zineb et al. 2016) and *Eucalyptus* spp. (Ferreira et al. 2008)
P. marcantiophytorum	*Herbertus sendtneri* (Guo et al. 2016)
P. pabuli	*P. cuspidatum* (Sun et al. 2013), *S. officinarum* (Varma et al. 2017) and *V. radiata* (Pandya et al. 2015)
P. taichungensis	*V. radiata* (Pandya et al. 2015)
P. poae	*Vitis vinifera* (Niem et al. 2020)
Sphingomonas panni	*Musa* sp. (Sekhar and Thomas 2015)
Staphylococcus capitis subsp. *capitis*	*G. hirsutum* and *Z. mays* (McInroy and Kloepper 1995)
S. epidermidis	*S. lycopersicum* (Nawangsih et al. 2011) and *Musa* sp. (Sekhar and Thomas 2015)
P. avellanae	*Corylus avellane* (Scortichini et al. 2006; Loreti et al. 2009)

The Simpson diversity index (D) calculated for both populations was 0.11 ± 0.02 for cultivated plants and 0.13 ± 0.07, whereas the Shannon index (H') was 2.22 ± 0.16 in the cultivated group and 2.19 ± 0.70 in the wild one. Finally, Pielou's evenness index (J') was 1 ± 0 in the cultivated plants and 1 ± 0 in the wild population (Fig. 4). No statistical differences were observed between groups for all the diversity indexes calculated.

Antagonism effect of endophytic bacteria against plant pathogens

Sixteen strains were tested for the ability to inhibit the growth of strawberry tree pathogens. Although most of the isolates caused a reduction in the growth of *Glomerella cingulata* after one week in culture, this antagonism effect was reduced over time (Figure 53 A-C). After 3 weeks in culture, the isolate Au06 (*B.* cereus) proved to be the most effective antagonist causing a reduction

in growth close to 30%. Several of the tested bacterial isolates had the opposite effect promoting the growth of *G. cingulata* (between 10% and 30%): Au3 (*P. humicus*), Au15 (*P. pabuli*), Au 21 (*B. simplex*), Au 47 (*P. etheri*), Au 53 (*P. taichungensis*), Au 61 (*B. taxi*) (Figure 53 C). In the case of *Phytophthora cinnamomi*, a slight reduction in growth (less than 20%) was observed after one week for most isolates (Figure 53 D). This antagonist effect was intensified after two weeks (Figure 53 E), but after three weeks in culture, the effect of the bacteria was barely noted, except for isolate Au06 (*B.* cereus), which caused a reduction in the growth of more than 40% (Figure 53 F). Most of the bacterial isolates caused a reduction in the growth of *M. aurantia* after three and six weeks in culture (Figure 53 G-H). After nine weeks, all the isolates proved to have an antagonist effect and caused a reduction in *M. aurantia* growth in some cases close to 40% (Figure 53 I). Overall, *B. cereus* (Au06) was the most effective antagonist of *A. unedo* fungal pathogens tested on this study as it was able to control their growth. It is important to note that the antagonism effect of *B. cereus* was caused at a distance and not by direct contact between microorganisms. (Figure 54).

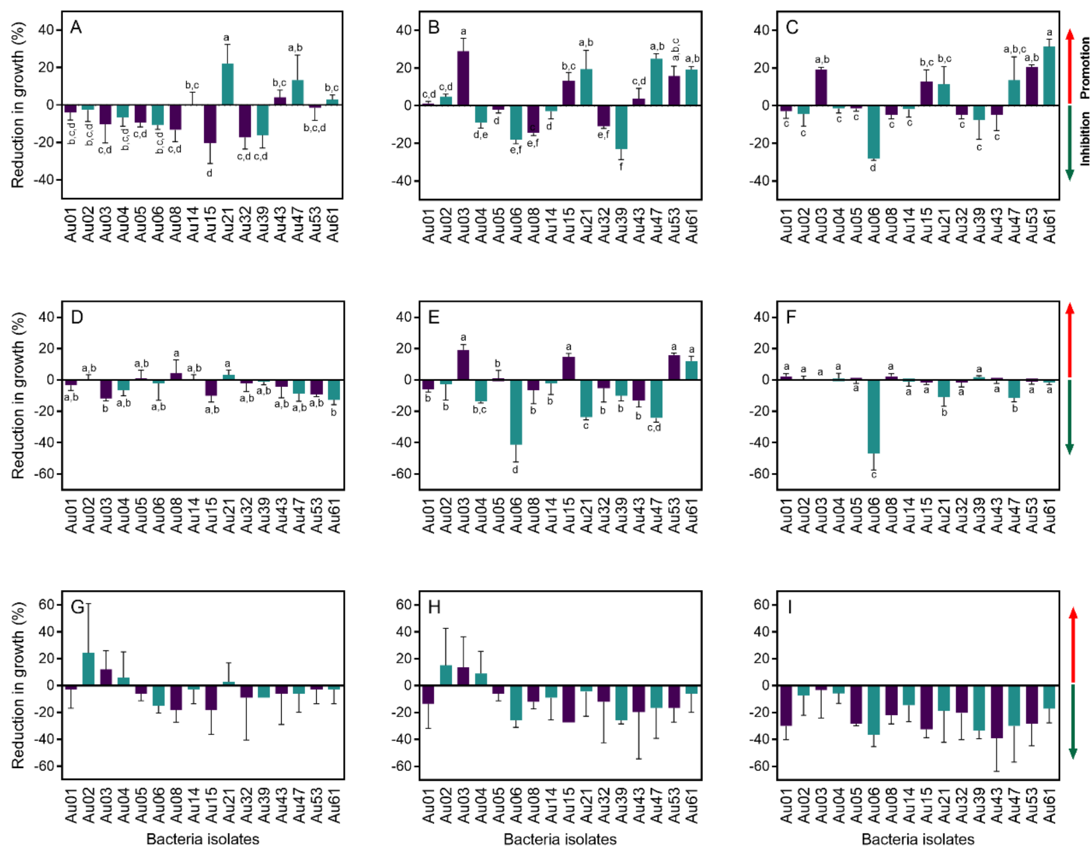

Figure 53. Antagonism assay between some of the endophytic bacteria isolated from *Arbutus unedo* and plant pathogens *Glomerella cingulata*, *Mycosphaerella aurantia* and *Phytophthora cinnamomi*. Reduction in growth (%) was calculated after 1, 2 and 3 weeks for *G. cingulata* and *P. cinnamomi*, and after 3, 6 and 9 weekd for *M. aurantia*: *G. cingulata* after 1 week (A); *G. cingulata* after 2 weeks (B); *G. cingulata* after 3 weeks (C); *M. aurantia* after 3 weeks (D); *M. aurantia* after 6 weeks (E); *M. aurantia* after 9 weeks (F); *Phytophthora cinnamomi* after 1 week (G); *Phytophthora cinnamomi* after 2 weeks (H); *Phytophthora*

cinnamomi after 3 weeks (I). Bacteria isolates: Au01 - *Pseudomonas avellanae*, Au02 - *Bacillus toyonensis*, Au03 - *Paenibacillus humicus*, Au04 - *Bacillus megaterium*, Au05 - *Bacillus toyonensis*, Au06 - *Bacillus cereus*, Au08 - *Bacillus toyonensis*, Au14 - *Bacillus safensis*, Au15 - *Paenibacillus pabuli*, Au21 - *Bacillus simplex*, Au32 - *Paenibacillus marchantiophytorum*, Au39 - *Pseudomonas poae*, Au43 - *Sphingomonas panni*, Au47 - *Paenibacillus etheri*, Au53 - *Paenibacillus taichungensis* and Au61 - *Bacillus taxi*. Means ± standard deviations, n = 3, different letters indicate significant differences between treatments ($P \leq 0.05$).

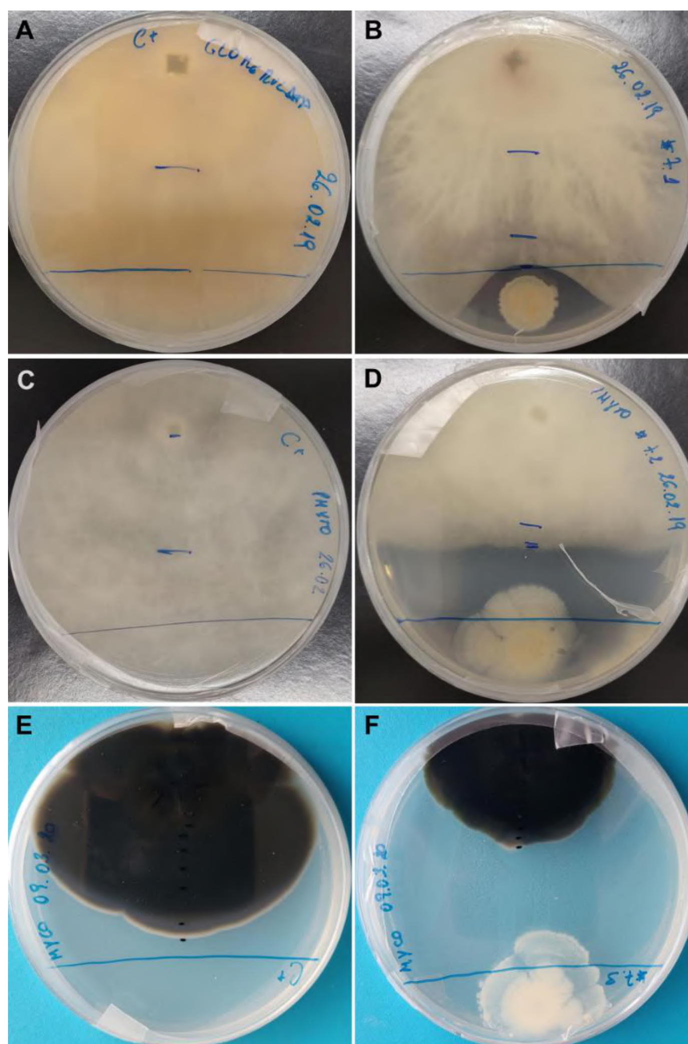

Figure 54. Antagonism test between *Bacillus cereus* (Au06) and *Glomerella cingulata*, *Phytophthora cinnamomi* and *Mycosphaerella aurantia*: *G. cingulata* (3 weeks) (A); *G. cingulata* and *B. cereus* (3 weeks) (B); *P. cinnamomi* (3 weeks) (C); *P. cinnamomi* and *B. cereus* (3 weeks) (D); *M. aurantia* (9 weeks) (E); *M. aurantia* and *B. cereus* (9 weeks) (F).

Plant growth-promoting potential of endophytic bacteria isolated from strawberry tree

From the 16 isolates tested, five were found to produce siderophores: Au01 (*P. avellanae*), Au06 (*B. cereus*), Au14 (*B. safensis*), Au39 (*P. poae*) and Au43 (*S. panni*) and four were able to solubilize phosphate: Au04 (*B. megaterium*), Au32 (*P. marchantiophytorum*), Au39 (*P. poae*) and Au61 (*Bacillus taxi*) (Table 5). Whereas most of the isolates tested were able to produce ammonia: Au01 (*P. avellanae*), Au02 (*B. toyonensis*), Au04 (*B. megaterium*), Au05 (*B. toyonensis*), Au06 (*B.

cereus), Au08 (*B. toyonensis*), Au14 (*B. safensis*), Au21 (*B. simplex*) and Au39 (*P. poae*) (Table 5), the production of IAA was only detected on six bacterial species: Au01 (*P. avellanae*), Au04 (*B. megaterium*), Au21 (*B. simplex*), Au39 (*P. poae*), Au43 (*S. panni*) and Au61 (*B. taxi*) (Table 5). IAA quantification revealed the production of this hormone by all the isolates, but considerable differences in the amount of IAA between bacterial species were found, with values ranging from 0.56 ± 0.43 for isolate Au32 (*P. marchantiophytorum*) and 10.98 ± 2.44 produced for Au01 (*P. avellanae*) (Table 5).

Table 5. The growth-promoting potential of the isolated endophytic bacteria based on siderophore, ammonia and IAA production and phosphate solubilization.

Isolate	Siderophores production	Phosphate solubilization	Ammonia production	IAA production	IAA µg mL^{-1}
Au01 (*Pseudomonas avellanae*)	+	-	2	+	10.98 ± 2.44
Au02 (*Bacillus toyonensis*)	-	-	2	-	2.57 ± 1.81
Au03 (*Paenibacillus humicus*)	-	-	0	-	1.90 ± 1.81
Au04 (*Bacillus megaterium*)	-	+	2	+	6.22 ± 4.69
Au05 (*Bacillus toyonensis*)	-	-	2	-	3.10 ± 0.83
Au06 (*Bacillus cereus*)	+	-	2	-	1.72 ± 1.75
Au08 (*Bacillus toyonensis*)	-	-	2	-	4.40 ± 5.30
Au14 (*Bacillus safensis*)	+	-	2	-	2.21 ± 0.59
Au15 (*Paenibacillus pabuli*)	-	-	0	-	3.07 ± 1.42
Au21 (*Bacillus simplex*)	-	-	2	+	5.21 ± 4.36
Au32 (*Paenibacillus marchantiophytorum*)	-	+	1	-	0.56 ± 0.43
Au39 (*Pseudomonas poae*)	+	+	2	+	4.68 ± 2.11
Au43 (*Sphingomonas panni*)	+	-	1	+	6.17 ± 4.34
Au47 (*Paenibacillus etheri*)	-	-	0	-	0.74 ± 0.03
Au53 (*Paenibacillus taichungensis*)	-	-	0	-	2.47 ± 2.33
Au61 (*Bacillus taxi*)	-	+	0	+	3.87 ± 1.76
Control (*Pseudomonas syringae*)	+	-	2	+	8.43 ± 1.11
Control (*Escherichia coli*)		+	2	-	0
Negative Control	-	-	0	-	0

Discussion

The two different media used in this study proved to be efficient for the isolation of strawberry tree endophytic bacteria. Although ABM2 medium is more complex when compared to 869, both had a common basal composition that was suitable for the recovery of bacteria. The difference observed on the number of isolates according to the pH of the medium was expected due to the influence of pH on shaping microbial communities. The higher number of isolates was obtained on pH 7 indicates most strawberry tree culturable endophytic bacteria are neutrophiles.

A considerable higher number of RAPD profiles was obtained when compared to the number of species identified which suggests a high inter-specific diversity. Another observation that reinforces this idea is the fact that no common RAPD profiles were found between replicates. The difference in the number of RAPD profiles and bacteria species between replicates on the cultivated plants suggests a more homogeneous microbiota within the population, whereas on the wild plants a higher heterogenicity seems to exist. Moreover, the total number of RAPD profiles as well as unique RAPD profiles were found to be higher on wild plants. The number of taxa identified in wild plants was also higher than in cultivated ones. Although these results suggest a higher diversity in the wild population when compared to the cultivated plants, diversity indexes were very similar between groups. Furthermore, Simpson and Pielou's indexes suggest low dominance of species and evenness among populations.

The differences observed between cultivated and wild plants may be due to genetic characteristics of the microorganisms and host plant, which is crucial to regulate the diversity and structure of the microbial community through physiological, morphological and chemical features (Whipps et al. 2008; Vorholt 2012; Turner et al. 2013; Compant et al. 2019). As mentioned before, agricultural practices might also influence microbiota. Nitrogen fertilization, for example, a common practice in modern intensive agriculture, proved to lead to a decline in plant microbiota diversity in maize (Manching et al. 2014). Moreover, either to promote growth and/or to facilitate fruit collection, orchard plants are pruned to obtain a specific plant architecture, which may also influence microbiota structure (Whipps et al. 2008).

The surrounding environment is an important source of microorganisms that might be transmitted horizontally and enter the plant through different paths integrating its microbiota (Wassermann et al. 2019). Thus, surrounding plants may constitute a local reservoir of bacteria and as plant diversity is hypothetically lower in an agriculture ecosystem when compared to a wild forest, this could lead to a decrease in plant microbiota diversity. Due to the lack of such niches on the cultivated population studied, the horizontal transmission between individuals might be compromised, whereas the microbiota diversity in the wild population might be promoted due to the existence of several other plant species. Still, the contribution of these bacteria reservoirs on plant microbiota structure and transmission of dominant bacteria species needs to be further evaluated, as such knowledge can be used to improve agricultural practices (*e.g.*, the inclusion of ecological corridors to promote local diversity).

Additionally, plant microbiota is also transmitted vertically by seeds or other propagation material. In this particular case, cultivated plants have been obtained by conventional seed germination. Thus, the origin of the seeds might be crucial to determine the microbiota composition of the plants. Nonetheless, future studies are required to evaluate the contribution of propagation material to the plant core microbiome.

P. humicus was found on most replicate samples while *P. taichungensis* was found on all the samples both from wild and cultivated plants. Because these plants are under different conditions, this result might indicate these two bacteria species are part of strawberry tree core microbiota, while the other species constitute the satellite microbiota. However, further studies with more plant genotypes growing on different environmental conditions are yet to be conducted to confirm this result.

A fierce competition for space and nutrient resources occurs in microbial communities, including between bacteria and fungi, through different mechanisms like cell signalling and antibiotic production. These complex interactions allow the coexistence or exclusion of some species (Vorholt 2012). On this work, several bacteria were able to antagonize fungi pathogens (*G. cingulata* and *M. aurantia*) and an oomycete (*P. cinnamomi*). The most promising results were obtained with *Bacillus cereus*, that was able to antagonize the three plant pathogens tested. Its antifungal effect has already been reported in the literature against *Fusarium oxysporum* f. sp. *Lycopersici* (Rocha et al. 2017). However, to our knowledge, this is the first report of its antifungal effect against *G. cingulata*, *M. aurantia* and *P. cinnamomi*. Several *B. cereus* strains were isolated from both populations (cultivated and wild), which seems to be an indicator of its importance and prevalence among strawberry tree populations. The antifungal effect of other bacteria, such as *B. safensis* against other plant pathogens (*Magnaporthe oryzae*) has also been reported in the literature (Rong et al. 2020). However, no antagonism effect of *B. safensis* was observed on this work on the pathogens tested.

Our characterization study also revealed the production of siderophores by five strawberry tree endophytic bacteria. The production of these low molecular weight compounds is an important competition mechanism, in particular under iron limiting conditions (Compant et al. 2005). Besides improving the nutritional status of bacteria, they also limit iron absorption by other organisms, such as fungi, which confers a competitive advantage and can be one of the mechanisms involved in biological control. Some of the isolates have also shown the ability to solubilize phosphate and produce ammonia which is important bacteria features, that enhances their nutritional status and have a significant effect on the competition with other microorganisms.

Also, bacteria might produce IAA and other plant hormones. IAA production enhances microbial fitness as it stimulates cell wall loosening and nutrient leakage (Vorholt 2012). As one of the most important plant hormones, IAA is involved in several plant signalling pathways and greatly affects plant growth and development. Thus, the production of IAA by microorganism might be of great benefit to plants (Zhao 2010). Although is fairly know most rhizobacteria can synthesize IAA, that is not the case for phyllosphere bacteria (Wagi and Ahmed 2019). Nonetheless, our results revealed all the tested bacteria can produce IAA, even though in small quantities. For instance, the amount of IAA produced by *B. cereus* (1.72 $\mu g\ mL^{-1}$) is much lower than that of 18 $\mu g\ mL^{-1}$ quantified by (Wagi and Ahmed 2019). This difference may be related to

the experimental conditions (*e.g.*, medium and temperature) or genetic factors. *B. megaterium*, *B. safensis* and *P. pabuli* have also been referred to in the literature as IAA producing bacteria (Varma et al. 2017; Dahmani et al. 2020).

This characterization study provides important insights about strawberry tree endophytic bacteria physiological mechanisms and possible ecological interactions with important implications on future biotechnological applications. Overall, endophytic bacteria have a great influence on plant resilience against biotic and abiotic stresses and a huge impact on plant health and development leading to an increase in agricultural production. Plant microbiota contributes to a reduction of greenhouse gases emissions and chemical inputs mostly due to its benefits on plant nutrition (*e.g.*, nitrogen fixation) and potential to reduce plant disease incidence (Whipps et al. 2008; Turner et al. 2013; Wassermann et al. 2019), leading to more sustainable agricultural practices. As pathogenic microorganisms are a chronic problem that affects food production, the dependency on agrochemicals is growing as agricultural production intensifies. Due to the negative impacts of agrochemicals (*e.g.*, high cost, low efficiency, development of pathogen resistance, environmental impacts), a reduction of chemical inputs is a desirable goal for worldwide agriculture making it urgent to develop alternative mitigation strategies. In this sense, biological control with bacteria can arise as an alternative reliable crop protection method (Compant et al. 2005). Furthermore, some bacteria species have also the potential for phytoremediation of contaminated soils (Wu et al. 2019). Metabolomic studies and the identification of plant genes that are crucial to the microbial composition as well as a better understanding of the microorganisms physiology and multitrophic interactions are key factors to elucidate the recruiting mechanisms and interactions between bacteria and host plants and develop further plant protection strategies (Vorholt 2012).

Such knowledge would have great repercussions on plant phenotyping and breeding. Due to its importance on plant phenotype, microbiome populations should be taken into account on plant selection. Moreover, as well as propagation, plant breeding may lead to an interaction disruption between the plant and its microbiota (Compant et al. 2019). Thus, the selection of plant genotypes with appetence to establish symbiotic relations with specific bacteria species and/or strains might be of high importance to maintain these beneficial interactions.

Due to *A. unedo* economic potential and ecological importance on the Mediterranean region, knowledge about plant tolerance mechanisms against biotic and abiotic stress must be considerably improved and plant protection strategies should be planned to ameliorate plant fitness. This study is the first step towards enhancing our understanding of *Arbutus unedo* microbiota and provides the first identification and characterization of its endophytic culturable bacteria. Although a slightly higher number of taxa and RAPD profiles was identified in the wild population when compared to the cultivated plants, no differences were obtained in terms of diversity indexes, which seems to indicate that, in this specific case, microbiota diversity is not compromised by agricultural practices. Among the several species isolated and identified, a *Bacillus cereus* strain (Au06) proved

its efficiency antagonizing three plant pathogens, *Glomerella cingulata*, *Phytophthora cinnamomi* and *Mycosphaerella aurantia*, revealing its ecological importance and potential as a biological control agent. However, this research has raised many questions in need of further investigation, such as the contribution of horizontal and vertical transmission of microorganisms into strawberry tree. The non-culturable microbiome is currently under investigation and future research will explore the potential of *B. cereus* (Au06) as a biocontrol agent.

CHAPTER 8: Seasonal variation of phenolic compounds in Strawberry tree (*Arbutus unedo* L.) leaves and inhibitory potential on *Phytophthora cinnamomi*

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Abstract

Arbutus unedo L. produces a wide range of secondary metabolites with bioactive properties and applications for food, cosmetic and pharmaceutical industries. Due to the antifungal activity of some secondary metabolites, they can play a key role in plant defense. In particular, arbutin and hydroquinone, two phenolic compounds typical of the Ericaceae species, might be especially important in plant defense against pathogens. Thus, in this work we (i) established a phenolic fingerprint of wild strawberry tree leaves, (ii) evaluated the effect of genotype and seasonal variation on phenolic metabolite composition, (iii) quantified arbutin and hydroquinone, and (iv) tested the antifungal effect of arbutin, hydroquinone and a methanol extract of *A. unedo* leaves against common strawberry tree pathogens. A total of 54 compounds were assigned by HPLC-PDA-ESI/MS^n, namely gallic acid derivatives, hydrolysable and condensed tannins, flavonoids, arbutin, hydroquinone and their derivatives, and significant differences on relative abundance of the phenolic compounds between individuals were verified. Hydroquinone and its glucoside arbutin were quantified and its antifungal and anti-oomycete effect against *A. unedo* pathogens evaluated. Arbutin concentrations ranged from 0.69 ± 0.05 to 22.97 ± 0.76 mg g^{-1} fresh weight of leaves. Hydroquinone was only detected in a few samples and its concentration ranged from 0.069 ± 0.004 mg g^{-1} to 0.604 ± 0.024 mg g^{-1} fresh weight. Both arbutin and hydroquinone induced a reduction in growth of *Glomerella cingulata* and both compounds were highly effective against *Phytophthora cinnamomi*, with a reduction in growth close to 80% at the higher arbutin concentration tested. Microplate tests showed that a methanol extract of *A. unedo* leaves displayed an antifungal activity with MIC (minimum inhibitory concentration) against *P. cinnamomi* at an arbutin equivalent concentration of 0.625 mg mL^{-1}, and was more active against *P. cinnamomi* than the arbutin standard (MIC=12 mg mL^{-1}), which suggests that a synergic effect occurs in the presence of other

metabolites existing in the extract. Moreover, extract containing an arbutin equivalent concentration of 2.5 mg mL^{-1} was lethal for this pathogen. Arbutin, their derivatives and/or other secondary metabolites play an important role in *A. unedo* defense mechanisms against biotic stress, opening the way to biotechnological applications, namely to use *A. unedo* leaf extracts as a natural phytosanitary agent. A genotype with the ability to produce high amounts of arbutin was also identified. The implications of these results on agroforestry management are discussed, as well as its relevance on plant phenotyping and selection for future breeding assays.

Keywords: antiphytofungal and anti-oomycete activities, arbutin, HPLC-PDA-MS^n; hydroquinone, phenolic metabolites

Introduction

A. unedo is a source of phytochemicals with biological activities and potential to be used by pharmaceutical, food and cosmetic industries (Boulanouar et al. 2013; Ziani et al. 2015; Morgado et al. 2018). Phenolic compounds have been identified, such as arbutin, hydroquinone, catechin, tannins, myricetin, kaempferol and quercetin derivatives, among others (Miguel et al. 2014; Coimbra et al. 2020). It is known that variations in plant secondary metabolites, both quantitative and qualitative, exist not only between species but also among genotypes of the same species through the influence of distinct environmental conditions or at different developmental stages (Verma and Shukla, 2015). The concentrations of these compounds in plant tissues are also strongly influenced by seasonal factors such as temperature, light and water availability, as well as by the chemical and physical properties of the soil, pathogen attacks, and many other factors (Pavarini et al. 2012; Yang et al. 2018).

Phenolic metabolites are involved not only in plant responses to various abiotic stress factors and in several physiological processes (Yang et al. 2018; Isah 2019), but they are also key players in the defense mechanisms against fungal pathogens causing leaf and root diseases (Witzell and Martín 2008). For this reason, the production and accumulation of phenolics has been considered a criterion for plant breeding (Ganthaler et al. 2017). Previous work showed that arbutin, hydroquinone and their derivatives, characteristic phenolics of Ericaceae species (Pavlović et al. 2009), can be useful in the control of plant pathogens due to their antimicrobial activity (Trevors and Basaraba 1980; Devillers et al. 1989; Kuźniak et al. 2015), leading to a reduction in pesticides use and implementation of environmentally friendly agroforestry management strategies. Several microorganisms have been recently found to cause diseases on strawberry tree leading to production constraints, namely *Alternaria* spp. (Thomma 2003), *Glomerella cingulata* (Polizzi et al. 2011), *Mycosphaerella* spp. (Romero-Martin and Trapero-Casas 2003) and *Phytophthora cinnamomi* (Moreira and Martins 2005; Moralejo et al. 2008). The most aggressive is *P.*

cinnamomi, a widespread invasive oomycete responsible for several plant diseases on different species worldwide (Sena et al. 2018).

The effect of seasonal variation and plant genotype on phenolic profiles remain unclear. Moreover, there is a lack of information concerning the relevance of arbutin and hydroquinone on *A. unedo* defense mechanisms. In the present context, in which the application of pesticides has been considerably reduced and advised against by regulatory authorities, it is important to develop alternative methods to help control plant pathogens. Thus, the identification of particular genotypes (chemotypes) containing higher amounts of specific secondary metabolites, may be relevant to breeding strategies aimed at developing new cultivars based on chemical phenotyping. Thus, the main objective of the present work was to obtain a phenolic fingerprint of wild strawberry trees and evaluate the effect of genotype and seasonal variation on phenolic composition. For this purpose, leaves of three different wild strawberry tree genotypes (selected based on their fruit quality production) were seasonally collected every two months for a year, and the chemical composition of their methanol extracts analyzed by HPLC-PDA and HPLC-PDA-ESI/MS^n. Additionally, arbutin and hydroquinone from the extracts were quantified and its antifungal and anti-oomycete activity, as well as that of a methanol extract, was evaluated against the following *A. unedo* common pathogens: *Alternaria alternata*, *Glomerella cingulata*, *Mycosphaerella* sp. and *Phytophthora cinnamomi*. Considering the ecological and growing economic importance of *A. unedo*, this work provides new insights into its chemical characterization and innovative plant selection strategies.

Material and Methods

Plant Material

Samples were collected from trees growing in the wild, in Chaves municipality (N 41º42'31.868'' W 7º26'32.506'', altitude 579 m), in the Northern region of Portugal and were selected based on their high-quality fruit production (data not shown). This area is characterized by cold winters (temperatures below 0 ºC during night and frost formation) and very hot and dry summers (temperatures between 30 ºC and 40 ºC). Although not regular, snowfall is common in most years. Samples were collected at six different periods throughout the year in order to evaluate different environmental stimuli: December (a), February (b), April (c), June (d), August (e) and October (f). Leaves were collected from three different trees (1, 2 and 3) and kept in sterile plastic bags at 4 ºC. Within 24 h plant material was ground with mortar and pestle in liquid nitrogen and stored at -80 ºC until the extract was prepared.

Extract Preparation

Samples were extracted with methanol (1:5, w:v, twice) by homogenizing for 5 min, at 20000 rpm (4472 *g*), on a T25 digital ULTRA-TURRAX® disperser (IKA, Staufen, Germany) and centrifuged at 5000 rpm (4696 *g*), 30 min, at 4 °C. Extracts were mixed and their volume made up

to 10 mL with methanol. The extraction yield was determined gravimetrically. Independent aliquots (3) of the methanol extract were evaporated at 40 ºC in a vacuum oven to constant weight.

Quantification of Total Phenolic Content

A modified technique from the Folin-Ciocalteu method was used (Attard 2013). Briefly, the extract (10 µL) was mixed with 100 µL of Folin-Ciocalteau reagent (Merck®, Darmstadt, Germany) and 80 µL of 1M sodium carbonate (Merck). Mixtures were then incubated (in the dark) at room temperature for 20 min, and the absorbance was measured at 630 nm in a SPECTRAmax PLUS 384 spectrophotometer (Molecular Devices, San Jose, CA, USA). Total phenolic content was determined from a standard curve (0-300 µg of gallic acid, $y = 0.0028x + 0.0575$, $R^2 = 0.9915$) and expressed as mg of gallic acid (Sigma-Aldrich, St. Louis, MO, USA) equivalents by g of leaf fresh weight, as mean ± standard deviation of three independent analyses.

HPLC Analyses

For HPLC assays aliquots of the methanol extracts (1 mL) were evaporated at 40 °C on a rotatory evaporator (Buchi R-215) under vacuum, and the dry residues dissolved in 10% aqueous methanol (2 mL), centrifuged at 2500 rpm (1000 *g*) for 15 min, 4 °C, and filtered with a PTFE (polytetrafluoroethylene) membrane filter (0.22 µm, Ø13 mm, Merck, Darmstadt, Germany).

HPLC-PDA-ESI/MS^n

Phenolic compounds were tentatively identified by High Performance Liquid Chromatograph (Finnigan Surveyor, THERMO) coupled to a Photodiode Spectrophotometer (PDA, Finnigan Surveyor, THERMO) and a Linear Ion Trap Mass Spectrometer (LIT-MS) (LTQ XL, Thermo Scientific). A LC column Waters Spherisorb ODS2 (3 µm, 150 x 2.1 mm) was preceded by a guard cartridge Waters Spherisorb ODS2 (5 µm, 10 x 4.6 mm) and separation was carried at 20 ºC. The mobile phase consisted of 2% aqueous formic acid (solvent A) and methanol (solvent B). The mobile phase profile used was 0-7 min, 0% B isocratically; 7-10 min, 0-5% B; 10-20 min, 5-25% B; 20-40 min, 25-30% B; 40-50 min, 30-50% B; 50-60 min, 50-80% B; 60-65 min, 80% B isocratically. The flow rate was 200 µL min^{-1}. The first detection was made with a PDA detector in a wavelength range of 200-500 nm, using 280 and 320 nm as preferred wavelengths. The second detection was made in the mass spectrometer. It was operated in the negative electrospray ionization (ESI) mode and programmed to perform a series of three scans: a full mass (MS) and a MS^2 and MS^3 of the most abundant ion. Collision gas was helium with a normalized collision energy of 35%. Nitrogen was used as nebulizing gas, with a sheath gas flow of 40 arbitrary units and the auxiliary gas flow of 5 arbitrary units. Capillary temperature and voltage were set at 275 ºC and -35.00 V, respectively. Source voltage was set at 5.00 kV. Data treatment was carried out with XCALIBUR software (Thermo Scientific).

HPLC-PDA Analysis

High Performance Liquid Chromatograph (HPLC) analysis was performed in a chromatograph GILSON equipped with a PDA detector (Gilson® Electronics SA, Villiers le Bel, France), using a Spherisorb S5 ODS2 column (250 × 4.6 mm i.d.; particle size, 5 µm; Waters® Corp., Milford, MA, USA) and a Nucleosil C18 guard cartridge (30 × 4 mm i.d.; particle size, 5 µm; Macherey-Nagel, Düren, Germany) at 25 °C. A mobile phase of 5% aqueous formic acid (solvent A) and methanol (solvent B) was used with 0% B isocratically (0-7 min), a discontinuous gradient of 0-5% B (7-10 min), 5-25% B (10-20 min), 25-30% B (20-40 min), 30-50% B (40-50 min) and 50-80% B (50-60 min), followed by an isocratic elution for 5 min, at a flow rate of 1 mL min^{-1}. Volumes of 100 µL were injected for all standards and extracts. Chromatographic profiles were acquired in the wavelength range 200–600 nm and recorded at 280 nm using the Unipoint® software (version 2.10, Gilson®). Arbutin and hydroquinone quantification was performed by calibration curves obtained from the arbutin (Sigma-Aldrich) standard (0.02 – 1.5 mg mL^{-1}, $y = 5*10^8 x + 8*10^6$, $R^2 = 0.9986$) and hydroquinone (Sigma-Aldrich) standard (4 – 275 µg mL^{-1}, $y = 1*10^9 x - 107823$, $R^2 = 1.0000$). The results were expressed as mg per g of leaf fresh weight and mean ± standard deviation of three independent analyses. Other phenolic compounds were evaluated using the peak areas at 280 nm. Three independent analyses and two technical replicates were performed for each extract.

Antifungal and Anti-oomycete Activity Assessment

The antifungal effect of the standards arbutin and hydroquinone was tested on 3 fungi (*Alternaria alternata* - Au01, *Glomerella cingulata* - Au26 and *Mycosphaerella* sp. - Au36) and an oomycete (*Phytophthora cinnamomi* - Au64) obtained from the Plant Biotechnology Laboratory (University of Coimbra) collection and previously isolated from the same strawberry tree individuals (1, 2 and 3). An adapted protocol described by (Kang et al. 2017) was used. Briefly, 5 mm mycelia discs from the margin of an actively growing colony (6 days old) were placed on 9 cm plates with PDA medium (Difco™ Potato Dextrose Agar: 4 g L^{-1} potato starch, 20 g L^{-1} dextrose, 15 g L^{-1} agar, Becton, Dickinson and Company, New Jersey, USA). Aqueous stock solutions of arbutin and hydroquinone standards were filtered (0.22 µm, PES membrane) and added to media after autoclaving at 121 ºC for 20 min and after it has been cooled down to 40-45 ºC, in equivalent concentrations to the amount range of these phenols in the 18 samples studied: 0.039-2.5 mg mL^{-1} for arbutin and 0.0125-0.1 mg mL^{-1} for hydroquinone. A control group without arbutin and hydroquinone was also included. After 6 and 12 days at 21 ºC in the dark, colony growth was measured (area in cm^2) using ImageJ2 software (Rueden et al. 2017) and growth reduction (GR) was calculated from three independent replicates according to (Royse and Ries 1978), using the formula: GR = (TG - CG) / CG *100, where TG is the growth of the colony with arbutin or hydroquinone and CG is the growth of the control group on PDA medium.

Activity on *P. cinnamomi* by arbutin and hydroquinone, as well as of the extract from tree number 3 at time 6 (sample 3f, October), was tested on a microplate assay to assess MIC (Minimum Inhibitory Concentration) and MLC (Minimum Lethal Concentration). Mycelial discs of 5 mm from the margin of a *P. cinnamomi* colony grown on PDA medium for 6 days were placed in a 24 well microplate with 1 mL PDB medium (Difco^{TM} Potato Dextrose Broth: 4 g L^{-1} potato starch, 20 g L^{-1} dextrose, Becton, Dickinson and Company, New Jersey, USA). Aqueous solutions of arbutin and hydroquinone standards were filtered (0.22 µm, PES membrane) and added to the medium after autoclaving to obtain concentrations ranging from 0.039 to 12 mg mL^{-1} and 0.0125 to 1.6 mg mL^{-1} of arbutin and hydroquinone, respectively. Microplates were incubated at 21 ºC in the dark, and results were recorded after 6 days.

To evaluate the activity of the extract from sample 3f, methanol was evaporated and the extract dry residue dissolved in distilled water, filtered and added to the medium in concentrations that ranged from 0.374 to 23.96 mg mL^{-1}, that contained arbutin concentrations equivalent to the amount range of these phenols in the 18 samples studied (from 0.039 to 2.5 mg mL^{-1}). After 6 days at 21 ºC, MIC was determined for the standards of arbutin and hydroquinone and for 3f extract. Mycelial plugs with no signs of growth were transferred to PDA medium to determine the MLC, defined as the lowest concentration showing 100% growing inhibition. All the experiments were carried out in triplicate against a control group growing on PDB medium.

Statistical analysis

All values are given as means ± standard deviation from three independent replicates. Total phenolic compounds, and arbutin and hydroquinone quantification were analyzed by two-way ANOVA (GraphPad Prism for Windows *v. 6.01*), followed by a Tukey´s multiple comparison test ($P < 0.05$). The antifungal and anti-oomycete effect of arbutin and hydroquinone on solid medium was analyzed by one-way ANOVA on each group independently and by a Tukey´s multiple comparison test ($P < 0.05$). A heatmap with dendrogram, and a correlation and a principal component analysis (PCA) were carried out using R software (R Core Team 2020) to evaluate the interaction and significance of chemical groups from the analyzed trees. A heatmap with data from all samples was constructed using the "Heatmap" function and the package "ComplexHeatmap" (Gu et al. 2016). The dendrogram within the heatmap was calculated with Euclidean distance as dissimilarity measure. To evaluate the interaction of the chemical classes a correlation was calculated using the "ggcor" function (Pearson correlation coefficients and pairwise observations) and the packages "GGally" (Schloerke et al. 2018) and "ggplot2" (Wickham 2016). Finally, data were classified with a PCA, using the "prcomp" function and the package "ggbiplot" (Vu 2011).

Total Phenols Quantification

Total phenols content ranged from 254.96 ± 13.86 mg g^{-1} leaf fresh weight in sample 2e to 495.24 ± 67.49 mg g^{-1} fresh weight in sample 3c. Higher content of total phenols was observed in samples 3c and 3f. Overall, for trees 1 and 2, statistically significant differences were not observed between them or overtime. However, samples 1f and 2e presented a lower quantity of phenolic compounds (Figure 1).

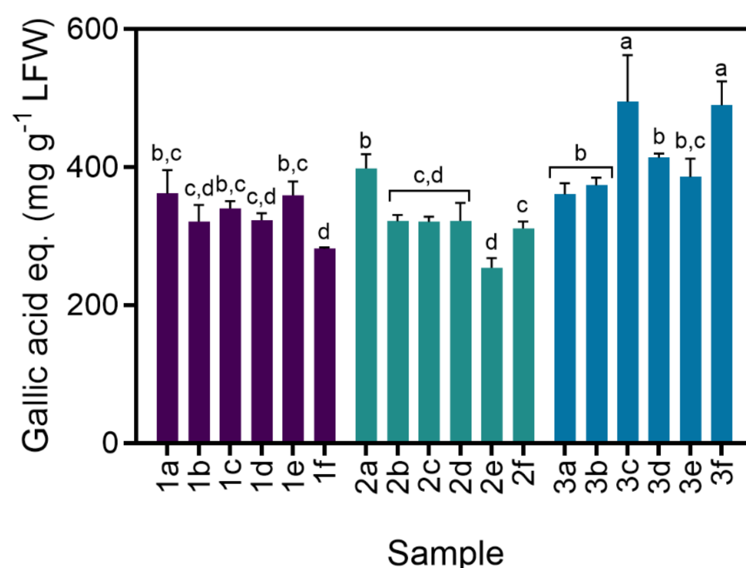

Figure 55. Total phenolic content of *A. unedo* leaves from samples of the trees 1, 2 and 3 throughout the year (a - December, b - February, c - April, d - June, e - August, f - October), expressed as equivalents of gallic acid (mg g^{-1} leaf fresh weight). Means ± SDs, n = 3, different letters indicate significant ($P \leq 0.05$) differences between samples.

HPLC-PDA-ESI/MS*ⁿ*

A total of 54 compounds were assigned for all the trees by using authentic standards or were tentatively identified based on literature (Table 6, Figure 56). Eighteen phytoconstituents were gallic acid derivatives and their identification was supported by ions at *m/z* 169 (deprotonated molecule of gallic acid) occurring in the MS^2 or MS^3 (peaks 3, 4, 6, 8, 9, 13, 14, 19, 20, 21, 27, 28, 29, 47) and/or ions through the resulting losses of the gallic acid molecule or its dehydrated molecule (-170 or -152 amu, respectively) (peaks 34, 36, 41, 42). Three gallic acid derivatives were shown to be combined with a hexose, that is usually glucose (peaks 3, 4, 6), two with quinic acid (peaks 8, 9), one with glucuronide acid or arbutin (peaks 13 and 19, respectively), six with shikimic acid (peaks 14, 20, 21, 27, 28, 41), one with flavan-3-ol (peak 29) and four with flavonol glycosides (peaks 34, 36, 42, 47).

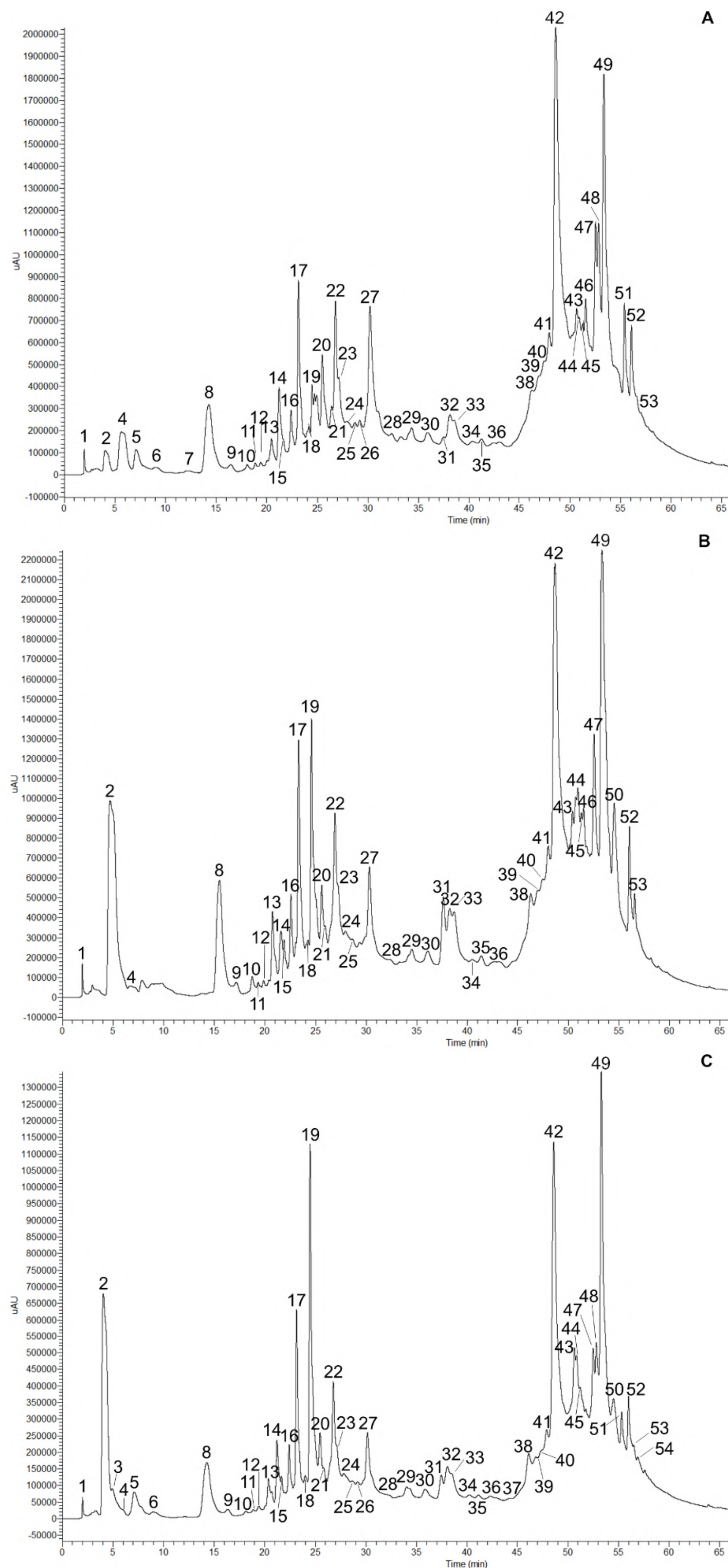

Figure 56. HPLC-PDA phenolic profiles of the samples: 1a (A); 2a (B) and 3a (C), recorded at 280 nm.

Gallic acid molecules were also present in the hydrolysable tannins, namely in gallotannins (peaks 33, 35) and ellagitannins (peaks 22, 23, 25, 26, 30, 32, 37). The gallotannins were characterized by successive loss of gallic acid (-170 amu) or gallic acid dehydrated (-152 amu) and ellagitannins by the additional occurrence of the ion at *m/z* 301, corresponding to the ellagic acid deprotonated molecule.

The flavan-3-ols (epi)gallocatechin, catechin and epicatechin (peaks 10, 17 and 18, respectively) and their polymers (condensed tannins or proanthocyanidins, peaks 7, 11, 12, 15, 16, 24) were also identified. Main ions in MS^2 and MS^3 of the polymers resulted from the Retro Diels Alder fission (RDA) and RDA+H_2O, originating losses of 152 or 168 and 170 or 186 amu for flavan-3-ol units with di- and tri-hydroxylated B-ring, respectively. Minor fragments at *m/z* 289 or 305, a result of an interflavanic bond cleavage were also observed in the mass spectra for di- or tri-hydroxylated lower unit.

Flavonoids were the most representative polyphenols in the extracts. They are of flavonol type and are in a glycoside form. According to their mass spectra, they were recognised as derivatives of myricetin (peaks 34, 36, 38-40), quercetin (peaks 42-46, 48-50, 54) and kaempferol (peaks 47, 51-53) based on their deprotonated molecules at *m/z* 317, 301 and 285, respectively, complemented by its fragmentation pattern, elution sequence and ultra-violet spectra.

Table 6. Compounds identified by HPLC-PDA-ESI-MS^n in the *Arbutus unedo* samples 1a, 2a, 3a.

Nº	**R_t** (min)[a]	**$\lambda_{máx}$** (nm)	**ESI-MSn[a]** [*m/z* (relative abundance,%)]			**Attempt to identify** (reference)	**1a**	**2a**	**3a**
			$[M-H]^-$	**MS^2**	**MS^3**				
1	2.70	238, 250	**191**	**173**, 127, 111, 109, 93, 85	155, **111**, 93	Quinic acid (Saldanha et al. 2013)	+	+	+
2	4.25	276	**317**	**271**	**161**, 109	Arbutin [b; *] (Sun et al. 2019)	+	+	+
3	4.94	284	**377**	**331**, 169, 151	313, 179, **169**, 161, 151, 125, 123	Galloyl hexoside [b] (Alberti et al. 2012)	-	-	+
4	6.03	277	**331**	271, 211, 193, **169**	**125**	Galloyl hexoside (Sobeh et al. 2019)	+	+	+
5	7.09	275	**479**	**433**	**323**, 221, 179	Caffeoyl arbutin [b] (Sun et al. 2019)	+	-	+
6	8.99	271	**331**	271, **169**	**125**	Galloyl hexoside	+	-	+

Nº	R_t (min)[a]	$\lambda_{máx}$ (nm)	ESI-MSn[a] [*m/z* (relative abundance,%)]			Attempt to identify	1a	2a	3a
			[M-H]⁻	MS²	MS³	(reference)			
						(Sobeh et al. 2019)			
7	12.16	272	**609**	483, **441**, 423,305	**423**	(epi)Gallocatechin-(epi)gallocatechin (Correia et al. 2006)	+	-	-
8	14.69	273	**343**	**191**, 169	173, 171, **127**, 111, 93, 85	Galloylquinic acid (Clifford et al. 2007)	+	+	+
9	16.68	272	**343**	**191**, 169	173, 171, **127**, 111, 93, 85	Galloylquinic acid (Clifford et al. 2007)	+	+	+
10	18.32	270	**305**	261, 221, 219, **179**, 165, 137, 125	**164**, 151, 137, 135	(epi)Gallocatechin (Hofmann et al. 2016)	+	+	+
11	19.02	276	**593**	467, **425**, 407, 289	**407**	(epi)Gallocatechin-(epi)catechin (Correia et al. 2006)	+	+	+
12	19.55	276	**593**	575, 467, 441, 425, **423**, 305	405, **297**, 285, 283, 243	(epi)Catechin-(epi)gallocatechin (Correia et al. 2006)	+	+	+
13	20.64	264	**345**	313, 299, **169**, 151, 125	**125**	Galloyl glucuronide (Zhang et al., 2010)	+	+	+
14	21.34	274	**325**	**169**, 125	**125**	Galloylshikimic acid (Wyrepkowski et al. 2014)	+	+	+
15	21.73	275	**577**	559, 451, **425**, 407, 289	**407**	(epi)Catechin-(epi)catechin (Correia et al. 2006)	+	+	+
16	22.45	278	**577**	559, 451, **425**, 407, 289, 287	**407**	(epi)Catechin-(epi)catechin (Correia et al. 2006)	+	+	+
17	23.20	278	**289**	**245**, 205, 179	227, **203**, 187, 161	Catechin *	+	+	+
18	24.02	278	**289**	**245**, 205, 179	227, **203**, 187, 161	Epicatechin *	+	+	+

Nº	Rt (min)[a]	λmáx (nm)	ESI-MSn[a] [*m/z* (relative abundance,%)]			Attempt to identify (reference)	1a	2a	3a
			[M-H]⁻	**MS²**	**MS³**				
19	24.51	276	**423**	**313**, 169	**169**, 151, 125	Galloyl arbutin (Wrona et al. 2019)	+	+	+
20	25.52	277	**477**	**325**	**169**, 125	Digalloylshikimic acid (Wyrepkowski et al. 2014)	+	+	+
21	25.91	276	**477**	**325**	**169**, 125	Digalloylshikimic acid (Wyrepkowski et al. 2014)	+	+	+
22	26.82	272	**633**	463, **301**, 275	301, 284, **257**, 229, 185	Galloyl-HHDP-hexoside (Sobeh et al. 2019)	+	+	+
23	27.17	276	**633**	463, **301**, 275	301, 284, **257**, 229, 185	Galloyl-HHDP-hexoside (Sobeh et al. 2019)	+	+	+
24	27.92	277	**577**	559, 451, **425**, 407, 289, 287	**407**, 273	(epi)Catechin-(epi)catechin (Correia et al. 2006)	+	+	+
25	28.66	276	**953**	**935**	765, 633, 463, 343, **301**	TriGG-DHHDP-glucose (Tuominen 2017)	+	+	+
26	29.20	276	**953**	**935**	765, 633, 463, 343, **301**	TriGG-DHHDP-glucose (Tuominen 2017)	+	-	+
27	30.22	273	**477**	**325**	**169**, 125	Digalloylshikimic acid (Wyrepkowski et al., 2014)	+	+	+
28	32.33	276	**477**	**325**	**169**, 125	Digalloylshikimic acid (Wyrepkowski et al., 2014)	+	+	+
29	34.28	276	**441**	331, **289**, 271, 169	247, **245**, 231, 205, 203, 179	(epi)Catechin gallate	+	+	+

Nº	R_t (min)[a]	$\lambda_{máx}$ (nm)	ESI-MSn[a] [*m/z* (relative abundance,%)]			Attempt to identify (reference)	1a	2a	3a
			[M-H]⁻	**MS²**	**MS³**				
						(Zengin et al. 2018)			
30	35.92	275	**785**	**633**	463, **301**, 275	Digalloyl-HHDP-hexoside (Abid et al. 2017)	+	+	+
31	37.50	259	**391**	**281**	**137**	Hydroquinone derivative	+	+	+
32	38.13	273	**953**	**935**, 909, 801, 783, 633, 463, 301	633, 589, **463**, 301	TriGG-DHHDP-glucose (Tuominen 2017)	+	+	+
33	38.56	275	**787**	635, **617**	**573**, 465, 447, 421, 403, 313	Tetra-galloyl-hexoside (Hofmann et al. 2016)	+	+	+
34	40.34	274, 355	**631**	**479**	**317**	Myricetin-*O*-galloyl) hexoside (Negri and Tabach 2013)	+	+	+
35	41.25	274	**787**	**635**, 617	483, **465**, 331, 313	Tetra-galloyl-hexoside (Gan et al. 2018)	+	+	+
36	42.40	275, 369sh	**631**	**479**	**317**	Myricetin-*O*-galloyl) hexoside (Negri and Tabach 2013)	+	+	+
37	44.20	275	**785**	633, 615, 465, **301**, 275, 249	301, **257**, 229, 201	Digalloyl-HHDP-hexoside (Abdulla et al. 2017)	-	-	+
38	46.22	269, 299sh, 357	**479**	**317**	287, 272, 192, **179**, 151	Myricetin-*O*-hexoside (Hofmann et al. 2016)	+	+	+
39	46.90	250, 273, 356	**449**	**317**	271, 192, **179**, 151	Myricetin-*O*-pentoside (Hofmann et al. 2016)	+	+	+

Nº	R_t (min)[a]	$\lambda_{máx}$ (nm)	ESI-MSn[a] [*m/z* (relative abundance,%)]			Attempt to identify	1a	2a	3a
			[M-H]-	**MS²**	**MS³**	(reference)			
40	47.46	249, 274, 354	**479**	**317**	272, 192, **179**, 151	Myricetin-*O*-hexoside (Hofmann et al. 2016)	+	+	±
41	47.96	250sh, 275	**629**	**477**	325, **289**, 263, 169, 137	Trigalloylshikimic acid (Singh et al. 2016)	+	+	+
42	48.63	265, 299s, 352	**615**	**463**, 301, 271	**301**	Quercetin galloyl-hexoside (Sobeh et al. 2019)	+	+	+
43	50.71	257, 299sh, 355	**463**	**301**	273, 257, **179**, 151	Quercetin-*O*-hexoside (Sobeh et al. 2019)	+	+	+
44	50.93	257, 299sh, 355	**463**	**301**	273, 257, **179**, 151	Quercetin-*O*-hexoside (Sobeh et al. 2019)	+	+	+
45	51.27	266, 299, 354	**463**	**301**	273, **179**, 151	Quercetin-*O*-hexoside (Sobeh et al. 2019)	+	-	+
46	51.75	252sh, 268, 355	**433**	**301**	273, 257, **179**, 151	Quercetin-*O*-pentoside (Sobeh et al. 2019)	+	+	+
47	52.54	263, 299sh, 351	**599**	447, **313**, 285	241, **169**, 125	Kaempferol galloyl-hexoside (Sobeh et al. 2019)	+	+	+
48	52.84	257, 268sh, 291, 299, 354	**433**	**301**	273, 257, **179**, 151	Quercetin-*O*-pentoside (Sobeh et al. 2019)	+	-	+
49	53.33	258, 291sh, 347	**447**	**301**	283, 273, 257, **179**, 151	Quercitrin * (Sobeh et al. 2019)	+	+	+
50	54.53	257, 291sh, 352	**583**	**463**, 301	**301**	Quercetin derivative	-	+	+

Nº	R_t (min)[a]	$\lambda_{máx}$ (nm)	ESI-MSn[a] [*m/z* (relative abundance,%)]			Attempt to identify	1a	2a	3a
			[M-H]⁻	**MS²**	**MS³**	(reference)			
51	55.36	265, 291sh, 347	**417**	**285**	267, **257**, 241, 239, 229, 213, 199, 197, 163	Kaempferol-*O*-pentoside (Jang et al. 2018)	+	-	+
52	56.04	265, 291sh, 344	**431**	**285**	267, **257**, 241, 239, 229, 213, 197, 163	Kaempferol-*O*-rhamnoside (Jang et al. 2018)	+	+	+
53	56.55	266, 343	**567**	447, 429, 327, **285**	285, 257, 241, **151**	Kaempferol-*O*-*p*-hydroxybenzoylglucoside (Zhang et al. 2018)	+	+	+
54	56.90	268, 361sh	**609**	**463**, 301	**301**	Quercetin coumaroyl-hexoside (Mariotto et al. 2008b)	-	-	+

Identification based on the UV-Vis spectra, molecular weight and fragmentation patterns, which are according to authors cited. The base peaks in MS spectra are in bold. [a] Rf mean values for the three samples [b] Proposal ion was [M - H + HCOOH]⁻ corresponding to adduct; [*] identification confirmed by authentic standards. HHDP – hexahydroxydiphenoyl. GG – galloylglucose, DHHD – dehydrohexahydroxydiphenoyl.

HPLC-PDA

HPLC analysis showed there was a seasonal variation in the profiles of phenolic compounds, as well as differences between individuals (Figure 57). Arbutin (AR), hydroquinone (HQ) and their derivatives (ARd and HQd, respectively) were more abundant on samples from tree 3, with an increasing concentration over time (Figure 57 A, Figure 58 A). In contrast, gallic acid derivatives and tannins were more abundant in samples from tree 1 (Figure 57 B, C). Although no significant differences were found in terms of flavonoid contents, some samples from tree 2 had higher amounts of these compounds. In general, higher amounts of quercetin derivatives (Qd), myricetin derivatives (Md) and kaempferol derivatives (Kd) were detected in samples from tree 2, whereas flavan-3-ols were present in higher concentrations in tree 3 (Figure 58 A). A high positive correlation was found between the amount of arbutin, hydroquinone and their derivatives, and the flavan-3-ols concentration. A similar relationship was also verified between gallic acid derivatives and tannins (Figure 58 B). A chemical pattern for each tree was also found through a principal component analysis (PCA), with samples forming three clusters and grouped according to the 3 genotypes analysed (Figure 58 C). Samples from genotype 1 were grouped according to the contribution of gallic acid derivatives and tannins. Genotype 2 was grouped by the quercetin derivatives (Qd), myricetin derivatives (Md) and kaempferol derivatives (Kd) whereas the samples

from genotype 3 by arbutin derivatives and flavan-3-ols. The classes of phenolic compounds that contributed the most to the total variance are arbutin (AR), hydroquinone (HQ), their derivatives (ARd and HQd) and gallic acid derivatives (GSa and GQa). In fact, arbutin, hydroquinone and their derivatives, flavan-3-ols (Flv-3-ols) and galloyl hexosides (GH) strongly influenced principal component 1 (PC1), while the other variables had higher influence on PC2 (Figure 58 C). In the 18 samples studied, arbutin concentrations ranged from 0.69 ± 0.05 mg g^{-1} of leaf fresh weight in sample 1a to 22.97 ± 0.76 mg g^{-1} fresh weight in sample 3f (Figure 59 A). Hydroquinone was only detected in sample 2a and in five samples from tree 3, with concentrations ranging from 0.069 ± 0.004 mg g^{-1} to 0.604 ± 0.024 mg g^{-1} leaf fresh weight for the samples 3c and 2a, respectively (Figure 59 B).

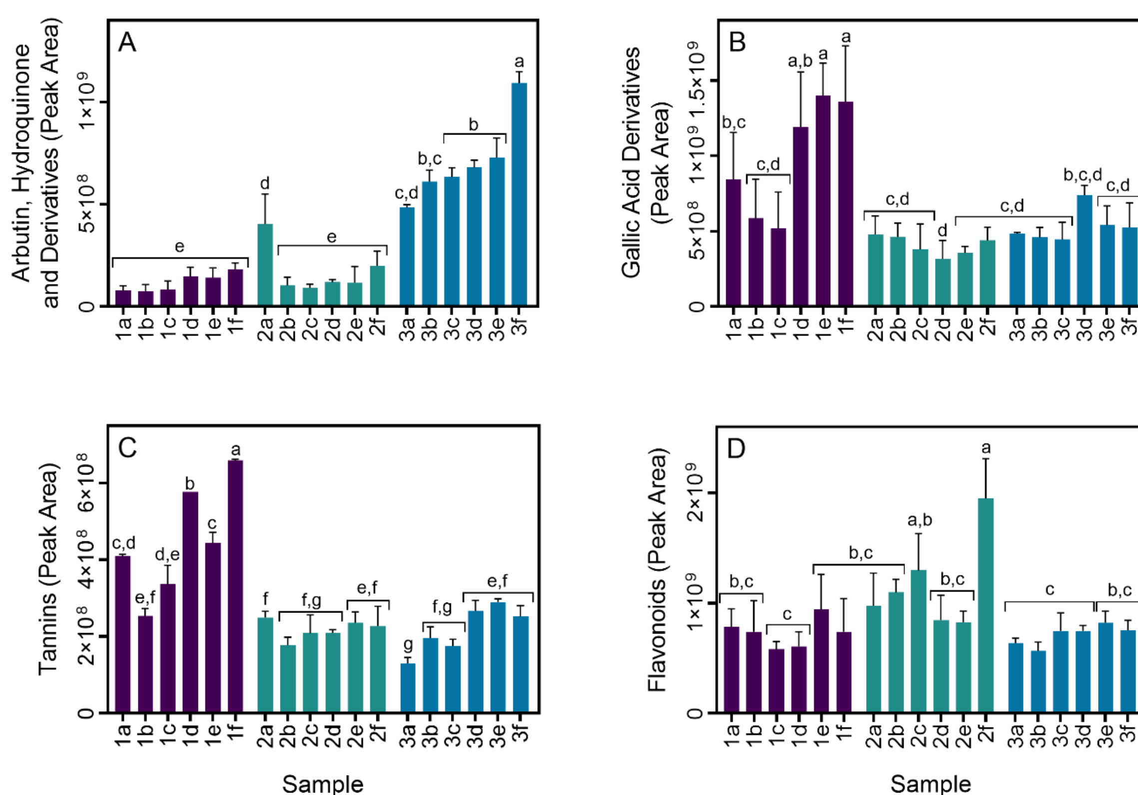

Figure 57. Relative concentration (peak area) of compounds from different classes of phenolic compound of *A. unedo* leaves from the 18 samples analyzed, from three genotypes (trees 1, 2 and 3) and throughout the year (a - December, b - February, c - April, d - June, e - August, f - October): Arbutin, hydroquinone and derivatives (A); gallic acid derivatives (B); tannins (C) and flavonoids (D). Means ± SDs, n = 3, different letters indicate significant ($P \leq 0.05$) differences between samples.

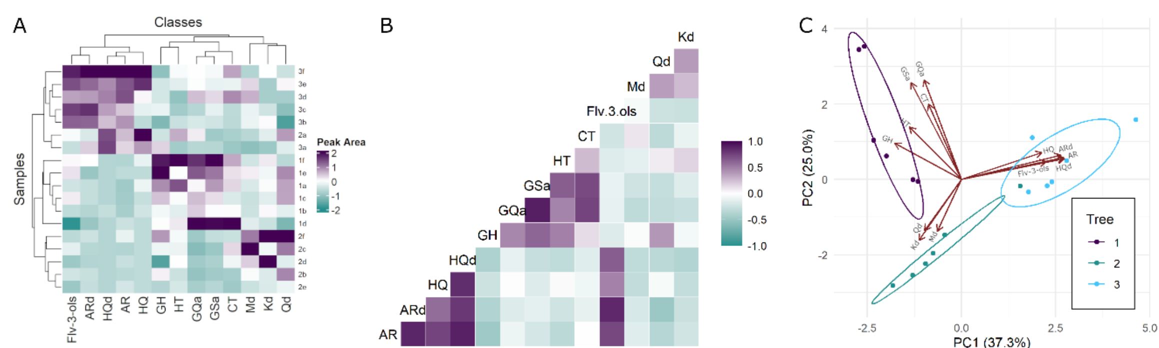

Figure 58. Analysis of the classes of phenolic compounds identified: Heatmap from the 18 samples analyzed belonging to the three genotypes (1, 2 and 3) (A); Pearson correlation of the chemical classes (B); principal component analysis (PCA) biplot (C). AR – arbutin, ARd – arbutin derivative, HQ – hydroquinone, HQd – hydroquinone derivative, GH – galloyl hexosides, GQa – galloylquinic acids, GSa – galloylshikimic acids, HT – hydrolysable tannins, CT – condensed tannins, Flv-3-ols – flavan-3-ols, Md – myricetin derivatives, Qd – quercetin derivatives, Kd – kaempferol derivatives.

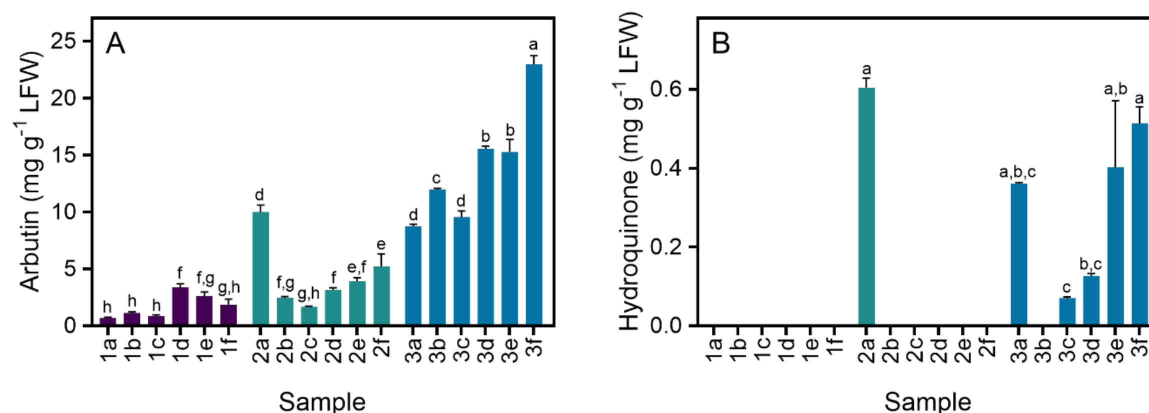

Figure 59. Concentrations (mg g^{-1} leaf fresh weight) of arbutin (A) and hydroquinone (B) of *A. unedo* leaves for samples of the trees 1, 2 and 3 throughout the year. Means ± SDs, n = 3, different letters indicate significant ($P \leq 0.05$) differences between samples.

Antifungal and Anti-oomycete Activity

For assayed concentrations, both arbutin and hydroquinone standards led to a promotion of *A. alternata* growth after 6 and 12 days (Figure 60 A-B). However, in general, both compounds showed antifungal effect against *G. cingulata*. This effect was more obvious for arbutin, with reductions in growth close to 20% (Figure 60 C-D). The development of *Mycosphaerella* sp. was only slightly reduced by arbutin after 6 days, and the higher concentration of hydroquinone, although no statistical differences were observed (Figure 60 E-F). Both compounds were highly effective against *P. cinnamomi*, with a reduction in growth close to 80% in the higher concentrations of arbutin and 60% with hydroquinone after 6 and 12 days (Figure 60 G-H). A dose-response effect was observed for these compounds.

In the microplate tests, extract from the 3f sample showed anti-oomycete activity against *P. cinnamomi*, presenting a MIC at 5.99 mg mL^{-1} and a MLC at 23.96 mg mL^{-1}, equivalent to arbutin and hydroquinone concentrations of 0.625 / 0.014 mg mL^{1} and 2.5 / 0.056 mg mL^{-1}, respectively according to the assessment by HPLC (Table 7). However, arbutin and hydroquinone standards showed a lower anti-oomycete activity against *P. cinnamomi*, presenting MIC at 12 mg mL^{-1} and 0.1 mg mL^{-1}, respectively (Table 7).

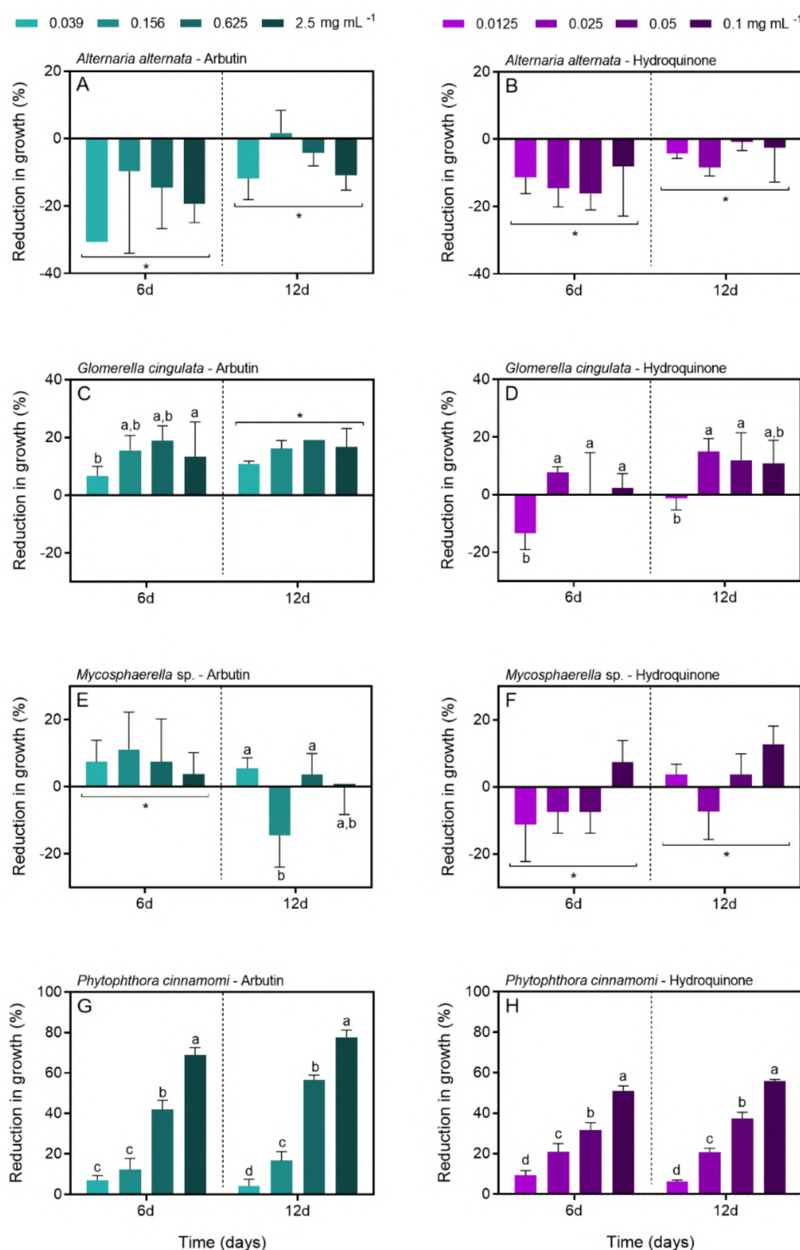

Figure 60. Antifungal activity of arbutin and hydroquinone (mg mL^{-1}) on a plate assay after 6 and 12 days, expressed as reduction in growth of *A. unedo* pathogens: *Alternaria alternata* (A-B); *Glomerella cingulata* (C-D) *Mycosphaerella* sp. (E-F); and *Phytophthora cinnamomi* (G-H). Means ± SDs, n = 3, for each group different letters indicate significant ($P \leq 0.05$) differences between treatments. * no statistical differences were found. 6d: 6 days, 12d: 12 days.

Table 7. Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC), determined in a microplate assay with Potato Dextrose Broth Medium, for *Arbutus unedo* extract (sample 3f), arbutin and hydroquinone on the plant pathogen *Phytophthora cinnamomi*.
*Arbutin and **hydroquinone concentrations in the extract from sample 3f.

Treatment	**MIC** (mg mL^{-1})	**MLC** (mg mL^{-1})
Extract	5.99 [0.625*, 0.014**]	23.96 [2.5*, 0.056**]
Arbutin	12.0	>12.0
Hydroquinone	0.1	>1.6

Discussion

Polyphenol profiles of *A. unedo* leaves were mainly composed of arbutin, hydroquinone and their derivatives, gallic acid derivatives, tannins and flavonoids like quercetin, kaempferol and myricetin derivatives. A similar polyphenolic composition of the leaves has been described before (Tavares et al. 2010; Coimbra et al. 2020). However, and as far as it is known, the presence of caffeoyl arbutin is now reported for the first time in *A. unedo*. This arbutin derivative was previously found in other plant species (Xu et al. 2015; Sun et al. 2019) including *Vaccinium dunalianum* (Zhao et al. 2008), also an Ericaceae.

Seasonal fluctuations in metabolite concentrations could be due to abiotic factors such as climatic conditions, soil fertility and light intensity. Previous studies have shown that the stage of plant development and climatic conditions play a crucial role in the concentration of secondary metabolites, whereas nutrient availability may also be determinant for the production of polyphenols like flavonoids, namely quercetin, kaempferol and their derivatives (Verma and Shukla 2015). Plants also release and accumulate other secondary metabolites, including simple phenols and triterpenoids, in response to light radiation (Yang et al. 2018). However, in strawberry tree, no relationship was found between the concentrations of the chemical groups analyzed and a specific time of year and an apparently random fluctuation was observed. Moreover, as plants sampled in this study were growing in the same area, the environmental conditions are similar, suggesting that abiotic factors may have only a minor influence on phenols production. Differences in their profiles were more evident between individuals, indicating a strong genetic component in plant metabolite production. These results are in line with those obtained by (Orak et al. 2019; Xia et al. 2019), who also reported the influence of genotype on the phenolic composition of *Olea europaea* and *Vitis* sp. The identification of genotypes producing high amounts of specific phenols ecologically relevant and/or with biotechnological applications has become a promising line of research. In the case of *A. unedo*, the three genotypes analyzed showed a specific chemical

composition with strong differences among them. Thus, genotype 1 produced the highest amounts of gallic acid derivatives and tannins, whereas genotype 2 produced more flavonoids. In turn, arbutin, hydroquinone and their derivatives were particularly abundant in genotype 3. The levels of arbutin in all trees were higher than those reported by other authors in strawberry tree (Fiorentino et al. 2007). Although the quantity of arbutin in tree 1 (1.63 - 8.42 mg g^{-1} fresh leaf weight) was in the same range of the values reported by (Jurica et al. 2015, 2018), arbutin concentration was higher in trees 2 and 3, with particularly high values in the latter. On the other hand, hydroquinone was not found in most of the samples, a result also reported by Jurica et al. (2015).

Differences observed in plant metabolite qualitative and quantitative composition between samples can also be due to biotic stresses. Several studies have correlated the production of certain metabolites, such as flavonoids and other phenols, with a role on plant defense. It has been reported that secondary metabolites have an effect on plant pathogens, thus inducing the increase of their concentrations when the plant is infected. Some of these compounds referred to in the literature are catechins (Girardi et al. 2014) and flavonoids (Sherwood and Bonello, 2013; Vargas et al., 2013 Mierziak et al., 2014; Ravensdale et al., 2014(Sherwood and Bonello 2013; Vargas et al. 2013; Mierziak et al. 2014; Ravensdale et al. 2014), and more specifically kaempferol (Padmavati et al. 1997; Galeotti et al. 2008; Bollina et al. 2010; Gunnaiah et al. 2012; Velasco et al. 2013) and quercetin (Padmavati et al. 1997; Parvez et al. 2004; Ali et al. 2012). These substances have antimicrobial effect against diverse microorganisms, including bacteria and fungi, like *Pseudomonas syringae*, *Xanthomonas* spp., *Fusarium* spp., *Cladosporium* spp., and *Botrytis cinerea*. Likewise, an isoflavone of *Glycine max* has been identified to have antifungal effect against *Phytophthora sojae* (Subramanian et al. 2005). As discussed above, hydroquinone, arbutin and their derivatives might also play an important role in plant defense against pathogens. Hydroquinone showed antimicrobial effect against *Pseudomonas fluorescens* (Trevors and Basaraba 1980), and arbutin strongly reduced the symptoms of angular leaf spot disease on cucumber leaves caused by *Pseudomonas syringae* pv. *lachrymans* (Kuźniak et al. 2015), through the formation of toxic substances and by inducing the expression of Pathogenesis-Related Gene 1 (PR1), a salicylic acid signaling marker. This hormone strongly influences plant growth and development and can regulate sugar metabolism and affect plant photosynthetic performance. Furthermore, it is mainly recognized as a key signaling compound mediating defense against pathogens, and some responses to abiotic stress as well (Kuźniak et al. 2015). In addition, arbutin may play a key role on cell membrane stabilization under stress (Oliver et al. 2001) and was also involved in the resistance to fire blight in pear (Powell and Hildebrand 1970). In the current study, arbutin and hydroquinone standards proved to be effective in reducing the growth of two plant pathogens, *G. cingulata* and *P. cinnamomi*, reinforcing the involvement and importance of these two phenols on plant defense mechanisms. However, the extract from *A. unedo* leaves was more effective than arbutin in inhibiting *P. cinnamomi*, probably due to a synergistic effect of other

phenolic compounds, namely kaempferol and quercetin derivatives. These polyphenols are the main components of the extract from the trees analyzed in this study (*e.g.*, peaks 17, 19, 42 and 49), which have showed antifungal and anti-oomycete effect, as mentioned before. The presence of arbutin derivatives in the extract, such as caffeoyl arbutin and galloyl arbutin, as well as hydroquinone derivatives, might also have had an essential contribution to the antimicrobial effect of the extract. Still, further work needs to be done in order to confirm the direct effect of these pathogens on *A. unedo* metabolite production.

The importance of secondary metabolites on plant stress tolerance mechanisms and their potential for several biotechnological applications justify the increasing interest on their production, and on understanding the complex pathways regulating their biosynthesis. Furthermore, plant phenotyping is the basis of any selection and breeding program. In this sense, the results achieved in *A. unedo* provide a first approach towards selection of individuals based on chemical features. Thus, tree 3 produced high levels of arbutin, a crucial characteristic to obtain high amounts of the compound itself or that can be useful in breeding strategies to develop cultivars combining high productivity with biotic stress tolerance. This work also provides insights on the ability of specific phenolic compounds to control pathogenic organisms that affect *A. unedo*, and opens the way to better explore their potential role on alternative defense strategies against plant pathogens. In particular, it was reported for the first time in this work that the extract from *Arbutus unedo* leaves, as well as arbutin and hydroquinone, show antifungal and anti-oomycete effects on strawberry tree pathogens, especially on the invasive and widely spread *P. cinnamomi*. Besides their pharmaceutical and cosmetic applications, it seems that arbutin and their derivatives, as well as the extract from *A. unedo* leaves, have the potential to be used as natural pesticides. The extract and/or specific phenolics might be used in the formulation of pesticides that can be applied in the soil or in the plant root system before plantation to prevent the spread of *P. cinnamomi*. At a time when there is a worldwide trend to reduce the application of synthetic pesticides this is particularly relevant. However, a lot of work has still to be carried out and the chemotyping of a large number of *A. unedo* genotypes is something to be pursued in the near future, not only due to its relevance for breeding but also to better understand the dynamic changes of plant secondary metabolites and to facilitate its biotechnological application.

Conclusions and future directions

The present study was undertaken to establish a solid research base for a breeding program in strawberry tree. In order to do so, three main lines of research were followed on this thesis: i) development and improvement of micropropagation protocols, evaluation of the resistance to drought of micropropagated plants and study of microbiome in the *in vitro* plants and changes after acclimatization, ii) breeding and selection of resistant genotypes and study of the tolerance mechanism under water limitation, and iii) structural and functional characterization of culturable endophytic bacteria and fungi, chemical fingerprint and antimicrobial activity of the plants extract and endophytes against phytopathogens and their relevance for plant defence against biotic agents.

In this work, the first detailed morpho-histological study of the somatic embryogenesis process is presented for the first time. The performance of strawberry tree plants under water deficit obtained by different micropropagation methods was also evaluated for the first time. The study of the microbiome in micropropagated plants is also a novelty in research in strawberry tree, and one of the few in general, especially because a culture-independent technique was used. The metabolic response of plants under drought conditions was also carried out for the first time as well as the functional characterization and evaluation of the antagonistic capacity of fungi and endophyte bacteria. Finally, although the chemical characterization of strawberry tree available in the literature is already very detailed, the seasonality effect on the chemical composition and antifungal effect of the leaves extract in phytopathogens was evaluated for the first time.

Overall, several breakthroughs have been accomplished on this research that will have an immediate impact on research and agricultural practices. Regarding the micropropagation techniques, somatic embryogenesis revealed to be very effective in terms of induction rates. However, it is genotype dependent, which means not all genotypes can be propagated by this method. Furthermore, probably due to several anomalies found on somatic embryos, conversion rates into fully developed plants are still low. For this reason, the use of this method for mass propagation is still not advisable. On the other hand, micropropagation in liquid medium, either by shoot proliferation or organogenesis proved to be highly efficient, with higher propagation rates when compared to the same propagation technique using solid medium. Besides, this method is more efficient in terms of time and resources required, because a jellifying agent is not used and the preparation of the explants is faster. Although plants produced by this method showed signs of hyperhydricity, which is common on continuous immersion systems, they recover their normal phenotype and presented a normal physiological performance under water restriction conditions. Thus, this method is highly recommended as the best option for the mass propagation of strawberry tree selected genotypes. The microbiome communities of the *in vitro* revealed to be quite diverse. Once again, genotype proved to be a driver of microbiome composition. However, when plants were acclimatized under the same conditions, the microbiome composition became similar between

genotypes, revealing the importance of horizontal transmission. Besides refuting the idea of axenic cultures, this discovery will have an impact on plant selection solely based on its phenotype and the use of micropropagation techniques for the propagation of superior genotypes. Because plant microbiome might be responsible for some of its host features, these characteristics might be lost when plants are micropropagated. Furthermore, due to the contribution of horizontal transmission of microorganisms, a plant selection strategy exclusively based on plant phenotype might not be adequate. One strategy that could work around this problem is the manipulation of the microbiome, either *in vitro* or *in vivo*, by the addition of specific microorganisms. Other drawback that has been found concerns the high contamination rates during *in vitro* establishment. A reasonable approach to tackle this issue could be to use prolonged sterilization protocols or biocides such as plant preservative mixture that have revealed to be quite effective.

The research has also shown that strawberry tree pollen has great viability and plant growth regulators can be used to improve or impede pollen germination. However, breeders should be careful when using these promotors, as they can lead to morphogenic processes in flowers and leaves causing several morphologic anomalies. Furthermore, pollen viability rapidly decreases, thus it is advisable to use only fresh collected pollen, whose viability has been checked, when hybridization is intended. The assays carried out in the lab and in the field indicate there are no self-incompatibilities mechanisms in place, something that facilitates the hybridization process. However, breeders can experience other challenges. The flowering time is prone to adverse weather conditions which can cause a negative effect on artificial pollination success. Furthermore, the long period required for fruit development delays even further the obtention of new generations, something that is already long on tree species. On this study, several hybrid plants were obtained, and three genotypes were selected (14, 15 and 18), based on physiological parameters, for its ability to cope with drought. One of the more significant findings to emerge from this study is the phenotypic plasticity under drought and after recovery. The genotypes tested showed different sensitivity to drought and were able to adopt different mechanisms. The drought sensitive genotypes close the stomata earlier to avoid water loses as they are not able to activate efficient repairing mechanism, whereas tolerant genotypes are able to activate a metabolic response which includes the up-regulation of key stress related hormones (*e.g*., abscisic and salicylic acids) and the production of secondary metabolites that are essential for ROS scavenging and repairing mechanisms. Thus, plants are able to maintain stomata open for longer periods maintaining normal CO_2 assimilation rates. Other important finding is that the recover ability of plants after a period under water restriction is also strongly genotype-dependent. Plant provenance seems to have a minor role on performance under stress but it is worth to explore.

The investigation of the endophytic culturable microbiome has shown that strawberry tree harbours a diverse community of bacteria and fungi playing an important role on plant defence mechanisms against pathogens. The results of this study indicate that some of the fungi endophytes

are able to antagonise strawberry tree pathogens, such as *Phytophthora cinnamomi*, by producing an enzymatic cocktail, whereas some bacteria strains showed ability to solubilize phosphate and to produce ammonia, siderophores and indol-3-acetic acid, which besides its antagonism effect against pathogens might also promote plant growth. The microorganisms with the most promising results in terms of antagonism ability are *Trichoderma atroviride* and *Bacillus cereus* that have already been identified in other plant species as biocontrol agents. As mentioned before, microbiome manipulation might be something to consider, and some of the microorganisms identified in this work would certainly grant competitive advantages to the plants, such as the ability to antagonise pathogens. However, breeders and plant producers should proceed with caution, as host-microorganism compatibility should be assured, otherwise an adverse response can be obtained when some beneficial microorganisms turn virulent. Other important factor that might also be relevant on defence against biotic agents concerns certain plant metabolites. In particular, phenolics such as arbutin and its precursor hydroquinone that are typical of Ericaceae species, show anti-fungal activity against *P. cinnamomi* and other common strawberry tree pathogens. Besides demonstrating its potential to be used as natural fungicides, this result points to a selection based on the plant chemical profile as a way to obtain orchards with intrinsic mechanisms to deal with pathogens. Furthermore, some of these bioactive compounds are used by the pharmaceutical and cosmetic industries, mainly as anti-septic and skin clear agents, and the selection of genotypes that are able to produce higher amounts of these compounds can add an extra value to the producers. Due to the vast range of applications of these compounds, especially arbutin, biotransformation assays are currently being carried out in order to develop an alternative production method of arbutin, using strawberry tree cell suspension cultures.

Overall, these results suggest that although several micropropagation methods (including somatic embryogenesis) can be used for the mass propagation of strawberry tree, shoot proliferation and/or organogenesis in liquid medium are currently the best options, producing plants with proven adequate performance under drought stress. Furthermore, apart from minor difficulties, hybrid plants can be obtained in field conditions. The current data also highlighted the idea that genotype is highly relevant for plant performance under drought, not only during the stress period but also recovering. Tolerant genotypes put in place a complex metabolomic response, involving key hormones and several phenolic compounds. Other breakthrough is the complex microbiome found in the plants that along with some plant phenolics are involved in several resistance mechanisms against biotic stress. For this reason, plant microbiome and chemical composition should be taken into account on plant selection.

Future directions

Although these findings are expected to have an impact in strawberry tree research and improve farming and forestry practices in the near future, several questions remain to be answered. Further work needs to be done to improve somatic embryos conversion into fully develop plants. Future research could usefully explore how microbiome communities evolve between *in vivo* and

in vitro conditions at the beginning of the micropropagation process. This could help us improve micropropagation and acclimatization rates and eventually manipulate the microbiome composition. Future studies to determine the contribution of horizontal and vertical transmission of microorganisms into strawberry tree, and the integration of beneficial endophytes (such as *T. atroviride* and *B. cereus*) on strawberry tree breeding program and orchard management are warranted. Regarding plant selection and breeding, a continuous effort is needed that would eventually make possible the establishment of a breeding orchard and the obtention of certified varieties. In order to facilitate the selection process, the identification of metabolic markers would be of great help. Thus, an in-depth comprehension of the response pathways of plants under drought is necessary, which would require additional targeted metabolomic analyses. More insights about the influence of plant provenance and possible epigenetic mechanisms should also be further explored. The chemotyping of a large number of *A. unedo* genotypes should also be conducted as a mean of broad selection and to better understand the links between phenolics concentrations and abiotic and biotic stimuli.

During this research work, the greatest bottleneck identified is probably the lack of background on strawberry tree genetics. Apart from some molecular markers that have been made available and the chloroplast genome sequencing, no in-depth genomic studies have been carried out so far, and the information available is certainly not sufficient to provide background for fundamental biology studies nor for a molecular based selection. Thus, continued efforts are needed to minimized this problem, although significant progress will not be attained, unless a concerted effort between academic institutions and producers across the country is intensified. Researchers from other southern European countries as well as from North Africa should also be involved. Taking into account the great amount of research items produced in recent years on strawberry tree, and the high number of researchers involved, the establishment of a research consortium could generate an enormous output and represent a headway on strawberry tree research that would benefit the agro-forestry sector, especially in countries with large areas of marginal soils, such as Portugal, Spain and many others around the Mediterranean basin.

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