Maria Filomena Figueiredo Nazaré Gomes

Strategies for the improvement of *Arbutus unedo* L. (strawberry tree): *in vitro* propagation, mycorrhization and diversity analysis

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Universidade de Coimbra

Maria Filomena Figueiredo Nazaré Gomes

Orientador:

Prof. Doutor Jorge Manuel Pataca Leal Canhoto

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To Beatriz and Eugénio!

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1/2 MS - Murashige and Skoog macronutrients reduced at half-strength plus micronutrients (Murashige and Skoog, 1962) and organic compounds of the Fossard medium (De Fossard *et al*., 1974)
2-iP - N6-(2-isopentenyl) adenine
A T - Purine nucleotide basis of DNA: adenine, thymine
AJS - Serra da Arrábida's provenance
AL - Serra de Alvéolos's provenance
AL01 to AL07 - Selected adult plants from Oleiros
AM - Arbuscular mycorrhizal symbiosis or VA or VAM - Vesicular arbuscular mycorrhizae
AND - Anderson macronutrients (Anderson, 1984), Murashige and Skoog micro nutrients (Murashige and Skoog, 1962) and organic compounds of the Fossard medium (De Fossard *et al*., 1974)
ArcSRootRate - Arcsine transformation of rooting rate
Au1 to Au27 - Genotypes identification for fingerprinting (27)
BA - Benzyladenine
bp - Base pairs
BVN - Algarve, Serra do Caldeirão's provenance
C - Coimbra's provenance
C G - Pyrimidine nucleotide basis of DNA: cytosine, guanine
C1 and ESAC5 - Spontaneous adult plants
C1C - Mycorrhiza control treatment using C1 clonal plants, under nursery conditions
C1M - Mycorrhiza vegetative inocula treatment (mycelium) using C1 clonal plants, under nursery conditions
C1S - Mycorrhiza dry sporocarps treatment using C1 clonal plants under nursery conditions
CA - Charcoal
cc - Clamp-connections
Co - Collenchyma cells
Cu - Cuticle
CZ - Cambial zone
DNA - Deoxyribonucleic acid; cpDNA- plastidial DNA; mtDNA - mitochondrial DNA
dNTP's - Term referring to the 4 deoxyribonucleotides: dATP, dCTP, dGTP and dTTP
DR - Diarch root
ECM – Ectomycorrhizae; ENDO - endomycorrhizae
Ep - Epidermis
ERM - Ericoid mycorrhizae
ESAC - Coimbra's provenance from Escola Superior Agrária de Coimbra
F Null - Null allele frequencies
FAA - Formalin/acetic, acid/ethyl, alcohol by volume (5:5:90 v/v)
FAM, HEX, or NED - Fluorescently labeled forward primers
Fis - Fixation index
FS - Fossard macronutrients (De Fossard *et al*., 1974), Murashige and Skoog micro nutrients (Murashige and Skoog, 1962) and organic compounds of the Fossard medium (De Fossard *et al*., 1974)
GCC - Growth culture chamber
GH - Greenhouse
GMA - Gerês, Mata de Albergaria's provenance
H – Hyphae
He - Expected heterozygosity
HN - Hartig net
Ho - Observed heterozygosity
HPN - Algarve, São Marcos da Serra's provenace
HWE - Hardy-Weinberg equilibrium
IAA - Indole-3-acetic acid
IBA - Indole-3-butyric acid
IM - Serra do Açor's provenance
IM1 to IM6 - Selected adult plants from Serra do Açor
IRe - Shoots transferred to the inoculated substrate with the mycorrhizal fungi, after root induction and a preliminary root expression (2 weeks)
IRi – Shoots transferred to the inoculated substrate with the mycorrhizal fungi, just after root induction period
ITS – Internal Transcribed Spacer
JF3 - Selected adult plant from Pióddão
KIN - Kinetin
LD - Linkage disequilibria
LLR - Length of the longest root
LSR - Length of the shortest root
M - Mantle
M1.* to M11.* - SSR primer pairs identification
Med. Shoot Prolif - Shoot proliferation media
$MgCl_2$ - Magnesium chloride
MMN - Modified Melin Norkrans culture medium
MS - Murashige and Skoog (Murashige and Skoog, 1962)
Na - Number of alleles
NAA - 1-Naphthalene acetic acid
NI - Control plants cultured in non inoculated substrate
NPK - Nitrogen, phosphor, potassium
NR - Number of roots per shoot
NUC - Neglected or underutilized crops
OM - Orchid mycorrhizae
OPC - Operon Technologies Kit C, sequences of the arbitrary primers
Pal - Palisade parenchyma
PAS - Serra da Gardunha s provenance
PCA - Principal component analysis
PCR - Polymerase chain reaction
PGR(s) - Plant growth regulator(s)
Ph - Phloem; Phs - secondary phloem
Pi - Pith zone
PIC - Polymorphic information content
r - Correlation coefficient
R - Ray cells
R^2 - Coefficient of determination
RAPD - Random amplified polymorphic DNA marker

rc - Root cap
RD - Root development medium
RD-days - Number of days on root development medium
Rep. - Replications
rhz - Root hair zone
RI-days - Number of days on root induction medium
RM - Rooting induction medium, Knop macronutrients (Gautheret, 1959), Murashige and Skoog micronutrients without potassium iodine (Murashige and Skoog, 1962) and organic compounds of the Fossard medium without riboflavin (De Fossard *et al*., 1974)
SAM - Shoot apical meristem
Sc - Shoot tissues showing differentiating fibers
SE - Standard error
SF - Number of shoots formed per test tube
SL - Shoot length
SNX - Number of shoots formed per test tube for further multiplication
Sp - Spongy parenchyma
SSR - Microsatellite markers or simple sequence repeats or variable number of tandem repeats (VNTR) or simple tandem repeat (STR)
St - Stomata
Taq polymerase - Enzyme originally isolated from the bacteria *Thermus aquaticus*
TDZ - Thidiazuron
Tr - Trichome; glandular (gt) or non-glandular (ng) trichomes
UPGMA - Unweighted pair group with arithmetic average method
UV - Ultraviolet light
V - Vascular tissues
Xy - Xylem; Xys - secondary xylem
Zt - Zeatin

GENERAL SUMMARY

Arbutus unedo grows spontaneously around the Mediterranean basin. The species is drought tolerant and able to regenerate following forestry fires making it quite interesting for forestation programs in Mediterranean regions. Fruits are used to make jellies and a spirit which represents the main income for owners. Considering the sparse information about the potential of this fruit tree to be propagated *in vitro*, a project to clone selected trees based on their fruit production was initiated. The role of several factors on propagation was evaluated and studies concerning the mycorrhization and genetic diversity analysis were also performed.

Shoot apices from epicormic shoots showed higher rates of *in vitro* establishment. Of the three basal media tested, the Fossard medium with the micronutrients of the Murashige and Skoog medium gave the highest rates of multiplication. Kinetin (8.9 µM) gave the best results although not different (P>5%) from those obtained with other cytokinins such as benzyladenine or zeatin. Thidiazuron or 1-naphthaleneacetic acid promoted callus growth and had a deleterious effect on the multiplication rate. The genotype was also a factor affecting *in vitro* multiplication and the conditions used for shoot multiplication greatly influenced further behaviour of shoots during the rooting phase. In this phase, the inclusion of an auxin significantly increased rooting yields. Anatomical studies data indicated that adventitious roots had a deep origin in the shoot stem, probably from the cambial zone. From the five substrates tested during acclimatization, perlite without fertilizer gave the best survival rate (98.0±1.23%) after 4 months. Mycorrhization was tested *in vitro* and under nursery conditions. Arbutoid mycorrhizae were observed *in vitro* 1 month after inoculation indicating compatibility between *A. unedo* and *Pisolithus tinctorius*. Both mycorrhizae inocula treatments tested in nursery (vegetative inocula and dry sporocarps) improved plant growth compared to control plants and seedlings after 20 months in a field trial (P>5%). The genetic diversity in 27 *Arbutus unedo* genotypes was assessed by molecular markers (RAPD and SSR). The RAPD primers generated 124 bands from which 57.3% showed to be polymorphic. Eleven SSR primers first tested in *Vaccinium* (an Ericaceae) were previously selected. Five SSR *loci* were polymorphic displaying a 75% mean expected heterozygosity which is a higher value than that observed with RAPDs (27%). Thus, the Lynch (1990) similarity coefficient revealed a similarity among trees higher for RAPD than SSR, respectively of 83% and 21% (Mantel test: r= 0.64; P<0.001; r=0.75; P<0.001, respectively). No genotypes could be grouped according to their geographical origin for both markers.

Some of the produced plants are now in the field for further studies. The data presented in this thesis have provided protocols concerning the *in vitro* propagation of adult plants, mycorrhizal synthesis and allowed the identification of five polymorphic molecular markers (SSR), used on genetic diversity analysis of 27 genotypes. The results are discussed and further research envisaged.

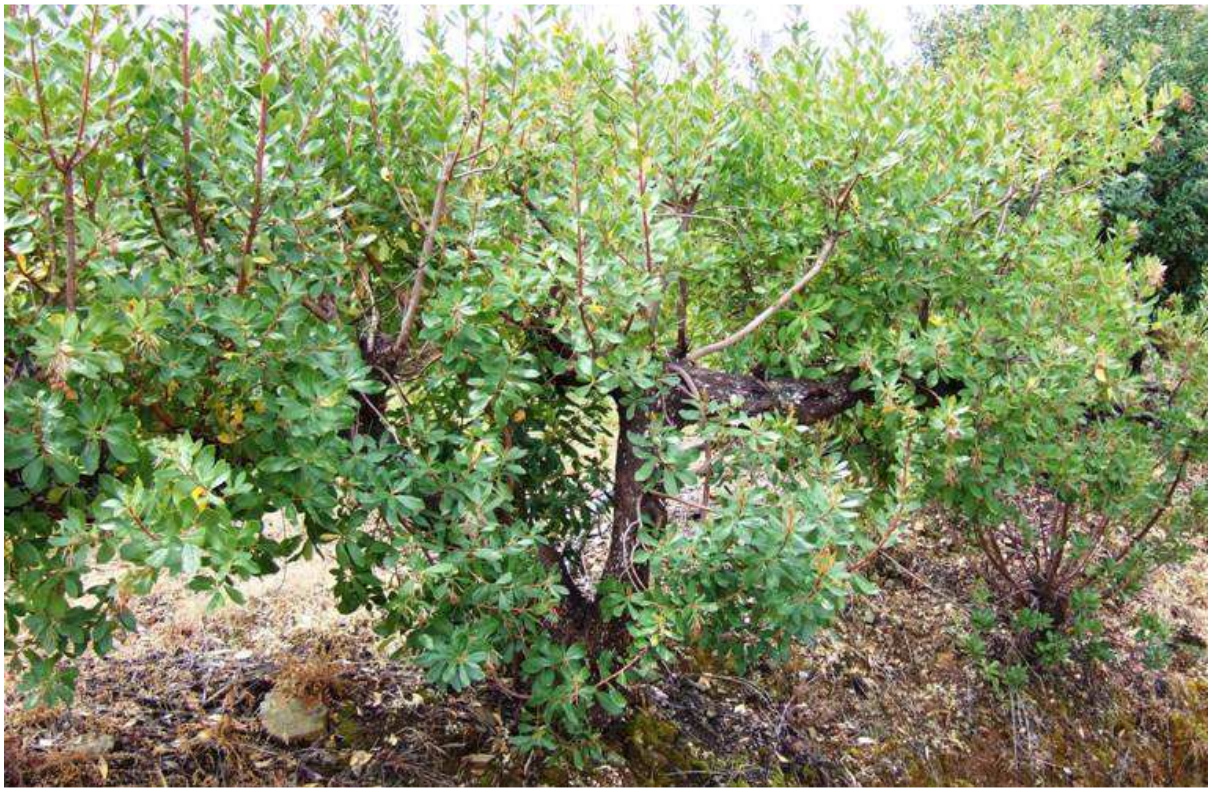
RESUMO GERAL

A espécie *Arbutus unedo* cresce espontaneamente em áreas Mediterrânicas. Trata-se de uma árvore tolerante à secura e com forte capacidade regenerativa após a ocorrência de incêndios florestais, o que a torna interessante para programas de florestação na região Mediterrânica. Os frutos são usados na produção de compota e aguardente. Esta corresponde à principal fonte de rendimento para os proprietários. Considerando a escassa informação acerca do potencial desta espécie frutícola para a propagação *in vitro*, foi iniciado um projecto para a propagação de árvores seleccionadas de acordo com a sua produção de fruto. O efeito de diversos factores na propagação foi avaliado e estudos relativos à micorrização e à diversidade genética foram efectuados.

Os ápices meristemáticos de rebentos epicórmicos apresentaram as melhores taxas no estabelecimento *in vitro*. De três meios de cultura base testados, o meio de Fossard adicionado de micronutrientes de Murashige e Skoog apresentou as maiores taxas de multiplicação. A citocinina cinetina (8.9 µM) permitiu obter as melhores taxas de multiplicação mas sem diferenças significativas relativamente à benziladenina ou zeatina. Tidiazurão ou o ácido 1-naftaleno acético induziram a formação de calos tendo um efeito nefasto na multiplicação. O genótipo foi também um dos factores que interferiu na multiplicação, tendo-se igualmente verificado que as condições testadas na multiplicação influenciaram o comportamento dos rebentos durante a fase de enraizamento. A inclusão de uma auxina aumentou significativamente o enraizamento. Estudos anatómicos mostraram que as raízes adventícias têm uma origem profunda no caule, provavelmente na zona cambial. De cinco substratos testados na aclimatização, a perlite sem fertilizantes foi a que permitiu obter uma maior taxa de sobrevivência após 4 meses (98.0±1.23%). A micorrização foi testada *in vitro* e no viveiro. Micorrizas arbutóides foram observadas *in vitro* 1 mês após a inoculação, mostrando a compatibilidade entre *A. unedo* e *Pisolithus tinctorius*. Ambos os tratamentos com inóculo testados no viveiro (inóculo vegetativo e caldo esporal) melhoraram o crescimento das plantas relativamente ao controlo e plantas de semente, 20 meses após a

instalação do ensaio de campo (P>5%). A diversidade genética entre 27 genótipos de *Arbutus unedo* foi avaliada com recurso a marcadores moleculares (RAPD e SSR). No caso dos RAPDs os primers utilizados geraram 124 bandas, com 57,3% de polimorfismo. Onze primers SSR de *Vaccinium* (uma Ericaceae) foram previamente seleccionados. Cinco SSR *loci* mostraram polimorfismo, com uma heterozigocidade esperada média de 75%, um valor superior ao obtido com RAPDs (27%). Assim, o coeficiente de similaridade de Lynch (1990) mostrou uma similaridade entre as árvores maior com os marcadores RAPD do que com os marcadores SSR, respectivamente de 83% e 21% (Mantel test: r= 0.64; P<0.001; r=0.75; P<0.001, respectivamente). Nenhum genótipo pôde ser agrupado de acordo com a sua origem geográfica, para ambos os marcadores.

Algumas das plantas produzidas foram instaladas em ensaios de campo. Os dados apresentados nesta tese permitiram estabelecer protocolos para a propagação *in vitro* de plantas adultas, para o estabelecimento de micorrizas e a identificação de cinco marcadores moleculares (SSR) polimórficos, utilizados na avaliação da diversidade genética entre 27 genótipos. Os resultados obtidos são discutidos e analisados em termos de perspectivas futuras.



1 - Introduction

1 INTRODUCTION

1.1 *ARBUTUS UNEDO* L., A BRIEF DESCRIPTION

The genus *Arbutus* is included in the Ericaceae, a family of small trees or shrubs, usually evergreen, with actinomorphic flowers, superior ovary and the fruit being a capsule, berry or drupe (Tutin *et al.*, 1972). *Arbutus unedo* is an evergreen species usually a bushy shrub or a small tree up to 12 m (Pedro, 1994). The bark is fissured and it is peeling off in small flakes, mostly dull brown. The leaves are oblong-lanceolate, usually 2-3 times as long as wide, and serrate to subentire, glabrous with a petiole 10 mm or less (Fig. 1A). The inflorescence is a drooping panicle which appears in autumn (Fig. 1A). The fruit, a round berry (c. 20 mm diameter) is covered with conical papillae, with its color changing from yellow and scarlet to deep crimson (Fig. 1B) as ripening occurs (Tutin *et al.*, 1972).

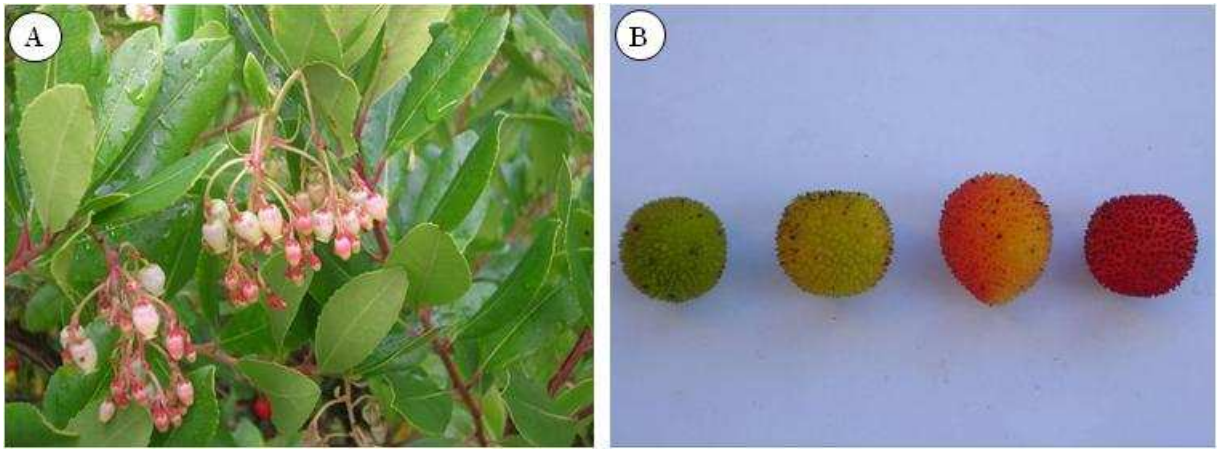


Figure 1 – Two aspects of strawberry tree. A - Leaves oblong-lanceolate, serrate to subentire, glabrous with a petiole 10 mm or less. The inflorescence is a drooping panicle. B - The fruit, a berry with its color changing from yellow to deep crimson.

Arbutus together with other five genera (*Arctostaphylos*, *Arctous*, *Comarostaphylis*, *Ornithostaphylos*, and *Xylococcus*) is included in the Arbutoideae sub-family. The Arbutoideae is a distinct and natural group within the Ericaceae, based on fruit and flower morphology, as well as in anatomical and phytochemistry features (Hileman *et al.*, 2001). *Arbutus* comprises about 11 to 20 species (depending on the author) distributed from West coast of north America through Mexico and Central America, Western Europe, the Mediterranean region, northern Africa and parts of the Middle East (Hileman *et al.*, 2001; Kron *et al.*, 2002b; Torres *et al.*, 2002). Three species of *Arbutus* are distributed around the Mediterranean basin: *A. unedo*, *A. andrachne*, and *A. x andrachnoides*, a hybrid between *A.*

unedo and *A. andrachne*. *A. canariensis* species is endemic of the Macaronesia region, namely to the Canary Islands (Hileman *et al.*, 2001). Torres *et al*. (2002) refer other Mediterranean species such as *A. pavarii* and another hybrid *A.* x *androsterilis* (*A. unedo* x *A. canariensis*, in the Canary Islands). The remaining species of *Arbutus* occur in the Western Hemisphere. Of these, *A. xalapensis*, *A. texana*, *A. peninsularis*, *A. tessellata*, *A. arizonica*, *A. occidentalis*, and *A. madrensis* have a Neotropical distribution with *A. xalapensis* being the most widespread and variable. *A. menziesii* extends along the West Coast of North America (Hileman *et al.*, 2001; Kron *et al.*, 2002b). According to Hileman *et al.* (2001) the Arbutoideae are dry-adapted and sclerophyllous taxa and most of the diversity in the group is in regions of Mediterranean climate and in western North America. Exceptions to this distribution include the circumarctic *Arctous alpina*, circumboreal *Arctostaphylos uva-ursi*, and four species of *Arbutus* that occur in Mediterranean regions of Europe, North Africa, and the Middle East, comprising *A. unedo*. Beyond the Arbutoideae sub-family, other 6 sub-families have been described, namely the Ericoideae, Cassiopoideae, Vaccinioideae, Styphelioideae, Monotropoideae and Enkanthoideae (Hileman *et al.*, 2001; Kron *et al.*, 2002a). Considering the phylogenetic relationships among the Ericaceae the subfamily Arbutoidea is closer to the Vaccinioideae which includes the important economic genus *Vaccinium* and is widespread in temperate and tropical zones, and the Ericoideae comprising *Rhododendron* also an economically important taxa used mostly as ornamental (Hileman *et al.*, 2001; Kron *et al.*, 2002b). *Vaccinium* and *Rhododendron* have been the subject of intense breeding programs in which micropropagation and genetic diversity studies have been applied (Debnath, 2007; Kosola *et al.*, 2007; Hancock *et al.*, 2008; Bassil *et al.*, 2010; Eeckaut *et al.*, 2010; Hirai *et al.*, 2010; Wang *et al.*, 2010).

Arbutus unedo, commonly known as strawberry tree, is found in western, central and southern Europe, north-eastern Africa and the Canary Islands and western Asia. Its progression in the temperate area of Europe occurs from the north of the Iberian Peninsula (Fig. 2), along the west coast, reaching its most septentrional limit in the northwest of Ireland Island (Tutin *et al.*, 1972; Torres *et al.*, 2002). *A. unedo* distribution is mostly typical of Mediterranean sclerophyllous and laurel-like vegetation, mainly in coastal and inland areas with benign climates, where either frost or summer dryness are not very intense (Torres *et al.*, 2002; Godinho-Ferreira *et al.*, 2005).

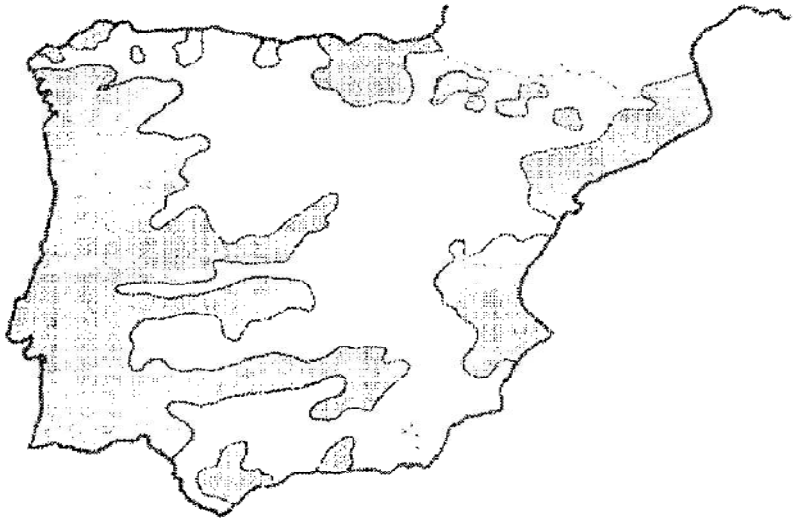


Figure 2 – *Arbutus unedo* distribution in the Iberian Peninsula (Source: Torres *et al*., 2002).

In Europe, it grows mainly in the Mediterranean basin: Portugal, Spain, France, Italy, Albania, Croatia, Bosnia, Montenegro, Greece, Turkey and the Mediterranean islands (Torres *et al.*, 2002). *A. unedo* distribution ranges from an altitude of 20 to 1000 m (Torres *et al.*, 2002; Celikel *et al.*, 2008). As far as the soil is concerned, it prefers siliceous or decarbonated substrata (Torres *et al.*, 2002; Godinho-Ferreira *et al.*, 2005). The tree can grow on alkaline and relatively acidic (pH 5–7.2) soils (Celikel *et al.*, 2008). In Portugal it is found in wide range of soil types according to their lithology origin (Ricardo and Veloso, 1987; Godinho-Ferreira *et al.*, 2005). *A. unedo* is broadly distributed, from Atlantic climate areas in the North to dry and arid areas in the South, occupying about 15.500 ha (Godinho-Ferreira *et al.*, 2005). According to Pedro (1994) *Arbutus unedo* rarely constitutes dominant stands being more common in patchy bush-like communities or in natural stands dominated by oaks. The species appears naturally in different phytosociological alliances, the cork-oak woodlands (*Sanguisorbo-Quercetum suberis*), the strawberry-tree dominated scrub (*Arbuto-Cistetum populifolii*) in the South, the pedunculate oak-woodlands (*Rusceto-Quercetum roboris*) in the North, including the oak-woodlands (*Arisaro-Quercetum broteroi* and *Arbuto-Quercetum pyrenaicae*) and strawberry dominated scrub (*Phillyreo-Arbutetum unedonis*) in the Centre of the country. Extensively populated areas of *A. unedo*, tough patchy, occur mainly in the Southern mountainous regions (Serra de Monchique and Caldeirão). A fragmented-like distribution is common in the central and northern regions due to intensive forestation programs with *Pinus pinaster* and *Eucalyptus globulus* that form closed canopy stands. In recent years, the area of *A. unedo* has increased as a consequence of recurrent fires,

ecosystem degradation and abandoned farmland, and large patches of shrub-like formations can be found in different areas (Meireles *et al.*, 2005).

1.2 ARBUTUS UNEDO, ECONOMIC AND BIOLOGICAL RELEVANCE

According to the International Centre for Underutilized Crops (www.icuc-iwmi.org/) and the Global Facilitation Unit for Underutilized Species (www.underutilized-species.org) this species falls into the category of neglected or underutilized crops (NUC). Therefore, it is an underestimated fruit tree, with different possible commercial usages from processed and fresh fruit production to ornamental, pharmaceutical and chemical industrial applications (Pedro, 1994; Ayaz *et al.*, 2000; Mereti *et al.*, 2002; Celikel *et al.*, 2008; Zizzo *et al.*, 2010).

Strawberry tree is a valuable ornamental plant due to its attractive red fruits appearing in fall and winter (Fig. 3), and the pinkish-white flowers occurring in the autumn, often simultaneously (Celikel *et al.*, 2008).

Figure 3 - The pinkish to white flowers and the red fruits occurring simultaneously in the autumn.

This species is characterized by an extreme rusticity and a wide morphological and phenological variability, which can facilitate the selection of accessions to develop new ornamental products particularly for gardening, environmental restoration and providing a good alternative for floriculture in the temperate areas (Zizzo *et al.*, 2010).

A. unedo flowers are a significant source of nectar and pollen for bees (Dalla Serra *et al.*, 1999; Neppi, 2001; Celikel *et al.*, 2008; Pajuelo, 2008) and different methods have been

developed for the identification of homogentisic acid (HA) in honey which is used as a marker for the origin of strawberry tree honey (Scanu *et al.*, 2005; Spano *et al.*, 2006).

Fruits of this species are processed into traditional products such as jam, marmalade, wine, alcohol and liqueur (Martins *et al.*, 1999; Galego *et al.*, 2001; Galego, 2006; Martins, 2006; Celikel *et al.*, 2008). In Portugal a considerable part of the fruit production is used to make a type of very alcoholic spirit called medronheira which represents the main income for forestry owners (Galego *et al.*, 2001; Galego, 2006). When eaten, fresh fruits are a good source of antioxidants (flavonoids, anthocyanins, ellagic acid and its diglucoside derivative) as well as of vitamins C and E and carotenoids (Pawlowska *et al.*, 2006; Demirsoy *et al.*, 2007; Pallauf *et al.*, 2008; Oliveira *et al.*, 2010a; Yavaşer *et al.*, 2010). Mature *A. unedo* fruits are still characterized by the high ratio of sugars/acids and the high content of phenolic acids, terpenoids compounds, mineral elements, and tannin (Ayaz *et al.*, 2000; Özcan and HacIseferogullarI, 2007; Pallauf *et al.*, 2008).

The leaves of *A. unedo* have been used in folk medicine because of their antiseptic, diuretic and astringent properties (Kıvçak *et al.*, 2001), and in the chemical industry due to its high tannin content (Celikel *et al.*, 2008). Some authors have also pointed out that leaves also have a strong antioxidant activity (Kivçak and Mert, 2001; Pabuccuoglu *et al.*, 2003; Oliveira *et al.*, 2009; Sá *et al.*, 2010). *A. unedo* together with *Hypericum empetrifolium*, *Pistacia terebinthus*, and *Cistus parviflorus* in a total of 42 species were indicated as the most promising plant species having antibacterial activities (Kaçar, 2008). According to El Haouari *et al.* (2007) *A. unedo* extracts show antiaggregant action and might be used for the treatment and/or prevention of cardiovascular diseases.

The interest of *Arbutus unedo* is not purely economic. In cultural terms there are numerous references to this tree in books, songs, popular stories and romances and an *A. unedo* tree is even present in the symbol of the city of Madrid.

From an ecological perspective since it is fire resistant (Fig. 4) and, due to its pioneer status, strawberry tree is very interesting for land recovery and desertification avoidance (Pedro, 1994; Piotto *et al.*, 2001). Besides, it contributes to maintain biodiversity, helps to stabilize soils and survives well in marginal lands (Piotto *et al.*, 2001; Godinho-Ferreira *et al.*, 2005). The strawberry tree is also important because it can contribute to CO_2 storage and to the biomaterials production (Fenning and Gartland, 2007). Moreover, *A. unedo* may contribute to the discontinuity of the forest biomass due to monocultures of pines and eucalypts, particularly in the centre and north regions of Portugal, a situation responsible for

the high number of fires and high fire intensity that all summers occur in these areas of the country (S. Silva and Harrison, 2010).

Figure 4 – *Arbutus unedo* regeneration following a forest fire (Piódão, 2005).

As previously stated, *A. unedo* is an under-exploited species in Portugal and other Mediterranean countries (Greece, Italy and Turkey). Fruits, leaves and shoots have been collected from spontaneous field-growing individuals without particular concerns about the quality of the plant material used (Pedro, 1994; Ayaz *et al.*, 2000; Mereti *et al.*, 2002; Celikel *et al.*, 2008; Zizzo *et al.*, 2010). However, in the last years, the situation has changed and forest owners and forest associations are becoming increasingly interested in *A. unedo* as a fruit or ornamental species, with the consequent demand of high-quality plant material (Celikel *et al.*, 2008; Gomes and Canhoto, 2009; Zizzo *et al.*, 2010). Unfortunately, they are unable to find this kind of trees since breeding programs have never been applied to strawberry tree. As a consequence, farmers potentially interested in *A. unedo*, drive their attention towards other species that can assure them more interesting profits. Whether they are really interested in strawberry tree, the only solution is to start from uncertified plant material, generally propagated from wild-growing trees of unknown genotype and quality. This last situation could be extremely disappointing for farmers since its expectations may not be achieved due to the poor quality of the starting material. The main purpose of this study is to develop a consistent breeding program. Tree species, with their long-life cycles, heterozygosity and difficulties to be vegetatively regenerated are not easy to breed. In spite of this, interesting results have been obtained in the improvement of other fruit producing plants suggesting that the same might occurs with strawberry tree.

1.3 MICROPROPAGATION OF WOODY PLANTS

Plant propagation is a key factor for tree breeding. The method of propagation applied to a given species must reflect the purposes of the multiplication process. Through sexual propagation and seed production genetic diversity is promoted and new genetic combinations can be selected. On the other hand, asexual methods of propagation are useful when the genetic characteristics of a particular genotype or individual elite tree must be maintained. Hybrids and sterile genotypes are also usually propagated through this process. Several examples of the application of asexual propagation methods to tree breeding have been described in the literature such as in *Eucalyptus*, *Populus* and some softwood trees as *Pseudotsuga menziesii* and *Picea abies* (Myburg *et al.*, 2007; Rae *et al.*, 2007; White *et al.*, 2007; George and Debergh, 2008; Canhoto, 2010). Seeds possess several advantages as a method of propagation: (a) they are often produced in large numbers thus reducing costs when compared to vegetative propagation on nursery; (b) in many species in which orthodox seeds are produced they can be easily stored for large periods without any loss of viability; (c) they possess dispersal mechanisms that facilitate the colonization of habitats; (d) virus and microorganisms causing diseases are not usually transmitted to the progeny through seeds (George and Debergh, 2008).

Many important crop plants are vegetatively propagated and grown as clones. Suitable methods for vegetative propagation have been developed. Cuttings and grafting are the most traditional 'macropropagation' techniques used in forestry trees. *In vitro* techniques have the following advantages over traditional methods: (a) cultures are started with very small pieces of plants (explants); (b) only a small amount of space is required to maintain cultures or to greatly increase their number; (c) production can be uniform all the year round and is more independent of seasonal changes; (d) vegetatively-reproduced material can be often stored over long periods; (e) propagation is ideally carried out in aseptic conditions; (f) methods are available to eliminate virus from contaminated plants and (g) a more flexible adjustment of factors influencing vegetative regeneration is possible such as nutrient and growth regulator levels, light and temperature, hence increasing the propagation rate (George and Debergh, 2008; Canhoto, 2010). The chief disadvantages of *in vitro* methods for large-scale multiplication include: (a) the need of advanced skills for their implementation; (b) a specialised and expensive production facility and (c) the present methods are labor intensive,

thus the cost of propagules is usually relatively high (George and Debergh, 2008; Chawla, 2009; Canhoto, 2010).

The methods available for *in vitro* micropropagation are the development of axillary shoots, the formation of adventitious shoots or somatic embryo development. Axillary shoot proliferation exploits the formation of new phytomers through the development of axillary meristems that can be further used to initiate new cycles of propagation through the culture of nodal segments or shoot tips (Chawla, 2009). Axillary shoot proliferation is a type of organized growth that can be stimulated *in vitro*, and hence used for plant cloning (George, 2008). *In vitro* shoot development may arise through the culture of shoot tips or shoot apical meristems (SAM) surrounded by a few leaf primordia in which the shoot apical meristem continues its normal development, or by the development of the often dormant axillary meristems (Chawla, 2009). The production of plants from axillary shoot proliferation has proved to be the most generally applicable and reliable method of true-to-type *in vitro* propagation (George and Debergh, 2008; Canhoto, 2010). In contrast, to axillary shoot proliferation, organogenesis induction occurs when SAMs are induced *de novo* from organs or tissues cultured *in vitro* (Chawla, 2009; Canhoto, 2010). The formation of adventitious shoots occurs either directly on pieces of tissue or organs (explants) removed from the mother plant, known as direct organogenesis, or indirectly from unorganised cells in callus or suspension cultures, known as indirect organogenesis (George and Debergh, 2008). In certain species, direct organogenesis can provide a reliable method for micropropagation, by increasing the final number of plantlets (Chawla, 2009). Several ornamental plants are at present propagated *in vitro* by direct organogenesis, such as *Achimenes*, *Euphorbia pulcherrima*, *Saintpaulia*, *Sinningia* and *Streptocarpu*s (George and Debergh, 2008; Castellanos *et al.*, 2010). Through indirect organogenesis other ornamental plants are propagated *in vitro*, such as *Anthurium andreanum*, *Freesia* and *Pelargonium* (George and Debergh, 2008; Maira *et al.*, 2010). However, this technique is more prone to yield off-types than axillary shoot proliferation (George and Debergh, 2008; Chawla, 2009). Propagation by all methods of indirect culture (organogenesis or somatic embryogenesis) carries a risk that the regenerated plants will differ genetically from each other and from the mother plant (Canhoto, 2010). On the other hand, in some crop plants, the genetic differences between plants derived from callus and suspension cultures may be interesting as a new source of selectable variability for plant breeding (Ptak, 2010; Winkelmann, 2010). Somatic embryos are often initiated directly upon explanted tissues (direct somatic embryogenesis). Embryogenesis has a great potential for mass propagation. However, due to the lack of clonal

stability the commercial application of this technology remains limited (Canhoto, 2010). Although plants can be regenerated from embryos directly initiated *in vitro*, and may be present in sufficient numbers for limited plant production in breeding programmes, the number of primary embryos per explant will usually be inadequate for large scale cloning (George and Debergh, 2008; Chawla, 2009). Other problems related with somatic embryogenesis are the asynchronous growth and the high number the abnormal somatic embryos usually formed (Canhoto, 2010). Somatic embryogenesis probably provides the way for tissue culture methods of plant propagation to be economically deployed on extensively planted field crops and forest trees. Thus, this method of propagation offers advantages suggesting that it will be increasingly used for plant cloning in the future (George and Debergh, 2008; Canhoto, 2010).

1.3.1 Factors affecting *in vitro* micropropagation

An analysis of the literature shows that several factors can affect micropropagation. Among them the genotype of the donor plants, the type and concentrations of plant growth regulators (PGRs) and the culture media seem to be the most relevant (George and Debergh, 2008; Machakova *et al.*, 2008; Chawla, 2009; Canhoto, 2010). Quite a few nutrients/substances are required for growth. These include inorganic nutrients, a carbon source, plant growth regulators (PGRs), and organic nutrients (George and De Klerk, 2008; Canhoto, 2010). Carbohydrates play an important role in tissue culture, both as an energy and carbon source and as osmotic agent. Sucrose is almost universally used for micropropagation purposes since it is a sugar usually metabolizable by plant tissues both *in vitro* and in natural conditions (George and De Klerk, 2008; Thorpe *et al.*, 2008). Several authors have pointed out that sugars are not only important as carbon sources, but they may also affect morphogenic processes (Machakova *et al.*, 2008). The inorganic nutrients are added to the culture media as salts. In weak aqueous solutions salts dissociate into cations and anions which are then absorbed by plant tissues Part of the nutrients, and especially the oligoelements, may also be added via impurities present in other components of the media as is the case of agar (George and De Klerk, 2008). When trying to find media formulations suitable for different plant species, two important factors must be considered: the total amount of nitrogen in the medium and the ratio of nitrate to ammonium ions (Canhoto, 2010). Small amounts of several organic compounds can improve growth and morphogenesis

of plant tissue cultures. These are mainly some vitamins and amino acids. The particular concentration of these compounds changes with the species and the purposes of the *in vitro* culture method, and probably reflects the synthetic capacity of the explant (Thorpe *et al.*, 2008). Vitamins are compounds required by animals in very small amounts. Many of the same substances are also needed by plant cells as essential intermediates or metabolic catalysts. However intact plants, unlike animals, are able to produce their own requirements. Cultured plant cells and tissues can, however, become deficient in some factors; growth and survival is then improved by their addition to the culture medium (Thorpe *et al.*, 2008). The success of plant tissue culture as a means of plant propagation is deeply influenced by the nature of the culture medium used (George and De Klerk, 2008; Chawla, 2009). The most commonly formulation used for plant tissue culture is that of Murashige and Skoog (1962) initially developed to optimize growth of tobacco callus (George and De Klerk, 2008). Several culture media have been tested for *in vitro* propagation of Ericaceae species, such as: (1) Anderson (Anderson, 1984) for *Arbutus unedo* (Mendes, 1997), *Rhododendron* (Anderson, 1984; Almeida *et al.*, 2005; Eeckaut *et al.*, 2010) and *Vaccinium* sp. (Gajdošová *et al.*, 2007; Ostrolucká *et al.*, 2007); (2) Woody Plant Medium (Lloyd and McCown, 1980) for *Arbutus andrachne* (Bertsouklis and Papafotiou, 2007), *Arbutus unedo* (Gonçalves and Roseiro, 1994; Mereti *et al.*, 2002), *Arbutus xalapensis* (Mackay, 1996), *Kalmia latifolia* (Lloyd and McCown, 1980), *Rhododendron* (Eeckaut *et al.*, 2010) and *Vaccinium* sp. (Gonzalez *et al.*, 2000); (3) Murashige and Skoog (Murashige and Skoog, 1962) for *Arbutus unedo* (Gonçalves and Roseiro, 1994), *Rhododendron* (Eeckaut *et al.*, 2010) and *Vaccinium* sp. (Debnath and McRae, 2001; Debnath, 2003); (4) Economou and Read medium (Economou and Read, 1984) for *Azalea* (Eeckaut *et al.*, 2010) and (5) Zimmerman and Broome, Z2-medium (Zimmerman and Broome, 1980) for *Vaccinium cylindraceum* (Pereira, 2009).

PGRs included in the culture media are particularly important for plant micropropagation (George and Debergh, 2008; Machakova *et al.*, 2008; Chawla, 2009; Canhoto, 2010). There are several recognized classes of PGRs. Until recently only five groups were recognized: auxins, cytokinins, gibberellins, ethylene and abscisic acid. In the last decade it became clear that other compounds can influence plant development. Among these compounds, brassinosteroids, jasmonic acid, oligosaccharins and systemin have been matter of intense research (Machakova *et al.*, 2008). To promote axillary shoot formation, and reduce apical dominance in shoot cultures, one or more cytokinins are generally added to the medium during the multiplication phase (Chawla, 2009; Canhoto, 2010). The compounds that are

most frequently used are: kinetin, benzyladenine (BA), zeatin, and N6-(2-isopentenyl) adenine (2-iP) (Chawla, 2009). More recently, thidiazuron (TDZ), a cytokinin-like compound has been intensively tested and has been used for the micropropagation of some woody plants (Van Staden *et al.*, 2008). This substituted phenylurea has been shown to stimulate axillary shoot growth on species where BA and 2-iP showed to be ineffective, as, for example, in *Acer saccharinum* (Preece *et al.*, 1991). In some species, the presence of an auxin together with cytokinin promotes axillary shoot proliferation by stimulating cell division, bud initiation and growth (Machakova *et al.*, 2008; Van Staden *et al.*, 2008; Chawla, 2009; Canhoto, 2010). The auxin/cytokinin ratio in culture media has an important role on several aspects of *in vitro* morphogenesis (Machakova *et al.*, 2008; Canhoto, 2010). The role of cytokinin and auxin has been found to be effective in shoot multiplication of *Eucalyptus nitens* (Gomes and Canhoto, 2003), *Ficus anastasia* (Al Malki and Elmeer, 2010) and *Tilia platyphyllos* (Chalupa, 2003) among other species. The required concentration of each type of PGR differs according to the species, the cultural conditions and the compounds used. Interactions between the two PGR (auxin/cytokinin) are often complex, and more than one combination of PGR is likely to produce optimum results (Van Staden *et al.*, 2008).

Cytokinins appear to be necessary for plant cell division. Subculture of the tissue onto a medium containing a cytokinin may induce the cells to divide synchronously (Van Staden *et al.*, 2008). The inclusion of cytokinins in the medium to promote shoot proliferation has been commonly used for several members of the Ericaceae family, such as: *Arbutus andrachne* (Bertsouklis and Papafotiou, 2007), *Arbutus unedo* (Gonçalves and Roseiro, 1994; Mendes, 1997; Mereti *et al.*, 2002), *Arbutus xalapensis* (Mackay, 1996), *Rhododendron* sp. (Almeida *et al.*, 2005; Eeckaut *et al.*, 2010) and *Vaccinium* sp. (Debnath and McRae, 2001; Gajdošová *et al.*, 2007; Ostrolucká *et al.*, 2007). These studies will be discussed on chapter three. As would be expected the specific cytokinin and its concentration varies considerably within species and genotypes that have been tested. This feature is also reported for Ericaceae species, *e.g.* for V*accinium. vitis-idaea* (Gajdošová *et al.*, 2007) and for *Vaccinium corymbosum* (Ostrolucká *et al.*, 2007). In members of the Ericaceae, isopentenyladenine (2iP) is often used in micropropagation assays (Mendes, 1997). However, generally the most commonly used cytokinin for axillary shoot proliferation is benzyladenine (BA). Most species respond well to BA and when the concentration is suitable, many axillary shoots will proliferate and elongate, without any adventitious shoot formation (Preece, 2008). Levels of cytokinins too high may induce adventitious shoot formation (Chawla, 2009) with a high number of small shoots difficult to elongate (Van Staden *et al.*, 2008). Other negative

consequence of an excess of cytokinins is the formation of vitrified shoots with impaired growth (Kotsias and Roussos, 2001). These negative aspects of cytokinin action have been reported by several authors in different species, including the Ericaceae *Vaccinium myrtillus* and *V. vitis-idaea* (Jaakola, 2001) and *Vaccinium macrocarpon* (Debnath and McRae, 2001). High cytokinin concentrations on the multiplication medium may decrease further root primordia induction and development. More than one subculture on a cytokinin-free medium may be required until the level of cytokinin within the tissues has been sufficiently reduced to permit an effective rooting (Tornero *et al.*, 2009).

Auxins control many physiological and developmental processes in plants. Just to mention a few, it is well known the role of auxins on stimulating differentiation of vascular tissues, in controlling the differentiation of buds and roots, and in the induction of somatic embryogenesis (Machakova *et al.*, 2008; Canhoto, 2010). At the cellular level, auxins control basic processes such as cell division and cell elongation. Thus, they are involved both in the formation of unorganised cell proliferations (callus) and meristems which develop into defined organs as adventitious roots or shoots (Machakova *et al.*, 2008; Chawla, 2009). Therefore, they are linked to root induction process. The induction of rhizogenesis usually requires a treatment with auxin. For each species or genotype is relevant to choose the appropriate auxin, its concentration and induction period to promote growth without inducing callus formation (Meiners *et al.*, 2007). However, root development does not need auxin stimulation, on the contrary auxins inhibit root development (Kotsias and Roussos, 2001). Thus, depending on other PGRs present in the medium, changes in auxin concentrations may modify the type of growth, *e.g.*, stimulation of root formation may switch to callus induction (Tornero *et al.*, 2009). As a result, each tissue culture system is unique, and the effects of different concentrations of PGR must be tested for each case individually and only to some extent can the results be reasonable extrapolated to other culture systems (Machakova *et al.*, 2008; Canhoto, 2010).

The success of any *in vitro* propagation method is highly dependent of an effective phase of acclimatization in order to ensure that a considerable number of plants can survive when transferred to soil and natural conditions (Ziv, 1986). Several strategies have been developed to enhance the survival rates following *in vitro* propagation (Hazarika, 2003). These include the stimula6tion of autotrophic cultures; the use of plant growth retardants; the reduction of the humidity levels; application of antitranspirants and promotion of a simultaneous rooting and acclimatization (Preece and Sutter, 1991). To stimulate autotrophic cultures two

strategies have been used. In some cases the pressure of oxygen is reduced, hence reducing the photorespiration rate (Sharma *et al.*, 1999). In other situations the amount of sugars present in the medium is reduced or completely eliminated, while the photosynthetic photon flux and the carbon dioxide pressure are increased (Hazarika, 2003). Several growth retardants can be used in micropropagation to reduce damage due to wilting without displaying deleterious side effects. Paclobutrazol is active as a growth retardant (inhibiting shoot growth) in a broad spectrum of species, and may also regulates various metabolic processes such as gibberellins biosynthesis (Hazarika *et al.*, 2000). Plant growth retardants generally induce a shortening of the internodes and have some additional effects, such as a reduction in leaf size, stimulation of chlorophyll synthesis, and thickening of roots (Al-Bahrany, 2002). According to Hazarika (2003) the use of antitranspirants to reduce water loss during acclimatization has produced mixed results. These have not proved to be useful in promoting *ex vitro* survival probably due to phytotoxicity and interference with photosynthesis. The low deposition of surface wax, stomata abnormalities and an interrupted cuticle are typical anatomical features of herbaceous plants growing under conditions of abundant moisture. This typical *in vitro* anatomy can be prevented by increasing the vapor-pressure gradient between the leaf and the atmosphere (Isutsa *et al.*, 1994). Lowering of the relative humidity *in vitro* has been done experimentally in several ways, such as through the application of desiccants, by coating the medium with oils or oil-derived compounds, (c) by opening culture containers, adjusting culture closures or using special closures that facilitate water loss, or by cooling container bottoms. Increasing the sugar or agar concentration, or adding osmotic agents such as polyethylene glycol to the medium will also lower the relative humidity and, in some cases, serve the same purpose as desiccants (Hazarika, 2003). Sharma *et al*. (1999) reported that acclimatization and hardening in tea micropropagation could be optimized using CO_2 enrichment and light conditions in specially designed hardening chambers.

1.4 MYCORRHIZAL SYNTHESIS

Mycorrhizal fungi are symbionts in roots of most woody plants. These associations vary in structure and functions, but the most widespread interaction is the arbuscular mycorrhizal (AM) symbiosis. It has been estimated that more than 80% of all terrestrial plants form this type of association (Goltapeh *et al.*, 2008). These fungi are a critical component in agricultural systems because they promote plant growth, earlier flowering and fruiting, plant

water stress tolerance, and plant health through antagonistic and competitive effects on pests and pathogens (Smith and Read, 1997; Peterson and Massicotte, 2004; Fortin *et al.*, 2008; Gobert and Plassard, 2008; Goltapeh *et al.*, 2008; Oliveira *et al.*, 2010b). This colonization may also enhance resistance to biotic and abiotic stresses promoting plant growth in harsh conditions, hence facilitating ecological restoration (Wu *et al.*, 2004; Fortin *et al.*, 2008; Goltapeh *et al.*, 2008; Oliveira *et al.*, 2010b).

According to the work of Goltapeh *et al.*(2008) hyphae and arbuscules were found in fossils of the early Devonian and the use of molecular markers indicated that Glomales were detected in the fossil record so early as 350–460 million years ago suggesting that mycorrhizae could have contributed to the successful colonization of land by plants (Honrubia, 2009a). Almost all green land plants live in symbiosis with mycorrhizal fungi and the data available show that only in a few species of Cruciferae and Chenopodiaceae mycorrhizae seem not to be present (Read, 2001; Fortin *et al.*, 2008; Honrubia, 2009a). However, it should be noted that if the association between the host and the collective mycorrhizae fungi community is not compatible, it can affect plant development and crop yield (Goltapeh *et al.*, 2008).

1.4.1 Different types of mycorrhiza

Several authors have classified the mycorrhizae in different types as a function of the morphological aspect of the association and the fungi involved (Smith and Read, 1997). Thus, the following types have been considered (Fig. 5): (1) arbuscular (bryophytes and vascular plants, about 70% of the species), (2) ectomycorrhizae (gymnosperms and angiosperms woody plants, circa 5% of the species), (3) ectendomycorrhizae (rare, in *Pinus*), (4) arbutoid mycorrhizae (in three genus of Ericaceae), (5) ericoid mycorrhizae (found mainly in Ericaceae), (6) orchid mycorrhizae (Orchidaceae), and (7) monotropoid mycorrhizae (found in three Ericaceae genus - *Monotropa*, *Pterospora* and *Sarcode*).

Species from the genus *Arbutus,* as *A. unedo* establish arbutoid mycorrhizae resulting from the association with some species of basidiomycetes (Fortin *et al.*, 2008). Ericoid mycorrhizae (ERM) are formed by ascomycete fungi and occur only in the two closely related orders of Ericales and Diapensiales. In the former they are found in major genera of the family Ericaceae, such as *Calluna*, *Erica*, *Gaultheria*, *Kalmia* (sheep laurels), *Ledum* (Labrador tea), *Rhododendron*, *Vaccinium*, as well as in the genus *Empetrum* (Empetraceae)

and in the genera *Dracophyllum*, *Epacris*, *Richea* and *Styphelia* of the Epacridaceae. In the Diapensiales ERM have been detected in the species *Diapensia lapponica* of the Diapensiaceae family (Read, 2001).

Orchid mycorrhizae (OM) are formed exclusively by basidiomycetes. The genus *Rhizoctonia*, which also includes a number of economically important plant pathogens, was considered to contain all the major OM forming fungi (Read, 2001). Both autotrophic and myco-heterotrophic species occur in the Orchidaceae family. Roots of autotrophic orchids become colonized by fungi, and these mainly supply the host with mineral nutrients. Myco-heterotrophic species are associated with fungi that provide hyphal links to neighboring autotrophic plants, through which they obtain photosynthates (Peterson and Massicotte, 2004). A wider range of fungi, including some that form ectomycorrhizae with trees (members of Russulaceae and Thelephoraceae) and the virulent tree pathogen *Armillaria mellea*, are now known also to produce OM (Read, 2001).

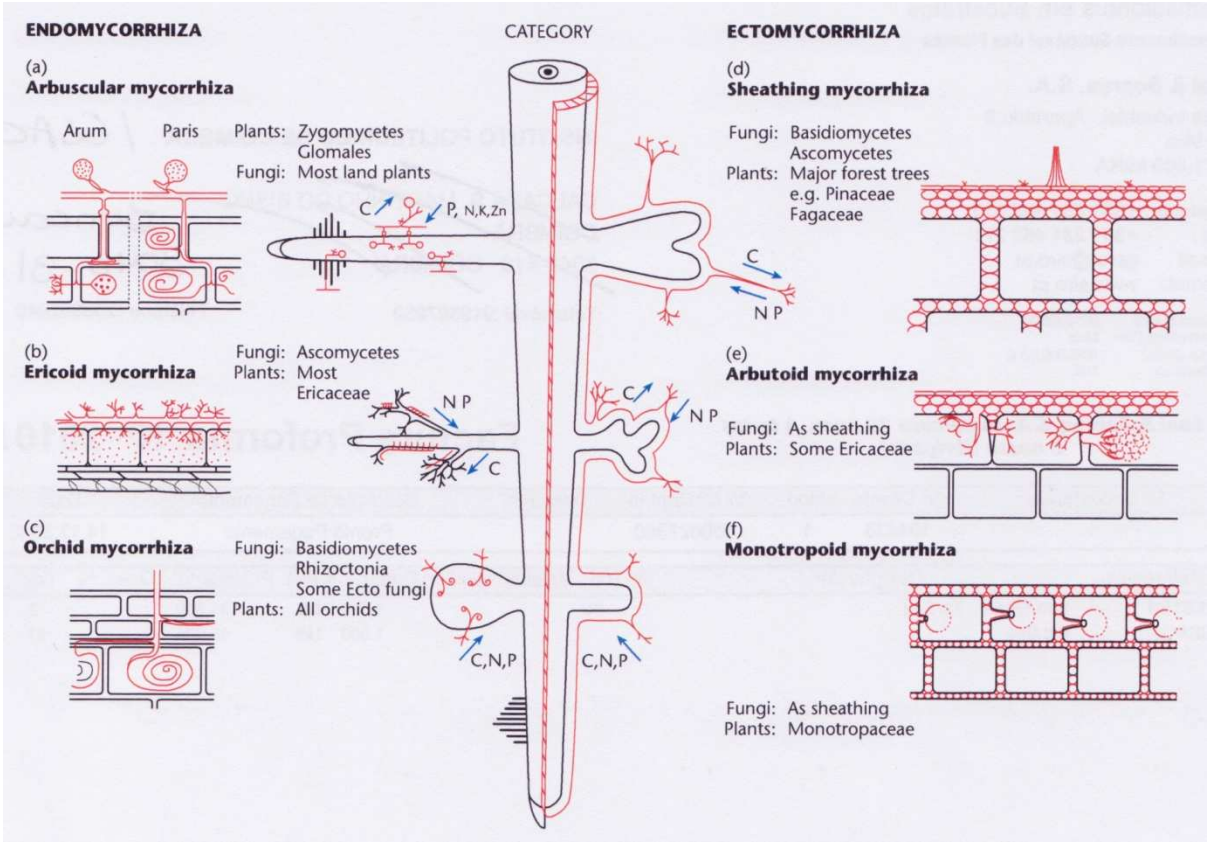


Figure 5 - Diagrammatic representation of six types of mycorrhiza. Fungal tissues are shown in red, plant tissues in black. In each type the diagnostic fungal structures are shown, the nature and directions of the main nutrient movements are indicated, and the main groups of fungi and plants involved are listed. C, carbon (sugars); N, nitrogen; P, phosphorus; K, potassium; Zn, zinc. The sizes of the letters indicate the relative importance of the transfer process within the mycorrhizal type. (Source: Read, 2001).

A very large number (c. 6000 species) of fungi, both basidiomycetes and ascomycetes, can form ectomycorrhizae (ECM) with woody plants (Fortin *et al.*, 2008). Important edible mushrooms including truffles (*Tuber* spp., ascomycete), cêpes (*Boletus edulis*, basidiomycete) and chanterelle (*Cantharellus* spp., basidiomycete) are all formed by ECM fungi. The importance of this symbiosis lies in the fact that the species involved are preponderant in some of the world's most important forestry systems. Thus, members of the Pinaceae such as *Abies*, *Larix*, *Picea*, and *Pinus* are dominant in boreal forests while the flowering plant families Betulaceae (e.g. *Betula*, *Corylus*), Fagaceae (e.g. *Fagus*, *Nothofagus*, *Quercus*), and Myrtaceae (e.g. *Eucalyptus*), which occupy wide areas of temperate and subtropical forests, all form ECM (Read, 2001). The dominant family of tropical rainforests of southeast Asia, the Dipterocarpaceae, also is associated to ECM species. In addition to their global importance as dominants of forest ecosystems, these families include most of the world's most valuable timber species (Read, 2001). The reproductive structures of ECM fungi are normally large and occur either above ground (epigeous), e.g. mushrooms, or below ground (hypogeous, including truffles, *Tuber* spp.). These are connected to the roots of their tree associates by extensive networks of hyphae, some of which form long linear aggregates called rhizomorphs. At the root surface these hyphae form compact sheathing mantles that completely envelop the root surface, covering its apex and enclosing that part which would otherwise produce root hairs (Read, 2001; Fortin *et al.*, 2008). The fitness of ECM fungi is hence intrinsically linked to the symbiosis and few, if any of them, have the ability to live in the soil free of their symbionts. Given the appropriate sugars, however, most ECM can be isolated and grown *in vitro*. ECM plants become dominant in environments where acidic organic residues accumulate at the soil surface. The fungi facilitate mobilization of nitrogen, which is often the major growth-limiting nutrient in ECM forests and, at the same time, contribute to the decomposition processes (Read, 2001). The ectomycorrhiza (ECM) show a mantle (sheathing mycorrhiza) and an intercellular mycelium called Hartig net (Read, 2001; Fortin *et al.*, 2008).

The ectendomycorrhiza, according to Peterson and Massicotte (2004) should be placed in a separate category, or considered as a modified ectomycorrhiza. The fungi involved in these associations are only a few species of ascomycete that colonize mostly *Pinus* spp. and *Larix* spp. roots. They show a mantle, Hartig net and intracellular hyphal complexes which develop within epidermal and cortical cells (Peterson and Massicotte, 2004).

The endomycorrhizae (ENDO) include the arbuscular (AM), ericoid (ERM) and orchid mycorrhizae (OM). All of them show intracellular hyphae, arbuscules or vesicula.

Arbuscular mycorrhizae (AM), synonymous of vesicular arbuscular mycorrhizae (VA or VAM) are formed by zygomycete fungi of the Glomales order. Dispersal of these species is achieved by single spores or by spores produced collectively in sporocarps. This kind of mycorrhizal colonization is considered to be the rule in most plants (Corkidi *et al.*, 2008; Fortin *et al.*, 2008; Honrubia, 2009a). Some plants with fibrous root systems, e.g. grasses, establish AM colonization. Beyond the nutritional role, other main advantage of this kind of mycorrhizae may result from a better tolerance towards pathogenic fungi. In fact, experiments performed with plants possessing AM have shown a better survival rate over the control when they were infected by root pathogens such as *Fusarium, Pythium* and *Phytophthora* (Read, 2001).

Arbuscular mycorrhizae were the first type to be described, as early as 1842 (Nageli, 1842). Frank (1885) gave the name "mycorrhiza" to the weird (at that time) association between temperate forest tree roots and ectomycorrhizal fungi. Janse (1897) called the intramatrical spores "vesicles" and, a few years later, Gallaud (1905) named the other commonly observed intracellular structures "arbuscules". The name "vesicular arbuscular mycorrhizae" became then established and persisted until recently. These structures are now known as arbuscular mycorrhizae (AM). Gallaud (1905) made very precise observations of the arbuscules, and concluded, that it is entirely surrounded by a host membrane, an observation later confirmed by electron microscopy analysis.

1.4.2 Function and potential applications of mycorrhiza

Nitrogen is a very abundant element in earth atmosphere. However it exists in a chemical form which is not readily available for plants. Following C, O and H, nitrogen is the most abundant element in plants, being present in macromolecules as nucleic acids and proteins. Nitrogen is also a component of alkaloids and of molecules like chlorophyll (Gobert and Plassard, 2008). In soil, from which plants usually obtain nitrogen as nitrate (usually) a strong competition between microorganisms and plants occurs for N acquisition (Gobert and Plassard, 2008; Goltapeh *et al.*, 2008). To overcome limitations of nitrogen uptake, plants have developed specific strategies for N acquisition. These include several types of traps developed by carnivorous plants; associations with bacteria in the roots of leguminous plants (*Rhizobium*); associations between other plants and microorganisms, such as bacteria (*Burkholderia, Azospirillum*), actinomycetes of the genus *Frankia* and mycorrhizae (Fortin *et*

al., 2008; Gobert and Plassard, 2008; Wong-Villarreal and Caballero-Mellado, 2009). Mycorrhizal symbiosis may also play a crucial role in barren lands such as primary succession sites, where the availability of nutrients is scarce (Wu *et al.*, 2004). According to Wu *et al.* (2004) a model of primary succession suggests that non or facultative mycotrophic are the first colonizing plants, followed by obligatory mycotrophic plants, which colonize the habitats only after arbuscular mycorrhizal (AM) populations have been established. However, post-eruption, the re-establishment of AM seems to occur quickly and, as a consequence, all plants of successional vegetation are mycotrophic. Thus, the authors suggest that the mycorrhizal status and the progress of vegetative succession would vary with different environmental conditions in terms of water and nutrient restrictions.

All mineral and nutrient exchanges between the host cell (carbohydrate production) and the fungal cells (water, phosphorus and nitrogen uptake) are thought to take place in the fungal–plant interface (Smith and Read, 1997). In the case of the arbutoid mycorrhizae of *Arbutus unedo*, there are three possible sites for nutrient exchange (Peterson and Massicotte, 2004): at the interface between the inner mantle hyphae and the tangential cell wall of epidermal cells, at the interface between Hartig net hyphae end epidermal cells, and at the interface between hyphal complexes and epidermal cell cytoplasm. In addition, Smith and Read (1997) pointed out that, as in ectomycorrhizae, the sheath on the roots of arbutoid mycorrhizae may have not only a storage function, but may also act as a boundary between the plant and the soil.

Underutilized plants, as *Arbutus unedo*, usually grow in marginal lands or impoverished habitats where they are subjected to various stress conditions including drought, salinity, low levels of nutrients and extreme pH values. For these plants, the establishment of mycorrhizae could be an important advantage to compete and survive in these hard environments (Goltapeh *et al.*, 2008). Forest owners can also benefit from this through the recovery of marginal lands and by increasing revenues.

Different inoculation methods have been applied *in vitro* or directly under greenhouse or nursery conditions to achieve mycorrhization. Mycorrhizal synthesis experiments *in vitro* are particularly useful to determine fungus-plant host compatibility and to elucidate the cellular, physiological and biochemical aspects of plant fungi interactions (Águeda *et al.*, 2008). Several reports have pointed out that mycorrhization improved the survival and acclimatization of woody micropropagated plants (Oliveira *et al.*, 2003; Martins, 2010).

Other studies focused on the management of nursery practices for mycorrhizal fungi application concerning to the reduction in fertilization, biocide treatments, irrigation and type of substrate (Parladé *et al.*, 2004; Rincón *et al.*, 2005; Corkidi *et al.*, 2008; Oliveira *et al.*, 2010b). Many different methods have been used to propagate AM fungi, but all of them involve growing the fungi in association with a living root system. AM fungi are obligate symbionts and cannot complete their life cycle without a host plant. The procedures for AM fungi propagation have progressed from the relatively simple pot culture technique to more complex hydroponic, aeroponic, and *in vitro* propagation systems on root cultures (Corkidi *et al.*, 2008; Oliveira *et al.*, 2010b). Mycorrhizal inoculum can be propagated outdoors or indoors in greenhouse conditions with high light intensity and soil temperatures ranging from 15 to 30 °C (Corkidi *et al.*, 2008; Oliveira *et al.*, 2010b). Nutrients can be incorporated in the growing medium or applied periodically as diluted nutrient solutions. At the nursery, for large-scale plant inoculation with AM fungi, Corkidi *et al.* (2008) used a dry pot culture material with spores, hyphae, and root fragments colonized by an isolate of *Glomus intraradices*. The AM fungi present in commercial inoculants are not always specified, but most of them contained different isolates of *Glomus intraradices*, a highly infective species of woody and herbaceous plants growing in a wide range of conditions (Corkidi *et al.*, 2008). Soilless media have lower bulk density, provide better aeration, and allow control over the substrate chemical composition (Parladé *et al.*, 2004). The most appropriate combination of substrate and ECM fungus for *Q. ilex* growth and nutrition was peat-based compost and inoculated *Hebeloma mesophaeum* (Oliveira *et al.*, 2010b). Materials that have been used for mycorrhizal AM inoculum propagation include expanded clay, turface (calcined montmorillonite clay), charcoal; rockwool, glass beads, sand of different particle sizes, clay brick granules, perlite, and different combinations of bark, peat, pumice, perlite, and vermiculite (Corkidi *et al.*, 2008). The impact on plant performance and ectomycorrhizal community after aforestation of abandoned farmland with conifer seedlings ECM inoculated in nursery conditions showed, one year after, that natural mycorrhizal infections prevailed in the inoculated root systems, and introduced mycorrhizae were seldom found (Menkis *et al.*, 2007). The propagation of native fungi is relevant and development program for screening and selecting effective mycorrhizae fungi isolates and using them in production of plant species is a must for the establishment of mycorrhizae and further impact on plant performance (Rai, 2001; Parladé *et al.*, 2004; Corkidi *et al.*, 2008).

Several studies have been reported the mycorrhization on Ericaceae species, such as inoculation of *Rhododendron* cultivars *in vitro* with different strains of ericoid

endomycorrhizae (Eccher and Martinelli, 2010); influence of ericoid endomycorrhizae inoculated *in vitro* on rooting of *Vaccinium corymbosum* (Eccher and Noé, 2002); influence of ericoid mycorrhizal fungi in *V. macrocarpon* in hydroponics conditions (Kosola *et al.*, 2007) and influence of selected mycorrhizal fungi inoculated on different varieties of *V. corymbosum* propagated *in vitro* (Noé *et al.*, 2002). Species from the genus *Arbutus, Arcostaphylos and Pyrola* establish arbutoid mycorrhizae (Fortin *et al.*, 2008). Molina and Trappe (1982) found a lack of specificity in hosts forming arbutoid mycorrhizae. Other experiments showed that ectomycorrhizal fungi form arbutoid mycorrhizae and consequently arbutoid mycorrhizae are most closely related to ectomycorrhizae than to ericoid mycorrhizae (Molina and Trappe, 1982; Smith and Read, 1997). These studies will be discussed on chapter four. To our knowledge, there are no previous works showing arbutoid mycorrhizae between *A. unedo* and *Pisolithus tinctorius* synthesized *in vitro*.

1.5 GENETIC DIVERSITY STUDIES USING DNA MARKERS

Response to selection depends ultimately on the breath of genetic diversity available to the breeder. Without a genetic diversified pool the selection of *Plus* trees tends to be difficult. To evaluate genetic diversity, molecular markers are effective tools (Bell *et al.*, 2008). Classical approaches, such as comparative anatomy, physiology and embryology, were employed in genetic analysis to determine inter and intra species variability. However, molecular markers have rapidly overtaken these classical strategies (Joshi *et al.*, 2009).

According to (Chawla, 2009) the polymorphism can uncover among individuals and populations differentiation at three different levels: (1) the phenotype, such as visible characters (morphological markers); (2) the sequence of proteins or secondary compounds, such as terpenes and flavonoids, (biochemical markers); and (3) the DNA nucleotide sequence – directly or indirectly - (molecular markers). Morphological markers correspond, in general, to visually score qualitative traits, either dominant or recessive. The markers used in the first genetic map (in *Drosophila*, 1988), were phenotypic traits scored by visual observation of the flies morphological characteristics. In plants these markers have been associated to morphological characters, including genes for dwarfism, albinism and leaf and fruit morphology (Chawla, 2009). Biochemical markers are proteins or secondary compounds produced by gene expression. Monoterpenes were the first biochemical markers in trees and were used for taxonomic studies in pines. The small number of monoterpene markers and their dominant expression limited their utility (White *et al.*, 2007). The groups of biochemical

markers that have most frequently been used are the isozymes (multiple molecular forms of an enzyme exhibiting similar or identical catalytic properties), frequently referred to as allozymes. A pair of isozymes may differ in one single amino acid, which often leads to a difference in their electric charge. Consequently, it is possible to separate them after migration on a gel with an applied voltage (Eriksson *et al.*, 2006). These markers are generally codominant, which means that the different forms of a marker should be detectable in diploid organisms to allow discrimination of homo and heterozygotes. The polymorphism of isozymes is rather poor within cultivated species (Chawla, 2009).

DNA markers correspond to a DNA sequence that is readily detected and whose inheritance can be easily monitored (Chawla, 2009). DNA markers are mainly DNA segments that can be distinguished by gel electrophoresis following amplification. DNA can be originated from the cell nucleus (nuclear DNA), from mitochondria (mtDNA) or plastids (cpDNA) (Eriksson *et al.*, 2006). DNA sequence information is transmitted among generations. Therefore, DNA is potentially the most accurate source of genetic information, compared to biochemical markers (isozymes), which are products of gene expression, thus an indirect and insensitive method of detecting variation in DNA (Wang and Szmidt, 2001; Schlötterer, 2004).

According to White *et al.* (2007) the following characteristics are desirable for an ideal DNA marker: 1) to be highly polymorphic; (2) display co-dominance; (3) show an high frequency through the genome; (4) to have a neutral behaviour (to environmental conditions or management practices); (5) the costs must be reasonable; (6) the assays must be easy and fast to perform; (7) the data must be reproducible and (8) the marker must allow for an easy exchange of data between laboratories. It is difficult to find a molecular marker that meets all these criteria. Depending on the type of the study to be undertaken, a marker system can be identified that fulfil at least a few of the above criteria (Chawla, 2009). In the following paragraphs a general description of the two markers used in this work is given.

Random amplified polymorphic DNA (RAPD) markers have been the most widely used molecular marker type in forest trees to date. The RAPD marker system is easy to apply as no prior DNA sequence information is needed for designing PCR primers (White *et al.*, 2007). Primers are usually just 10 base pairs long (10-mers) and are of random sequence. The reactions products are submitted to electrophoresis and the bands are visualized by staining agarose gel with ethidium bromide, and seen under UV light (Chawla, 2009). There are several thousands of primers commercially available, all with a different 10-base sequence,

which in theory will all amplify different regions of the target genome, which means that a large number of amplified sequences might be identified by this technique. Therefore, the RAPD marker system has the potential to randomly survey a large portion of the genome for the presence of polymorphisms (White *et al.*, 2007). RAPD markers compared to SSRs (microsatellites) do not require either species-specific probe libraries or previous knowledge of the genome. Thus, the work can be conducted on a large variety of species where such probe libraries are not available, which make them easier to apply than SSRs (Chawla, 2009). Although RAPD markers are easy and quick to use, they have several disadvantages, such as: (1) problems with marker reproducibility across laboratories (due to low annealing temperatures, which make them not specific), (2) the segments amplified are dominant and as a result RAPD polymorphisms are inherited as dominant-recessive characters, and (3) difficulties to analyse and to automate (Schlötterer, 2004; Eriksson *et al.*, 2006; White *et al.*, 2007; Chawla, 2009). Several studies have successfully reported genetic diversity analysis using RAPDs markers on woody plants such as in *Argania spinosa* (Majourhat *et al.*, 2008), *Castanea sativa* (Seabra *et al.*, 2001), *Olea europea* (Besnard *et al.*, 2001), *Prunus armeniaca* (Hurtado *et al.*, 2001), *Prunus dulcis* (Martins *et al.*, 2001), *Prunus persica* (Quarta *et al.*, 2001) and *Vitis* sp. (Regner *et al.*, 2001) among many others.

Also in Ericaceae RAPDs have been used in studies of genetic diversity analysis such as on characterization of *Rhododendron* (Jain *et al.*, 2000; Milne and Abbott, 2008), on genetic analysis of *Vaccinium angustifolium* (Burgher *et al.*, 1998; Burgher-Maclellan and Mackenzie, 2004), on genetic diversity of *V. macrocarpon* (Debnath, 2007), on clonal structure of *V. myrtillus* populations (Albert *et al.*, 2003, 2004), on *V. uliginosum* populations (Albert *et al.*, 2005) and on assessment of genetic diversity within *Vaccinium* spp. and hybrids (Levi and Rowland, 1997). Also in Ericaceae RAPDs have been used on fingerprint for the flower type in the ornamental crop *Calluna vulgaris* (Borchert *et al.*, 2008; Borchert and Hohe, 2009), for conservation purposes of *Leucopogon obtectus* (Zawko *et al.*, 2001), on analysis of *Vaccinium* inflorescences (Vander Kloet and Dickinson, 2005) and on assessment of novelties resulting in a new species of *Vaccinium* (Vander Kloet and Paterson, 2000). To our knowledge, two recent studies have been reported about *A. unedo* genetic diversity analyses: 1) the characterization of 38 *A. unedo* genotypes by RAPD (Lopes *et al.*, 2010) and 2) about genetic diversity in nine *A. unedo* populations in the distribution area of the species in Tunisia using RAPD (Takrouni and Boussaid, 2010). These studies will be discussed on chapter five.

Microsatellites (SSRs) are regions of DNA containing short segments (2-6/8 base pairs), repeated a variable number of times. Such repetitions are called tandem repeats. Thus, SSRs are sometimes referred to as "variable number of tandem repeats" (VNTR) or simple tandem repeat (STR). They occur all over the genome, mainly in non-coding regions of DNA (Eriksson *et al.*, 2006), which make them insensitive, in general, to natural selection and thus neutral by nature (White *et al.*, 2007). Since the number of tandem repeats at a *locus* can vary greatly, SSRs markers are among the most polymorphic genetic markers, being highly informative due to the a large number of alleles that can be identified (Schlötterer, 2004). SSRs are codominant markers and a very large number of variants may occur (highly polymorphic). Moreover, they are useful for fingerprinting (identification of single individuals) studies, genetic diversity studies, as well as, for gene flow analyses (Schlötterer, 2004; Bassil *et al.*, 2006; Eriksson *et al.*, 2006; White *et al.*, 2007).

According to (Schlötterer, 2004; Väli *et al.*, 2008) microsatellites have some limitations that impair a wider application of this type of markers. One of the drawbacks is the complex and heterogeneous mutation pattern usually displayed by microsatellites which introduces ambiguities in further data analysis. Genotyping errors may occur because of stutter bands and technical artifacts such as allelic dropouts, null alleles, false alleles and size homoplasy. The allelic drop-outs, which occur when the amount of DNA is insufficient, do not allow a complete PCR amplification. Therefore, it is only amplified the shorter of the two alleles, consequently underestimating the heterozygosity (Ribeiro, 2003). Microsatellite null alleles are heterozygotes alleles undetected or scored by SSRs which frequency is considered as a systematic error. Therefore, they also underestimate the heterozygosity (Ribeiro, 2003). This systematic error is commonly encountered in studies of population genetics (Chapuis and Estoup, 2007), and can be caused by insertions/deletions (indels) in SSR flanking regions (Ribeiro, 2003). False alleles occur when the true allele is misgenotyped because of factors such as PCR or electrophoresis artifacts or human errors in reading and recording data, situations that can lead to substantial overestimation of census size (Johnson and Haydon, 2007). The essential difference between the effects of the two classes of errors (allelic dropout and false alleles), as far as inference is concerned, is that both homozygotes and heterozygotes potentially contain false alleles, but only homozygotes can be suspected of allelic dropout (Johnson and Haydon, 2007). The size homoplasy (the occurrence of nonhomologous fragments of the same size) occurs when two allelic lineages converge on the same size, due to the mutation process as opposed to ancestry (Ribeiro, 2003).

According to White *et al.* (2007) the SSR length polymorphisms at individual loci can be detected by PCR, using locus-specific flanking region primers of known sequence, but their identification is a very expensive and time-consuming process usually requiring the construction and screening of a genomic library. However, it is easy to apply when markers have already been developed. As pointed out by White *et al.* (2007) once a pair of primers is developed to amplify the SSR region, it must be determined whether there is any polymorphism for the SSR and whether the banding pattern on gels (or peaks on an automated capillary electrophoresis) has genetic interpretations (namely are not stutter bands or technical artifacts). SSRs have been applied in cross species amplification studies, by assuming that the primer binding sites are sufficiently conserved in a related species to allow amplification using primers designed for a different but phylogenetically related species (Goldstein and Schlötterer, 1999). Known primers are not likely to amplify the same locus across related taxa, unless the flanking regions where priming sites are located are highly conserved (Ellegren, 1992), which happens, usually in closely related species (Kijas *et al.*, 1995). Therefore, the success of cross amplification diminishes with increasing species divergence (Whitton *et al.*, 1997).

Different studies have been reported for genetic diversity analysis of woody plants using SSRs markers. Examples are in *Argania spinosa* (Majourhat *et al.*, 2008); *Actinidia, Olea* and *Prunus* (Cipriani *et al.*, 2001); in *Eucalyptus dunnii* (Marcucci Poltri *et al.*, 2003); in *P. pinaster* (Fernandes *et al.*, 2008) and in *Vitis* sp. (Borrego *et al.*, 2001; Filippetti *et al.*, 2001; Regner *et al.*, 2001) among others. Genetic diversity analysis in Ericaceae species using SSRs markers has been carried out on assessment of genetic diversity in species such as: *Monotropa hypopitys* (Klooster *et al.*, 2008), *Rhododendron simsii* (Tan *et al.*, 2009), *Vaccinium corymbosum* (Bassil *et al.*, 2006; Wiedow *et al.*, 2007) and within *Vaccinium* spp. and hybrids (Levi and Rowland, 1997; Boches *et al.*, 2005; Bassil *et al.*, 2010). Also in Ericaceae SSRs markers have been carried out on micropropagated *Vaccinium* plants for the assessment of genetic stability among donor plants and tissue culture regenerates (Debnath, 2010), as well as, on studies about the mating system evolution, patterns of pollen flow and the process of natural hybridization in *Phyllodoce aleutica* and *P. caerulea* (Kameyama *et al.*, 2006). These studies will be discussed on chapter five. As far as it is known previous studies related to genetic diversity analysis using SSRs in *A. unedo* have never been published.

1.6 OBJECTIVES

As stated in the introduction, *A. unedo* fits in the concept of NUC species due to under exploitation from an agronomic perspective. However, farmers are becoming increasingly interested in this culture as a fruit crop, with the resulting demand of high-quality plant material for orchards. Considering this scenario it is of the utmost importance to develop a consistent breeding program to improve genetically strawberry tree, giving to this species a condition identical to other popular fruit crops as the related blueberries or bilberries of the genus *Vaccinium*. We are aware that the genetic improvement of a tree species is a high-demanding, never ending objective. In fact, tree species, with their long-life cycles, heterozygosity and difficulties to be vegetatively regenerated are not easy to breed.

The long-term objective of this research is to develop strawberry tree cultivars that can be delivered to the farmers to increase fruit productivity. As could be easily understandable, a task like this is not feasible during the limited period of time available to conclude a PhD thesis. However, tree breeding is the result of several steps toward the final goal which is the development of new cultivars. These steps include selection of *Plus* trees, crosses and selection of progenies, application of effective protocols of plant propagation and evaluation of the produced material in different environments. Since the lack of information about strawberry tree was almost absolute we decided to concentrate our efforts a) in the optimization of protocols for *in vitro* propagation of *A. unedo* from selected trees chosen based on their fruit productivity and quality; b) on a survey of the genetic variability occurring in this species and c) in the development of protocols of mycorrhization that could improve plant acclimatization and further growth in the field.

To select the appropriate trees a great help has been obtained from forest owners and techniques of the Ministry of Agriculture which have been involved in the selection of *Plus* trees in different regions of the country based on fruit productivity and quality evaluation.

Once the trees were selected the first objective was to develop reliable protocols of propagation of these trees. Cloning the selected adult plants by cuttings or grafting has proved to be difficult. Therefore, we directed our objectives to development of protocols that could be applied to the propagation of adult material.

Thus, the first set of experiments (chapter 2) aimed to develop a protocol for an efficient *in vitro* propagation of *A. unedo* adult trees, through axillary shoot proliferation. The effect of several culture media was analysed. Selected genotypes were tested as well as the conditions for root formation and acclimatization. Anatomical studies were performed to better understand some steps of the regeneration process.

The second set of experiments (chapter 3) was designed to analyse the effect of plant growth regulators and genotype on the micropropagation of adult trees of *A. unedo*. Different plant growth regulators (four cytokinins and one auxin) were tested. Several selected genotypes were also tested to find those which are more amenable for *in vitro* cloning. In long-term breeding programmes the selection of high responsive genotypes can be used to transfer their regeneration potential to more recalcitrant ones.

The goal of the third set of experiments (chapter 4) was to test the ability of basidiomycete fungi to form arbutoid mycorrhizae with *A. unedo*. Pure cultures syntheses of *P. tinctorius* and *L. deliciosus* were tested *in vitro* conditions. Nursery assays with *P. tinctorius* were also performed and a field trial was later on established. The inoculation effect on plant growth was assessed in nursery and in a field trial. Histological studies were performed. Molecular assays complemented this work.

The purpose of the last chapter (chapter 5) was to develop an appropriate set of RAPD and SSR markers suitable for fingerprinting *A. unedo* trees (27) from nine different provenances of Portugal. Some of them (6 genotypes) were selected based on fruit production and had been already propagated *in vitro*. A set of random amplified polymorphic DNA (RAPD) and some of the microsatellite markers (SSR) previously developed for *Vaccinium* were tested in *A. unedo*.

Part of this research was already published in international journals and presented in national and international meetings while some data were submitted for publication as indicated at the beginning of the chapters. Also important, cloned plants have been regularly provided to farmers and orchards with *A. unedo* plants have been established.

1.7 REFERENCES

Águeda, B., Parladé, J.F., Fernández-Toirán, L.M., Cisneros, O., Miguel, A.M., Modrego, M.P., Martínez-Pena, F., Pera, J., 2008. Mycorrhizal synthesis between *Boletus edulis* species complex and rockroses (*Cistus* sp.). Mycorrhiza 18, 443-449.

Al-Bahrany, A.M., 2002. Effect of phytohormones on *in vitro* shoot multiplication and rooting of lime *Citrus aurantifolia* (Christm.) Swing. Sci. Hort. 95, 285-295.

Al Malki, A.A.H.S., Elmeer, K.M.S., 2010. Influence of auxin and cytokinine on *in vitro* multiplication of *Ficus anastasia*. Afr. J. Biotechnol. 9, 635-639.

Albert, T., Raspe, O., Jacquemart, A.L., 2003. Clonal structure in *Vaccinium myrtillus* L. revealed by RAPD and AFLP markers. Int. J. Plant Sci. 164, 649-655.

Albert, T., Raspe, O., Jacquemart, A.L., 2004. Clonal diversity and genetic structure in *Vaccinium myrtillus* populations from different habitats. Belgian J. Bot. 137, 155-162.

Albert, T., Raspe, O., Jacquemart, A.L., 2005. Diversity and spatial structure of clones in *Vaccinium uliginosum* populations. Can. J. Bot. 83, 211-218.

Almeida, R., Gonçalves, S., Romano, A., 2005. *In vitro* propagation of endangered *Rhododendron ponticum* L. subsp. *baeticum* (Boissier & Reuter) Handel-Mazzetti. Biodiv. Conservation 14, 1059-1069.

Anderson, W.C., 1984. A revised tissue culture medium for shoot multiplication of *Rhododendron*. J. Amer. Soc. Hort. Sci. 109, 343-347.

Ayaz, F.A., Kucukislamoglu, M., Reunanen, M., 2000. Sugar, non-volatile and phenolic acids composition of strawberry tree (*Arbutus unedo* L. var.*ellipsoidea*) fruits. J. Food Compos. Anal. 13, 171-177.

Bassil, N.V., Boches, P., Rowland, L., 2006. Genetic diversity in the highbush blueberry evaluated with microsatellite markers. J. Amer. Soc. Hort. Sci. 131, 674-686.

Bassil, N.V., Bunch, T., Nyberg, A., Hummer, K., Zee, F.T., 2010. Microsatellite markers distinguish Hawailan ohelo from other *Vaccinium* L. section *Myrtillus* species Acta Hort. 859, 81-88.

Bell, D.J., Rowland, L.J., Polashock, J.J., Drummond, A.A., 2008. Suitability of EST-PCR markers developed in highbush blueberry for genetic fingerprinting and relationship studies in lowbush blueberry and related species. J. Amer. Soc. Hort. Sci. 133, 701-707.

Bertsouklis, K.F., Papafotiou, M., 2007. *In vitro* propagation of *Arbutus andrachne* L. Acta Hort. 813, 477-480.

Besnard, G., Baradat, P., Bervillé, A., 2001. Olive cultivar identification using nuclear RAPDs and mitochondrial RFLP Acta Hort. 546, 317-324.

Boches, P.S., Bassil, N.V., Rowland, L.J., 2005. Microsatellite markers for *Vaccinium* from EST and genomic library. Mol. Ecol. 5, 657-660.

Borchert, T., Hohe, A., 2009. Identification of molecular markers for the flower type in the ornamental crop *Calluna vulgaris*. Euphytica 170, 203-213.

Borchert, T., Krueger, J., Hohe, A., 2008. Implementation of a model for identifying essentially derived varieties in vegetatively propagated *Calluna vulgaris* varieties. BioMed. Central Genetics doi:10.1186/1471-2156-9-56 9.

Borrego, J., Rodriguez, M.T., Martin, J.C., J, Cabello, F., Ibañez, J., 2001. Characterization of the most important Spanish grape varieties through isozyme and microsatellites analysis. Acta Hort. 546, 371-375.

Burgher-Maclellan, K., Mackenzie, K., 2004. An overview of RAPD analysis to estimate genetic relationships in Lowbush blueberry. Small Fruits Rev. 3, 295-305.

Burgher, K., Jamieson, A., Lu, X., McRae, K., 1998. Genetic relationship of 26 wild blueberry clones (*Vaccinium angustifolium*), as determined by random amplified polymorphic DNA analysis. Proc. Atlantic. Plant Tissue Culture Assoc. Ann. Mtg., New Brunswick, p. *Abst.* 7.

Canhoto, J.M., 2010. Biotecnologia vegetal, da clonagem de plantas à transformação genética. Imprensa da Universidade de Coimbra.

Castellanos, M., Power, J.B., Davey, M.R., 2010. Micropropagation of Poinsettia by organogenesis. In: Jain, S.M., Ochatt, S.J. (Eds.), Protocols for *in vitro* propagation of ornamental plants. Humana Press, New York, pp. 67-76.

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

Chalupa, V., 2003. *In vitro* propagation of *Tilia platyphyllos* by axillary shoot proliferation and somatic embryogenesis. J. For. Science 49, 537-543.

Chapuis, M., Estoup, A., 2007. Microsatellite null alleles and estimation of population differentiation. Mol. Biol. Evol. 24, 621-631.

Chawla, H.S., 2009. Introduction to plant biotechnology, 3rd ed. Science Publis., Inc., New Hampshire.

Cipriani, G., Marrazzo, M.T., Di Gaspero, G., Testolin, R., 2001. DNA microsatellite in fruit crops: isolation, length polymorphism, inheritance, somatic stability and cross-species conservation. Acta Hort. 546, 145-150.

Corkidi, L., Mike, E., Jeff, B., 2008. An introduction to propagation of arbuscular mycorrhizal fungi in pot cultures for inoculation of native plant nursery stock. Native plants J. 9, 29-38.

Dalla Serra, A., Franco, M.A., Mattivi, F., Ramponi, M., Vacca, V., Versini, G., 1999. Aroma characterization of Sardinian strawberry tree (*Arbutus unedo* L.) honey. Italian J. Food Science 11, 47-56.

Debnath, S.C., 2003. Improved shoot organogenesis from hypocotyl segments of Lingonberry (*Vaccinium vitis-idaea* L.) In Vitro Cell. Dev. Biol.-Plant 39, 490-495.

Debnath, S.C., 2007. An assessment of the genetic diversity within a collection of wild Cranberry (V*accinium macrocarpon* Ait.) clones with RAPD-PCR. Genet. Resour. Crop. Evol. 54, 509-517.

Debnath, S.C., 2010. Berry bioreactor micropropagation and molecular analysis for genetic fidelity. Acta Hort. 865, 43-51.

Debnath, S.C., McRae, K., 2001. An efficient *in vitro* shoot propagation of Cranberry (*Vaccinium macrocarpon* Ait.) by axillary bud proliferation. In Vitro Cell. Dev. Biol.-Plant 37, 243-249.

Demirsoy, H., Demirsoy, L., Celikel, G., Koyuncu, T., 2007. Effects of dried on some properties of strawberry tree fruits. Asian J. Chemistry 19, 1777-1782.

Eccher, T., Martinelli, M., 2010. Inoculation of *Rhododendron* cultivars *in vitro* with different strains of ericoid endomycorrhizae. Acta Hort. 865, 327-332.

Eccher, T., Noé, N., 2002. Influence of Ericoid endomycorrhizae inoculated *in vitro* on rooting and early growth of micropropagated plants of *Vaccinium corymbosum* L. Acta Hort. 574, 373-378.

Economou, A.S., Read, P.E., 1984. *In vitro* shoot proliferation of Minnesota deciduous azaleas. Hort. Sci. 19, 60-61.

Eeckaut, T., Janssens, K., De Keyser, E., De RieK, J., 2010. Micropropagation of *Rhododendron*. In: Jain, S.M., Ochatt, S.J. (Eds.), Protocols for *in vitro* propagation of ornamental plants. Springer, Dijon, pp. 141-152.

El Haouari, M., López, J.J., Mekhfi, H., Rosado, J.A., Salido, G.M., 2007. Antiaggregant effects of *Arbutus unedo* extracts in human platelets. J. Ethnopharmacology 113, 325-331.

Ellegren, H., 1992. Polymerase-Chain-Reaction (PCR) analysis of microsatellites - a new approach to studies of genetic relationships in birds. Auk 109, 886-895.

Eriksson, G., Ekberg, I., Clapham, D., 2006. An introduction to forest genetics. SLU. http://vaxt.vbsg.slu.se/forgen/, Uppsala.

Fenning, T., Gartland, K.M.A., 2007. Why forests need us, and more importantly, why we need them. IUFRO Tree Biotechnology-2007. ITQB, IBET, RAIZ, IUFRO, Açores, p. SV.3 (oral invited presentation).

Fernandes, L., Rocheta, M., Cordeiro, J., Pereira, S., Gerber, S., Oliveira, M.M., Ribeiro, M.M., 2008. Genetic variation, matting patterns and gene flow in a *Pinus pinaster* Aiton clonal seed orchard. Ann. For. Sci. 65, 1-9.

Filippetti, I., Silvestroni, O., Thomas, M.R., Intrieri, C., 2001. Genetic characterisation of Italian wine grape cultivars by microsatellite DNA analysis. Acta Hort. 546, 395-399.

Fortin, J.A., Plenchette, C., Piché, Y., 2008. Les mycorhizes la nouvelle révolution verte. Ed. Multimondes, Ed. Quae, Québec.

Frank, A.B., 1885. Ueber die auf Wurzelsymbiose beruhende Ernahrung gewisser Baume durch unterirdische Pilze. Ber Dtsch Bot Ges 3, 128-145.

Gajdošová, A., Ostrolucká, M.G., Libiaková, G., Ondrušková, E., 2007. Protocol for micropropagation of *Vaccinium vitis-idaea* L. In: Jain, S., Häggman, H. (Eds.), Protocols for Micropropagation of Woody Trees and Fruits. Springer, Berlin, pp. 457-464.

Galego, L., 2006. Valorização da aguardente de medronho. Jornadas do Mel, Medronho e Medronheira. C.M. da Pampilhosa da Serra, DRABL, LOUSAMEL, Pampilhosa da Serra, pp. 1-5.

Galego, L., Martins, A.N., Jesus, J.P., Nunes, J.P., Reis, E.P., 2001. Aguardente de medronho já tem legislação. 11º Congresso do Algarve, Albufeira, pp. 1-6.

Gallaud, J., 1905. Etude sur les mycorrhizes endotrophes. Rev Gen Bot 17, 5-48, 66-86,123-136, 223-249, 313-325, 425-433, 479-500.

George, E.F., 2008. Plant tissue culture procedure - background 1. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 1-28.

George, E.F., De Klerk, G.-J., 2008. The components of plant tissue culture media I : macro and micro-nutrients. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 65-114.

George, E.F., Debergh, P.C., 2008. Micropropagation: uses and methods. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 29-64.

Gobert, A., Plassard, C., 2008. The beneficial effect of mycorrhizae on N utilization by host-plant: myth or reality? In: Varma, A. (Ed.), Mycorrhiza Genetics and Molecular Biology, Eco-function, Biotechnology, Eco-physiology, Structure and Systematic. 3rd ed. Springer, Berlin, pp. 209-240.

Godinho-Ferreira, P.G., Azevedo, A.M., Rego, F., 2005. Carta da tipologia florestal de Portugal Continental. Silva Lusitana 13, 1-34.

Goldstein, D.B., Schlötterer, C., 1999. Microsatellites, evolutions and apllications. Oxford Univ. Press, N. York.

Goltapeh, E.M., Danesh, Y.R., Prasad, R., Varma, A., 2008. Mycorrhizal fungi: what we know and what should we know? In: Varma, A. (Ed.), Mycorrhiza Genetics and Molecular Biology, Eco-function, Biotechnology, Eco-physiology, Structure and Systematics. 3rd ed. Springer, Berlin, pp. 3-27.

Gomes, F., Canhoto, J.M., 2003. Micropropagation of *Eucalyptus nitens* Maiden (Shining gum). In Vitro Cell. Dev. Biol.-Plant 39, 316-321.

Gomes, F., Canhoto, J.M., 2009. Micropropagation of strawberry tree (*Arbutus unedo* L.) from adult plants. In Vitro Cell. Dev. Biol.-Plant 45, 72-82.

Gonçalves, J.C., Roseiro, R.J., 1994. Establishment and *in vitro* multiplication of *Arbutus unedo* L. seedlings. VIII International Congress of Plant Tissue and Cell Culture, Firenze, Italy, p. *Abst* 20.

Gonzalez, M.V., Lopez, M., Valdes, A.E., Ordas, R.J., 2000. Micropropagation of three berry fruit species using nodal segments from field-grown plants. Ann. App. Biol. 137, 73-78.

Hancock, J.F., Lyrene, P., Finn, C.E., Vorsa, N., Lobos, G.A., 2008. Blueberries and Cranberries. Temperate Fruit Crop Breeding, pp. 115-150.

Hazarika, B.N., 2003. Acclimatization of tissue-cultured plants. Curr. Sci. 85, 1704-1712.

Hazarika, B.N., Parthasarathy, V.A., Nagaraju, V., 2000. Paclobutrazol induced biochemical changes in microshoots of *Citrus* species. Folia Hortic. 12, 69-77.

Hileman, L.C., Vasey, M.C., Thomas Parker, V., 2001. Phylogeny and Biogeography of the Arbutoideae (Ericaceae): Implications for the Madrean-Tethyan Hypothesis. Systematic Botany 26, 131-143.

Hirai, M., Yoshimura, S., Ohsako, T., Kubo, N., 2010. Genetic diversity and phylogenetic relationships of the endangered species *Vaccinium sieboldii* and *Vaccinium ciliatum* (Ericaceae). Plant Syst. Evol. 287, 75-84.

Honrubia, M., 2009. Las micorrizas: una relación planta-hongo que dura más de 400 millones de años. Anales del Jardín Botánico de Madrid 66, 133-144.

Hurtado, M.A., Badenes, M.L., Llácer, G., Westman, A., Beck, E., Abbott, G.A., 2001. Contribution to apricot genetic analysis with RFLP, RAPD and AFLP markers. Acta Hort. 546, 417-420.

Isutsa, D.K., Pritts, M.P., Mudge, K.W., 1994. Rapid propagation of blueberry plants using *ex-vitro* rooting and controlled acclimatization of micropropagules. Hortscience 29, 1124-1126.

Jaakola, L., 2001. Effect of N^6-isopentenyladenine concentration on growth initiation *in vitro* and rooting of bilberry and lingonberry microshoots. Plant Cell Tis. Org. Cult. 66, 73-77.

Jain, A., Pandit, M.K., Elahi, S., Bhaskar, A., Kumar, V., 2000. Reproductive behaviour and genetic variability in geographically isolated populations of *Rhododendron arboreum* (Ericaceae). Curr. Sci. 79, 1377-1381.

Janse, J.M., 1897. Les endophytes radicaux de quelques plantes Javanaises. Ann Jardin Bot Buitenzorg 14, 53-201.

Johnson, P.C.D., Haydon, D.T., 2007. Maximum-likelihood estimation of allelic dropout and false allele error rates from microsatellite genotypes in the absence of reference data. Genetics 175, 827-842.

Joshi, S.P., Ranjekar, P.K., Gupta, V., 2009. Molecular markers in plant genome analysis (1-19). In: Yeatman, C.W. (Ed.), In Plant Genetic Resources: a conservation imperative Plant Molecular Biology Group.

Kaçar, D., 2008. Screening of some plant species for their total antioxidant and antimicrobial activities Master of Sciences in Biotechnology and Bioengineering, Izmir.

Kameyama, Y., Kasagi, T., Kudo, G., 2006. Eight microsatellite markers for sympatric alpine shrubs, *Phyllodoce aleutica* and *P. caerulea* (Ericaceae). Mol. Ecol. 6, 402-404.

Kijas, J.M.H., Fowler, J.C.S., Thomas, M.R., 1995. An evaluation of sequence tagged microsatellite site markers for genetic analysis within *Citrus* and related species. Genome 38, 349-355.

Kivçak, B., Mert, T., 2001. Quantitative determination of [alpha]-tocopherol in *Arbutus unedo* by TLC-densitometry and colorimetry. Fitoterapia 72, 656-661.

Kıvçak, B., Mert, T., Denizci, A.A., 2001. Antimicrobial activity of *Arbutus unedo* L. Fabad J. Pharm. Sci. 26, 125-128.

Klooster, M.R., Hoenle, A.W., Culley, T.M., 2008. Characterization of microsatellite *loci* in the myco-heterotrophic plant *Monotropa hypopitys* (Ericaceae) and amplification in related taxa. Mol. Ecol. Resources 9, 219-221.

Kosola, K.R., Workmaster, B.A.A., Spada, P.A., 2007. Inoculation of cranberry (*Vaccinium macrocarpon*) with the ericoid mycorrhizal fungus *Rhizoscyphus ericae* increases nitrate influx. New Phytologist 176, 184-196.

Kotsias, D., Roussos, P.A., 2001. An investigation on the effect of different plant growth regulating compounds in *in vitro* shoot tip and node culture of lemon seedlings. Sci. Hort. 89, 115-128.

Kron, K.A., Powell, E.A., Luteyn, J.L., 2002a. Phylogenetic relationships within the blueberry tribe (Vaccinieae, Ericaceae) based on sequence data from MATK and nuclear ribosomal ITS regions, with comments on the placement of *Satyria*. Amer. J. Botany 89, 327-336.

Kron, K.A., Stevens, P.F., Crayn, D.M., Anderberg, A.A., Gadek, P.A., C.J., Q., Luteyn, J.L., 2002b. Phylogenetic classification of Ericaceae: molecular and morphological evidence. The Botanical Review 68, 335-423.

Levi, A., Rowland, L.J., 1997. Identifying blueberry cultivar and evaluating their genetic relationships using randomly amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR)-anchored primers. J. Amer. Soc. Hort. Sci. 122, 74–78.

Lloyd, G.B., McCown, B.H., 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Proc. Int. Plant Prop. Soc. 30, 421-427.

Lopes, L., Sá, O., Baltazar, J.L., Pereira, J.A., Baptista, P., 2010. Analysis of molecular and morphological diversity of *Arbutus unedo* in the interior North and Center of Portugal. In: SPG (Ed.), XXXV Jornadas Portuguesas de Genética. Univ. do Minho, Braga, p. *Abst* 76.

Machakova, I., Zazimalova, E., George, E.F., 2008. Plant growth regulators I: Introduction; auxins, their analogues and inhibitors. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 175-204.

Mackay, W.A., 1996. Micropropagation of *Texas madrone*, *Arbutus xalapensis* HBK. Hortscience 31, 1028-1029.

Maira, O., Alexander, M., Vargas, T.E., 2010. Micropropagation and organogenesis of *Anthurium andreanum* Lind cv Rubrun. In: Jain, S.M., Ochatt, S.J. (Eds.), Protocols for *in vitro* propagation of ornamental plants. Humana Press, New York, pp. 3-14.

Majourhat, K., Jabba, Y., Hafidi, A., Martínez-Gómez, P., 2008. Molecular characterization and genetic relationships among most common identified morphotypes of critically endangered rare Moroccan species *Argania spinosa* (Sapotaceae) using RAPD and SSR markers. Ann. For. Sci. 65, 1-6.

Marcucci Poltri, S.N., Zelener, N., Rodriguez Traverso, J., Gelid, P., Hopp, H.E., 2003. Selection of a seed orchard of *Eucalyptus dunnii* based on genetic diversity criteria calculated using molecular markers. Tree Physiology 23, 625-632.

Martins, A., 2010. Mycorrhizal inoculation of Chestnut seedlings: effect on survival and growth after transplantation. Acta Hort. 866, 325-334.

Martins, A.N., 2006. Produção de aguardente de medronho: resultados do trabalho realizado no Algarve. Jornadas do Mel, Medronho e Medronheira. C.M. da Pampilhosa da Serra, DRABL, LOUSAMEL, Pampilhosa da Serra, pp. 1-2.

Martins, A.N., Varela, J.J.M., Mendes, M.L.A., Gonçalves, G.G., Lima, A.P., Carvalheiro, C.A.R., Jesus, J.P., Almeida, V.R., Galego, L., Carvalho, M.M.R., 1999. O medronheiro e a aguardente de medronho. DRAALG & EST/UALg, Faro.

Martins, M., Farinha, A., Ferreira, E., Cordeiro, V., Monteiro, A., Tenreiro, R., Oliveira, M., 2001. Molecular analysis of the genetic variability of Portuguese almond collections Acta Hort. 546, 449-452.

Meiners, J., Schwab, M., Szankowski, I., 2007. Efficient *in vitro* regeneration systems for *Vaccinium* species. Plant Cell Tis. Org. Cult. 89, 169-176.

Meireles, C., Gonçalves, P., Rego, F., Silveira, S., 2005. Estudo da regeneração natural das espécies arbóreas autóctones na Reserva Natural da Serra da Malcata. Silva Lusitana 13, 217 - 231.

Mendes, M.L.A., 1997. Multiplicação vegetativa *in vitro* de medronheiro. Tese de Mestrado. ISA-UTL, Lisboa.

Menkis, A., Vasiliauskas, R., Taylor, A.F.S., Stenlid, J., Finlay, R., 2007. Afforestation of abandoned farmland with conifer seedlings inoculated with three ectomycorrhizal fungi-impact on plant performance and ectomycorrhizal community. Mycorrhiza 17, 337-348.

Mereti, M., Grigoriadou, K., Nanos, G.D., 2002. Micropropagation of the strawberry tree, *Arbutus unedo* L. Sci. Hort. 93, 143-148.

Milne, R.I., Abbott, R.J., 2008. Reproductive isolation among two interfertile *Rhododendron* species: low frequency of post-F-1 hybrid genotypes in alpine hybrid zones. Mol. Ecol. 17, 1108-1121.

Molina, R., Trappe, J.M., 1982. Lack of mycorrhizal specificity by the Ericaceous hosts *Arbutus menziesii* and *Arctostaphylos uva-ursi*. New Phytol. 90, 495-509.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant. 15, 473-497.

Myburg, A.A., Potts, B.M., Marques, C.M., Kirst, M., Gion, J.-M., Grattapaglia, D., Grima-Pettenatti, J., 2007. Eucalypts. In: C. Kole, C. (Ed.), Genome mapping and molecular breeding in plants. Springer-Verlag, Berlin Heidelberg, pp. 115-160.

Nageli, C., 1842. Pilze im Innern von Zellen. Linnaea 16, 278-285.

Neppi, M., 2001. Alberi ed arbusti della flora mellifera della regione mediterrânea. In: Piotto, B., Noi, A. (Eds.), Propagazione per seme di alberi e arbusti della flora mediterranea. Dipart. Prev. Risanam. Ambient., Manuale ANPA (Agenz. Naz. per la Protezi. dell' Ambiente), pp. 44-49.

Noé, N., Eccher, T., Borra, M., 2002. The use of selected mycorrhizae for quality improvement of Highbush Blueberry (*Vaccinium corymbosum* L.) plant production Acta Hort. 574, 387-392.

Oliveira, I., Coelho, V., Baltasar, R., Pereira, J.A., Baptista, P., 2009. Scavenging capacity of strawberry tree (*Arbutus unedo* L.) leaves on free radicals. Food and Chemical Toxicology 47, 1507-1511.

Oliveira, I., Pereira, J.A., Bento, A., Baptista, P., 2010a. Actividade sequestrante de radicais livres do medronho (*Arbutus unedo* L.). Actas Portuguesas Hortic. 16, 173-178.

Oliveira, P., Barriga, J., Cavaleiro, C., Peixe, A., Potes, A.Z., 2003. Sustained *in vitro* root development obtained in *Pinus pinea* L. inoculated with ectomycorrhizal fungi. Forestry 76, 579-587.

Oliveira, R.S., Franco, A.R., Vosátka, M., Castro, P.M.L., 2010b. Management of nursery practices for efficient ectomycorrhizal fungi application in the production of *Quercus ilex*. Symbiosis DOI 10.1007/s13199-010-0092-0.

Ostrolucká, M.G., Gajdošová, A., Libiaková, G., Hrubíková, K., Bezo, M., 2007. Protocol for micropropagation of selected *Vaccinium*. In: Jain, S.M., Häggman, H. (Eds.),

Protocols for Micropropagation of Woody Trees and Fruits. Springer, Berlin, pp. 445-455.

Özcan, M.M., HacIseferogullarI, H., 2007. The Strawberry (*Arbutus unedo* L.) fruits: Chemical composition, physical properties and mineral contents. J. Food Engineering 78, 1022-1028.

Pabuccuoglu, A., Kivcak, B., Bas, M., Mert, T., 2003. Antioxidant activity of *Arbutus unedo* leaves. Fitoterapia 74, 597-599.

Pajuelo, A.G., 2008. Singularidades dos méis de urzes e o caso particular do mel de medronho. II Jornadas do Mel, Medronho e Medronheira, C.M. da Pampilhosa da Serra, DRABL, LOUSAMEL, Pinhais do Zêzere, Pampilhosa da Serra.

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

Parladé, J., Pera, J., Luque, J., 2004. Evaluation of mycelial inocula of edible *Lactarius* species for the production of *Pinus pinaster* and *P. sylvestris* mycorrhizal seedlings under greenhouse conditions. Mycorrhiza 14, 171-176.

Pawlowska, A.M., Marinella, D.L., Braca, A., 2006. Phenolics of *Arbutus unedo* L. (Ericaceae) fruits: identification of anthocyanins and gallic acid derivatives J. Agric. Food Chem. 54, 10234-10238.

Pedro, J.G., 1994. Portugal Atlas do Ambiente. Notícia Explicativa II.6 Carta da distribuição de figueira e medronheiro. D.G.A., Ministério do Ambiente e Recursos Naturais, Lisboa.

Pereira, M.J., 2009. Reversion to juvenility: the use of epicormics in the micropropagation of mature wild shrubs of *Vaccinium cylindraceum* Smith (Ericacaeae). *Arquipélago*. Life and Marine Sciences 26, 63-68.

Peterson, R.L., Massicotte, H.B., 2004. Exploring structural definitions of mycorrhizas, with emphasis on nutrient-exchange interfaces. Can. J. Bot. 82, 1074-1088.

Piotto, B., Piccini, C., Arcadu, P., 2001. La ripresa della vegetazione dopo gli incendi nella regione mediterrânea. In: Piotto, B., Noi, A. (Eds.), Propagazione per seme di alberi e arbusti della flora mediterranea. Dipart. Prev. Risanam. Ambient., Manuale ANPA (Agenz. Naz. per la Protezi. dell' Ambiente), pp. 32-38.

Preece, J., 2008. Stock plant physiological factors affecting growth and morphogenesis. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 403-422.

Preece, J.E., Huetteman, C.A., Ashby, W.C., Roth, P.L., 1991. Micro- and cutting propagation of silver maple. I. Results with adult and juvenile propagules. J. Amer. Soc. Hortic. Sci. 116, 142-148.

Preece, J.E., Sutter, E., 1991. Acclimatization of micropropagated plants to the greenhouse and field. In: Debergh, P.C., Zimmerman, R.H. (Eds.), Micropropagation: Technology and Application. Kluwer Academic Pub., Dordrecht, pp. 71-79.

Ptak, A., 2010. Somatic embryogenesis in *in vitro* culture of *Leucojum vernum* L. . In: Jain, S.M., Ochatt, S.J. (Eds.), Protocols for *in vitro* propagation of ornamental plants. Humana Press, New York, pp. 223-234.

Quarta, R., Dettori, M.T., Verde, I., Marchesi, U., Palombi, M.A., 2001. Characterization and evaluation of genetic diversity in peach germplasm using RAPD and RFLP markers. Acta Hort. 546, 489-496.

Rae, A.M., Street, N.R., Rodríguez-Acosta, M., 2007. *Populus* Trees. In: Kole, C. (Ed.), Genome mapping and molecular breeding in plants. Springer-Verlag, Berlin Heidelberg, pp. 1-28.

Rai, M.K., 2001. Current advances in mycorrhization in micropropagation. In Vitro Cell. Dev. Biol.-Plant 37, 158-167.

Read, D.J., 2001. Mycorrhiza. Encyclopedia of life sciences. John Wiley & Sons, Ltd., www.els.net.

Regner, F., Stadlbauer, A., Eisenheld, C., 2001. Molecular markers for genotyping grapevine and for identifying clones of traditional varieties. Acta Hort. 546, 331-341.

Ribeiro, M.M., 2003. Ferramentas de informática para análise de resultados. In: INETI, C.r. (Ed.), I Encontro sobre Microssatélites e Genética de Populações. INETI. , Lisboa.

Ricardo, C.P., Veloso, M.M., 1987. Features of seed germination in *Arbutus unedo* L. In: Tenhunen, *et al.* (Eds.), Plant response to stress, functional analysis in Mediterranean ecosystems. Springer- Verlag, Berlin, pp. 565-572.

Rincón, A., Parladé, J., Pera, J., 2005. Effects of ectomycorrhizal inoculation and the type of substrate on mycorrhization, growth and nutrition of containerized *Pinus pinea* L. seedlings produced in a commercial nursery. Ann. For. Sci. 62, 817-822.

S. Silva, J., Harrison, S.P., 2010. Humans, climate and land cover as controls on European fire regimes. In: S. Silva, J., Rego, F., Fernandes, P., Rigolot, E. (Eds.), Towards integrated fire management – outcomes of the European project fire paradox. European Forest Institute, Joensuu, pp. 49-59.

Sá, O., Malheiro, R., Baltasar, J.L., Pereira, J.A., Baptista, P., 2010. Actividade antioxidante de folhas de medronheiro (*Arbutus unedo* L.) de 19 genótipos provenientes da região de Bragança. Actas Portuguesas Hortic. 16, 179-183.

Scanu, R., Spano, N., Panzanelli, A., Pilo, M.I., Piu, P.C., Sanna, G., Tapparo, A., 2005. Direct chromatographic methods for the rapid determination of homogentisic acid in strawberry tree (*Arbutus unedo* L.) honey. J. Chromatography A 1090, 76-80.

Schlötterer, C., 2004. The evolution of molecular markers - just a matter of fashion? Nat. Rev. Genet. 5, 63-69.

Seabra, R.C., Simões, A.M., Baeta, J., Salomé Pais, M., 2001. Evaluation of Portuguese chestnut stands by RAPDs. For. Snow Landsc. Res. 76, 435-438.

Sharma, M., Sood, A., Nagar, P.K., Prakash, O., Ahuja, P.S., 1999. Direct rooting and hardening of tea microshoots in the field. Plant Cell Tis. Org. Cult. 58, 111-118.

Smith, S.E., Read, D.J., 1997. Mycorrhizal Symbiosis, 2nd ed. Academic Press, Harcourt Brace and Comp. Pub., San Diego.

Spano, N., Casula, L., Panzanelli, A., Pilo, M.I., Piu, P.C., Scanu, R., Tapparo, A., Sanna, G., 2006. An RP-HPLC determination of 5-hydroxymethylfurfural in honey: The case of strawberry tree honey. Talanta 68, 1390-1395.

Takrouni, M.M., Boussaid, M., 2010. Genetic diversity and population's structure in Tunisian strawberry tree (*Arbutus unedo* L.). Scientia Hortic. 126, 330-337.

Tan, X.-X., Li, Y., Ge, X.-J., 2009. Development and characterization of eight polymorphic microsatellites for *Rhododendron simsii* Planch (Ericaceae) Conserv. Genet. 10, 1553-1555.

Thorpe, T., Stasolla, C., Yeung, E.C., De Klerk, G.-J., Roberts, A., George, E.F., 2008. The components of plant tissue culture media II : organic additions, osmotic and pH effects, and support systems. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 115-174.

Tornero, O.P., C. Tallón, C., Porras, I., 2009. *Citrus limon* micropropagation: Effect of different phytohormones on multiplication and rooting. Acta Hort. 839, 57-62.

Torres, J.A., Valle, F., Pinto, C., Garcia-Fuentes, A., Salazar, C., Cano, E., 2002. *Arbutus unedo* communities in southern Iberian Peninsula mountains. Plant Ecol. 160, 207-223.

Tutin, T.G., Heywood, V.H., Burges, N.A., Valentine, D.H., Walters, S.M., Webb, D.A., 1972. Flora europea Vol. 3. Cambrige Univ. Press, Cambrige.

Väli, U., Brandström, M., Johansson, M., Ellegren, H., 2008. Insertion-deletion polymorphisms (indels) as genetic markers in natural populations. BMC Genetics 9: 8, doi: 10.1186/1471-2156-1189-1188.

Van Staden, J., Zazimalova, E., George, E.F., 2008. Plant growth regulators II: cytokinins, their analogues and inhibitors. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 205-226.

Vander Kloet, S.P., Dickinson, T.A., 2005. RAPD typification: phenetic analysis of *Vaccinium* inflorescences. Bot. J. Linnean Society 148, 445-457.

Vander Kloet, S.P., Paterson, I.G., 2000. RAPD assessment of novelties resulting in a new species of *Vaccinium* L. (Ericaceae) from Vietnam. Bot. J. Linnean Society 134, 575-586.

Wang, N., Qin, Z.C., Yang, J.B., Zhang, J.L., 2010. Development and characterization of 15 microsatellite *loci* for *Rhododendron delavayi* Franch. (Ericaceae). Hortscience 45, 457-459.

Wang, X.-R., Szmidt, A.E., 2001. Molecular markers in population genetics of forest trees. Scan. J. For. Res. 16, 199-220.

White, T.L., Adams, W.T., Neale, D.B., 2007. Forest Genetics. CAB Internacional Oxfordshire.

Whitton, J., Rieseberg, L.H., Ungerer, M.C., 1997. Microsatellite *loci* are not conserved across the *Asteraceae*. Mol. Biol. Evolution 14, 204-209.

Wiedow, C., Buck, E.J., Gardiner, S., Scalzo, J., 2007. Genetic diversity within a blueberry (*Vaccinium* spp. and hybrids) germplasm collection at HortResearch. Plant and Animal Genome Conference, San Diego, p. *Abst* 218.

Winkelmann, T., 2010. Clonal propagation of *Cyclamen persicum* via somatic embryogenesis. In: Jain, S.M., Ochatt, S.J. (Eds.), Protocols for *in vitro* propagation of ornamental plants. Humana Press, New York, pp. 281-290.

Wong-Villarreal, A., Caballero-Mellado, J., 2009. Rapid identification of nitrogen-fixing and legume-nodulating *Burkholderia* species based on PCR-16S rRNA species-specific oligonucleotides. Systematic Appl. Microbiology, DOI 10.1016/j.syapm.2009.10.004.

Wu, B., Isobe, K., Ishii, R., 2004. Arbuscular mycorrhizal colonization of the dominant plant species in primary successional volcanic deserts on the southeast slope of Mount Fuji. Mycorrhiza 14, 391-395.

Yavaşer, R., Çetinyürek, F., Uygun, D.A., Karagözler, A.A., 2010. Investigation of antioxidant properties of *Arbutus unedo* L. fruit 6th Conference Aromatic and Medicinal Plants of Southeast European Countries, CMAPSEEC, Antalya, Turkey, p. *Abst* 102.

Zawko, G., Krauss, S.L., Dixon, K.W., Sivasithamparam, K., 2001. Conservation genetics of the rare and endangered *Leucopogon obtectus* (Ericaceae). Mol. Ecol. 10, 2389 - 2396.

Zimmerman, R.H., Broome, O.C., 1980. Blueberry micropropagation. Conference on nursery production of fruit plants through tissue culture. Applications and feasibility. USA Department of Agriculture. Science and Education Administration. Agricultural Research Results-NE-11, Beltsville, Maryland, pp. 44-47.

Ziv, M., 1986. *In vitro* hardening and acclimatization of tissue culture plants. In: Withers, L.A., Alderson, P.G. (Eds.), Plant Tissue Culture and its Agricultural Application. Butterworth, London, pp. 187-196.

Zizzo, G., Aprile, S., La Mantia, A., S., L., 2010. The biodiversity of *Arbutus unedo* L. (Ericaceae Fam.) to develop new floricultural crops for the temperate areas. 28th International Horticultural Congress, Science and Horticulture for People, ISHS, Lisboa, p. *Abst* 417.

2 - Micropropagation of strawberry tree (*Arbutus unedo* L.) from adult plants

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Gomes, F., Lopes M L, Santos, T., Canhoto, J.M., 2009. Micropropagation of selected trees of *Arbutus unedo* L. trough axillary shoot proliferation and somatic embryogenesis. Acta Hort. 839: 111-116.

2 MICROPROPAGATION OF STRAWBERRY TREE (*ARBUTUS UNEDO* L.) FROM ADULT PLANT

2.1 ABSTRACT

Arbutus unedo L. is a species of strawberry tree, widely represented in the Mediterranean climates of southern Europe. Fruits are used to make jellies and a spirit called “medronheira.” Shoot apices and nodal segments from epicormic and coppiced shoots of adult plants were used for plant propagation. Shoot apices from epicormic shoots, which were developed in a growth chamber, showed higher rates of *in vitro* establishment. The results also indicated that shoot apices are more effective for plant establishment than nodal segments, with rates of establishment significantly higher after 12 weeks of culture. Of the three basal media used in combination with 9.0 μM benzyladenine and 0.087 M sucrose, the Fossard medium with the micronutrients of the Murashige and Skoog medium gave the highest rates of multiplication, especially when the parameter analysed was the number of shoots formed per test tube for further multiplication. When shoot apices from selected adult plants (AL01–AL06) were tested, the multiplication rate was not significantly different among the plants. However, in the conditions tested, shoots from the clones AL01, AL02, and AL03 showed better development, whereas shoots from AL04, AL05, and AL06 showed an impaired development and could not be rooted. Rooting was achieved in all the conditions tested, even in the absence of auxin. The inclusion of an auxin significantly increased root formation, whereas the addition of charcoal did not improve root formation. Rooted plantlets were acclimatized. From the five substrates tested during acclimatization, perlite without fertilizer gave the best survival rate (98.0±1.23%) after 4 months. Some of the produced plants are now in the field for further study. The anatomical data indicate that adventitious roots had a deep origin in the shoot stem, most probably from the cambial zone and/or from phloem cells.

Keywords: acclimatization; anatomical studies, epicormic shoots; rooting; shoot proliferation.

RESUMO

Arbutus unedo L. é uma espécie com ampla distribuição no sul da Europa, em regiões de clima Mediterrânico. Os frutos são utilizados para a produção de compotas e de uma aguardente conhecida como “medronheira”. Ápices meristemáticos e segmentos nodais de rebentos epicórmicos e rebentos de touça de plantas adultas foram utilizados para a propagação das plantas. Ápices meristemáticos de rebentos epicórmicos provenientes da câmara de crescimento mostraram maiores taxas de estabelecimento *in vitro*. Os resultados também indicaram que os ápices meristemáticos são mais eficientes no estabelecimento que os segmentos nodais, apresentando taxas significativamente superiores após 12 semanas de cultura. Dos três meios base testados, adicionados de 9,0 µM BA e de 0,087 M sacarose, o meio de cultura de Fossard com os micronutrientes de Murashige and Skoog apresentou as maiores taxas de multiplicação, em particular quando o parâmetro estudado foi o número de rebentos formados por tubo para posterior multiplicação. Quando foram testados ápices meristemáticos de plantas adultas seleccionadas (AL01 – AL06), a taxa de multiplicação não mostrou diferenças significativas entre os clones. Contudo, nas condições testadas, os clones AL01, AL02 e AL03 mostraram um melhor desenvolvimento comparativamente com AL04, AL05 e AL06, não tendo estes últimos produzido rebentos para o enraizamento, no mesmo período de tempo. O enraizamento, no primeiro ensaio, foi observado em todas as condições testadas, incluindo na ausência da auxina. A adição de auxina aumentou significativamente a taxa de enraizamento, o que não se observou com a adição de carbono activo ao meio de cultura. As plântulas enraizadas foram aclimatizadas. Dos cinco substratos testados na aclimatização, a perlite sem fertilizante permitiu a obtenção da maior taxa de sobrevivência após 4 meses (98,0±1,23%). Algumas das plantas produzidas estão no campo para avaliação futura. A caracterização anatómica sugere que as raízes adventícias têm uma origem profunda no rebento, mais provavelmente da zona cambial e/ou das células do floema.

Palavras-chave: aclimatização; enraizamento; estudos anatómicos; multiplicação por gomos axilares; rebentos epicórmicos.

2.2 INTRODUCTION

The genus *Arbutus* (Ericaceae) includes about 20 species from which *Arbutus unedo*, commonly known as strawberry tree, is the most interesting from an economic point of view. Strawberry tree seems to be native to Ireland, southern Europe and the western Mediterranean region and grows spontaneously in several countries around the Mediterranean basin in rocky and well-drained soils (Piotto *et al.*, 2001). The plant is an evergreen shrub or small tree (rarely exceeding 3 m) with a spreading habit, and grey-brown bark (Heywood, 1993). The small white blueberry-like flowers are assembled in panicles about 5 cm long. The spherical bright red fruits are warty and about 2 cm inch in diameter. They take a year to ripen and, during several months of the year, both flowers and fruits are present in the same tree.

Populations of *A. unedo* can be uniform but in most cases this species grows associated with other trees (Neppi, 2001), in particular with some species of the family *Fagaceae* (e.g. *Quercus suber*). The fruits are edible and have been traditionally used to make a strong tasting spirit called “medronheira”. They can also be used to make preserves and a very good type of honey (Neppi, 2001). Fruits are collected from October to December and each tree produces an average of 7-10 kg. In general, 10 kg are necessary to produce 1 l of the drink (Cardoso, 2004). The bark has been used to tan leather and the plant has been used in folk medicine. For instance, the fruits and leaves have been used as an astringent, diuretic, antirheumatic, antidiarrheal, and against urinary infections (Pabuccuoglu *et al.*, 2003; Cardoso, 2004). More recent usages are related with biomass for energy production and floriculture since young branches make very attractive floral bouquets (Metaxas *et al.*, 2004).

From an ecological perspective, *A. unedo* is also an interesting plant. As a species characteristic of Mediterranean ecosystems it contributes to maintain the biodiversity of the fauna, helps to stabilize soils avoiding erosion, has a strong regeneration capacity following fires and survives quite well in poor soils. Additionally, it can withstand low temperatures and is tolerant to drought (Piotto *et al.*, 2001; Godinho-Ferreira *et al.*, 2005).

The selection of the most interesting trees, based on fruit production/quality or any other characteristic, is relevant for its economic use. Considering this general overview, and the importance that alternative crops are assuming in the scope of the agriculture policy of the European Union, it is of great importance to start a long-term program for the improvement of *A. unedo* in Portugal based on the genetic diversity occurring among natural populations of different regions (Torres *et al.*, 2002).

The propagation of the selected strawberry trees is particularly important. Seed propagation does not assure the genetic stability and particular characteristics can be lost. Assays of vegetative propagation can be made by conventional vegetative propagation methods such as rooting of cuts as well as by micropropagation methods (Hartman *et al.*, 1997). However, the frequencies of rooting are quite low especially when mature cuttings are used (Mereti *et al.*, 2002; Metaxas *et al.*, 2004). Micropropagation may be a valuable alternative when: (a) conventional propagation is difficult to achieve, (b) problems of rejuvenation persist, and (c) pressure to increase multiplication rates occurs (McComb and Bennett, 1986; Gomes and Canhoto, 2003). Several micropropagation techniques such as somatic embryogenesis (Chawla, 2009), organogenesis (Arezki *et al.*, 2000) and axillary shoot proliferation (Jain and Häggman, 2007) have been applied for *in vitro* propagation of woody plants. Among these methods, axillary shoot proliferation is the most widely used for Ericaceae clonal propagation. Members of this family that have been successfully micropropagated include *Arbutus xalapensis* (Mackay, 1996), *Kalmia latifolia* (Lloyd and McCown, 1980), *Oxydendrum arboreum* (Banko and Stefani, 1989), *Rhododendron* (Anderson, 1984; Almeida *et al.*, 2005) and several species of the genus *Vaccinium* (Isutsa *et al.*, 1994; Gajdošová *et al.*, 2007; Ostrolucká *et al.*, 2007; Ratnaparkhe, 2007). Previous studies in strawberry tree have shown that *in vitro* propagation from juvenile material could be accomplished (Gonçalves and Roseiro, 1994; Mereti *et al.*, 2002). However, as with seeds, juvenile explants are of unknown genotype making difficult the propagation of selected trees, which can only be achieved through the propagation of adult plants. Previous reports of *A. unedo* micropropagation are scarce. As far as it is known, the only report in which micropropagation of *A. unedo* has been achieved from adult material was that of Mendes (1997). Therefore, the development of a protocol for an efficient *in vitro* propagation of this species from adult trees is necessary. In this paper we describe a reliable and reproducible method to propagate adult trees of *A. unedo* through axillary shoot proliferation. To achieve this goal the effect of several culture media was analysed. Moreover, previously selected genotypes were tested as well as the conditions for root formation and acclimatization. To better understand some steps of the regeneration process anatomical studies have also been performed.

2.3 MATERIAL AND METHODS

2.3.1 Plant material

Shoots from spontaneous field-growing adult plants were collected to obtain the explants used in the experiments. Young shoots (5-10 cm length) were gathered in the autumn from 2 to 3-month-old coppices of a tree from Coimbra (C01) sprouting in a burned area during the previous summer (August). Woody branches with 30-40 cm length and 0.4-2.5cm diameter were collected from another spontaneous adult plant (ESAC05) and from 6 adult plants selected for its high fruit productivity and growing in an orchard (AL01 to AL06). Woody branches from ESAC05 and AL01 to AL06 plants were stimulated to produce epicormic shoots which were the source of the explants used for *in vitro* culture establishment.

2.3.2 Surface-sterilization procedures and culture establishment

For explant sterilization coppiced shoots were defoliated, dipped in a fungicide (dichlofluanid, Euparene, 120 mgl^{-1}) for 2 min, rinsed with running tap water and placed in a 20 % sodium hypochloride solution (5 % active chlorine) containing 2-3 drops of Tween 20, for 18 min. After three washes in sterile distilled water, they were inoculated on the establishment medium. Epicormic shoots were surface-sterilized in a 75% ethanol solution for 2 minutes, dipped in a 10 % sodium hypochlorite solution (5 % active chlorine) for 3 to 6 minutes, and then washed in sterile distilled water for three times.

Woody branches were washed under running tap water, dipped in a fungicide (dichlofluanid, Euparene, 120 mgl^{-1}) for 10 min and rinsed with distilled water. Following this treatment, the branches were transferred to jars containing 1.5 l of sterilised sand and watered with distilled-sterilised water and placed into a greenhouse or in a growth culture chamber at 25 ± 1 ºC under a 16h photoperiod provided by cool-white fluorescent lights (40 $\mu mol\ m^{-2}\ s^{-1}$) to promote epicormic shoot development. The branches placed in the growth chamber were covered with transparent polythene plastic to keep a high degree of humidity. To promote epicormic shoot development, branches placed in the greenhouse were sprayed with a solution of benzyladenine (BA, 9.0 µM) three times a week and/or covered with polypropylene plastic bags for 2 months. Following this period, epicormic shoot development

was evaluated by the number of produced shoots and shoot length. The epicormic shoots thus obtained where then used for *in vitro* establishment.

Shoot apices (< 2 mm) and nodal segments (10-20 mm) were used as explants to establish the cultures. Explants from coppices of C01 tree were used. Explants were incubated individually and subcultured every 3 weeks in test tubes (nodal segments) or Petri dishes (shoot apices). To avoid phenol oxidation and tissue necrosis explants were placed in a growth chamber at 25 ± 1ºC, under dark conditions for a week and then transferred to light (16h photoperiod, 40 µmol m^{-2} s^{-1}). Test tubes (Sigma, 25 x 150 mm) containing 12 ml of culture medium and covered with plastic caps were used. The medium for culture establishment (AND) consisted of Anderson salts (Anderson, 1984), MS micro nutrients (Murashige and Skoog, 1962) and organic compounds of the FS medium (De Fossard *et al.*, 1974). Sucrose 0.087 M and 9.0 µM BA were added. The medium was jellified with agar ($7gl^{-1}$) and the pH adjusted to 5.7 prior to autoclaving (121 °C, 20 min.). The best conditions for epicormic shoot development were further applied to obtain epicormic shoots from clones AL01-AL06 which were then used as source of the explants for *in vitro* establishment. For all the assays the number of established explants as well as the frequencies of necrosed and contaminated explants was taken after 1 and 12 weeks of culture.

2.3.3 Shoot multiplication

Established explants were transferred to test tubes (Sigma, 25 x 150 mm) containing the same medium and subcultured every 3 weeks until enough stock material was available to perform further experiments. Since *in vitro* plant establishment from the C01 plant was easily achieved and a large number of shoots from this material was obtained, this clone was used to test the effect of three culture media on shoot multiplication: 1) The Anderson medium above described (AND); 2) the same medium containing as major salts the MS macronutrients reduced at half-strength (1/2 MS) and 3) the same medium with the major salts of the FS medium (FS). After 12 weeks (4 subcultures at 3-week intervals) of culture the multiplication rate was determined per test tube by evaluation of the following parameters: a) maximum shoot length (SL) and b) the number of shoots formed per test tube for further multiplication (SNX). The best culture medium was then tested on shoot multiplication of the six selected adult clones (AL01 – AL06).

2.3.4 Rooting

Two different approaches were tested for root induction of the formed shoots: a) a protocol involving two-steps and a b) one-step rooting protocol. In the first case, shoots were transferred to a root induction medium containing the auxin indole-3-butyric acid (IBA), for a short period to induce root primordia. After the induction period, shoots were transferred to a new medium (development medium) without auxin to promote root and shoot development. Trying to reduce time and costs associated with the rooting phase a simpler assay was performed in which one-step rooting was also tested. In this case shoots were transferred and maintained in a rooting medium for 6 weeks containing the auxin indole-3-acetic acid (IAA) at 5.7; 10 and 17.1 µM and compared to the control (without auxin).

When rooting was carried out using a two-steps protocol, two different assays were performed. In the first situation the following parameters were tested: a) IBA concentration during the induction period, and b) addition of charcoal following the auxin treatment. In both assays test tubes (Sigma, 25 x 150 mm) containing 12 ml of culture medium and covered with plastic caps were used. Micropropagated shoots (14–20 mm) of the C01 clone growing in the AND proliferation medium were transferred to the rooting induction medium (RM), which consisted of Knop macronutrients (Gautheret, 1959), MS micronutrients without potassium iodine and FS organics without riboflavin. Sucrose was added to the culture media at concentrations of 0.044 or 0.087 M for the induction and development medium, respectively. Root induction was assayed in darkness, and three treatments with IBA were tested. In one set of experiments rooting was induced on RM medium containing 9.8 or 24.7 µM IBA for six days and compared to the control (without IBA). In another experiment, shoots were dipped in a solution containing 9.8 mM IBA for 15s. In both cases, auxin treatment was followed by culture on the same RM medium without IBA for shoot and root development. The role of charcoal (1.5% w/v) on root development was also evaluated. A total of 240 shoots was tested (ten shoots × three replications × four auxin treatments) comprising the control × two development media (with or without charcoal). Owing to the high number of shoots required, this assay was accomplished 6 months after C01 culture establishment. Root formation was analysed by the percentage of rooted shoots after 5 weeks of culture on the RM medium. The best conditions for root induction were further tested in the selected clones (AL01–AL03).

In a second experiment, shoots of the same clone (C01) were used to test the effect of the two different shoot proliferation culture media (FS and AND) as well as a broad range of IBA concentrations on rooting. The procedures were similar to those described previously. Root induction was assayed on RM medium with IBA, on dark conditions, for 7 days. Shoots were then subcultured (5 weeks) on the same medium without growth regulators and containing charcoal (1.5 % w/v) to promote root development. This assay was accomplished 15 months after C01 culture establishment. For root induction, five IBA treatments were used (2.5; 4.9; 9.8; 24.6; 49.2 µM for 1 week) plus the control. A total of twelve treatments were tested: two shoot proliferation media (FS and AND) and 6 different IBA concentrations including the control, and 30 shoots (ten shoots × three replications) were used per treatment (a total of 360 shoots). Root formation was evaluated after 5 weeks of culture on the RM medium and the following parameters were assessed: percentage of rooted shoots, number of roots per shoot (NR), length of the longest root (LLR), length of the shortest root (LSR), and final shoot length (SL). When apical shoot necrosis or callus formation at the cut end of the shoot were observed they were also registered.

When one-step rooting was tested, micropropagated shoots (14–20 mm) of the AL03 clone growing in the FS proliferation medium were used. In this assay jars with a volume of 500 ml were used. Trying to stimulate root development a mixture of peat and perlite (1:4, v/v), previously sterilized (121 °C, 60 min.), was used (70 ml substrate per jar). In order to avoid a possible toxic effect of auxin on rooted shoots, the weaker auxin IAA was used instead of IBA. Three IAA concentrations (5.7; 10 and 17.1 µM) in the rooting medium (70 ml of RM with 0.087 M sucrose) were tested and compared with the control. The addition of charcoal (1.5 % w/v) to the culture medium was also tested. A total of 8 treatments were evaluated: 4 IAA treatments (4 different IAA levels, including the control) and 2 charcoal conditions (with *versus* without). Jars containing the substrate and the culture medium were autoclaved at 121 °C for 20 min. Shoots were isolated and transferred to the substrate (5 shoots per jar). A total of 320 shoots was tested: 10 shoots x 4 replications x 4 IAA treatments (including the control) x 2 charcoal conditions (with *versus* without). Shoots were cultured in these conditions for 6 weeks, and at the end of this period, root formation was evaluated by the parameters indicated before: percentage of rooted shoots; number of roots formed per shoot (NR); length of the longest root (LLR); length of the shortest root (LSR); and final shoot length (SL).

2.3.5 Acclimatization

Following agar removal with tepid tap water, rooted plantlets were dipped in a fungicide solution (benomil, Benlate 0.6 gl^{-1}) and transferred to containers (60 x 40 cm).

In a first set of experiments a total of 154 C01 plantlets rooted according to the two-steps method were acclimatized. The aim of this acclimatization experiment was to evaluate the effect of IBA treatment on the number of the survival plants after acclimatization procedures. For plantlet acclimatization a mixture of sand and *Siro 30* (1:1 v/v) previously sterilised was used as a substrate. *Siro 30* is a commercial substrate of composted pine bark and peat (70:30%; v/v) supplemented with “Osmocote" slow release fertilizer (4%; v/v). The containers were covered with plastic to maintain a high degree of humidity and were placed on an irrigation sheet in a greenhouse. The levels of humidity were gradually decreased by raising the covertures after 3 to 4 weeks. The covertures were totally removed after 1.5 month. Half a month later plants were transferred to individual containers (220 cm^3) and, at that moment, the survival rate was recorded (2 months). As substrate a mixture of peat, vermiculite and perlite (1:1:1.5; v/v) was used. A slow release fertilizer (20:9:11 NPK + 2Mg) was added to the substrate (2.6 g/L). Following this treatment the plants were transferred to the nursery. Two months later the number of surviving plants was evaluated (plant survival after 4 months of acclimatization). A similar protocol was tested to acclimatize plantlets of the clones AL01, AL02 and AL03 (in a total of 229 plantlets). In this situation perlite (100%) without fertilizer was used as substrate and the plants were weekly (during a month) sprayed with Knop solution. The survival rate was recorded 2 and 4 months after acclimatization. Following this period plants were planted in an orchard in the Centre of Portugal (Estreito, Oleiros).

In the second set of experiments different substrates were tested. A total of 600 C01 plantlets were transferred to containers similar to those used in the first experiment (60x40 cm or 30 x 25 cm, covered with plastic bags) and placed in the greenhouse. Five substrates supplemented with a slow release fertilizer (20:9:11 NPK + 2Mg; 26 g/10L) were used, namely: a) two mixtures of perlite and peat (70: 30% or 50: 50% v/v); b) sand, composted pine bark and peat (50: 35: 15% v/v); c) perlite (100%) with fertilizer; and d) perlite (100%) without fertilizer. When perlite 100% without fertilizer was used, a macronutrients Knop

solution was also weekly sprayed for a month. The survival rate was recorded 2 and 4 months after acclimatization, as previously described**.** Following this period plants were established for cutting production and for clonal trials.

2.3.6 Anatomical studies

Samples (shoots and leaves) from *in vitro* propagated plantlets and from field-growing plants were fixed in formalin/acetic acid/ethyl alcohol by volume (FAA, 5:5:90, v/v) dehydrated in a graded ethyl alcohol/chloroform series, and embedded in paraffin wax. Sections (10–12μm) were stained by the safranine-light method as described in Guimarães *et al.* (1988).

2.3.7 Statistical analysis

The multiplication experiments started with 30 shoots per treatment comprising at least three replicas of 10 explants. Variables were recorded after each one of the 4 subcultures at 3-week intervals. For rooting experiments 30 or 40 shoots per treatment (for two-steps and one-step rooting, respectively) were tested.

All quantitative data expressed as percentages were first submitted to arcsine transformation and the means corrected for bias before a new conversion of the means and standard error (SE) back into percentages (Zar, 1996). Statistical analysis was performed by ANOVA (STATISTICA 6) and the significantly different means ($P \leq 0.05$ or $P \leq 0.01$) were identified by using the Duncan's test (Duncan, 1955). A principal component analysis (PCA) was carried out when thought it might lead to a better perception of the results. For the principal components analysis all variables were analysed in order to obtain a better understanding of the interactions between them and of the significance level of each variable for the total variance.

2.4 RESULTS

2.4.1 Culture establishment

Woody branches from the ESAC05 tree (Fig. 1*A*) showed epicormic shoot development after 15 days in the growth culture chamber (Fig. 1*B*). Epicormic shoot development from branches maintained in the greenhouse developed after 2 months, much slower than those developed in the culture chamber.

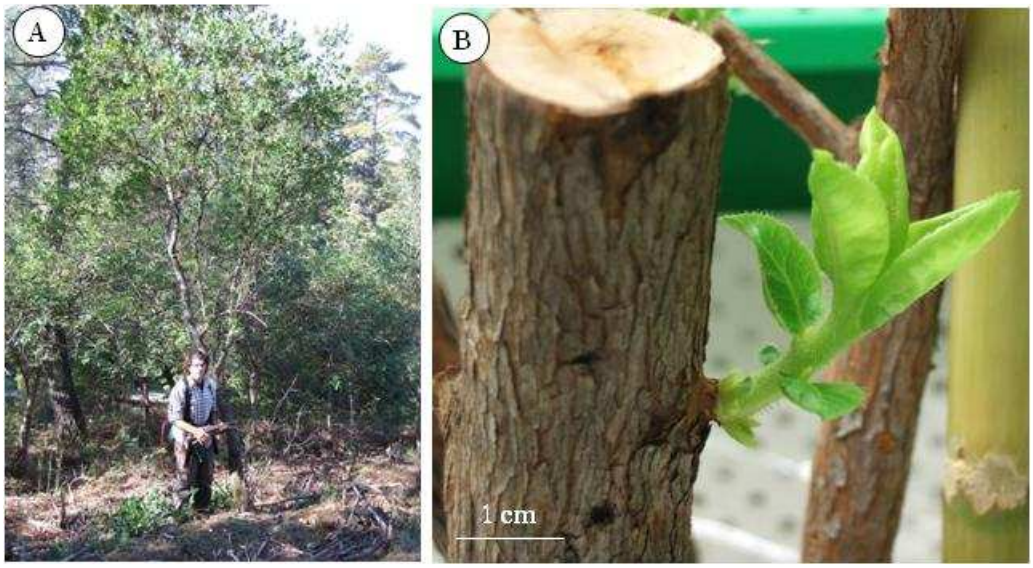

Figure 1 - Tree ESAC05 (A) and branches (B) showing epicormic shoot development after 15 days in the growth culture chamber.

The number of shoots per branch was not affected by the treatment applied, since the values obtained for the different situations were not significantly different (Table 1; Appendix Table 1). The simultaneous use of BA and uncovered branches gave shorter shoots than covered branches treated with BA (Table 1). Significant differences were only observed when shoot length was the factor analysed. In this case shoot elongation (1.75±0.31 cm) was more effective when branches were simultaneously covered with plastic and treated with BA. (Table 1).

Table 1 - Effect of BA (9.0 µM) and plastic covertures (covered or uncovered) on epicormic shoot development (ESAC05 plant) in branches placed in the green house for two months.

Treatment of the branches	Nº of shoots per branch	Shoot length (cm)
Covered and BA	4.0 ± 0.58 [a]	1.75 ± 0.31 [a]
Covered	4.3 ± 2.03 [a]	0.81 ± 0.39 [ab]
Uncovered	3.0 ± 1.73 [a]	0.69 ± 0.49 [ab]
Uncovered and BA	1.0 ± 1.0 [a]	0.07 ± 0.07 [b]

In each column values (mean ± SE) followed by different letters are significantly different (P≤0.05).

A higher frequency of necrosis and contamination were noted for epicormic shoots grown in the greenhouse than for epicormic shoots grown in the growth chamber (Table 2). As a consequence, the number of established explants (12 weeks) was higher when explants were formed in the growth culture chamber (Table 2).

Table 2 - Establishment of shoot apices from epicormic shoots formed in the growth culture chamber (GCC) or in the greenhouse (GH), after 12 weeks of culture on the AND medium. The percentages of contaminated, necrosed and surviving explants after one week of culture are indicated.

Plant	Epicormic shoot formation	Nº of inoculated explants	One week of culture			Established explants (12 weeks)	
			Survival (%)	Necrosis (%)	Contamination (%)	Nº	(%)
ESAC05	GCC	29	79.3	0	20.7	20	68.9 [a]
	GH	25	48.0	16.0	36.0	10	40.0 [b]
AL 01	GCC	101	53.5	11.9	34.6	40	39.6 [c]
AL 02	GCC	114	64.9	11.4	23.7	74	64.9 [a]
AL 03	GCC	82	76.8	10.9	12.2	44	53.7 [ab]
AL 04	GCC	41	68.2	7.3	24.3	21	51.2 [bc]
AL 05	GCC	16	81.2	6.3	12.5	2	12.5 [d]
AL 06	GCC	22	36.4	0	63.6	3	13.6 [d]
Total AL explants		376				184	48.9

In each column values (mean ± SE) followed by different letters are significantly different (P≤0.01).

The analysis of explant establishment for the six adult selected trees (AL01–AL06) showed significant statistical differences; the AL02 and AL03 gave the highest percentage (64.9% and 53.7%,respectively) of established shoots after 12 wk of culture (Table 2; Appendix Table 2). When shoot apices (Figs. 2*A*, *B*) and nodal segments (Fig. 2*C*) were compared for explant establishment the results showed that the levels of contamination were similar (Table 3).

Figure 2 - Established cultures of *A. unedo* on AND medium. A and B – Established cultures from shoot apices after 8 and 30 days, respectively. C – Established cultures from nodal segments after 15 days.

However, the levels of necrosis were significantly lower in the shoot apices (Table 3; Appendix Table 3). Additionally, the number of explants that survived and were established after 12 wk was significantly higher for shoot apices than for nodal segments (Table 3; Appendix Table 3).

Table 3 - Effect of the type of the explant on survival, contamination, and necrosis.

Type of explant	One week of culture			Established explants (%) 12 weeks
	Survival explants (%)	Necrosed explants (%)	Contaminated explants (%)	
Shoot apices	38.65±9.78[a]	8.44±4.46[a]	52.91±9.89[a]	26.13±8.20[a]
Nodal segments	4.49±2.63[b]	43.04±11.05[b]	52.47±11.21[a]	2.55±1.78[b]

In each column values (mean ± SE) followed by different letters are significantly different (P≤0.05).

2.4.2 Shoot multiplication

The effect of three different culture media on the multiplication of the C01 clone (Fig. 3*A*, Table 4) showed that the FS medium gave a higher number of shoots formed per test tube for further multiplication (1.99±0.11) than the other two media (1/2-MS and AND). No significant difference was observed in shoot length between FS and 1/2-MS media, but shorter shoots were noted on AND medium (Table 4).

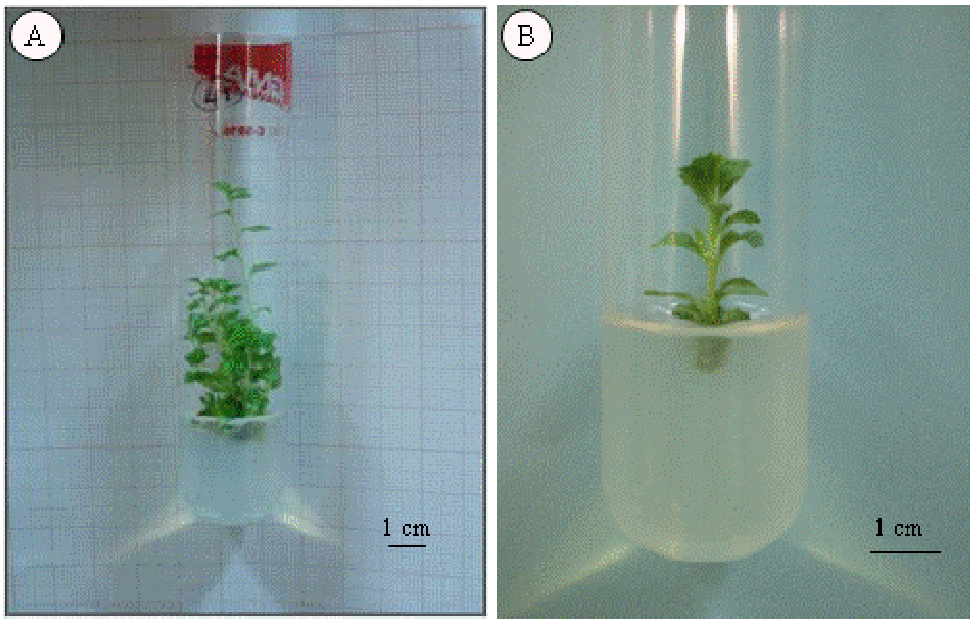

Figure 3 - *A. unedo* propagation from shoot apices. A - Cluster of shoots after 21 days on the FS medium containing 9.0 µM BA. B - Shoot just before transference to the rooting medium.

The differences were particularly clear when the parameter analysed was the number of shoots formed per test tube for further multiplication (SNX) with the results obtained in the FS medium being statistically different ($P \leq 0.01$) from the other two media (Table 4; Appendix Table 4). No significant difference was observed in shoot length between FS and 1/2-MS media, but shorter shoots were noted on AND medium ($P \leq 0.01$).

Table 4 - Effect of culture media on the multiplication rate evaluated by the number of shoots formed per test tube for further multiplication (SNX) and shoot length (cm) after four subcultures, at 3-week intervals, in the same culture medium.

Culture Media	SNX	Maximum shoot length (cm)
FS	1.99 ± 0.11 [a]	3.30 ± 0.11 [a]
1/2 MS	1.47 ± 0.10 [b]	3.00 ± 0.11 [a]
AND	1.34 ± 0.07 [b]	2.45 ± 0.10 [b]

Explants (shoot apices) of the clone C01 were used. In each column values (mean ± SE) followed by different letters are significantly different ($P \leq 0.01$).

The FS medium was further used to test the multiplication potential of the six adult clones (AL01– AL06), and the results showed no significant statistical differences among the clones (Table 5; Appendix Table 5). After five subcultures at 3-week intervals, shoots able to be rooted (Fig. 3*B*) were only obtained from the clones AL01, AL02, and AL03. Clones AL04, AL05, and AL06 were unable to produce shoots elongated enough to be used in the rooting assays (Table 5).

Table 5 - Multiplication rates of 6 adult selected clones (AL01 - AL06) during the first five subcultures, at 3-week intervals.

Sub culture	AL 01		AL 02		AL 03		AL 04		AL 05		AL 06	
	Test tubes	SNX	Test tubes	SNX	Test tubes	SNX	Test tubes	SNX	Test tubes	SNX	Test tubes	SNX
1 st	40	1.0	74	1.1	44	1.1	21	1.2	2	1.0	3	1.0
2 nd	40	1.3	79	1.3	50	1.4	26	1.0	2	0.5	3	1.0
3 rd	50	1.2	102	1.3	69	2.2	27	1.5	1	1.0	3	1.0
4 th	59	2.4	128	1.2	152	1.1	40	0.9	1	2.0	3	1.0
5 th	140		153		174		35		2		3	
Multip. Rate	1.45±0.31 [a]		1.20±0.05 [a]		1.47±0.25 [a]		1.16±0.13 [a]		1.13±0.31 [a]		1.00±0.0 [a]	
Shoots*	40		38		11		0		0		0	

Values (mean ± SE) followed by the same letter are not significantly different. FS medium was used for shoot multiplication. The multiplication rate was evaluated by the number of shoots formed per test tube for further multiplication (SNX). The number of test tubes was evaluated at the end of each subculture.
*Total number of shoots able to be rooted after the fifth subculture.

2.4.3 Rooting

In the first rooting assay using a two-steps protocol clone 01 was used for root induction (Fig. 4*A*). The shoots used were obtained from 6-month-old cultures after *in vitro* culture establishment. The results showed that shoots were able to root in all tested conditions, even when no auxin was used (Table 6; Appendix Table 6). Rooted shoots showed neither callus formation at the shoot base nor apical necrosis (Fig. 4*B*).

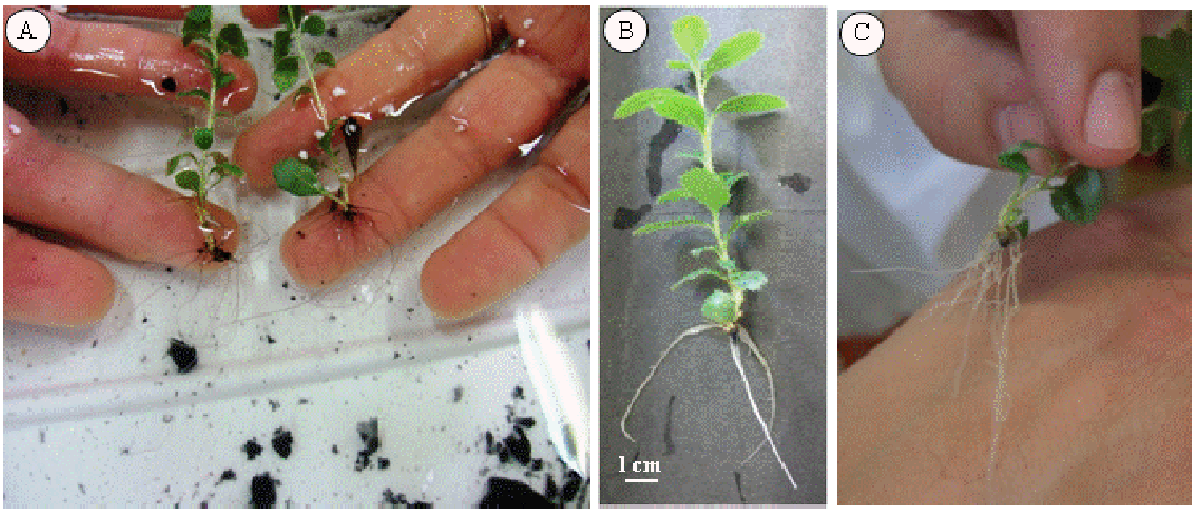

Figure 4 - Rooting of *A. unedo*. A and B - Rooted plants showing a well developed root system without callus and shoots without apical necrosis. C - Rooted shoot developed in RM containing charcoal.

High frequencies of root induction (between 76.7% and 93.3%) were achieved either after a treatment with 24.7 µM IBA for 6 days or by an auxinic pulse (15 s dipping in 9.8 mM IBA) followed by root development on a medium containing (1.5%) charcoal or in a charcoal-free medium (Table 6). The ANOVA analysis showed that the only factor affecting root formation was the IBA treatment and that there is no interaction between IBA and charcoal on root formation (Appendix Table 6). However, it was found that when charcoal was added to the development medium the adventitious root system displayed a higher number of roots (Fig. 4*C*) when compared with the treatments without charcoal where only one root was usually formed. According to these results it was decided to include charcoal in the subsequent assays.

Table 6 - Effect of several treatments on root formation after five weeks in the development medium.

IBA (µM)	Auxin treatment	Development medium	Rooting (%)
--	--	charcoal	40.00±20.0 ^{bc}
--	--	--	53.33±3.3 ^{abc}
9.8	6 days	charcoal	30.00±15.3 ^{c}
9.8	6 days	--	43.33±28.5 ^{abc}
24.7	6 days	charcoal	93.33±6.7 ^{a}
24.7	6 days	--	83.33±16.7 ^{ab}
9.8 x 10^3	Pulse (15 s)	charcoal	93.33±3.3 ^{ab}
9.8 x 10^3	Pulse (15 s)	--	76.67±3.3 ^{abc}

In each column values (mean ± SE) followed by different letters are significantly different (P≤0.01).

In the ensuing experiments shoots (a total of 317) from the clones AL01, AL02 and AL03 were tested for rooting in a medium containing 24.7 µM IBA for 6 days followed by culture in a charcoal containing medium (1.5% w/v) without IBA. Shoot rooting varied between 78.1 ± 6.7 % for the clone AL02 to 66.9±3.8 % for the clone AL01 whit the clone AL03 showing intermediate values 72.3±0.7. However, no statistically significant differences were found between these three AL clones and the C01 clone rooted in the same conditions.

On a second assay using the two-step rooting method, shoots of the same clone (C01) were used to test the effect of the two different proliferation culture media (FS and AND) as well as a broad range of auxin concentrations. Experiments were performed with shoots subcultured for 15 months on the culture media above-mentioned. The data showed that for

all parameters evaluated significant differences between treatments occurred. The percentage of rooted shoots was affected ($P \leq 0.01$) by several factors such as IBA concentration, type of shoot proliferation medium tested, and interaction between these two factors (Appendix Table 7). Best results were observed when AND and FS media were used in combination with IBA at concentrations of 49.2; 24.6 and 9.8 µM (Table 7). In general, root formation increased when higher IBA concentrations were tested (Fig. 5) and, in several treatments, it was found that 100% of rooting occurred. The results also indicated that the length of the longest root (LLR) was affected by IBA concentration ($P \leq 0.01$) and by the interaction between the two factors ($P \leq 0.05$; Appendix Table 7). However, it should be noted that except for the treatments in which no IBA was used (controls), no statistical significantly differences were found among the other treatments (Table 7).

Table 7 - Effect of the interaction between shoot proliferation media (FS and AND) and IBA on the percentage of rooted shoots and on the length of the longest root (LLR, mm).

Proliferation medium x IBA (µM)	Rooting (%)**	LLR (mm)*
FS x 0 µM	9.1 ± 0.5 ^{d}	16.7 ± 7.22 ^{b}
AND x 0 µM	5.6 ± 2.8 ^{d}	17.5 ± 2.50 ^{b}
FS x 2.5 µM	57.6 ± 13.8 ^{c}	35.7 ± 3.61 ^{a}
AND x 2.5 µM	85.6 ± 3.1 ^{b}	41.9 ± 2.34 ^{a}
FS x 4.9 µM	43.9 ± 4.0 ^{c}	34.9 ± 3.33 ^{a}
AND x 4.9 µM	83.3 ± 3.3 ^{b}	42.0 ± 1.95 ^{a}
FS x 9.8 µM	93.3 ± 3.3 $^{a\,b}$	40.8 ± 1.86 ^{a}
AND x 9.8 µM	100.0 ± 0.0 ^{a}	37.2 ± 1.34 ^{a}
FS x 24.6 µM	96.7 ± 3.3 ^{a}	35.8 ± 1.85 ^{a}
AND x 24.6 µM	100.0 ± 0.0 ^{a}	41.3 ± 1.11 ^{a}
FS x 49.2 µM	100.0 ± 0.0 ^{a}	37.1 ± 1.27 ^{a}
AND x 49.2 µM	100.0 ± 0.0 ^{a}	39.4 ± 1.14 ^{a}

In each column values (mean ± SE) followed by different letters are significantly different ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$).

The PCA analysis (Fig. 5) can explain 71.9% of total variance observed and points out to a positive interaction between rooting and IBA treatment (with high factor loadings, associated to Factor 1; Fig. 5). The variance explained by Factor 2 is about 25%, presumably due to the number of replications used (Fig. 5).

Variable	Factor Loadings (Un Extraction: Principal (Marked loadings are Factor 1	Factor 2
Med.Shoot Prolif	0,18185	0,14544
IBA	0,95139	-0,04570
Rep.	-0,01723	-0,98831
ArcSRootRate	0,96877	0,00000
Expl.Var	1,87703	1,00000
Prp.Totl	0,46925	0,25000

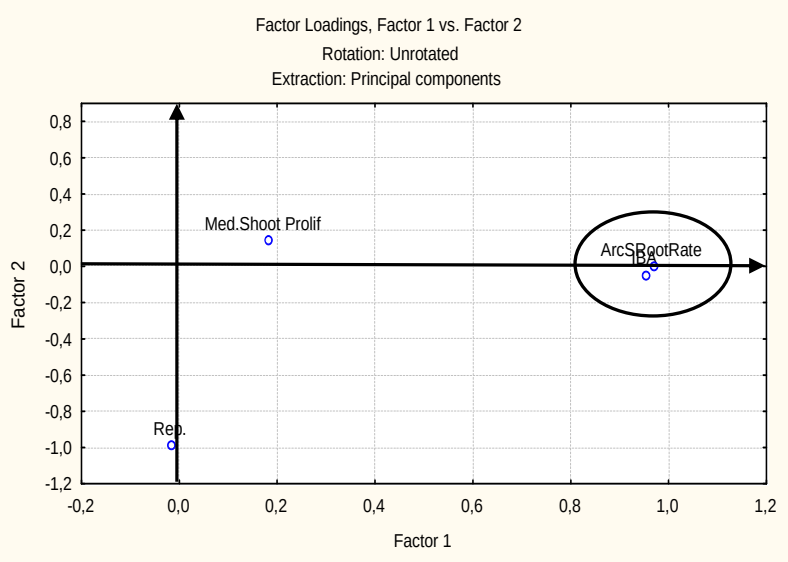

Figure 5 – PCA analysis of the effect of different rooting treatments. The variables analysed were shoot proliferation media, IBA treatments, replications and rooting percentage (submitted to arcsine transformation).

The variables number of roots (NR), length of the shortest root (LSR) and final shoot length (SL) were affected (P≤0.01) only by IBA concentration (Appendix Table 8). The number of roots, a parameter relevant for further acclimatization showed the highest value (19.4±1.28; P≤0.01) when the highest IBA concentration was used (Table 8). On media without IBA the parameters LSR and SL reached the minimum values (Table 8).

Table 8 – Effect of IBA treatments on the number of roots (NR), length of the shortest root (LSR, mm) and final shoot length (SL, mm).

IBA treatments	Number of roots	LSR (mm)	Shoot length (mm)
0 µM	1.4 ± 0.40 ^{d}	1.6 ± 1.60 ^{d}	17.0 ± 1.73 ^{b}
2.5 µM	2.6 ± 0.23 $^{c\,d}$	14.9 ± 1.94 ^{ab}	25.7 ± 1.25 ^{a}
4.9 µM	4.6 ± 0.69 $^{c\,d}$	19.2 ± 1.91 ^{a}	23.8 ± 1.58 ^{a}
9.8 µM	6.4 ± 0.55 $^{c\,d}$	16.3 ± 1.08 ^{ab}	21.2 ± 0.79 ^{ab}
24.6 µM	11.4 ± 0.87 ^{b}	10.9 ± 0.78 ^{bc}	25.8 ± 1.20 ^{a}
49.2 µM	19.4 ± 1.28 ^{a}	8.93 ± 0.55 ^{c}	26.6 ± 1.07 ^{a}

In each column values (mean ± SE) followed by different letters are significantly different (P≤0.01).

The PCA analysis (Fig. 6) can account for a total of 55.5% variance and, once again, points out to a positive interaction between the number of roots (NR) and IBA treatment (with high factor loadings, associated to Factor 1; Fig. 6). The variance explained by Factor 2 is about 23.6% and shows a weak interaction between the length of the longest and shortest root (LLR and LSR, respectively).

Variable	Factor Loadings (Unrc Extraction: Principal c (Marked loadings are Factor 1	Factor 2
Med. Shoot Prolif.	-0,06874(	0,433501
IBA	-0,81322(	-0,138631
RootsNb	-0,90319(	0,039217
LLRmm	-0,08628(	0,787200
LSRmm	0,45327(	0,572691
ShootLength mm	-0,46731(	0,511822
Expl.Var	1,91311(	1,418300
Prp.Totl	0,31885(	0,236383

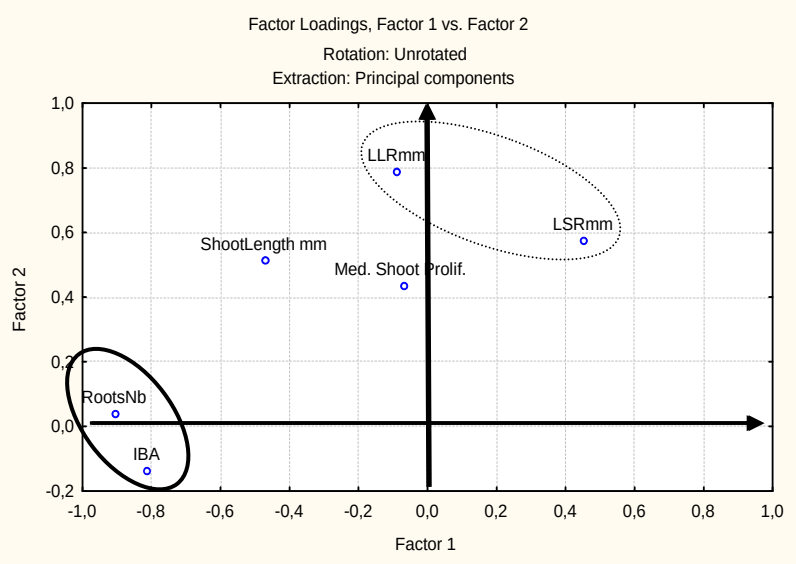

Figure 6 - PCA evaluation of the effect of different treatments on rooting. The following variables were analysed: shoot proliferation media, IBA treatments, number of roots (NR), length of the longest root (LLR), length of the shortest root (LSR) and the final shoot length (SL).

In the attempts to achieve root formation through the one-step protocol shoots of the AL03 were used. In these experiments 3 treatments with IAA (5.7; 10 and 17.1 µM) were compared to the control. The effect of the inclusion of charcoal in the rooting medium was also tested. The results showed that for all the parameters assessed no significant differences were found among the IAA treatments with the highest rooting percentage (55.0±10.41%) being obtained when IAA was used at the highest concentration without charcoal (Table 9). However, the interaction between the two factors (IAA x charcoal) contributed to significant differences for all variables, with the exception to the length of the shortest root (Appendix Table 9). Worth to mention is the fact that the average rooting rate obtained in these experiments was considerable lower (38.8±2.80%) than that achieved with the same clone in a previous experiment (72.3±0.7%).

Table 9 - Effect of the interaction between IAA treatments and charcoal (CA) on the percentage of rooted shoots, number of roots (NR), length of the longest root (LLR, mm) and final shoot length (SL, mm).

IAA µM	X	CA	Rooting (%)*	Number of roots*	LLR (mm)**	SL (mm)*
0 µM	X	--	32.5 ± 4.79 abc	1.9 ± 0.31 b	2.5 ± 0.55 c	1.9 ± 0.49 a
0 µM	X	CA	42.5 ± 9.46 abc	2.1 ± 0.30 b	4.8 ± 0.96 abc	1.7 ± 0.45 a
5.7 µM	X	--	35.0 ± 6.45 abc	3.7 ± 0.36 a	6.9 ± 0.85 a	3.5 ± 0.49 a
5.7 µM	X	CA	52.5 ± 6.29 ab	1.9 ± 0.29 b	2.8 ± 0.57 bc	1.8 ± 0.68 a
10 µM	X	--	30.0 ± 4.08 bc	3.1 ± 0.47 a	6.0 ± 0.59 a	2.9 ± 0.41 a
10 µM	X	CA	37.5 ± 4.79 abc	1.6 ± 0.19 b	2.4 ± 0.45 c	1.4 ± 0.19 a
17.1 µM	X	--	55.0 ± 10.41 a	3.0 ± 0.31 a	4.9 ± 0.71 abc	2.7 ± 0.50 a
17.1 µM	X	CA	25.0 ± 6.45 c	1.5 ± 0.25 b	5.3 ± 1.65 ab	3.4 ± 1.39 a

In each column values (mean ± SE) followed by different letters are significantly different (* P≤0.05; ** P≤0.01).

The results clearly indicate that the number of roots (NR) was higher (P≤0.05) on media containing only IAA (Table 9). The length of the longest root (LLR) was affected by charcoal (P≤0.05) and by the interaction between these two factors (P≤0.01; Appendix Table 9). Once again, the results clearly point out to a positive effect of IAA alone. Both variables NR and LLR, when compared to other assays formerly accomplished (two steps rooting, Figs. 7 *A*, *B*) showed rather low values (Figs. 7 *C*, *D*). The length of the shortest root (2.6±0.21 mm) showed to be independent of any factor or interaction (Appendix Table 9). Rooted shoots showed neither callus formation at the shoot cut neither end nor apical necrosis.

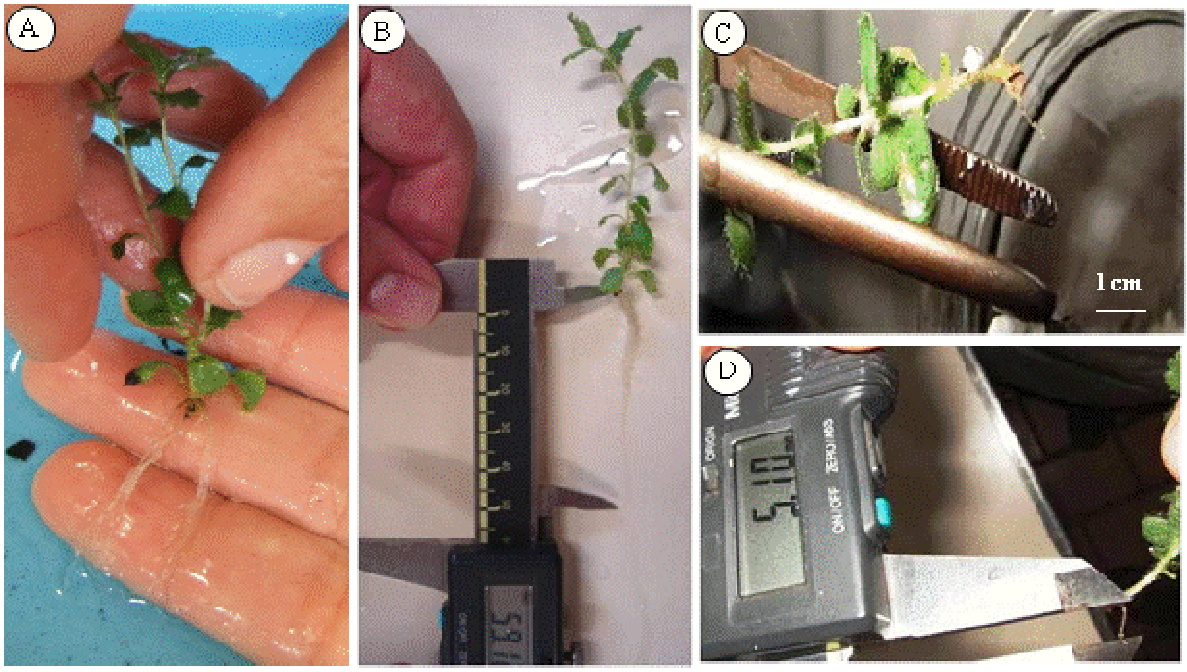

Figure 7 - Rooting of *A. unedo* shoots. A and B - Rooted shoots by the two-steps rooting method. C and D - Shoots rooted through the one-step rooting method.

2.4.4 Acclimatization

In a first experiment a total of 154 plants (C01) obtained from the two-step rooting method were acclimatized. The main objective of this acclimatization experiment was to evaluate if the IBA treatment applied during the rooting phase interfered with further plant development. Three induction IBA treatments were tested and compared with the control (9.8 or 24.7 µM for 6 days or dipping on 9.8 mM solution for 15s). The acclimatization of the rooted shoots (Fig. 8*A*) in the greenhouse (Fig. 8*B*) showed that 87.7 ± 6.4% and 84.4 ± 4.6% of the plantlets have survived after 2 and 4 months in the greenhouse, respectively. The results indicated that acclimatization was not affected by the type and treatment with IBA (P> 0.05; Appendix Table 10). Apical necrosis was never observed during acclimatization. Plantlets from clones AL01, AL02, and AL 03 showed the following rates of plant survival: AL01 (98.2% of plants acclimatized), AL02 (85% acclimatization), and AL03 (94.4% acclimatization). Following acclimatization, plants were placed in a nursery (Fig. 8*C*). Some plants from C01 and AL01-AL03 clones were planted in a field trial, 17 and 24 months after *in vitro* establishment, respectively (Fig. 8*D*). Some of these plants have already produced fruits and keep on growing in the field. (Fig. 8*E*).

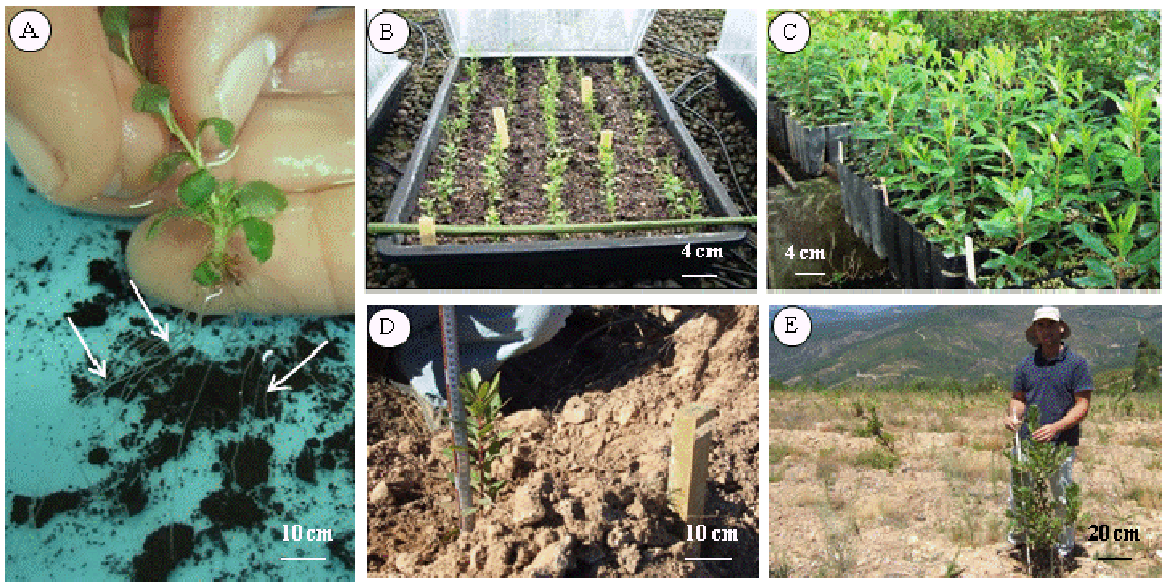

Figure 8 - Acclimatization and field-growth of *A. unedo* micropropagated plants: A – Plant showing a well-developed root system (arrows). B - Rooted plants during the early stages of acclimatization in the greenhouse (30 days). C – Four-month-old plants growing in the nursery. D - Measurement of plant initial height, just after plantation in a field trial. E - Measurement of plant height of 2-year-old *A. unedo* plants in a field trial.

In the second experiment 600 C01 plantlets were used to test the role of different substrates on plant acclimatization (Figs. 9*A*, *B*). After humidity gradually reduction (Fig. 9*C*) plants showed a good development (Figs. 9*D*, *E*). Plant survival was recorded after 2 and 4 months upon transfer to the substrate (Figs. 9*F*, *G*; Table 10).

Table 10 - Effect of the different substrates on acclimatization.

Acclimatization Substrate Composition		Volume (%)	Survival rate (%) (2 months)			Survival rate (%) (4 months)		
I	perlite; peat [(1)]	70:30	90.9 ± 2.11	a		69.7 ± 9.96	b	
II	perlite; peat [(1)]	50:50	95.0 ± 5.00	a		73.8 ± □.0	b	
III	perlite [(1)]	100	0.0 ± 0.00	b		0.0 ± 0.00	c	
IV	perlite	100	98.7 ± 0.98	a		98.0 ± 1.23	a	
V	sand; composted pine bark, peat [(1)]	50:35:15	84.6 ± 4.84	a		69.2 ± 3.66	b	
	Mean		87.7 ± 3.23			79.02 ± 3.34		

In each column values (mean ± SE) followed by different letters are significantly different (P≤0.01).
[(1)] added slow release fertilizer (2.6 g/1 l).

The frequency of acclimatization showed to be dependent of the substrate used (Appendix Table 11). Thus, when fertilizer was added to perlite 100%, the plantlets showed necrosis, probably due to the high amount of nutrients released. However, plants growing on the same substrate without fertilizer showed the highest survival rate (Table 10; Fig. 9*D*).

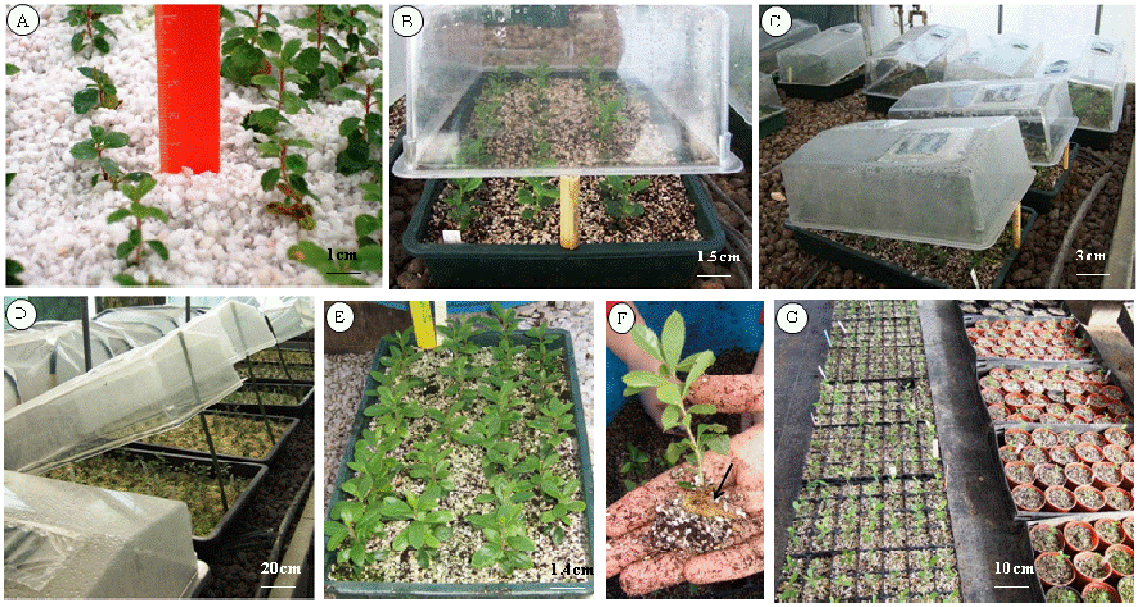

Figure 9 – Acclimatization of C01 plantlets on different substrates. A - Perlite 100%. B - Peat and perlite. C - Reduction of the humidity level by raising gradually the covertures. D and E - Plant development after 4 weeks in two different substrates. F - Plant at the time of being transfer to a new container showing a well developed root system (arrow). G - Plants growing in individual containers.

When the survival rate was evaluated in 4-month-old plants the data confirmed that the best results (and significantly different, Table 10) were achieved with perlite 100% without fertilizer (98.0±1.23%). These plants were further established for cutting production and for clonal trials (Fig. 9*G*).

2.4.5 Anatomical studies

2.4.5.1 Leaf structure

The structure of leaves was analysed at different stages of the micropropagation process trying to find how the culture conditions could affect shoot and plantlet development. The cross sections made on shoot leaves indicated that during the multiplication phase the mesophyll was poorly differentiated (Fig. 10). Sections through the midrib showed the midvein with small amounts of xylem and phloem cells. The mesophyll displayed a reduced number of supporting tissues and no sclerenchyma cells could be found (Fig. 10*A*). Trichomes were usually present in both leaf surfaces (Fig. 10*A*). Transverse sections through the leaf blade showed the presence of two differentiating parenchyma: the palisade parenchyma in the upper surface and a broad spongy parenchyma at the opposite surface. Minor veins and the respective vascular cells were already present (Fig. 10*B*). In general the cells possessed thin walls, lignification was reduced and the cuticle usually thin or incipient (Fig. 10*B*).

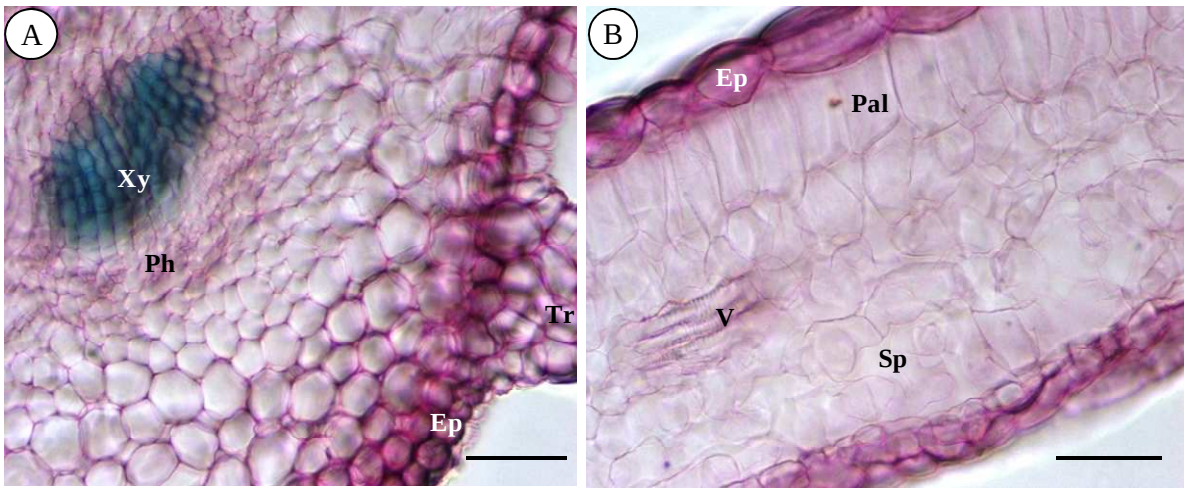

Figure 10 – Cross sections made in leaves during the multiplication phase. A – Midrib zone showing the midvein with the vascular tissues xylem (Xy) and phloem (Ph) and the lower epidermis (Ep). Part of a trichome (Tr) can be seen on the lower right. B – Section through the leaf blade. The two developing parenchyma (Pal and Sp) can be observed as well as a minor vein (V). Bar indicates 50 µm.

During the rooting phase, after 15 days in the root development medium, the structure of the leaves (Fig. 11) was similar to that observed during the multiplication phase. However, some features showed to be different. Thus, it was found that the vascular system appeared now more developed, the midrib thicker (Fig. 11*A*), and the cells of the palisade parenchyma more elongated but still reduced to a one layer thick (Fig. 11*B*). Some cells surrounding the vascular tissues showed signals of lignification (Fig. 11*C*).

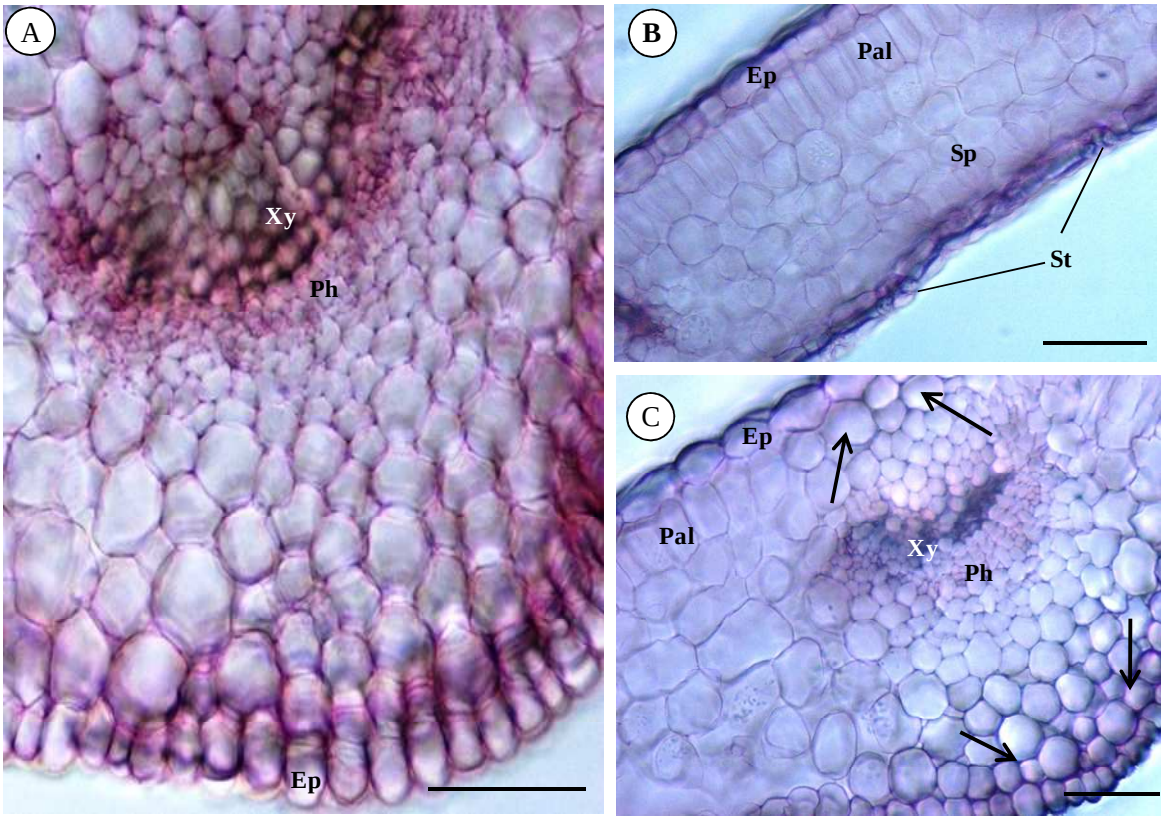

Figure 11 – Leaf cross sections during the rooting phase. A – Cross through the midrib showing the vascular tissues xylem (Xy) and phloem (Ph) and the lower epidermis (Ep). B - Transverse section across the leaf blade showing the palisade (Pal) and the spongy (Sp) parenchymas. Two stomata (St) can be seen in the lower epidermis. C – As in A showing the lignifications of some cells surrounding the vascular tissues and beneath the lower epidermis (arrows). Bar indicates 50 µm.

Following acclimatization and transfer to soil conditions the structure of the leaves deeply changed displaying the usual features commonly found in field-growing dicotyledonous plants. Leaves were much thicker (Fig. 12*A*) and two layers of palisade parenchyma could be observed (Fig. 12*A*) instead of the only layer observed during *in vitro* culture. The amount of xylem and phloem in the midvein was much higher than during *in vitro* culture (Fig. 12*A*). The cells surrounding the vascular tissues showed clear signals of lignification (Fig. 12*A*).

The midrib was more developed showing collenchyma cells (Figs. 12*A*, *C*). A cambial zone could be detected, as well as secondary vascular tissues and ray cells (Fig. 12*B*). Epidermal cells showed a thick cuticle (Fig. 12*C*) in both epidermis and some cells accumulated a stainable material presumably of phenolic origin (Fig. 12*C*).

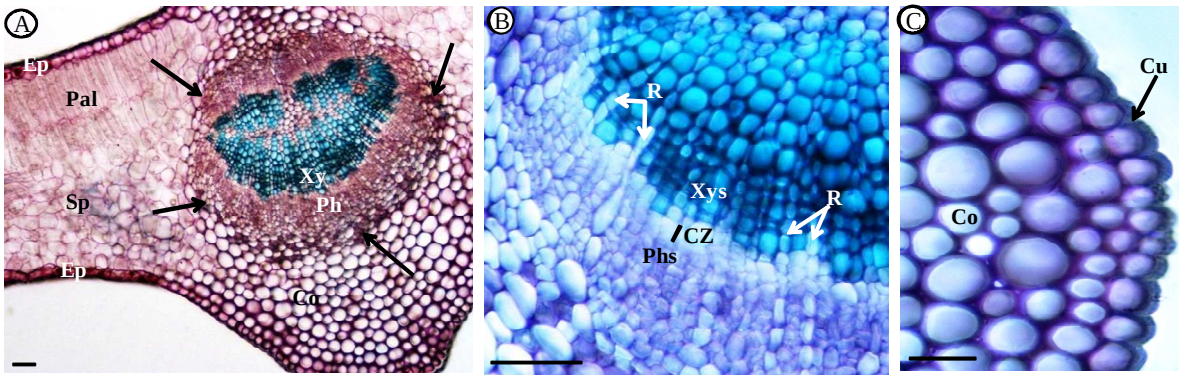

Figure 12 - Cross sections made in leaves one year after acclimatization. A - Section through the central part of a leaf showing two layers of palisade parenchyma (Pal). Note the development of the vascular tissues (Xy and Ph), the presence of lignified cells surrounding the vascular tissues (arrows) and collenchyma cells (Co). B - Section through the midvein showing the cambial zone (CZ) and the development of secondary xylem and phloem (Xys and Phs). Ray cells (R) can be seen. C - Part of the lower surface at the midrib showing the thick cuticle (Cu). Some collenchyma (Co) cells under the epidermis (Ep) are also visible. Bar indicates 50 µm.

2.4.5.2 Adventitious root formation

Cross sections made on *in vitro* formed shoots before adventitious root induction showed the occurrence of two types of trichomes at the periphery: large glandular trichomes and less developed non-glandular trichomes (Fig. 13*A*). Below the epidermis several layers of large parenchyma cells were present building the cortex region (Fig. 13*A*). In the central part of the stem a pith zone of parenchyma cells was surrounded by a more or less continuous ring of vascular tissues (Fig. 13*A*). Some larger cells which appear to be differentiating fibres were seen in close contact with the primary phloem (Figs. 13*A*, *B*). In the cortex parenchyma cells starch grains could be seen (Fig. 13*B*). Secondary growth was already initiated as indicated by the presence of a cambial zone between the secondary xylem and phloem (Figs. 13*B*, *C*).

Shoot cross sections at the basal cut end of the shoots where roots usually appeared showed the initiation of root primordia, eight days after root induction (Figs. 13 *D*, *E*). In the central part of the stem a pith zone of parenchyma cells containing large amounts of starch grains could be observed (Fig. 13 *D*). The anatomical data seem to indicate that adventitious roots had a deep origin in the stem, presumably from the cambial zone and/or from phloem cells (Fig. 13*E*).

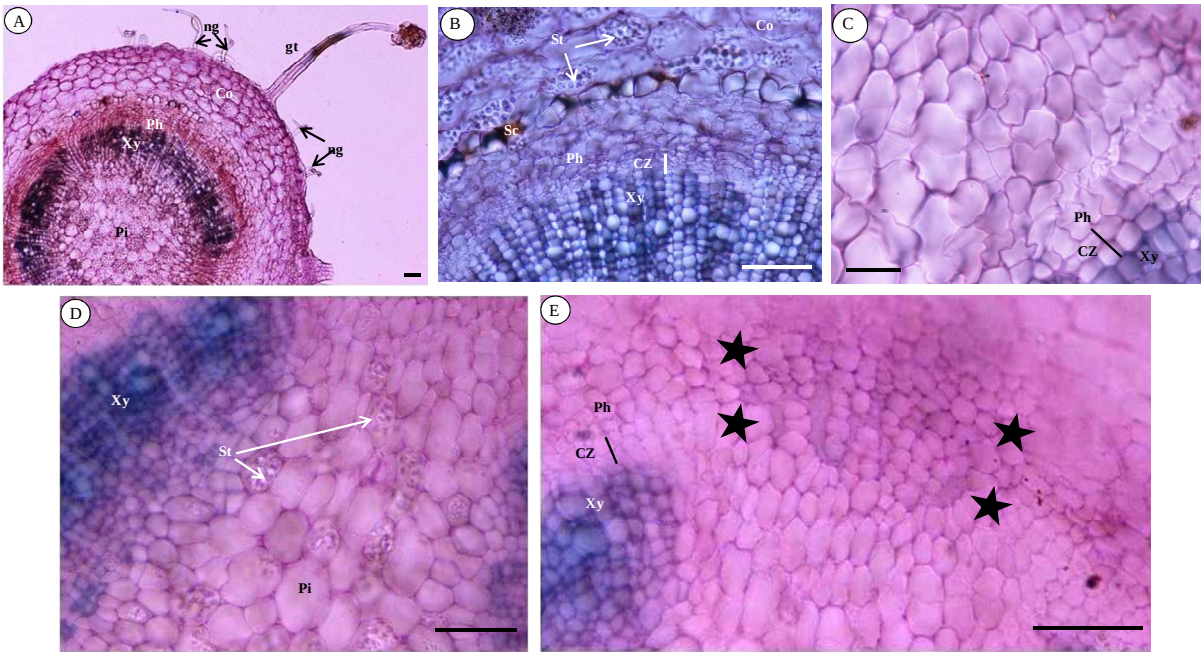

Figure 13 - Cross section of shoots before and 8 days after root induction (A-C and D-E, respectively). A- Cross section of a shoot before root induction showing glandular (gt) and non-glandular (ng) trichomes; the cortex region (Co) and the pith zone (Pi) surrounded by a ring of vascular tissues (Xy and Ph). B – A more amplified view of the shoot tissues showing differentiating fibers (Sc), which were in contact with the secondary phloem (Ph). Note the cambial zone (CZ) and starch in the cortex parenchyma cells; C – Cambial zone (CZ) developing secondary phloem (Ph) and xylem (Xy). D - Zone of adventitious root formation. Cells accumulating large amounts of starch grains (St) can be seen. E – Cell divisions in the cambial zone (CZ) and in phloem (Ph) cells (between stars) were the adventitious roots presumably have origin. Bar indicates 50 µm.

Transverse sections made on shoots (8 days after transfer to root development medium) showed that the earlier stages of root differentiation came along with incipient callus resulting from the proliferation of cortex and/or epidermal cells (Figs. 14*A*, *B*). However, these small calli never showed an effective proliferation and usually could not be visible to the naked eye. Following root primordia formation, the developing roots proliferated trough the cortex until they appeared at the shoot surface (Figs. 14 *C*, *D*; 15 days after transfer to root development medium). In general more than one root was formed per shoot starting from different points of the cambial zone. In some sections it was evident that the vascular system of the adventitious roots was in close association with the vascular tissues of the shoot (Fig. 14 *D*). Roots originating from epidermal or subepidermal cells were never observed.

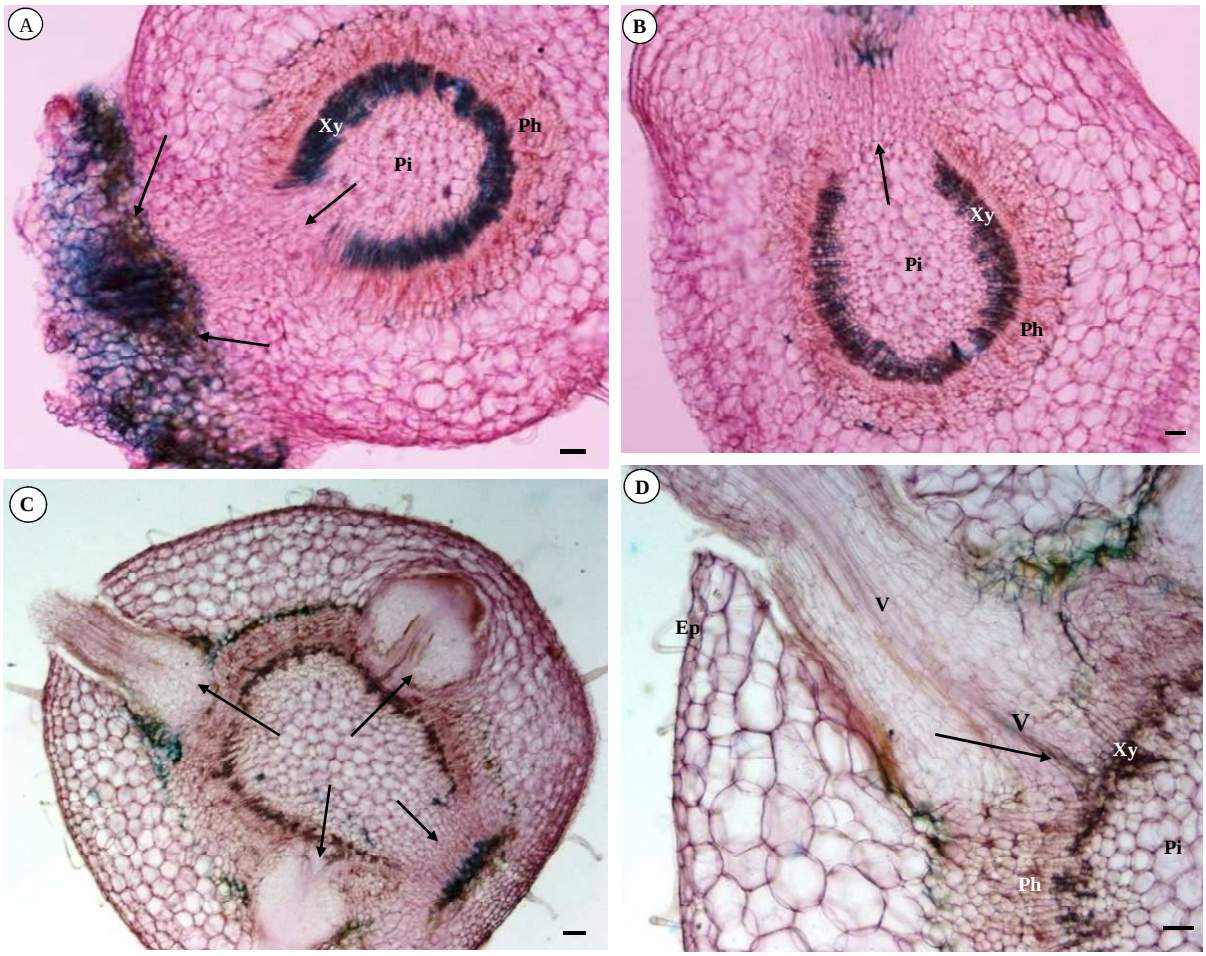

Figure 14 - Cross sections of rooted shoots. A – Root initiation (arrow) from the cambial zone showing cell proliferation (incipient callus, two arrows zone) at the root surface. B – Root initiation (arrow) from the cambial zone after 8 days on a root development medium. C – Transverse section of a shoot showing 4 developing roots (arrows) at different developmental stages. D – Detail of a developing adventitious root. The connection between the root and the vascular tissues of the shoot can be seen (arrow); (Xy - xylem; Ph - phloem; Pi - pith; V – vascular tissues). Bar indicates 100 µm in A- C and 50 µm in D.

2.5 DISCUSSION

2.5.1 Culture establishment

Juvenile explants are often used for the micropropagation of woody species due to the difficulties encountered when explants from adult plants are used for cloning (Rathore *et al.*, 2004). However, juvenile plants are usually of unknown genotype, making difficult the cloning of adult selected trees (Hacket, 1985). This is also the case in *Arbutus unedo* in which previous assays of micropropagation used potted plants growing in the greenhouse (Gonçalves and Roseiro, 1994; Mereti *et al.*, 2002). Partially successful attempts to propagate *A. unedo* from adult plants were obtained by Mendes (1997). However, the results showed that the multiplication rate was greatly impaired by the high number of contaminated cultures and the reduced establishment period. Moreover, plant establishment could only be achieved during early spring, thus reducing the efficiency of the establishment stage. Our results showed that epicormic shoots from branches of adult trees are a good source of explants (shoot apices and nodal segments) for the propagation of mature trees (ESAC05 and AL01 to AL06). This is particularly true when the branches were maintained in a growth culture chamber (covered with plastic), probably because the controlled light and temperature were more adequate for shoot growth. In the greenhouse, the epicormic shoot development was enhanced when branches were sprayed with BA. The role of cytokinins on promoting axillary shoot proliferation and their ability to stimulate shoot development is known (Chawla, 2009). The induction of epicormic shoot development from large stem segments of adult trees is a suitable source of explants because a reduction in the levels of contamination can be achieved and less injury is inflicted in trees than when they are cut down to force coppiced shoots with juvenile characteristic (Preece, 2008). Several authors have applied this strategy to obtain juvenile explants from adult trees (Preece, 2008). Recently another species of the Ericaceae family (*Vaccinium cylindraceum*) was also propagated through axillary shoot proliferation from epicormic stems (Pereira, 2009).

On his earlier work Murashige (1974) indicated that plant regeneration through axillary shoot proliferation involves several steps and that the success of each step is conditioned by the rate of success in the previous phase. The *in vitro* establishment of explants which can be further used in the multiplication phase is quite relevant. The results obtained with *A. unedo* showed that the levels of explant establishment could reach values over 60%, mainly when explants from the plants ESAC05 and AL02 were used. However, explants from other plants

(AL05 and AL06) displayed considerably lower rates of establishment and, in some cases, the percentages of explant establishment after 12 weeks of culture were lower than 15%. Contamination of the explants contributed to the reduced number of established plants from AL06 explants, and senescence in culture was responsible for the reduced number of established plants from AL05 explants. It does not seem plausible that the difference between explant establishment in *A. unedo* could be attributed to the genotype. Instead, it seems more reasonable to improve the conditions of disinfection and culture of the explants in the cases in which the rate of success is too low.

The data obtained indicated that shoot apices are more effective for explant establishment than nodal segments. Similar results have been found in other woody species studied in our lab such as *Ceratonia siliqua* (Panteleitchouk, 2002), *Eucalyptus nitens* (Gomes and Canhoto, 2003) and *Leucadendron laureolum* x *L. salignum* cv. (Ferreira *et al.*, 2003). Since the levels of contamination were similar in the two explant types, the better performances of shoot apices are due to the lower rates of tissue senescence. The reason for this behaviour is not clear; it is possible that nodal segments, due to their larger size, exude a higher number of phenolic compounds, which inhibited plant growth upon oxidation. At the time of culture, the shoot apical meristem is not a dormant structure and is surrounded by several leaf primordia. These produce PGRs which promote cell division and elongation (auxins) and reduce senescence (cytokinins). These characteristics may explain the better performance of this type of explant. However, other factors may also be involved, since in some species nodal segments are very effective for plant micropropagation (Gonzalez *et al.*, 2000; Ibañez *et al.*, 2005). This study suggests that further experiments on *A. unedo* micropropagation should preferentially use shoot apices as explants.

2.5.2 Shoot multiplication

The best results for shoot multiplication were obtained with FS medium, especially when number of shoots formed per test tube for further multiplication (SNX) by the clone C01 was evaluated. The three basal media used in our experiments differed only in the composition of the macronutrients and the results obtained must necessarily reflect these differences. The AND medium has reduced levels of macronutrients that might explain the reduced rates of shoot multiplication. FS and MS media have similar compositions to FS medium possessing reduced levels of nitrogen both in the form of NH_4^+ and NO_3^-. The amount and type of nitrogen can influence *in vitro* morphogenic processes (Young and Cameraon, 1985; Tsai and

Saunders, 1999; Dal Vesco and Guerra, 2001), but in the case of *A. unedo* more assays are necessary to evaluate the role of nitrogen on *in vitro* propagation. Another possibility is that the higher levels of sodium present in the FS medium can have a role on shoot proliferation. Sodium is a trace element important for some plants (Salisbury and Ross, 1992) but its role on the micropropagation process has not been evaluated. Finally, we can speculate that these variations in the mineral composition can interfere with membrane transport and affect the pH of plant cells thus influencing the *in vitro* response (Niedz, 1994). However, it should be noted that experiments performed with *A. unedo* and other Ericaceae indicate that different culture media, other cytokinins and sucrose were also able to promote shoot proliferation. Thus, Gonçalves and Roseiro (1994), Roseiro (1994) and Mereti *et al.* (2002) showed that WPM (Lloyd and McCown, 1980) combined with BA gave the best rates of shoot proliferation whereas Mendes (1997) pointed out that and Anderson medium (Anderson, 1984) and the cytokinin N6-(2-isopentenyl) adenine (2-iP) were particularly effective to propagate adult material. In *Vaccinium corymbosum*, a member of the strawberry tree family, high sucrose levels in the propagation medium increased shoot proliferation (Cao *et al.*, 2003). Some of these factors such as sucrose and other cytokinins are now being tested with *A. unedo* in order to increase the success of the micropropagation process. Our data suggests that shoot multiplication might be influenced by the genotype of the explants. Although we could not find statistical differences in the multiplication rate of the clones, the data showed that some (AL04, AL05, and AL06) were difficult to propagate, since they formed shoots that were unable to elongate. On the other hand, shoots from clones AL01, AL02, and AL03 displayed a normal growth, which makes them more interesting both for shoot multiplication and rooting. The role of the genotype in several morphogenic processes occurring *in vitro* is well established, it may be related with the levels of endogenous growth regulators in the explants of different genotypes (Bhau and Wakhlu, 2001; Gajdošová *et al.*, 2007). In our case, it is possible that the slow-growing shoots possess unbalanced levels of endogenous cytokinins and/or auxins, which in the tested conditions, were inhibitory for shoot growth. If this is the case, the manipulation of auxin and cytokinin concentration or the inclusion of other growth regulators, such as gibberellins, may result in a more effective growth of these shoots, thus promoting the rates of multiplication (Hansen *et al.*, 1999).

The cross sections made on shoot leaves showed that during the multiplication phase the mesophyll was poorly differentiated. Most cells of the mesophyll were of the spongy parenchyma type showing more or less enlarged spaces between them. Our observations agree with the report of Ziv and Chen (2008), who refer that leaves of several tissue cultured

plants showed to have poorly differentiated tissues with a very thin palisade tissue. The cross sections of *A. unedo* leaves from *in vitro* shoots also showed a thin cuticle and limited vascular development. During the rooting phase, some cells surrounding the xylem showed signals of lignification. Taken together the observations made on *A. unedo* leaves are not very distinct from the features observed in other species which, according with Preece and Sutter (1991) and Ziv and Chen (2008), include the presence of underdeveloped vascular tissues, occurrence of many starch grains in some cell types and reduced lignification. The specific *in vitro* environment where the shoots develop, in particular the high humidity levels present in the culture vessels, is presumably responsible for these anatomical features. One year after acclimatization, *A. unedo* leaves displayed a normal anatomy with the development of two layers of palisade parenchyma, well developed veins as well as a prominent cuticle indicating that the process of acclimatization was effective.

2.5.3 Rooting

Rooting of micropropagated shoots is a crucial step for the success of the propagation process (George and Debergh, 2008; Chawla, 2009). Using the two-step rooting method, our data showed that shoots of *A. unedo*, C01 clone (6-month-old cultures after *in vitro* establishment) has a high rooting ability since shoots rooted even in media without auxins. However, a treatment of the shoots for six days with IBA (24.7µM) or a pulse of 15 s at high IBA concentrations ($9.8x10^3$ µM) considerably increased the rates of root formation (88.3±8.3% and 85.0±4.3%, respectively) over the control (46.7±9.5%). In all plantlets obtained, callus formation at the shoot base or apical necrosis was never observed. Similar frequencies of induction were obtained by Meretti *et al*. (2002), when explants from potted plants growing in the greenhouse were tested. The same authors also showed that IBA is more effective than IAA on root formation. An auxin shock proved to be effective in the assays carried out by Mackay (1996) in *Arbutus xalapensis* and by Mendes (1997) in *Arbutus unedo*.

A two-step rooting method and shoots of the same clone (C01) were used to test the effect of the two different proliferation culture media (FS and AND) as well as a broad range of auxin concentrations. These shoots were from 15-month-old cultures after *in vitro* establishment. The results indicated that the percentage of rooted shoots was affected by IBA concentration, shoot proliferation basal medium and the interaction between these two factors. The best results were observed when AND and FS were used in combination with

IBA at concentrations of 9.8, 24.6 and 49.2 µM with rooting rates ranging from 93.3% to 100%. When no IBA was added to the medium, rooting was considerably reduced ($P \leq 0.01$) showing rooting percentages of 5.6% and 9.1%, respectively for AND and FS medium. Mereti *et al*., (2002) and Mereti *et al*., (2003) using actively growing shoots of potted greenhouse plants of *A. unedo* have reported that the highest percentages of rooting were achieved in 10 µM IBA (92%) and 10 µM IAA (82%). Rœiro (1994) and Gonçalves and Roseiro (1994) using shoots of one-year-old potted greenhouse plants of *A. unedo* have shown that the highest percentages of rooting were achieved with 9.8 µM IBA (93%) although the results (80% rooting induction) did not significantly differ from those obtained on a medium with higher IBA concentrations (24.6 µM). When compared with the results we have obtained the differences may be due to the fact that in our experiments explants from adult trees were used. Moreover the coppiced (C01) or epicormic shoots (AL01 to AL06) that were used in our assays may have promoted a more effective rejuvenation thus promoting root development. According to Salisbury and Ross (1992) coppiced shoots are a suitable juvenile material since their origin is linked to dormant buds formed during earlier stages of plant development. However, other authors have pointed out that shoots arising from dormant buds of adult plants usually display a juvenile morphology although precocious flowering has been observed on plants derived from this type of explants (Preece, 2008) suggesting that coppiced shoots do not produce shoots so rejuvenated as usually thought. This feature can explain why shoots of the same clone C01 showed different rooting responses to auxin. Thus, younger shoots, from 6-month-old cultures possessed a high rooting ability, even in the control (46.7±9.5%), a situation that contrasts to the reduced levels of rooting (7.3±1.4%) obtained with 15-month-old shoots under the same conditions.

The higher cost of *in vitro* propagated plants is one of the reasons that have impaired the application of this technology to the propagation of many plant species at a commercial scale. Thus, all the technical improvements that can reduce costs during the micropropagation process are of particular interest. In *A. unedo* we have tried to reduce the labor and laboratory costs to achieve root formation using a one-step protocol in which different concentrations of IAA and charcoal were used. The results indicated that the mean rooting rate (38.8%) was lower than in the two-step method (72.3%). Best results (55.0%; $P \leq 0.05$) were obtained when IAA was used at the highest concentration without charcoal. Similar results were obtained for the number of roots per shoot ($P \leq 0.05$). Also in *A. unedo* Mereti *et al.* (2002) and Mereti *et al*. (2003) found that IAA was more effective for rooting than IBA. Using IAA these authors have obtained rooting percentages over 95%. Lower auxin concentrations or weaker auxins

are often used in attempts to induce adventitious roots in order to avoid callus formation or the toxic effects of stronger auxins (Chawla, 2009). However, endogenous auxins as IAA are more prone to be metabolized or inactivated by conjugation with endogenous organic compounds such as sugars and aminoacids in plant tissues than synthetic auxins what can explain the reduced percentages of rooting generally obtained with natural auxins (Machakova *et al.*, 2008).

In the case of charcoal we were unable to find a positive role ($P \leq 0.05$) for this substance on root formation both in the one-step and two-step rooting protocols. The only positive effect observed was the formation of an increased number of roots in the two-step protocol. It is well known that charcoal can absorb a large array of compounds from the culture medium or compounds produced and released to the medium by plant tissues that would affect morphogenic processes (Fridborg and Eriksson, 1975). This includes compounds such as phenolics, vitamins and other organic compounds, and PGRs (Eymar *et al.*, 2000). It is possible that in the case of *A. unedo* one-step rooting, charcoal can reduce the levels of auxin present in the culture medium to a suboptimal level that reduces adventitious root formation. The possibility that charcoal can absorb other compounds necessary for root formation cannot be ruled out. From the data already obtained it can be concluded that root induction is more effective when strawberry tree shoots are submitted to a two-step rooting protocol using IBA as has been also observed in other members of the Ericaceae family (Gonçalves and Roseiro, 1994; Roseiro, 1994; Mackay, 1996; Mendes, 1997; Eeckaut *et al.*, 2010). Root induction and development are complex processes that are influenced by a large number of factors, such as genotype, type and concentration of PGRs, and culture conditions (Bennett *et al.*, 1994; Mylona and Dolan, 2002). In this way, it is not surprising that the conditions to achieve root formation are widely variable between different species and in the same species or cultivar. The age of the explants used in our experiments (epicormic shoots of an adult tree), as well as the genotype may help to explain the differences between our results and the data obtained by other authors working with the same species.

Adventitious roots formed in micropropagated shoots may have different origins. Roots may be formed from more peripheral tissues such the epidermis or the cortex or may have a deeper origin from the phloem, the vascular cambium or even from pith tissues (Ziv and Chen, 2008). An origin from or near the vascular tissues is the ideal situation since the new roots are usually in close association with the vascular tissues of the stem thus avoiding problems related with the effective ascent of sap. Poor vascular connections between the developing root and the original shoot have been often found to contribute to the reduced

survival of tissue culture plantlets during their acclimatization (Smith *et al.*, 1991; Smith *et al.*, 1992; Ziv and Chen, 2008). The sections made on shoots of *A. unedo* following root induction indicated that the adventitious roots have its origin near the vascular tissues probably from the secondary phloem and/or from the vascular cambium. Roots originated from more peripheral tissues were never found. Root formation showed to be an asynchronous process with roots at different developmental stages being present in the same shoot. Root primordia were first detected at the end of the root induction treatment (8 days) and the first roots appeared at the shoot surface by the tenth day of culture on root development medium. Similar results were reported by Gonçalves *et al.* (1998) with *C. sativa* microcuttings, by Canhoto and Cruz (2000) in *Feijoa sellowiana,* and in some other species (Ziv and Chen, 2008). On these species the processes of initiation and development of adventitious roots were also asynchronous, but had a similar sequence of events.

The histological analysis performed in roots of *A. unedo* also showed that although an incipient callus could sometimes be formed, roots were never seen developing from proliferating callus a situation often occurring during *in vitro* rooting. The origin of the roots and the absence of callus formation are probably responsible by the high number of acclimatized plants obtained in *A. unedo*.

2.5.4 Acclimatization

The success of any process of plant cloning can be evaluated by the number of regenerated plants that can survive in field conditions, following acclimatization and hardening (Kirdmanee *et al.*, 1995). A substantial number of micropropagated plants are unable to survive following transference from *in vitro* to greenhouse or field conditions. This change of environment results in reduced levels of humidity and light intensity as well as exposition to several kinds of microorganisms that can be detrimental for plant survival (Hazarika, 2003). Our results with *A. unedo* showed that plant survival rates after 2 and 4 months ranged from 84% (first experiment) to 98% (second experiment) according to the different clones.

The acclimatization experiments revealed that the addition of a fertilizer to the substrate perlite is not recommended for *A. unedo*. On these conditions 100% of the plantlets exhibited necrosis of the shoot apice. However, perlite 100% without fertilizer gave the best frequencies of acclimatization indicating that the inhibitory effect is fertilizer-related. This fact might be linked to the high level of nutrient release from the fertilizer, due to the high temperatures and water content associated with the lower ability of perlite to retain cations.

Assays of acclimatization carried out by other authors working with *A. unedo* showed rates of acclimatization ranging from 68% after 3 months (Gonçalves and Roseiro, 1994; Roseiro, 1994) to 90% after 1 month (Mereti *et al.*, 2002). The use of perlite (100%) as substrate has some advantages when compared to other substrates such as composted pine bark or peat: a) perlite avoids the need of substrate sterilization, b) roots are well aerated and not submitted to an excess of water, and c) diseases caused by fungi like *Botrytis* sp. are usually not found, avoiding the need for fungicide application. However, there is also an important drawback related to the lack of nutrients. This can be worked out by spraying weekly the plants with a nutrient solution. Some of our plants have been planted in field (since November 2007), and their performances related to plant growth, fruit production and fruit quality will be evaluated and compared with others strawberry plants. Recently, it was found that some of the *in vitro* propagated plants growing in the field produced the first fruits, about three years after plantation.

Taken together our results show that the procedures adopted in this work are the basis of a reliable and reproducible protocol to the cloning of selected adult trees of *A. unedo*. However, the method's success is lessened by the high number of contaminations in some clones and the slow-growth of shoots during the multiplication phase. Therefore, attempts to reduce these factors through the refinement of the technique have been carrying out. Alternative ways of micropropagation through the formation and conversion of somatic embryos and organogenesis have also been carrying out with very promising results referred by Canhoto *et al.* (2007), Gomes *et al.* (2009) and on Chapter 5.

2.6 REFERENCES

Almeida, R., Gonçalves, S., Romano, A., 2005. *In vitro* propagation of endangered *Rhododendron ponticum* L. subsp. *baeticum* (Boissier & Reuter) Handel-Mazzetti. Biodiv. Conservation 14, 1059-1069.

Anderson, W.C., 1984. A revised tissue culture medium for shoot multiplication of *Rhododendron*. J. Amer. Soc. Hort. Sci. 109, 343-347.

Arezki, O., Boxus, P., Kevers, C., Gaspar, T., 2000. Hormonal control of proliferation in meristematic aglomerates of *Eucalyptus camaldulensis* Dehn. In Vitro Cell. Dev. Biol.-Plant 36, 398-401.

Banko, T.J., Stefani, M.A., 1989. *In vitro* propagation of *Oxydendrum arboreum* from mature trees. Hortscience 24, 683-685.

Bennett, I.J., McComb, J.A., Tonkin, C.M., Mcdavid, D.A.J., 1994. Alternating cytokinins in multiplication media stimulates *in vitro* shoot growth and rooting of *Eucalyptus globulus* Labill. Ann. Bot. 74, 53-58.

Bhau, B.S., Wakhlu, A.K., 2001. Effect of genotype, explant type and growth regulators on organogenesis in *Morus alba*. Plant Cell Tis. Org. Cult. 66, 25-29.

Canhoto, J.M., Cruz, G.S., 2000. Micropropagation of pineapple guava through organogenesis and axillary shoot proliferation. Acta Hort. 520, 109-118.

Canhoto, J.M., Lopes, M.L., Sequeira, J., Gomes, F., 2007. Somatic embryogenesis and organogenesis induction from adult trees of *A. unedo* and *A. canariensis*., IUFRO Tree Biotechnology-2007, Azores, p. *Abst* 12.

Cao, X., Fordahm, I., Douglas, L., Hammerschlag, F.A., 2003. Sucrose levels influences micropropagation and gene delivery into leaves from *in vitro* propagated highbush blueberry shoots. Plant Cell Tis. Org. Cult. 75, 255-259.

Cardoso, A.V.R., 2004. Historial recente da propagação vegetativa do medronheiro no Algarve, contribuição para a criação de um futuro parque de pés-mãe. Tese de Licenciatura. ISA - UTL, Lisboa.

Chawla, H.S., 2009. Introduction to plant biotechnology, 3rd ed. Science Publis., Inc., New Hampshire.

Dal Vesco, L.L., Guerra, M.P., 2001. The effectiveness of nitrogen sources in *Feijoa* somatic embryogenesis. Plant Cell Tis. Org. Cult. 64, 19-25.

De Fossard, R.A., Nitsch, C., Cresswell, R.J., Lee, H.C.M., 1974. Tissue and organ culture of *Eucalyptus*. N. Zeal. J. For. Sci. 4, 267-278.

Duncan, D.B., 1955. Multiple range and multiple F tests. Biometry 11, 1-42.

Eeckaut, T., Janssens, K., De Keyser, E., De RieK, J., 2010. Micropropagation of *Rhododendron*. In: Jain, S.M., Ochatt, S.J. (Eds.), Protocols for *in vitro* propagation of ornamental plants. Springer, Dijon, pp. 141-152.

Eymar, E., Alegre, J., Toribio, M., López Vela, D., 2000. Effect of activated charcoal and 6-benzyladenine on *in vitro* nitrogen uptake by *Lagerstroemia indica*. Plant Cell Tis. Org. Cult. 63, 57-65.

Ferreira, C., Dias, J.D., Canhoto, J.M., 2003. *In vitro* propagation of *Leucadendron laureolum* x *L. salignum* cv. Safari Sunset and cytological analysis of the regenerated plantlets. Acta Hort. 602, 29-38.

Fridborg, G., Eriksson, T., 1975. Effects of activated charcoal on growth and morphogenesis in cell cultures. Physiol. Plant. 34, 306-308.

Gajdošová, A., Ostrolucká, M.G., Libiaková, G., Ondrušková, E., 2007. Protocol for micropropagation of *Vaccinium vitis-idaea* L. In: Jain, S., Häggman, H. (Eds.), Protocols for Micropropagation of Woody Trees and Fruits. Springer, Berlin, pp. 457-464.

Gautheret, R.J., 1959. La culture des tissues végétaux, techniques et réalisations. Masson, Paris.

George, E.F., Debergh, P.C., 2008. Micropropagation: uses and methods. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 29-64.

Godinho-Ferreira, P.G., Azevedo, A.M., Rego, F., 2005. Carta da tipologia florestal de Portugal Continental. Silva Lusitana 13, 1-34.

Gomes, F., Canhoto, J.M., 2003. Micropropagation of *Eucalyptus nitens* Maiden (Shining gum). In Vitro Cell. Dev. Biol.-Plant 39, 316-321.

Gomes, F., Lopes, M.L., Santos, T., Canhoto, J.M., 2009. Micropropagation of selected trees of *Arbutus unedo* L. trough axillary shoot proliferation and somatic embryogenesis. Acta Hort. 839, 111-116.

Gonçalves, J.C., Diogo, G., Amâncio, S., 1998. *In vitro* propagation of chestnut (*Castanea sativa x Castanea crenata*): effects of rooting treatments on plant survival, peroxidase activity and anatomical changes during adventitious root formation Sci. Hort. 72, 265-275.

Gonçalves, J.C., Roseiro, R.J., 1994. Establishment and *in vitro* multiplication of *Arbutus unedo* L. seedlings. VIII International Congress of Plant Tissue and Cell Culture, Firenze, Italy, p. *Abst* 20.

Gonzalez, M.V., Lopez, M., Valdes, A.E., Ordas, R.J., 2000. Micropropagation of three berry fruit species using nodal segments from field-grown plants. Ann. App. Biol. 137, 73-78.

Guimarães, M.L., Cruz, G.S., Montezuma-de-Carvalho, J.M., 1988. Somatic embryogenesis and plant regeneration in *Cyphomandra betacea* (Cav.) Sendt. Plant Cell Tis. Org. Cult. 15, 161-167.

Hacket, W.P., 1985. Juvenility, maturation and rejuvenation in woody plants. Hort. Rev. 7, 109-155.

Hansen, E., Olsen, J.E., Junttila, O., 1999. Gibberellins and subapical cell divisions in relation to bud set and bud break in *Salix pentrana*. J. Plant Growth Reg 18, 167-170.

Hartman, H.T., Kester, D.E., Davies, J.F.T., Geneve, R.L., 1997. Plant propagation: principles and practices, 6th ed. Prentice-Hall, Inc., New Jersey.

Hazarika, B.N., 2003. Acclimatization of tissue-cultured plants. Curr. Sci. 85, 1704-1712.

Heywood, V.H., 1993. Flowering Plants of the World. B.T. Batsford Ltd., London.

Ibañez, A., Valero, M., Morte, A., 2005. Establishment and *in vitro* clonal propagation of the Spanish autochthonous table grapevine cultivar Napoleon: an improved system where proliferating cultures alternate with rooting ones Ann. Biol. 27, 211-220.

Isutsa, D.K., Pritts, M.P., Mudge, K.W., 1994. Rapid propagation of blueberry plants using *ex-vitro* rooting and controlled acclimatization of micropropagules. Hortscience 29, 1124-1126.

Jain, S.M., Häggman, H., 2007. Protocols for Micropropagation of Woody Trees and Fruits. Springer, Berlin.

Kirdmanee, C., Kitaya, Y., Kozai, T., 1995. Effects of CO_2 enrichment and supporting material *in vitro* on photoautotrophic growth of *Eucalyptus* plantlets *in vitro* and *ex vitro*. In Vitro Cell. Dev. Biol.-Plant 31, 144-149.

Lloyd, G.B., McCown, B.H., 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Proc. Int. Plant Prop. Soc. 30, 421-427.

Machakova, I., Zazimalova, E., George, E.F., 2008. Plant growth regulators I: Introduction; auxins, their analogues and inhibitors. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 175-204.

Mackay, W.A., 1996. Micropropagation of *Texas madrone, Arbutus xalapensis* HBK. Hortscience 31, 1028-1029.

McComb, J.A., Bennett, I.J., 1986. Eucalypts (*Eucalyptus* spp.). In: Bajaj, Y.P.S. (Ed.), Biotechnology in Agriculture and Forestry. Springer-Verlag, Berlin, pp. 350-362.

Mendes, M.L.A., 1997. Multiplicação vegetativa *in vitro* de medronheiro. Tese de Mestrado. ISA-UTL, Lisboa.

Mereti, M., Grigoriadou, K., Leventakis, N., Nanos, G.D., 2003. *In vitro* rooting of strawberry tree (*Arbutus unedo* L.) in medium solidified by peat - perlite mixture in combination with agar. Proc. First Int. Symp. on Acclimat. and Estab. of Microp. Plants, pp. 207-210.

Mereti, M., Grigoriadou, K., Nanos, G.D., 2002. Micropropagation of the strawberry tree, *Arbutus unedo* L. Sci. Hort. 93, 143-148.

Metaxas, D.J., Syros, T.D., Yupsanis, T., Economou, A.E., 2004. Peroxidases during adventitious rooting in cuttings of *Arbutus unedo* and *Taxus baccata* as affected by plant genotype and growth regulator treatment. Plant Growth Regul. 44, 257-266.

Murashige, T., 1974. Plant propagation through tissue culture. Ann. Rev. Plant Physiol. 25, 135-166.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant. 15, 473-497.

Mylona, P., Dolan, L., 2002. The root meristem. In: McManus, M.T., Weit, B.E. (Eds.), Meristematic Tissues in Plant Growth and Development. Sheffield Academic Press, Sheffield, pp. 279-292.

Neppi, M., 2001. Alberi ed arbusti della flora mellifera della regione mediterrânea. In: Piotto, B., Noi, A. (Eds.), Propagazione per seme di alberi e arbusti della flora mediterranea. Dipart. Prev. Risanam. Ambient., Manuale ANPA (Agenz. Naz. per la Protezi. dell' Ambiente), pp. 44-49.

Niedz, R.P., 1994. Growth of embryogenic sweet orange callus on media varying in the ratio of nitrate to ammonium nitrogen. Plant Cell Tis. Org. Cult. 39, 1-5.

Ostrolucká, M.G., Gajdošová, A., Libiaková, G., Hrubíková, K., Bezo, M., 2007. Protocol for micropropagation of selected *Vaccinium*. In: Jain, S.M., Häggman, H. (Eds.), Protocols for Micropropagation of Woody Trees and Fruits. Springer, Berlin, pp. 445-455.

Pabuccuoglu, A., Kivcak, B., Bas, M., Mert, T., 2003. Antioxidant activity of *Arbutus unedo* leaves. Fitoterapia 74, 597-599.

Panteleitchouk, A.V., 2002. Micropropagacao de alfarrobeira (*Ceratonia siliqua* L.) variedade Aida. Estudos quimicos e de microscopia. Tese de Mestrado. FCTUC, Coimbra.

Pereira, M.J., 2009. Reversion to juvenility: the use of epicormics in the micropropagation of mature wild shrubs of *Vaccinium cylindraceum* Smith (Ericacaeae). *Arquipélago*. Life and Marine Sciences 26, 63-68.

Piotto, B., Piccini, C., Arcadu, P., 2001. La ripresa della vegetazione dopo gli incendi nella regione mediterrânea. In: Piotto, B., Noi, A. (Eds.), Propagazione per seme di alberi e arbusti della flora mediterranea. Dipart. Prev. Risanam. Ambient., Manuale ANPA (Agenz. Naz. per la Protezi. dell' Ambiente), pp. 32-38.

Preece, J., 2008. Stock plant physiological factors affecting growth and morphogenesis. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 403-422.

Preece, J.E., Sutter, E., 1991. Acclimatization of micropropagated plants to the greenhouse and field. In: Debergh, P.C., Zimmerman, R.H. (Eds.), Micropropagation: Technology and Application. Kluwer Academic Pub., Dordrecht, pp. 71-79.

Rathore, J.S., Rathore, V., Shekhawat, N.S., Singh, R.P., Liler, G., Mahendra, P., Dagla, H.R., 2004. Micropropagation of Woody Plants. In: Srivastava, P.S., Narula, A., Srivastava, S. (Eds.), Plant Biotechnology and Molecular Markers. Anamaya Pub., New Dehli, pp. 195-205.

Ratnaparkhe, M.B., 2007. Blueberry. In: Kole, C. (Ed.), Genome mapping and molecular breeding in plants, Fruits and Nuts. Springer-Verlag, Berlin Heidelberg, pp. 217-227.

Roseiro, R.J.B., 1994. Micropropagação de medronheiro (*Arbutus unedo* L.). Relat. de Trabalho de Fim de Curso, Engº de Produção Florestal. ESACB, Castelo Branco, p. 59.

Salisbury, F.B., Ross, C.W., 1992. Plant Physiology. Wadsworth Publishing Company, Belmont, California, USA.

Smith, M.A.L., Eichorst, S.M., Rogers, R.B., 1992. Rhizogenesis pretreatments and effects on microcuttings during transition. Acta Hort. 319, 77-82.

Smith, M.A.L., Mcclell, M.T., Timerman, R., 1991. Anomalous root structure on woody plants *in vitro*. J. Environ. Hortic. 9, 61-64.

Torres, J.A., Valle, F., Pinto, C., Garcia-Fuentes, A., Salazar, C., Cano, E., 2002. *Arbutus unedo* communities in southern Iberian Peninsula mountains. Plant Ecol. 160, 207-223.

Tsai, C.-J., Saunders, J.W., 1999. Evaluation of sole nitrogen sources for shoot and leaf disc culture of suger beet. Plant Cell Tis. Org. Cult. 59, 47-56.

Young, M.J., Cameraon, J.S., 1985. Influence of growth regulators and nitrogen form on micropropagation of rabbiteye blueberries. Fruit Var. J. 39, 16-18.

Zar, J.H., 1996. Biostatistical Analysis (4th Ed.). Prentice-Hall, Upper Saddle River.

Ziv, M., Chen, J., 2008. The anatomy and morphology of tissue cultured plants. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 465-477.

APPENDIX

Appendix Table 1 – ANOVA: effect of BA (9.0 µM) pulverization and plastic covertures on epicormic shoot development: I - epicormic shoot length and II - number of shoots per branch.

I: Epicormic shoot length					
Source	SS	d.f.	MS	F	p
A: covertures	2.4277	1	2.4277	6.5748	0.0334*
B: BA	0.0709	1	0.0709	0.1906	0.6728
Interaction: A x B	1.8323	1	1.8323	4.9624	0.0565
Error	2.9539	8	0.3692		

II: Number of shoots per branch					
Source	SS	d.f.	MS	F	p
A: covertures	14.0833	1	14.083	2.2237	0.1742
B: BA	4.0833	1	4.0833	0.6447	0.4452
Interaction: A x B	2.0833	1	2.0833	0.3289	0.582
Error	50.6667	8	6.3333		

*Significant at P ≤ 0.05.

Appendix Table 2 – ANOVA: effect of origin of epicormic shoots (GCC *vs* GH) and explant source on culture establishment.

Effect of origin of epicormic shoots on culture establishment					
Source	SS	d.f.	MS	F	p
Shoots origin (GCC *vs* GH)	837.48	1	837.48	15.067	0.0037**
Error	500.27	9	55.59		

Effect of explant source (AL clones) on culture establishment					
Source	SS	d.f.	MS	F	p
Adult clones	3434.69	5	686.94	28.422	0.0001**
Error	749.24	31	24.17		

**Significant at P ≤ 0.01.

Appendix Table 3 – ANOVA: effect of the type of explant on the rates of A – necrosis, B- contamination, C - survival and D - established explants.

A – Necrosis, after 1 week (arcsine transformation)					
Source	SS	d.f.	MS	F	p
Explant type	5563.48	1	5563.48	6.9495	0.01446*
Error	19213.33	24	800.56		

B - Contamination, after 1 week (arcsine transformation)					
Source	SS	d.f.	MS	F	p
Explant type	34.02	1	34.02	0.02988	0.864206
Error	27321.03	24	1138.4		

C - Survival, after 1 week (arcsine transformation)					
Source	SS	d.f.	MS	F	p
Explant type	6317.09	1	6317.09	11.6432	0.00228**
Error	13021.34	24	542.56		

D - Established explants, after 12 weeks (arcsine transfor.)					
Source	SS	d.f.	MS	F	p
Explant type	3757.75	1	3757.8	7.7109	0.01047**
Error	11073.39	24	461.39		

**Significant at P ≤ 0.01; *Significant at P ≤ 0.05.

Appendix Table 4 – ANOVA: effect of culture media on shoot proliferation: A - on shoot length (SL) and B – on the number of shoots formed per test tube for further multiplication (SNX).

A: SL (mm)					
Source	SS	d.f.	MS	F	p
Culture media	31.628	1	15.814	14.523	0.00001**
Error	268.964	247	1.089		

B: Number of shoots formed/test tube for further multiplication (SNX)					
Source	SS	d.f.	MS	F	p
Culture media	21.9865	1	10.9933	10.0015	0.000067**
Error	271.4932	247	1.0992		

**Significant at P ≤ 0.01.

Appendix Table 5 – ANOVA: effect of the genotype during the first five subcultures at 3-week intervals on multiplication rate evaluated by SNX.

Source	SS	df	MSS	F ratio	Sig. level
Clone AL	0.6967	5	0.1393	0.7484	0.5978
Error	3.3512	18	0.1862		

Appendix Table 6 – ANOVA: effect of auxin treatment (IBA) and charcoal addition to the development medium on the percentage of rooted shoots.

Source	SS	d.f.	MSS	F	p
A: auxin (IBA)	6852.1	3	2284.05	5.5027	0.00861**
B: Charcoal (development medium)	0.83	1	0.83	0.0020	0.96482
Interaction: A x B	817.86	3	272.62	0.6568	0.59040
Error	6641.21	16	415.08		

**Significant at P ≤ 0.01.

Appendix Table 7 – ANOVA: effect of the shoot proliferation media (FS and AND) and IBA treatment on the following variables I - percentage of rooted shoots (arcsine transformation) and II - length of the longest root (LLR).

I: Rooting rate (arcsine transformation)

Source	SS	d.f.	MS	F	p
A : Shoot proliferation media	760.2	1	760.2	15.718	0.000576**
B: IBA	25118.8	5	5023.8	103.877	0.0000**
Interaction: A x B	1007.1	5	201.4	4.165	0.007257**
Error	1160.7	24	48.4		

II: LLR (mm)

Source	SS	d.f.	MS	F	p
A: Shoot proliferation media	279.4	1	279.4	3.019	0.083395
B: IBA	2203.1	5	440.6	4.761	0.000345**
Interaction: A x B	1050.3	5	210.1	2.27	0.047911*
Error	25358.6	274	92.5		

*Significant at P ≤ 0.05; **Significant at P ≤ 0.01.

Appendix Table 8 – ANOVA: effect of the shoot proliferation media (FS and AND) and IBA treatment on the following variables A - number of roots (NR), B - length of the shortest root (LSR), and C - final shoot length (SL).

A - Number of roots (NR)

Source	SS	d.f.	MS	F	p
Shoot proliferation media	75.19	1	75.192	1.6329	0.202386
IBA	11228.95	5	2245.789	48.7697	0.00000**
Shoot prolif. med. X IBA	129.05	5	25.81	0.5605	0.730265
Error	12617.38	274	46.049		

B - LSR (mm)

Source	SS	d.f.	MS	F	p
Shoot prolif. med. X IBA	23.48	1	23.48	0.2943	0.587894
IBA	4308.87	5	861.77	10.8022	0.00000**
Shoot prolif. med. X IBA	102.36	5	20.47	0.2566	0.93626
Error	21859.12	274	79.78		

C - SL (mm)

Source	SS	d.f.	MS	F	p
Shoot proliferation media	18.24	1	18.24	0.2488	0.618356
IBA	1366.67	5	273.33	3.7278	0.00278**
Shoot prolif. med. X IBA	601.13	5	120.23	1.6397	0.149635
Error	20090.72	274	73.32		

**Significant at P ≤ 0.01

*Significant at P ≤ 0.05

Appendix Table 9 – ANOVA: effect of the IAA treatments and addition of charcoal (CA) to the culture media on rooting of AL03 shoots according to the following variables rooting rate (%), number of roots (NR), length of the longest root (LLR), length of the shortest root (LSR) and final shoot length (SL).

Rooting rate (arcsine transformation)

Source	SS	d.f.	MS	F	p
IAA	144.42	3	48.14	0.6704	0.578494
CA	172.73	1	172.73	24.057	0.133984
IAA * CA	866.21	3	288.74	40.213	0.01884*
Error	1723.25	24	71.80		

Number of roots (NR)

Source	SS	d.f.	MS	F	p
IAA	97.95	3	32.649	19.249	0.129337
CA	416.58	1	416.583	245.612	0.0000**
IAA * CA	179.20	3	59.734	35.219	0.01727*
Error	1984.437	117	16.961		

Length longest root (LLR. mm)

Source	SS	d.f.	MS	F	p
IAA	37.26	3	12.421	11.705	0.324133
CA	44.38	1	44.381	41.823	0.043111*
IAA * CA	219.28	3	73.092	68.878	0.000261**
Error	1230.96	117	10.612		

Length shortest root (LSR. mm)

Source	SS	d.f.	MS	F	p
IAA	150.96	3	50.321	14.112	0.246053
CA	87.50	1	87.499	24.538	0.121393
IAA * CA	170.07	3	56.691	15.898	0.198796
Error	2710.03	76	35.658		

Shoot length (SL mm)

Source	SS	d.f.	MS	F	p
IAA	345.0	3	115.0	1.297	0.278970
CA	10778.2	1	10778.2	121.541	0.00000**
IAA * CA	731.4	3	243.8	2.749	0.045966*
Error	10286.8	117	88.7		

**Significant at $P \leq 0.01$

*Significant at $P \leq 0.05$

Appendix Table 10 – ANOVA: effect of IBA treatments and charcoal on the percentage of survival of C01 plants (C01).

Source	SS	d.f.	MS	F	p
A: auxin (IBA)	0.15841	3	0.0528	1.069	0.390004
B: Charcoal (elongation medium)	0.00319	1	0.00319	0.0645	0.802711
Interaction: A x B	0.12913	3	0.04304	0.8714	0.476355
Error	0.79034	16	0.0494		

**Significant at $P \leq 0.01$; * Significant at $P \leq 0.05$.

Appendix Table 11 – ANOVA: effect of different substrates on the percentage of survival C01 plants after 2 and 4 months in the greenhouse and nursery, respectively.

Survival rate 2 months (arcsine transformation)

Source	SS	d.f.	MS	F	p
Substrate	21952.83	4	5488.21	26.7294	0.0000**
Error	11703.53	57	205.33		

Survival rate 4 months (arcsine transformation)

Source	SS	d.f.	MS	F	p
Substrate	28375.96	4	7094	58.2853	0.00**
Error	6937.56	57	121.71		

*Significant at $P \leq 0.05$; **Significant at $P \leq 0.01$.

3 - Effect of plant growth regulators and genotype on the micropropagation of adult trees of *Arbutus unedo* L. (strawberry tree)

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3 EFFECT OF PLANT GROWTH REGULATORS AND GENOTYPE ON THE MICROPROPAGATION OF ADULT TREES OF *ARBUTUS UNEDO* L. (STRAWBERRY TREE)

3.1 ABSTRACT

Arbutus unedo grows spontaneously around the Mediterranean basin. The species is tolerant to drought and has a strong regeneration capacity following fires making it interesting for Mediterranean forestation programs. Considering the sparse information about the potential of this fruit tree to be propagated *in vitro*, a project to clone selected trees based on their fruit production was initiated a few years ago. The role of several factors on *A. unedo* propagation was evaluated. The results showed that 8.9 mM kinetin gave the best multiplication rates, although not significantly different from those obtained with benzyladenine or zeatin. The inclusion of thidiazuron or 1-naphthaleneacetic acid promoted callus growth and had deleterious effects on the multiplication rate. The genotype of the donor plants is also a factor interfering with the multiplication. The results also indicated that the conditions used for multiplication influenced the behaviour of shoots during the rooting phase.

Key words: cytokinins; multiplication; NAA; rooting.

RESUMO

Arbutus unedo cresce espontaneamente na bacia Mediterrânica. A espécie é tolerante à secura e apresenta uma forte capacidade de regeneração após a ocorrência de incêndios florestais, o que a torna interessante para os programas de florestação na região Mediterrânica. Considerando a reduzida informação que existe sobre o potencial desta espécie frutícola para a propagação *in vitro*, foi iniciado há poucos anos um projecto para a clonagem de árvores adultas seleccionadas com base na produtividade e qualidade do fruto. A função de vários factores na propagação de *Arbutus unedo* foi avaliada. Os resultados mostraram que com a utilização de cinetina a 8.9 µM se obtiveram as melhores taxas de multiplicação, embora sem diferenças significativas dos valores que foram obtidos com a utilização de benziladenina ou zeatina. A inclusão no meio de cultura de tidiazurão ou ácido 1-naftaleno acético promoveu o crescimento de calos e mostrou ter um efeito negativo na taxa de multiplicação. O genótipo das plantas mãe é um factor que interfere na multiplicação das culturas. Os resultados também indicaram que as condições utilizadas na fase de multiplicação influenciaram a resposta dos rebentos na fase de enraizamento.

Palavras chave: citocininas; enraizamento; multiplicação; NAA.

3.2 INTRODUCTION

The genus *Arbutus* (Ericaceae) includes about 20 species from which *Arbutus unedo,* commonly known as Strawberry tree, is the most interesting from an economic point of view. According to Piotto *et al*. (2001). Strawberry tree seems to be native to Ireland, southern Europe and the western Mediterranean region growing spontaneously as a bush or small tree in several countries around the Mediterranean basin in rocky and well-drained soils. This species can withstand low temperatures and is drought tolerant (Piotto *et al.*, 2001; Godinho-Ferreira *et al.,* 2005). Moreover, *A. unedo* easily regenerates after forest fires, a characteristic which is particularly important for forestation programs in southern European countries such as Greece, Italy, Portugal and Spain where fires are common during the dry season. As a species characteristic of Mediterranean ecosystems strawberry tree contributes to maintain the biodiversity and helps to stabilize soils, avoiding erosion (Neppi, 2001; Metaxas *et al.*, 2004). From an economic point of view, strawberry tree can be considered a neglected or underutilized crop (NUC) and has been included in the list of NUCs by the Global Facilitation Unit for Underutilized Species (G. F.U.U. Sp, 2008) since it is used in small scale in particular areas of Mediterranean countries. In Portugal, it grows in most of the country (Godinho-Ferreira *et al.,* 2005) often associated with other trees such as cork-oaks and maritime-pines but it is in the Algarve region and in the Centre that the fruits are most popular to make a spirit called “medronheira” which is the main income for small farmers. The fruits are usually picked up by local populations from spontaneously growing trees (culture in orchards is unusual) which are then abandoned in the field until the new period of fruit collection (late fall to early winter). The mature red berries can be eaten fresh or used to make jams. Due to an expanded belief that the fruits are reach in ethanol, they are consumed only in small amounts (*unedo* means “eat only one”) and therefore are seldom found in supermarkets. The bark has been used in tanning and the plant has been used in folk medicine (Pabuccuoglu *et al.,* 2003). The small white flowers take a year to ripen and, during several months, both flowers and fruits are present making the tree an attractive ornamental. Considering the increasing importance that alternative crops are assuming in the agricultural policy of the European Union, a project to select, characterize and clone selected adult trees based on their fruit production and quality was initiated by our group a few years ago.

The number of papers dealing with the *in vitro* cloning of *A. unedo* is scarce and the same is true for other members of the Ericaceae family. Members of this family that have been

successfully micropropagated include *Arbutus xalapensis* (Mackay, 1996), *Kalmia latifolia* (Lloyd and McCown, 1980), *Oxydendrum arboreum* (Banko and Stefani, 1989), *Rhododendron* (Anderson, 1984; Almeida *et al.*, 2005) and several species of the genus *Vaccinium* (Gajdošová *et al.*, 2007; Ostrolucká *et al.*, 2007; Ratnaparkhe, 2007). Previous studies on micropropagation of Strawberry tree, from juvenile material, have been reported by Gonçalves and Roseiro (1994), Mereti *et al*. (2003) and Mereti *et al*. (2002). More recently, we have established a protocol for the establishment and micropropagation of adult trees through axillary shoot proliferation (Gomes and Canhoto, 2009). In this work it was reported that shoot apices from epicormic shoots showed higher rates of *in vitro* establishment when compared with nodal segments. From the three basal medium used in combination with 9.0 µM BA, the FS medium (De Fossard *et al.*, 1974) gave the highest rates of multiplication and the inclusion of an auxin significantly increased root formation. Preliminary results about the induction and regeneration of adult plants by somatic embryogenesis in *A. unedo* have been also published (Gomes *et al.*, 2009). It is well known that several factors can affect *in vitro* micropropagation. Among these parameters are the genotype of the donor plants (George and Debergh, 2008; Chawla, 2009) and the plant growth regulators (PGRs) included in the culture media (Gomes and Canhoto, 2003; Machakova *et al.*, 2008). According to this we decided to analyse the effect of different cytokinins and one auxin on the propagation of *A. unedo* to find a suitable combination that can assure high rates of propagation. Several previously selected genotypes were also tested in order to find those which are more amenable for *in vitro* cloning. In long-term breeding programmes these genotypes can be used to transfer their regeneration potential to more recalcitrant genotypes.

3.3 MATERIAL AND METHODS

3.3.1 Establishment and propagation

The conditions for plant establishment have been described before (Gomes and Canhoto, 2009), on Chapter 2. Briefly, branches (30 – 40 cm length) of adult selected plants were collected in the field, disinfected with a fungicide (dipped in dichlofluanid, Euparene, 120 mgl^{-1} for 10 min.) and kept in a culture chamber covered with plastic bags to maintain a high humidity environment that stimulate epicormic shoot development. Shoot apices and nodal segments from these epicormic shoots were further used to establish *in vitro* cultures through its culture on a medium (AND) consisting of Anderson major salts (Anderson, 1984), Murashige and Skoog (MS) micro nutrients (Murashige and Skoog, 1962) and organic compounds of the FS medium (De Fossard *et al.*, 1974). Sucrose 0.087 M and 9.0 µM BA were added. The pH was adjusted to 5.7 before agar addition (7 gl^{-1}) and the media were then autoclaved (121 °C, 20 min.), being these procedures similar for all prepared culture media. Also, all the genotypes used were established in this way. To propagate the established material, three culture media were tested on shoot multiplication: 1) the Anderson medium above described (AND); 2) the same medium containing as major salts the MS macronutrients reduced at half-strength (1/2 MS) and 3) the same medium with the major salts of the FS medium (FS). After 12 weeks (4 subcultures at 3-week intervals) of culture the multiplication rate was evaluated by the number of clusters formed and by the maximum shoot length formed per test tube. For more details see Gomes and Canhoto (2009; Chapter 1).

3.3.2 Effect of plant growth regulators

Shoots (12 - 18 mm height) of the established explants were used to test the role of plant growth regulators (PGRs) on shoot multiplication. Cultures were placed in a culture chamber (16h photoperiod, 40 µmol m^{-2} s^{-1}) and test tubes (Sigma, 25 x 150 mm) containing 12 ml of culture medium and covered with plastic caps were used. To test the PGRs the FS medium described in the previous section was used and prepared following the same procedure. The effect of four cytokinins and one auxin (1-naphthalene acetic acid, NAA) on shoot multiplication was evaluated, during 4 subcultures at 4-week intervals. The experiments were

carried out with the genotype AL1 derived from an adult selected tree (Gomes and Canhoto, 2009) and the following assays were performed: 1) different BA concentrations were tested (0; 2.2; 4.4; 8.9; 17.8 µM); 2) the cytokinins kinetin (KIN), zeatin (Zt), thidiazuron (TDZ) and N^6-(2-isopentenyl) adenine (2-iP) were tested and compared with BA at the same concentration (8.9 µM); 3) NAA at different concentrations (0; 0.54; 1.34; 2.69 µM), was tested in combination with BA (8.9 µM). The multiplication rate was evaluated by the following variables assessed per test tube: 1) maximum shoot length (SL), 2) number of shoots formed (SF) and 3) the number of shoots formed per test tube for further multiplication (SNX). Other parameters assessed were: callus formation, necrosis and axillary shoot proliferation (referred as callus, necrosis and proliferation in the results).

3.3.3 Effect of the genotype

To evaluate the role of the genotype on the multiplication rate 10 selected adult genotypes were tested. FS was used as basal medium containing 9.0 µM of BA and 0.087 M sucrose. All the genotypes were from adult trees selected according to their fruit production and quality. These ten genotypes were obtained from 3 provenances from centre region of Portugal: Oleiros (AL2; AL3; AL4; AL6; AL7), Alva (IM1; IM2; IM4; IM6) and Pióda̋o (JF3). The multiplication rate was evaluated by the same variables described before and calculated per test tube.

3.3.4 Rooting and acclimatization

Shoots were rooted on a rooting induction medium containing Knop macronutrients (Gautheret, 1959), MS micronutrients without potassium iodide and FS organics without riboflavin. The auxin 3-indolebutyric acid (IBA: 14.8; 19.7 or 24.6 µM) was added to promote root differentiation. On a first assay the rooting potential of shoots formed on media containing different concentrations of BA was tested. In this case, IBA at the concentration of 24.6 µM was used. On a second set of experiments, shoots formed on media with different cytokinins were rooted in the presence of 14.8 µM IBA. Finally, on a third set of experiments shoots formed on media containing combinations of NAA and BA, or shoots from the different genotypes analysed were rooted on a medium containing 19.7 µM IBA. In general, root induction (RI) was carried out in darkness conditions for 7 days. However, other periods (5-10 days) of root induction were also tested (RI-days). Following root induction shoots

were transferred to a root development medium (RD) identical to the rooting medium but without IBA and with 1.5% charcoal. Nevertheless, other periods (22-48 days) of root development (RD-days) were also tested. After four to five weeks on this medium rooted plantlets were transferred to containers (covered) and placed into the greenhouse, as described elsewhere (Gomes and Canhoto, 2009). Rooting ability was evaluated using the following parameters: percentage of rooted shoots, number of roots formed per shoot (NR), length of the longest root (LLR), length of the shortest root (LSR), and final shoot length (SL). When apical shoot necrosis or callus formation at the cut end of the shoot were observed they were also registered.

3.3.5 Experimental design and statistics

The multiplication experiments started with 30 shoots per treatment comprising at least three replicas of 10 to 20 explants. Variables were recorded after each one of the four subcultures at four-week intervals. For rooting experiments 30 shoots per treatment were also tested. The rooting parameters were recorded after 5 weeks on medium without auxin and containing charcoal during acclimatization procedures and transference of the *in vitro* plants to the greenhouse as described by Gomes and Canhoto (2009). For statistics analysis (STATISTICA 6) complementary approaches were tested: an ANOVA was performed, and means were compared using the Duncan test ($P \leq 0.05$) (Duncan, 1955) followed by a multiple linear regression and a principal components analysis (PCA). The quantitative data expressed as percentages were first submitted to arcsine transformation and the means corrected for bias before a new conversion of the means and standard error (SE) back into percentages (Zar, 1996).

For the multiple linear regression analysis the effect of the different variables on the multiplication rate (effect of PGRs and genotype) was evaluated considering the number of shoots formed per test tube for further multiplication (SNX) as variable dependent. The results are displayed when multiple R^2 coefficient was higher than 0.67 (R=0.81). For the principal components analysis (PCA) all variables were analysed to achieve a better understanding of the interactions between them and its significance level for the total variance. The results are presented when the percentage of total variance explained by the two factors of PCA analysis was higher than 49%.

3.4 RESULTS

3.4.1 Effect of PGRs

When different BA concentrations were tested best results were achieved with 8.9 µM (Table 1; Appendix Table 1). When this concentration of BA was used the number of shoots formed per test tube for further multiplication (SNX) was significantly higher than in the other concentrations tested ($P<0.01$; Appendix Table 1).

Table 1 - Effect of different combinations of BA (experiment I), cytokinins (experiment II), combinations of NAA and BA (experiment III), and of the genotype (experiment IV) on the multiplication of *A. unedo* Multiplication was evaluated by the maximum shoot length and by the number of shoots formed per test tube for further multiplication (SNX).

Experiment	Variable	Shoot length (SL mm)	SNX
Experiment I BA (µM) N = 843	0 µM	20.11 ± 0.65 [a]	1.43 ± 0.04 [bc]
	2.2 µM	20.45 ± 0.66 [a]	1.49 ± 0.04 [b]
	4.4 µM	16.07 ± 0.64 [b]	1.28 ± 0.05 [c]
	8.9 µM	19.42 ± 0.54 [a]	1.75 ± 0.05 [a]
	17.8 µM	14.96 ± 0.69 [b]	1.33 ± 0.07 [c]
Experiment II Cytokinins (8.9 µM) N = 945	2-iP	23.21 ± 0.50 [b]	1.20 ± 0.03 [b]
	BA	21.44 ± 0.49 [c]	1.29 ± 0.04 [a]
	KIN	25.38 ± 0.44 [a]	1.31 ± 0.03 [a]
	TDZ	12.74 ± 0.39 [d]	1.01 ± 0.02 [c]
	Zt	24.89 ± 0.51 [a]	1.28 ± 0.03 [ab]
Experiment III NAA + BA 8.9µM N = 1283	0 µM	25.31 ± 0.54 [a]	2.27 ± 0.06 [a]
	0.54 µM	19.50 ± 0.39 [b]	1.80 ± 0.06 [b]
	1.34 µM	15.91 ± 0.52 [c]	1.48 ± 0.05 [c]
	2.69 µM	17.34 ± 0.56 [c]	1.43 ± 0.06 [c]
Experiment IV Genotypes N = 560	AL2	16.00 ± 0.65 [a]	1.71 ± 0.08 [ab]
	AL3	15.72 ± 0.57 [a]	1.48 ± 0.06 [b]
	AL4	12.64 ± 0.70 [b]	1.48 ± 0.13 [b]
	AL6	11.65 ± 1.24 [bc]	1.57 ± 0.21 [ab]
	AL7	12.70 ± 2.05 [b]	1.90 ± 0.23 [a]
	IM1	8.92 ± 0.88 [c]	0.93 ± 0.08 [d]
	IM2	12.88 ± 1.07 [b]	1.71 ± 0.24 [ab]
	IM4	11.34 ± 0.49 [bc]	1.36 ± 0.08 [bc]
	IM6	11.28 ± 0.74 [bc]	1.34 ± 0.11 [bc]
	JF3	9.85 ± 0.69 [bc]	1.00 ± 0.06 [cd]

Each value is the mean ± SE. In each treatment, values followed by different letters are significantly different.

The multiple regression analysis (Table 2 a) indicated that the dependent variable SNX increased with the concomitant increase of the variables SL, SF and BA (P<0.01). However, SL and SF showed a higher coefficient (0.658 and 0.451, respectively) than BA (0.098). The multiple regression accounts for 68.9% (R^2= 0.689) of the variance of the dependent variable (SNX). On the other hand, the variables necrosis and shoot proliferation gave non-statistically different (P>0.05) results. Callus formation was never observed during these assays.

Table 2 - Multiple linear regression analysis for shoot multiplication assessed by the dependent variable SNX and the following independent variables: number of subcultures, necrosis, proliferation, number of shoots formed (SF), maximum shoot length (SL) per test tube, and callus formation.

a) Concentration of BA µM

Statistic	Summary Value
Multiple R	0.8300
Multiple R²	0.6889
Adjusted R²	0.6867
F(6,836)	308.6052
p	0.0000
Std.Err. of Estimate	0.3875

Regression Summary for Dependent Variable: SNX (BA X AL1.sta)
R= .83002735 R²= .68894540 Adjusted R²= .68671295
F(6,836)=308.61 p<0.0000 Std.Error of estimate: .38755

N=843	Beta	Std.Err. of Beta	B	Std.Err. of B	t(836)	p-level
Intercept			0.535401	0.321380	1.66594	0.096099
BA	0.098694	0.020224	0.049210	0.010084	4.88011	0.000001
Subculture	-0.057959	0.021308	-0.036853	0.013549	-2.71998	0.006664
Necrosis	0.023398	0.023701	0.078847	0.079870	0.98720	0.323832
Proliferation	-0.054316	0.033067	-0.045589	0.027754	-1.64260	0.100842
SF-formed	0.451097	0.032544	0.574731	0.041464	13.86112	0.000000
SL-length	0.658314	0.025438	0.053329	0.002061	25.87933	0.000000

b) Effect of NAA addition

Statistic	Summary S Value
Multiple R	0.8186
Multiple R²	0.6701
Adjusted R²	0.6683
F(7,1275)	369.9413
p	0.0000
Std.Err. of Estimate	0.7092

Regression Summary for Dependent Variable: SNX (NAA X AL1.sta)
R= .81858498 R²= .67008138 Adjusted R²= .66827006
F(7,1275)=369.94 p<0.0000 Std.Error of estimate: .70917

N=1283	Beta	Std.Err. of Beta	B	Std.Err. of B	t(1275)	p-level
Intercept			-18.0241	1.833841	-9.82860	0.000000
NAA	0.103040	0.018579	0.1000	0.018037	5.54603	0.000000
Subculture	0.234935	0.020942	0.2263	0.020173	11.21828	0.000000
Necrosis	0.053691	0.016623	0.5771	0.178665	3.22992	0.001270
Proliferation	0.179976	0.017694	0.2408	0.023670	10.17154	0.000000
SF-formed	0.532377	0.018045	0.6825	0.023133	29.50251	0.000000
SL-length	0.468784	0.017620	0.0547	0.002056	26.60552	0.000000
callus	0.032754	0.021271	0.0845	0.054862	1.53985	0.123846

c) Effect of the genotype

Statistic	Summary Value
Multiple R	0.8189
Multiple R²	0.6706
Adjusted R²	0.6664
F(7,552)	160.5534
p	0.0000
Std.Err. of Estimate	0.4511

Regression Summary for Dependent Variable: SNX (Genotype X.sta)
R= .81891367 R²= .67061961 Adjusted R²= .66644268
F(7,552)=160.55 p<0.0000 Std.Error of estimate: .45108

N=560	Beta	Std.Err. of Beta	B	Std.Err. of B	t(552)	p-level
Intercept			1.151140	0.836645	1.37590	0.169411
Genotype	-0.097547	0.029674	-0.026269	0.007991	-3.28732	0.001076
Subculture	0.086869	0.030444	0.081721	0.028640	2.85338	0.004488
Necrosis	0.166336	0.030580	0.696983	0.128138	5.43931	0.000000
Proliferation	0.371883	0.027196	0.468467	0.034260	13.67405	0.000000
SF_formed	0.323954	0.029216	0.662036	0.059706	11.08832	0.000000
SL_length	0.601141	0.028436	0.072102	0.003411	21.13996	0.000000
callus	-0.017154	0.033793	-0.031296	0.061650	-0.50763	0.611912

PCA analysis (Fig. 1) shows that the factor 1 accounts for 42% of the total variance showing as significant variables SNX, SL-lenght, SF-formed and shoot proliferation and displaying factor loadings higher than 0.70. This type of analysis also indicated that the

variable necrosis affected negatively the culture propagation group (SNX; SL; SF). As it would be expected the variables SF-formed and shoot proliferation are closely related. Factor 2 contributes with 17.5% for the total variance being almost dependent of the variable subculture. Finally, it must be signaled that the multiplication, evaluated by SNX, was not dependent of the number of subcultures showing a factor loading of -0.045.

Variable	Factor Loadings (Unrotate Extraction: Principal comp (Marked loadings are > .7 Factor 1	Factor 2
BA	0.013087	0.095291
Subculture	-0.210944	-0.797533
Necrosis	0.623762	0.274702
Proliferation	-0.769050	0.463782
SF-formed	-0.786374	0.388164
SL-length	-0.753297	-0.373578
SNX	-0.872038	-0.045489
Expl.Var	2.971475	1.227995
Prp.Totl	0.424496	0.175428

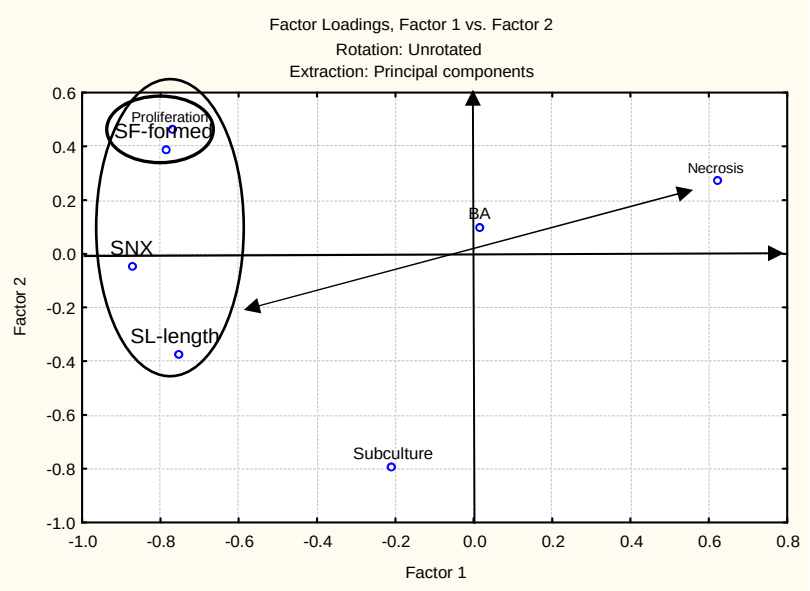


Figure 1 - Evaluation of the effect of different BA concentrations on shoot multiplication by PCA analysis considering the following variables: BA concentration, number of subcultures, necrosis, proliferation, callus formation, number of shoots formed/SF, maximum shoot length per test tube/SL, and SNX. (The variables are associated to each factor 1 and 2, according to the coefficients "factor loadings", presented on the left table. The highest factor loadings are marked whether they are correlated or are relevant to explain the total variance expressed by the factors 1 and 2).

When cytokinins were tested and compared with BA at the same concentration, KIN showed to be the most effective (Table 1, experiment II). In the presence of KIN shoot length reached an average of 25.38 ± 6.85 mm. However, this value was not significantly different from the results obtained with Zt (average shoot length of 24.89 ± 6.98 mm). Furthermore, the multiplication rate evaluated by the number of shoots formed per test tube for further multiplication (SNX) was not significantly different on the media containing KIN, BA or Zt (Table 1, experiment II; Appendix Table 1). Both Zt and KIN promoted shoot elongation, but BA also induced shoot proliferation (Figs. 2*A* to *C*). When TDZ was used, callus formation was often observed (Figs. 2*D*, *E*) whereas shoot growth was inhibited (12.74 ± 4.26 mm). Moreover, in the presence of TDZ, callus formation increased with the number of subcultures while and shoot growth was consistently impaired. For this set of experiments multiple regression analysis is not shown since a low coefficient (0.46 - the multiple R^2) was obtained.

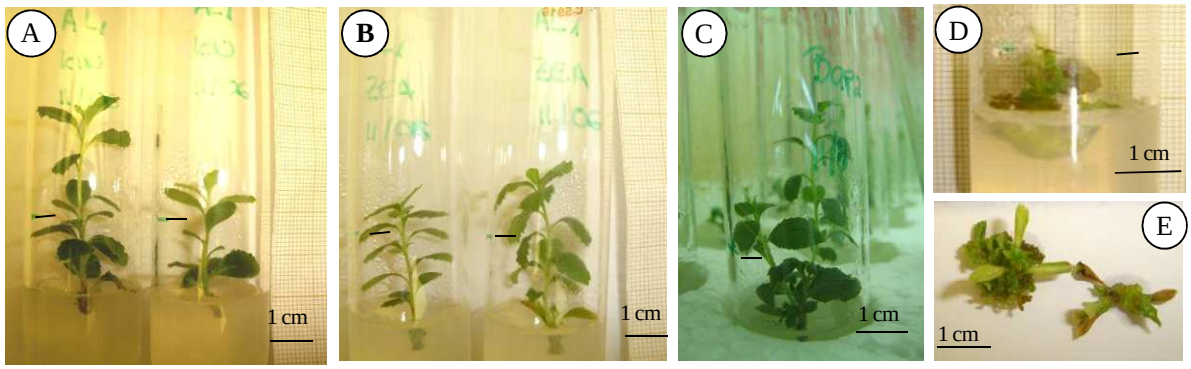


Figure 2 - Micropropagation of *A. unedo* on different culture conditions. A – Kinetin, B – Zeatin, C – BA and D-E – TDZ. When TDZ was used callus were often formed (D) and shoot growth inhibited (E). Marks on tubes indicate the initial shoot length.

PCA analysis (Fig. 3) shows that in this case the multiplication (SNX; factor 1) is more correlated with shoot elongation (SL) than with shoot formed (SF). Axilary shoot proliferation and subsquently shoot development was only observed when BA was tested (Fig. 2*C*). This feature is confirmed by the low factor loading associated to the variable SF (0.31; Fig. 3).

Variable	Factor Loadings (Unrota Extraction: Principal com (Marked loadings are > . Factor 1	Factor 2
CytoKinins	0.601138	0.499781
Subculture	-0.294305	0.285339
Necrosis	-0.119668	0.384346
Proliferation	0.106251	-0.204887
SF-formed	0.309309	-0.604010
SL-length	0.833882	-0.065191
SNX	0.719921	-0.352963
callus	-0.664813	-0.521342
Expl.Var	2.224886	1.286358
Prp.Totl	0.278111	0.160795

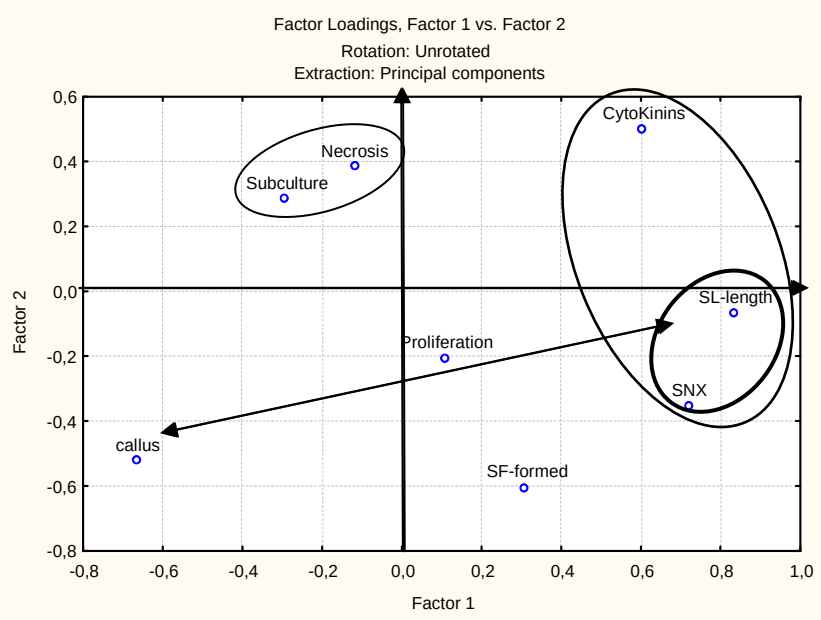


Figure 3 - Evaluation of the effect of different cytokinins (8.9 µM) on shoot multiplication by PCA analysis considering the following variables: type cytokinin, number of subcultures, necrosis, proliferation, callus formation, number of shoots formed/SF, maximum shoot length per test tube/SL, and SNX. For more details see Figure 1.

The data also indicate (Fig. 3) that multiplication (SNX) is also positively affected by cytotokinins whereas callus formation, induced by TDZ, has a negative effect. Then, Factor 1

of the PCA analysis (Fig. 3) shows that cytokinins promoted (factor loadings 0.60 and 0.72) mutiplication (SNX) while inhibiting callus formation (-0.66).

When the auxin NAA was included on the culture media, the results showed that NAA was unable to improve the multiplication rate (Table 1, experiment III; Appendix Table 1). Best results ($P<0.01$) were achieved on media without NAA (25.32 ± 12.44 mm SL; 2.27 ± 1.38 SNX). Moreover, NAA induced callus formation, and unorganized growth increased in the follow-up subcultures. When NAA was tested at concentrations higher than 1.34 µM, callus formation completely inhibited shoot growth and multiplication.

The multiple regression analysis performed for this assay (Table 2 b) contributes to 67% ($R^2= 0.67$) of the variance of the dependent variable (SNX). The values obtained for the variable callus showed to be not significant ($P>0.05$) while SL- length or SF- formed exhibited the highest coefficients (0.46 and 0.53, respectively). PCA analysis (Fig. 4) points out to a positive interaction between the variables multiplication (SNX), SL-length, SF-formed and proliferation (with high factor loadings for factor 1, Fig. 4). It also confirms that callus formation increases with the increasing number of subcultures (Fig. 4, factor 2).

Variable	Factor Loadings (Unrota Extraction: Principal con (Marked loadings are > . Factor 1	Factor 2
NAA	-0.305686	0.003153
Subculture	0.202049	0.867594
Necrosis	0.316080	-0.240767
Proliferation	-0.650479	-0.084097
SF-formed	-0.646643	-0.253336
SL-length	-0.618304	0.456174
SNX	-0.826147	0.345363
callus	0.365334	0.686008
Expl.Var	2.273733	1.679926
Prp.Totl	0.284217	0.209991

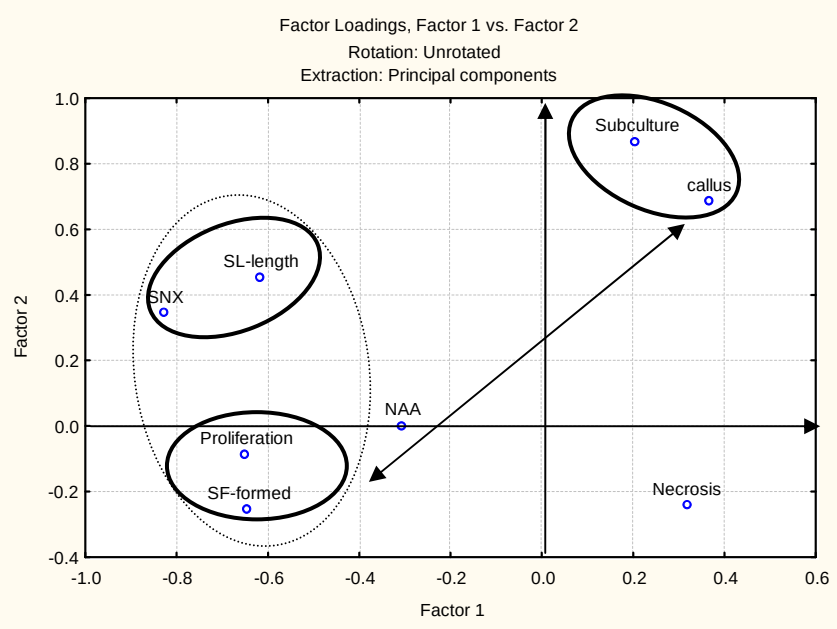


Figure 4 - Evaluation of the effect of different NAA concentration on shoot multiplication by PCA analysis considering the following variables: NAA concentration, number of subcultures, necrosis, proliferation, callus formation, number of shoots formed/SF, maximum shoot length per test tube/SL and SNX. For more details see Figure 1.

3.4.2 Effect of the genotype

When selected adult clones were tested (Table 1, experiment IV; Appendix Table 1), ANOVA analysis showed that the genotype significantly affects the multiplication rate ($P<0.01$) both in terms of shoot length (SL) and number of shoots (SNX) with some genotypes sharply giving better results than others. The multiple regression analysis of SNX (Table 2 C; $R^2=0.67$) showed significant differences ($P<0.01$) for all variables analysed with the only exception being callus formation. PCA analysis for multiplication assessment showed a positive interaction between the variables multiplication (SNX), SL-length, SF-formed and proliferation (Fig. 5, factor 1). The results also demonstrated that the genotype has an important factor loadings both in terms of multiplication (variables, which are pointed out by factor 1 SNX, SL, SF and proliferation), and callus formation (factor 2). Once again, PCA analysis indicates that when genotypes display callus formation they have a tendency to increase this feature in the next subcultures (Fig. 5).

Variable	Factor Loadings (Unrot Extraction: Principal co (Marked loadings are > Factor 1	Factor 2
Genotype	0.405668	0.510388
Subculture	-0.023829	0.782603
Necrosis	-0.670151	0.027956
Proliferation	0.686844	-0.005965
SF_formed	0.638103	-0.152445
SL_length	0.697233	-0.085439
SNX	0.828458	-0.118064
callus	0.099111	0.898894
Expl.Var	2.675466	1.726270
Prp.Totl	0.334433	0.215784

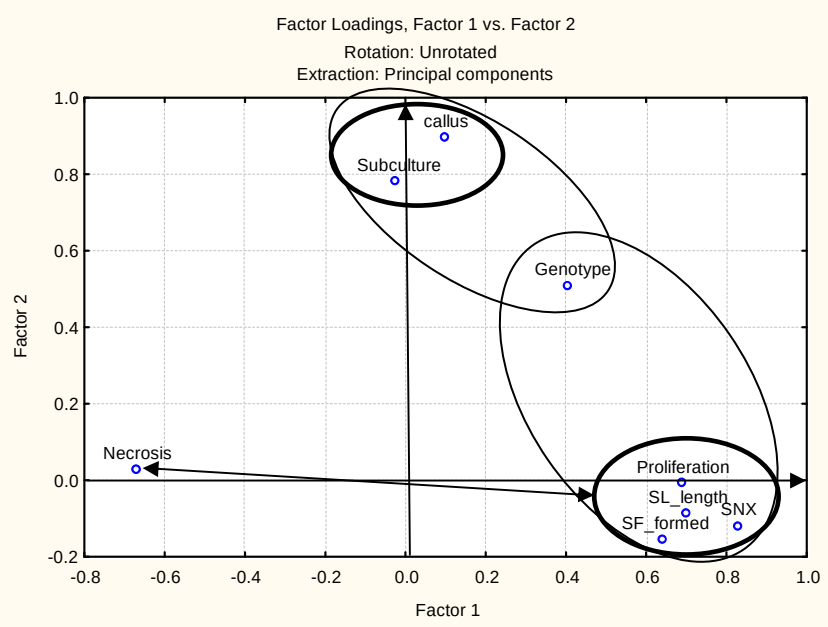


Figure 5 - Analysis of the effect of different genotype on shoot multiplication by PCA analysis considering the following variables: genotypes, number of subcultures, necrosis, proliferation, callus formation, number of shoots formed/SF, maximum shoot length per test tube/SL and SNX. For more details see Figure 1.

3.4.3 Rooting and acclimatization

When roots formed on media containing different concentrations of BA were induced to root on a IBA-containing medium for 7 days followed by transference to an auxin-free medium it was found that best frequencies of root formation were obtained with shoots formed on a medium containing 8.9 µM but the results were not significantly different from

those obtained with shoots grown in other BA concentrations (Table 3; Appendix Table 2). At this concentration (8.9 µM) shoot necrosis was never detected. When the parameter evaluated was the number of roots per shoot the results showed significant differences among the treatments with shoots formed on a medium containing BA 17.8 µM giving the best results (Table 3; Appendix Table 2). However, as indicated before, this medium gave poor results in terms of shoots for further multiplication (SNX) than the medium with 8.9 µM BA (see Table 1).

Table 3 - Rooting of shoots formed in different culture conditions. The following parameters were analysed: percentage of shoots forming roots, number of roots per shoot and final shoot elongation.

Conditions of shoot formation		Rooting (%)	Number of roots	Shoot length (mm)
BA (µM)	BA 0 µM	56.7 ± 10.4 [a]	6.6 ± 1.0 [ab]	51.65 ± 3.6 [a]
	BA 2,2 µM	53.3 ± 6.0 [a]	6.9 ± 0.6 [ab]	47.07 ± 3.0 [ab]
	BA 4,4 µM	53.3 ± 9.8 [a]	5 ± 0.6 [b]	43.34 ± 2.3 [ab]
	BA 8,9µM	60 ± 2.6 [a]	5.7 ± 0.8 [b]	40.64 ± 3.5 [b]
N = 150	BA 17,8µM	50 ± 6.8 [a]	8.7 ± 0.9 [a]	36.77 ± 4.9 [b]
NAA	0 µM	95.38 ± 3.4 [a]	7.53 ± 0.2 [a]	56.8 ± 0.8 [a]
+	0,54 µM	94.59 ± 4.8 [a]	7.09 ± 0.2 [a]	46.1 ± 1.1 [b]
BA 8,9µM	1,34 µM	80 ± 9.7 [a]	7.08 ± 0.5 [a]	45.81 ± 3.4 [b]
N = 120	2,69 µM	100 ± 0.0 [a]	7.07 ± 0.5 [a]	44.24 ± 1.5 [b]
	5 days	94.55 ± 1.3 [a]	7.6 ± 0.5 [b]	52.89 ± 1.4 [ab]
Days on	6 days	94.87 ± 2.1 [a]	6.99 ± 0.2 [b]	54.93 ± 1.0 [a]
RI	7 days	93.33 ± 2.6 [a]	6.46 ± 0.3 [b]	45.24 ± 1.8 [c]
	8 days	96.36 ± 1.7 [a]	7.27 ± 0.4 [b]	48.93 ± 2.1 [abc]
	9 days	86.67 ± 4.6 [a]	7.04 ± 0.5 [b]	47.96 ± 2.7 [bc]
N = 494	10 days	97.5 ± 1.0 [a]	9.04 ± 0.4 [a]	51.68 ± 1.2 [ab]
	AL1	88.12 ± 4.4 [a]	8.25 ± 0.4 [b]	38.23 ± 1.2 [a]
Genotype	AL2	89 ± 3.7 [a]	11.67 ± 2.1 [ab]	41.69 ± 2.4 [a]
	AL3	89.17 ± 4.6 [a]	15 ± 1.3 [a]	38.84 ± 2.7 [a]
N = 120	AL4	88.17 ± 4.8 [a]	12.5 ± 2.5 [ab]	39.52 ± 0.5 [a]
Days on	22 days	89.17 ± 4.7 [a]	8.13 ± 0.4 [b]	37.15 ± 1.3 [a]
RD	27 days	87.61 ± 4.1 [a]	7 ± 1.2 [b]	41.84 ± 4.4 [a]
N = 90	48 days	88.49 ± 5.1 [a]	12.14 ± 0.9 [a]	41.12 ± 1.9 [a]

Values are the mean ± SE. For each treatment, values followed by different letters are significantly different.

The results of the PCA analysis (Fig. 6) could explain 65.75 % of total variance observed and indicated that the number of roots (NR) is related to the length of the longest root (LLR), displaying a factor loadings higher than 0.70. The data also clearly show that shoot length is strongly affected by BA concentration even after rooting had occurred. In fact, when BA was

used at the highest concentration, shoot length attained its lowest value after acclimatization (Table 3). On these conditions, apical shoot necrosis was often observed (8.7%; P>0.05) as well as callus formation at the basis of the shoots. Considering these results it was decided to reduce the level of IBA in the next rooting assays. Thus, when shoots originated from media containing different cytokinins were rooted the concentration of IBA used was reduced to 14.8 µM instead of the 24.6 µM previously tested. This IBA drastic reduction (maintaining the same induction period of 7days) is likely responsible for the low rooting rate observed (average of 2.00 ±4.14 %). Therefore, on the following assays IBA 19.7 µM was used.

Variable	Factor Loadings (Unrota Extraction: Principal cor (Marked loadings are > Factor 1	Factor 2
BA	-0.097995	-0.699946
NRoots	0.766604	0.100591
LLRoot	0.838832	-0.247745
LSRoot	0.546832	-0.584358
Shoot-L	0.548494	0.695828
Expl.Var	1.900795	1.387070
Prp.Totl	0.380159	0.277414

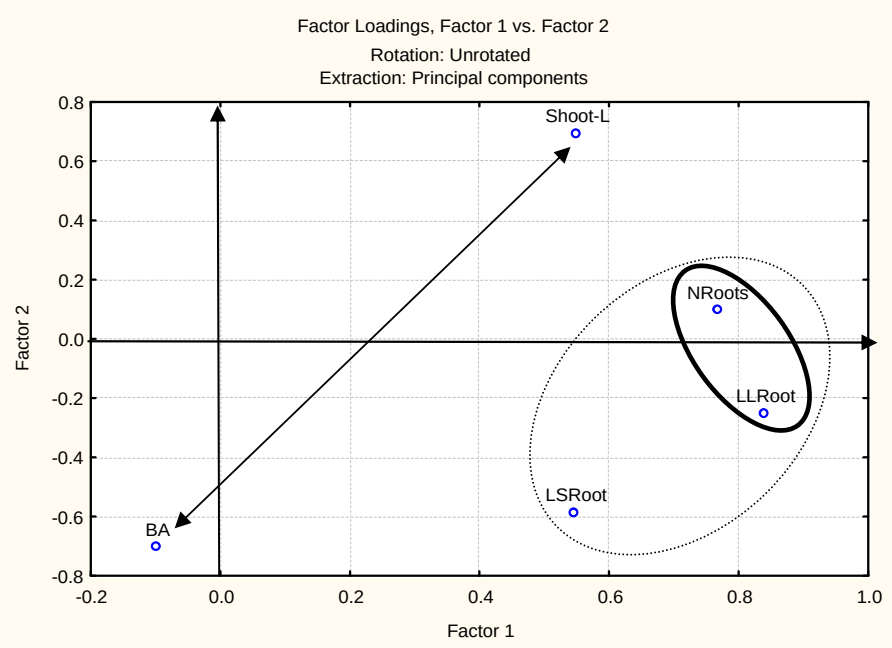


Figure 6 - Evaluation of the effect of BA concentration on rooting by PCA analysis considering the following variables: BA concentration, number of roots (NR), length of the longest root (LLR), length of the shortest root (LSR) and shoot length (SL). (The variables are associated to each factor 1 and 2, according to the coefficients, factor loadings, presented on the left table. For more details see Figure 1.

Rooting of shoots formed on a medium containing NAA (plus BA 8.9 µM) showed no significant differences either on the frequencies of rooting and on the number of roots per shoot (Table 3) with averages of 94.61% and 7.31, respectively. However, shoot length, measured after root induction and development (5 weeks), showed to be negatively influenced (P<0.01) by the presence of NAA in the multiplication medium (Table 3; Appendix Table 2). The period of root induction (5 to 10 days) had no impact on the percentage of rooted shoots (Table 3), however the number of roots per explant (9.04±3.95) was significantly higher when a ten-day period of exposition to auxin was used (Appendix Table 2).

Shoot genotype was another parameter tested for shoot rooting. The data obtained indicated that the rooting percentage as well as shoot length were not influenced by the genotype (Table 3). However, the number of roots per explant is genotype dependent ($P<0.01$; Appendix Table 2) with the genotypes AL3, AL4 and AL2 giving the best results (15.0, 12.5 and 11.7, respectively).

When different periods of root development (RD-days: 22 - 48) were tested before acclimatization, the data obtained indicated that the same parameters were not influenced by the root development period (Table 3; Appendix Table 2). However, on RD-48, the plantlets displayed a number of roots significantly higher (12.14±4.35) than on RD-22 or RD-27 (8.12±2.65 and 7.00±2.74, respectively; $P<0.01$, Appendix Table 2). In these assays, PCA analysis (Fig. 7) explained 57.2 % of total variance, showing that the number of roots (NR) is related to the length of the longest root (LLR), root development period (RD) and genotype (factor loadings $\geq$ 0.70). By contrast, more extensive callus formation is generally accompanied by a reduced root growth (LSR) and, in some cases, a complete inhibition of the root development.

Variable	Factor Loadings (Unrota Extraction: Principal con (Marked loadings are > Factor 1	Factor 2
Genotype	-0.696659	0.351342
Root Develop.	-0.879810	0.075352
Nroots	-0.765053	0.213427
LLRoot	-0.745316	-0.336106
LSRoot	-0.329308	-0.773908
SLength	-0.326994	0.236524
Callus	0.069809	0.663609
Expl.Var	2.620444	1.382893
Prp.Totl	0.374349	0.197556

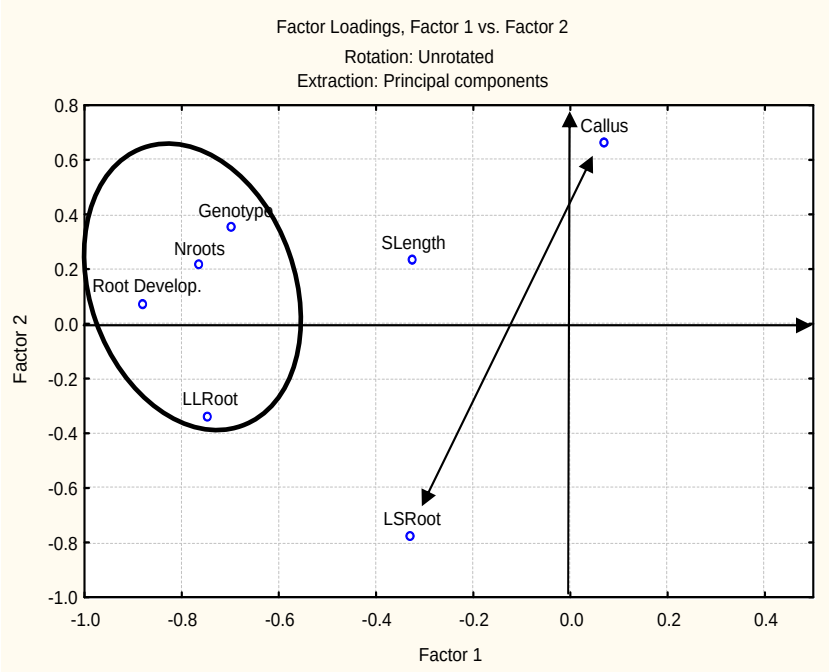


Figure 7 - Evaluation of the effect of different genotypes on rooting by PCA analysis considering the following variables: genotype, root development period, number of roots (NR), length of the longest root (LLR), length of the shortest root (LSR), shoot length (SL) and basal callus formation (callus). For more details see Figure 1.

3.5 DISCUSSION

The results so far obtained indicate that the multiplication rate evaluated by the number of shoots obtained per test tube that can be used for further multiplication (SNX) is dependent of the genotype and PGRs tested. When different BA concentrations were used best results were achieved with 8.9 µM. Cytokinins are usually used on the micropropagation media to stimulate axillary shoot proliferation (Van Staden *et al.*, 2008; Chawla, 2009; El-Agamy, 2009; Tornero *et al.*, 2009). However, the ideal concentrations are different from species to species and need to be established accurately to achieve effective rates of multiplication. Moreover, some problems related with the use of cytokinins have been pointed out such as callus growth, poor shoot growth and vitrification (Van Staden *et al.*, 2008; Chawla, 2009). In other members of the Ericaceae family, such as *Rhododendron ponticum* (Almeida *et al.*, 2005) and *Vaccinium macro*carpon (Debnath and McRae, 2001) cytokinins have been also commonly included in the culture media to promote shoot proliferation. Our data indicated that there are differences in the effect of the different cytokinins analysed. Thus, it was found that KIN was the most effective in promoting shoot growth whereas TDZ, a urea-derived cytokinin, induced callus proliferation in the explants. This situation must be avoided since these calli can display organogenic potential leading to the production of adventitious shoots that can exhibit some kind of variability. Experiments performed with *A. unedo* and other members of the Ericaceae family indicate that beyond cytokinins other factors such as culture media composition and sucrose can also influence shoot proliferation. Gonçalves and Roseiro (1994) and Mereti *et al*. (2002) showed that WPM (woody plant medium) combined with BA gave the best rates of shoot proliferation. According to Mendes (1997) the Anderson medium and the cytokinin 2-iP were particularly effective to propagate adult material. In *Vaccinium macrocarpon* (Debnath and McRae, 2001) the highest rates of shoot production were obtained when nodal segments were cultured on a medium supplemented with 12.3-24.6 µM of 2-iP without auxin. Also, in *Vaccinium myrtillus* and *Vaccinium vitis-idaea* (Jaakola, 2001) higher rates of micropropagation were obtained when the cytokinin 2-iP was used at concentrations of 49.2 µM and 24.6 µM, respectively. The best results of multiplication were obtained in the WPM salts with MS vitamins and 25 mM 2-iP for *Vaccinium corymbosum* L. cv. Berkeley from nodal segments of adult field grown plants (Gonzalez *et al.*, 2000). Our results showed that the auxin NAA was unable to increase the multiplication rate. Moreover, inclusion of the NAA in the multiplication phase induced callus formation and this feature

increased with the number of subcultures. Some species may require a low concentration of auxin in combination with high levels of cytokinin to increase shoot proliferation (Van Staden *et al.*, 2008). Nevertheless, this does not seem to be the case in *Arbutus unedo* or in related species as our results and those obtained in *Arbutus xalapensis* (Mackay, 1996) might suggest. The observation that both the auxin NAA and the cytokinin TDZ are able to induce callus formation in *A. unedo* seems to indicate, as pointed out by other authors, that TDZ may act through the modification of the endogenous levels of auxins increasing the amounts of indol-3-acetic acid or other endogenous auxin-like compounds that promote cell proliferation (Visser *et al.*, 1992; Maxwell, 2007; Sedlak and Paprstein, 2009). Callus formation may be interesting if they have the ability to undergo shoot formation without loss of the genetic uniformity of the regenerated plants. This possible alternative for plant regeneration in strawberry tree needs to be analysed in more detail. For now it was only observed that on the conditions tested shoot formation was never recorded on these calli. In some sporadic situations, morphologically abnormal underdeveloped leaves were seen arising from the calli but further shoot growth was impaired. However, this kind of observation indicates that the calli thus obtained are able to organize meristematic regions showing its potential for future research.

Our results also showed that the genotype is another important factor involved on shoot proliferation of *A. unedo*. It is well known that *in vitro* culture is highly dependent on the genotype of donor material. In fact different types of morphogenic responses *in vitro* such as somatic embryogenesis, organogenesis, shoot proliferation, rooting and microspore embryogenesis are strongly determined by the genotype of the explants (Bhau and Wakhlu, 2001; Gajdošová *et al.*, 2007; Ostrolucká *et al.*, 2007; Gahan and George, 2008). This seems to indicate that specific genetic combinations found in some genotypes are more prone to undergo a particular type of morphogenesis than others. In our experiments we found that shoot multiplication and callus formation were highly genotype-dependent. Because these factors are also highly dependent of the PGRs present in the culture media it is plausible to assume that different genotypes possess different levels of endogenous auxins and/or cytokinins that influence their behaviour *in vitro*. Experiments of somatic embryogenesis induction in this species carried out at our lab also showed that somatic embryo formation is dependent of the genotype (data not published). Although these results need to be supported by a more detailed analysis they point out to a strong variability in *A. unedo* genotypes in what concerns *in vitro* culture. However, it should be referred that many of the published genotype effects may in fact result from less understood interactions between the culture

environment and the genetic background of the explant. A genotype displaying a low multiplication rate can be only the result of deficient culture conditions that can be improved to achieve a better response. Further research on *A. unedo* micropropagation is necessary to better understand the role of the genotype on *in vitro* morphogenesis.

Rooting is a crucial step to the success of micropropagation. Without an effective root system plant acclimatization will be difficult and the rate of plant propagation may be severely affected (Gonçalves *et al.*, 1998). In a previous paper we established the conditions for *in vitro* rooting in *A. unedo* (Gomes and Canhoto, 2009). In that paper the role of the multiplication conditions on rooting was evaluated. According to the conditions now tested it can be concluded that a ten day period of root induction is the more suitable for *A. unedo* whereas plant acclimatization should not be carried out before 35-40 days on the root development medium. The statistic analysis performed showed that the frequency of root formation is not affected by previous multiplication conditions. However, when the number of roots formed per explant was the variable considered the results showed a strong influence of factors such as the concentration of BA, genotype, and the periods of root induction and development. Metaxas *et al*. (2004) have also found that the genotype and growth regulators are the main factors involved on root formation in cuttings of *A. unedo*. The eventual role of the genotype was already discussed and what was stated then can also apply to the results obtained during the rooting phase. A remarkable result obtained in *A. unedo* was the observation that shoots produced on higher cytokinin containing medium are more amenable to root induction than shoots obtained with the lowest concentrations of BA. A review of the literature clearly points out to a negative effect of cytokinins on shoot rooting (Van Staden *et al.*, 2008) although a positive role has been occasionally referred (Nemeth, 1979; Bennett *et al.*, 1994). Once again these results may be explained by the complex interactions between endogenous and exogenous growth regulators (mainly auxins and cytokinins) occurring during *in vitro* cultures. Only a time-course evaluation of the growth regulators present in the explants during root morphogenesis can bring some information about these interactions. However, other compounds such as ethylene and phenolics may also be involved (Machakova *et al.*, 2008). Finally it should be noted that the tendency of some genotypes to produce callus at the basis of the shoots is a major drawback for further rooting since it can avoid the establishment of normal connections between the vascular system of the forming roots and the shoot.

Assays of acclimatization carried out by authors working with *A. unedo* showed that plantlets obtained *in vitro* are easily acclimatized (Gonçalves and Roseiro, 1994; Mereti *et*

al., 2002; Gomes and Canhoto, 2009) and the same is true for other members of the Ericaceae family, such as several *Vaccinium* species (Isutsa *et al.*, 1994). The conditions used for acclimatization in our experiments seem also to work well allowing the regeneration of a large number of individuals from different genotypes which are now growing in the field.

The results so far obtained on the micropropagation of *A. unedo* have established the conditions to clone selected trees and to propagate them in large scale to be evaluated in the field for fruit quality and productivity. Following that analysis the more indicated genotypes will be produced in large scale to be distributed to the farmers interested in this crop. This is part of our strategy for breeding strawberry tree making it a competitive species for fruit production. These selected clones are now being characterized by molecular markers (microsatellites) and conserved *in vitro* and in the field in a germplasm bank. These genotypes are also being evaluated for its potential to undergo somatic embryogenesis and the first preliminary results about somatic embryo formation in strawberry were recently published (Gomes *et al.*, 2009).

3.6 REFERENCES

Almeida, R., Gonçalves, S., Romano, A., 2005. *In vitro* propagation of endangered *Rhododendron ponticum* L. subsp. *baeticum* (Boissier & Reuter) Handel-Mazzetti. Biodiv. Conservation 14, 1059-1069.

Anderson, W.C., 1984. A revised tissue culture medium for shoot multiplication of *Rhododendron*. J. Amer. Soc. Hort. Sci. 109, 343-347.

Banko, T.J., Stefani, M.A., 1989. *In vitro* propagation of *Oxydendrum arboreum* from mature trees. Hortscience 24, 683-685.

Bennett, I.J., McComb, J.A., Tonkin, C.M., Mcdavid, D.A.J., 1994. Alternating cytokinins in multiplication media stimulates *in vitro* shoot growth and rooting of *Eucalyptus globulus* Labill. Ann. Bot. 74, 53-58.

Bhau, B.S., Wakhlu, A.K., 2001. Effect of genotype, explant type and growth regulators on organogenesis in *Morus alba*. Plant Cell Tis. Org. Cult. 66, 25-29.

Chawla, H.S., 2009. Introduction to plant biotechnology, 3rd ed. Science Publis., Inc., New Hampshire.

De Fossard, R.A., Nitsch, C., Cresswell, R.J., Lee, H.C.M., 1974. Tissue and organ culture of *Eucalyptus*. N. Zeal. J. For. Sci. 4, 267-278.

Debnath, S.C., McRae, K., 2001. An efficient *in vitro* shoot propagation of Cranberry (*Vaccinium macrocarpon* Ait.) by axillary bud proliferation. In Vitro Cell. Dev. Biol.-Plant 37, 243-249.

Duncan, D.B., 1955. Multiple range and multiple F tests. Biometry 11, 1-42.

El-Agamy, S.Z., 2009. *In vitro* propagation of some Grape root stocks. Acta Hort. 839, 125-132.

G. F.U.U. Sp, 2008. Web site of the Global Facilitation Unit for Underutilized Species, www.underutilized-species.org/ Nov./2009.

Gahan, P.B., George, E.F., 2008. Adventitious regeneration. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 355-401.

Gajdošová, A., Ostrolucká, M.G., Libiaková, G., Ondrušková, E., 2007. Protocol for micropropagation of *Vaccinium vitis-idaea* L. In: Jain, S., Häggman, H. (Eds.), Protocols for Micropropagation of Woody Trees and Fruits. Springer, Berlin, pp. 457-464.

Gautheret, R.J., 1959. La culture des tissues végétaux, techniques et réalisations. Masson, Paris.

George, E.F., Debergh, P.C., 2008. Micropropagation: uses and methods. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 29-64.

Godinho-Ferreira, P.G., Azevedo, A.M., Rego, F., 2005. Carta da tipologia florestal de Portugal Continental. Silva Lusitana 13, 1-34.

Gomes, F., Canhoto, J.M., 2003. Micropropagation of *Eucalyptus nitens* Maiden (Shining gum). In Vitro Cell. Dev. Biol.-Plant 39, 316-321.

Gomes, F., Canhoto, J.M., 2009. Micropropagation of strawberry tree (*Arbutus unedo* L.) from adult plants. In Vitro Cell. Dev. Biol.-Plant 45, 72-82.

Gomes, F., Lopes, M.L., Santos, T., Canhoto, J.M., 2009. Micropropagation of selected trees of *Arbutus unedo* L. trough axillary shoot proliferation and somatic embryogenesis. Acta Hort. 839, 111-116.

Gonçalves, J.C., Diogo, G., Amâncio, S., 1998. *In vitro* propagation of chestnut (*Castanea sativa x Castanea crenata*): effects of rooting treatments on plant survival, peroxidase activity and anatomical changes during adventitious root formation Sci. Hort. 72, 265-275.

Gonçalves, J.C., Roseiro, R.J., 1994. Establishment and *in vitro* multiplication of *Arbutus unedo* L. seedlings. VIII International Congress of Plant Tissue and Cell Culture, Firenze, Italy, p. *Abst* 20.

Gonzalez, M.V., Lopez, M., Valdes, A.E., Ordas, R.J., 2000. Micropropagation of three berry fruit species using nodal segments from field-grown plants. Ann. App. Biol. 137, 73-78.

Isutsa, D.K., Pritts, M.P., Mudge, K.W., 1994. Rapid propagation of blueberry plants using *ex-vitro* rooting and controlled acclimatization of micropropagules. Hortscience 29, 1124-1126.

Jaakola, L., 2001. Effect of N^6-isopentenyladenine concentration on growth initiation *in vitro* and rooting of bilberry and lingonberry microshoots. Plant Cell Tis. Org. Cult. 66, 73-77.

Lloyd, G.B., McCown, B.H., 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Proc. Int. Plant Prop. Soc. 30, 421-427.

Machakova, I., Zazimalova, E., George, E.F., 2008. Plant growth regulators I: Introduction; auxins, their analogues and inhibitors. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 175-204.

Mackay, W.A., 1996. Micropropagation of *Texas madrone, Arbutus xalapensis* HBK. Hortscience 31, 1028-1029.

Maxwell, P.A.J., 2007. The mode of action of thidiazuron: auxins, indoleamines and ion channels in the regeneration of *Echinacea purpurea* L. Plant Cell Rep. 26, 1481-1490.

Mendes, M.L.A., 1997. Multiplicação vegetativa *in vitro* de medronheiro. Tese de Mestrado. ISA-UTL, Lisboa.

Mereti, M., Grigoriadou, K., Leventakis, N., Nanos, G.D., 2003. *In vitro* rooting of strawberry tree (*Arbutus unedo* L.) in medium solidified by peat - perlite mixture in combination with agar. Proc. First Int. Symp. on Acclimat. and Estab. of Microp. Plants, pp. 207-210.

Mereti, M., Grigoriadou, K., Nanos, G.D., 2002. Micropropagation of the strawberry tree, *Arbutus unedo* L. Sci. Hort. 93, 143-148.

Metaxas, D.J., Syros, T.D., Yupsanis, T., Economou, A.E., 2004. Peroxidases during adventitious rooting in cuttings of *Arbutus unedo* and *Taxus baccata* as affected by plant genotype and growth regulator treatment. Plant Growth Regul. 44, 257-266.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant. 15, 473-497.

Nemeth, G., 1979. Benzyladenine-stimulated rooting in fruit-tree rootstocks cultured *in vitro*. Z. Pflanzenphysiol. 95, 389-396.

Neppi, M., 2001. Alberi ed arbusti della flora mellifera della regione mediterrânea. In: Piotto, B., Noi, A. (Eds.), Propagazione per seme di alberi e arbusti della flora mediterranea. Dipart. Prev. Risanam. Ambient., Manuale ANPA (Agenz. Naz. per la Protezi. dell' Ambiente), pp. 44-49.

Ostrolucká, M.G., Gajdošová, A., Libiaková, G., Hrubíková, K., Bezo, M., 2007. Protocol for micropropagation of selected *Vaccinium*. In: Jain, S.M., Häggman, H. (Eds.), Protocols for Micropropagation of Woody Trees and Fruits. Springer, Berlin, pp. 445-455.

Pabuccuoglu, A., Kivcak, B., Bas, M., Mert, T., 2003. Antioxidant activity of *Arbutus unedo* leaves. Fitoterapia 74, 597-599.

Piotto, B., Piccini, C., Arcadu, P., 2001. La ripresa della vegetazione dopo gli incendi nella regione mediterrânea. In: Piotto, B., Noi, A. (Eds.), Propagazione per seme di alberi e arbusti della flora mediterranea. Dipart. Prev. Risanam. Ambient., Manuale ANPA (Agenz. Naz. per la Protezi. dell' Ambiente), pp. 32-38.

Ratnaparkhe, M.B., 2007. Blueberry. In: Kole, C. (Ed.), Genome mapping and molecular breeding in plants, Fruits and Nuts. Springer-Verlag, Berlin Heidelberg, pp. 217-227.

Sedlak, J., Paprstein, F., 2009. *In vitro* proliferation of newly bred Czech pear cultivars. Acta Hort. 839, 87-92.

Tornero, O.P., C. Tallón, C., Porras, I., 2009. *Citrus limon* micropropagation: Effect of different phytohormones on multiplication and rooting. Acta Hort. 839, 57-62.

Van Staden, J., Zazimalova, E., George, E.F., 2008. Plant growth regulators II: cytokinins, their analogues and inhibitors. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 205-226.

Visser, C., Qureshi, J.A., Gill, R., Saxena, P.K., 1992. Morphoregulatory role of thidiazuron: substitution of auxin and cytokinin requirement for the induction of somatic embryogenesis in *Geranium* hypocotyl cultures. Plant Physiol. 99, 1704-1707.

Zar, J.H., 1996. Biostatistical Analysis (4th Ed.). Prentice-Hall, Upper Saddle River.

APPENDIX

Appendix Table 1 – ANOVA: effect of different combinations of BA (experiment I), cytokinins (experiment II), combinations of NAA and BA (experiment III), and of the genotype (experiment IV) on the multiplication of *A. unedo* Multiplication was evaluated by the maximum shoot length (SL mm) and by the number of shoots formed per test tube for further multiplication (SNX).

Number of shoots formed per test tube for further multiplication (SNX)						Shoot length (SL mm)					
Experiment I BA (µM)						**Experiment I** BA (µM)					
Source	SS	d.f.	MS	F	p	Source	SS	d.f.	MS	F	p
BA	30.641	4	7.660	17.209	0.000**	BA	4713.7	4	1178.4	17.387	0.000**
Error	373.020	838	0.445			Error	56797.2	838	67.8		
Experiment II Cytokinins (8.9 µM)						**Experiment II** Cytokinins (8.9 µM)					
Source	SS	d.f.	MS	F	p	Source	SS	d.f.	MS	F	p
CytoKinins	10.428	4	2.607	14.483	0.000**	CytoK.	17802.9	4	4450.7	98.870	0.00**
Error	169.559	942	0.180			Error	42405.1	942	45.0		
Experiment III NAA + BA 8.9µM						**Experiment III** NAA + BA 8.9µM					
Source	SS	d.f.	MS	F	p	Source	SS	d.f.	MS	F	p
NAA	154.391	3	51.464	36.789	0.00**	NAA	18531.6	3	6177.2	63.679	0.00**
Error	1789.193	1280	1.399			Error	124166.3	1280	97.0		
Experiment IV Genotypes						**Experiment IV** Genotypes					
Source	SS	d.f.	MS	F	p	Source	SS	d.f.	MS	F	p
Genotype	37.9559	9	4.2173	7.654	0.000**	Genoty.	3778.57	9	419.84	11.589	0.000**
Error	303.0423	552	0.5510			Error	19924.89	552	36.23		

*Significant at $P \leq 0.05$; **Significant at $P \leq 0.01$.

Appendix Table 2 – ANOVA: effect of different culture conditions on shoots rooting.

Rooting (%)						**Number of roots**						**Final Shoot Length (mm)**					
Conditions of shoot formation: BA (µM)						**Conditions of shoot formation:** BA (µM)						**Conditions of shoot formation:** BA (µM)					
Source	SS	d.f.	MS	F	p	Source	SS	d.f.	MS	F	p	Source	SS	d.f.	MS	F	p
BA (µM)	64.77	4	16.19	0.06936	0.9953	BA	119.717	4	29.929	3.01	0.023*	BA	2048.7	4	512.2	2.644	0.000**
Error	3501.96	15	233.46			Error	735.954	74	9.945			Error	14335.1	74	193.7		
Cond. of shoot formation: NAA + BA 8.9µM						**Cond. shoot formation**: NAA + BA 8.9µM						**Cond. shoot formation**: NAA + BA 8.9µM					
Source	SS	d.f.	MS	F	p	Source	SS	d.f.	MS	F	p	Source	SS	d.f.	MS	F	p
NAA (µM)	1085.6	3	361.9	1.894	0.1354	NAA	24.52	3	8.17	0.79	0.4977	NAA	14723.4	3	4907.8	27.903	0.000**
Error	19100.8	100	191.0			Error	5044.71	490	10.30			Error	86184.4	490	175.9		
Conditions of shoot induction: Ri (days)						**Conditions of shoot induction**: Ri (days)						**Conditions of shoot induction**: Ri (days)					
Source	SS	d.f.	MS	F	p	Source	SS	d.f.	MS	F	p	Source	SS	d.f.	MS	F	p
Ri (days)	313.4	5	62.7	0.158	0.9065	Ri	328.14	5	65.63	6.76	0.000**	Ri	6528.0	5	1305.6	6.751	0.000**
Error	19873.0	50	397.5			Error	4741.09	488	9.72			Error	94379.8	488	193.4		
Genotype						**Genotype**						**Genotype**					
Source	SS	d.f.	MS	F	p	Source	SS	d.f.	MS	F	p	Source	SS	d.f.	MS	F	p
Genotype	492.15	3	164.05	0.6009	0.6267	Genot.	314.268	3	104.756	10.67	0.000**	Genot.	67.43	3	22.48	0.272	0.8451
Error	3276.21	12	273.02			Error	687.083	88	7.808			Error	5775.06	88	65.63		
Cond. shoot and root development: RD (days)						**Cond. shoot & root development:** RD (days)						**Cond. shoot & root development:** RD (days)					
Source	SS	d.f.	MS	F	p	Source	SS	d.f.	MS	F	p	Source	SS	d.f.	MS	F	p
RD (days)	899.35	2	449.68	1.8092	0.1978	RD	261.530	2	130.765	12.55	0.000**	RD	285.98	2	142.99	1.827	0.1684
Error	3728.25	9	414.25			Error	739.821	71	10.420			Error	5556.51	71	78.26		

*Significant at $P \leq 0.05$; **Significant at $P \leq 0.01$.

4 - Mycorrhizal synthesis between Basidiomycete fungi and *Arbutus unedo L.*

Part of this chapter was submitted to an international journal

Parts of the results were also presented as a poster in the following meeting:

Gomes, F., Santos, V., Sorzabalbere, I., Ponce Díaz, A., San Martin, E.P., Canhoto, J.M., 2008. Efeito da inoculação com *Pisolithus tinctorius* na sobrevivência de plântulas micropropagadas de *Arbutus unedo* L., I Congresso Luso-Espanhol de Fixação de Azoto, Fundamentos e Aplicações agronómicas e ambientais da fixação de azoto e microrganismos benéficos para as plantas. INRB, I.P. & SEFIN, Estoril, p. Abst 20.

4 MYCORRHIZAL SYNTHESIS BETWEEN BASIDIOMYCETE FUNGI AND *ARBUTUS UNEDO L.*

4.1 ABSTRACT

The objective of this study was to evaluate fungus-plant host compatibility during *in vitro* conditions between *Arbutus unedo* clones and *Pisolithus tinctorius* or *Lactarius deliciosus*. Several sets of experiments were carried out by transfer of induced to root shoots to previously inoculated substrate in vessels or test tubes used as containers. In nursery, two inoculation treatments with *P. tinctorius* were tested and compared to control plants: vegetative inocula produced in liquid medium and dry sporocarps. In a field trial, the nursery inoculation treatments were compared to fertilized plants (seedlings and selected clones). Plant height was evaluated 4 and 20 months later, in the nursery and in the field trial, respectively. Arbutoid mycorrhizae were observed *in vitro* 1 month after inoculation indicating compatibility between *A. unedo* and *P. tinctorius*. Shoots cultured for 2 weeks on the root development medium following root induction gave origin to plantlets displaying enhanced growth as well as increased survival rate after acclimatization (P>5%). Secondary and branched roots were observed in inoculated plantlets. Root development and mycorrhization was higher in test tubes when compared with assays performed in vessels. *L. deliciosus* showed reduced *in vitro* growth compared to *P. tinctorius*. The arbutoid mycorrhizae were described based on morphological and anatomical characters. The observations showed the presence of a mantle, Hartig net, and intracellular hyphal complexes confined to the epidermal root cells. *Thelephora* and *Hebeloma* mycorrhizae, two types of highly competitive and widespread mycorrhizae on nurseries were analysed by molecular markers techniques in previously inoculated plants, 12 or 17 months after acclimatization or *in vitro* inoculation, respectively. Both mycorrhizae inocula treatments tested in nursery improved plant growth compared to control plants and seedlings (P>5%). Mycorrhization may help to reduce fertilizers and biocides application thus contributing to more friendly environmental agricultural practices and to a decrease of productivity costs. Further studies are needed to ensure fungal persistence, as well as, fungal strains selection for their aggressiveness under nursery conditions, which is a requirement for a successful implementation of these methods. Mycorrhizal synthesis with edible fungi can improve not only the success of plant transplantation to field conditions, but also can represent another forest income, contributing to the development of local communities.

Key words: Arbutoid mycorrhizae; Ericaceae; field growth; *in* and *ex vitro* inoculation.

RESUMO

O objectivo deste estudo foi avaliar a compatibilidade fungo-planta hospedeira entre clones de *Arbutus unedo* e *Pisolithus tinctorius* ou *Lactarius deliciosus,* em condições *in vitro*. Diversos ensaios foram estabelecidos pela transferência de rebentos, após indução de enraizamento, para substrato previamente inoculado, em frascos ou tubos testados como contentores. No viveiro foram testados dois tratamentos de inoculação com *P. tinctorius* e comparados com plantas controlo: micélio produzido em meio líquido e caldo esporal. A altura das plantas foi avaliada no viveiro e num ensaio em campo, após 4 e 20 meses, respectivamente. *In vitro*, micorrizas arbutóides foram observadas um mês após a inoculação com *P. tinctorius.* A expressão do sistema radicular durante 2 semanas, após a indução do enraizamento melhorou o desenvolvimento das plântulas, bem como a taxa de sobrevivência na aclimatização (P>5%). Nas plantas inoculadas foi observado o desenvolvimento de um sistema radicular ramificado. A utilização de tubos de ensaio foi mais favorável ao desenvolvimento da raiz e à micorrização que a utilização frascos. *L. deliciosus* mostrou um crescimento *in vitro* inferior a *P. tinctorius.* As micorrizas arbutóides foram descritas com base nas características morfológicas e anatómicas. As observações mostraram um manto espesso, a rede de Hartig e complexos de hifas intracelulares, ambos confinados às células da epiderme. *Thelephora* e *Hebeloma*, fungos micorrízicos competitivos e normalmente existentes em viveiros, foram identificados por marcadores moleculares em plantas previamente inoculadas, 12 ou 17 meses após a aclimatização ou inoculação *in vitro*, respectivamente. Ambos os tratamentos com micorrizas testados em estufa melhoraram o crescimento das plantas relativamente às plantas controlo e de semente (P>5%). As micorrizas podem contribuir para a redução da aplicação de fertilizantes e biocidas, prática que poderá reduzir o custo da planta e a poluição ambiental. Serão necessários mais estudos para assegurar a persistência do fungo inoculado, bem como a selecção de estirpes pela sua agressividade em viveiro, facto este que será necessário para viabilizar a implementação do método. A utilização de micorrizas associada a fungos comestíveis, poderá melhorar a transplantação das plantas, mas também funcionar como mais um recurso económico, contribuindo para a fixação da população nas zonas rurais.

Palavras-chave: ensaio de campo; Ericaceae; inoculação *in* e *ex vitro*; micorrizas arbutóides.

4.2 INTRODUCTION

Species of *Arbutus* (Ericaceae) form mycorrhizae with a broad range of fungal partners (Massicotte *et al.*, 1993). Ultrastructural studies classified as arbutoid mycorrhizae those formed by plants of *Arbutus*, *Arctostaphylos* and *Pyrola* (Smith and Read, 1997). Structurally, this kind of mycorrhizae resembles the ectendomycorrhizae since they possess a mantle, Hartig net (HN), and form intracellular hyphal complexes. However, they differ from that type of mycorrhizae as the intracellular hyphal complexes are restricted to the epidermal cells (Peterson and Massicotte, 2004). Massicotte *et al.* (1993) added to the characteristics of arbutoid mycorrhizae the presence of a variable fungal sheath.

Previous works have shown that fungi involved in the formation of arbutoid mycorrhizae are also able to form ectomycorrhizae in association with other plant hosts (Molina and Trappe, 1982; Massicotte *et al.*, 1993), as is the case of the broad host range basidiomycete *Pisolithus tinctorius*. *Lactarius deliciosus*, is a basidiomycete (Russulaceae) able to form ectomycorrhizae with trees, particularly conifers. According to Parladé *et al.* (2004) the reports about mycorrhizae formation by *L. deliciosus* are, to some extent, contradictory. Considering observations on fruiting behavior, it seems that most species of *Lactarius* are late-stage ectomycorrhizae colonizers since sporocarps are generally observed in old forest stands. However, other studies have reported the identification of this species in young (0 – 5 years old) *Pinus sylvestris* plantations. Furthermore, pure culture inoculation studies demonstrate that this fungal species readily colonizes the root system of pines under aseptic conditions (Parladé *et al.*, 2004). Based on these data, some authors have began to ponder the mycorrhizal inoculation of tree species with edible *Lactarius* as an effective way to increase forest productivity in Mediterranean forest areas in which the natural resources are scarce or very focused. Unlike *L. deliciosus*, which has as host mainly conifers (Martins, 2004), *P. tinctorius* (Syn. *P. arhizus*) is a widespread basidiomycete fungi able to establish ectomycorrhizae with a large diversity of hosts (Cairney and Chambers, 1997). However, there are evidences reporting some degree of specificity in the interaction between host root and *P. tinctorius* strains. For instance, it has been reported that *P. tinctorius* isolated from carpophores collected in association with *Pinus* spp. are poor colonizers of *Eucalyptus* spp. (Cairney and Chambers, 1997).

Earlier studies had already shown the lack of specificity in hosts forming arbutoid mycorrhizae (Molina and Trappe, 1982). Other experiments also indicated that *Arbutus*

menziesii and *Arctostaphylos uva-ursi* were able to develop arbutoid mycorrhizae in association with several fungi which also can form ectomycorrhizae, including *Cenococcum geophilum, Hebeloma crustiliniforme, Laccaria laccata, Lactarius sanguifluus, Piloderma bicolour, Pisolithus tinctorius, Poria terrestris* var. *subluteus, Rhizopogon vinicolor* and *Thelephora terrestris* (Smith and Read, 1997). Molina and Trappe (1982) previously observed that *Arbutus menziesii* and *Arctostaphylos uva-ursi* are broadly receptive towards fungal associates, in particular to those linked to ectomycorrhizae. From these studies it became clear that the plant host plays a crucial role in regulating the development and structure of mycorrhizae and that the same type of fungi may originate different types of mycorrhizae according to the plant species that was colonized (Rai, 2001; Mirabelli *et al.*, 2009). Studies performed by Richard *et al.* (2005) point also in this direction since they have indicated that two mycorrhizal hosts, *A. unedo* and *Quercus ilex* growing in an old Mediterrean forest dominated by *Quercus ilex*, tended to share a few ectomycorrhizal species (<15% of the ectomycorrhizal diversity). The molecular analysis performed by these authors enabled the identification of 28 taxa (81% of the characterized mycorrhizae), being the family Thelephoraceae the most represented; the genera *Russula*, *Cortinarius, Laccaria*, all of the Russulaceae family, and members of the Clavulinaceae were also present. Interactions between fungal strain and plant species in other Ericaceae such as *Rhododendron* sp. and *Vaccinium corymbosum* also showed that the type of fungal strain used affects mycorrhization and further plant growth (Noé *et al.*, 2002; Eccher and Martinelli, 2010). Taken together all these data indicate that the selection of the most effective fungal strains for their endurance under nursery and field conditions is a prerequisite for the successful establishment of mycorrhizae (Rai, 2001; Parladé *et al.*, 2004).

Nitrogen is often a limiting factor for plant growth and production. Mycorrhizal fungi can uptake and use a variety of nitrogen sources such as organic compounds (amino acids and oligopeptides), and enhance mineral uptake as ammonium and nitrate forms (Gobert and Plassard, 2008). This capacity is particularly advantageous for woody plants conferring them a selective advantage in natural conditions since not only water and mineral (e.g. nitrogen and phosphorous) uptake increase but root growth is buffered against biotic and abiotic stresses (Smith and Read, 1997; Harrison, 1999). In orchards, mycorrhization is no less important since plant growth can be stimulated and, therefore, crop production (Gobert and Plassard, 2008). *Arbutus unedo* (strawberry tree) grows spontaneously in Mediterranean ecosystems usually associated with other trees such as pines and oaks, and fruit production represents the main income for farmers interested in this species. From an ecological perspective, it

contributes to the biodiversity, helps to stabilize soils, has a strong regeneration capacity following fires and survives well in harsh environments (Piotto *et al.*, 2001). Due these characteristics it has been used in the colonization of marginal lands and to prevent the propagation of forestry fires. For the last five years we have been involved in a long-term breeding program which the main goal is to select and propagate high quality trees converting the underutilized strawberry tree into a valuable crop. In close collaboration with farmers trees were selected based on fruit production/quality and protocols for the propagation of these adult trees through axillary shoot proliferation (Gomes and Canhoto, 2009) and somatic embryogenesis (Gomes *et al.*, 2009) were developed. As aforementioned, mycorrhizae can improve plant adaptation and tolerance to stress environmental conditions. In this way, mycorrhizal synthesis experiments are useful to determine fungus-plant host compatibility (Águeda *et al.*, 2008). The present study is a first approach to induce mycorrhizae formation between *Arbutus unedo* and *P. tinctorius* (Pisolithaceae) or *L. deliciosus* (Russulaceae), types of symbiosis that do not seem to occur in natural stands (Richard *et al.*, 2005). These two species were selected because both are able to form ectomycorrhizae in association with other plant hosts. Moreover *P. tinctorius* is a broad host range basidiomycete and *L. deliciosus* is an edible species belonging to the cosmopolitan genus *Lactarius* which has been intensively marketed in many countries of Europe, Asia and northern Africa (Parladé *et al.*, 2004). According to Molina and Trappe (1982) arbutoid mycorrhizae are most closely related to ectomycorrhizae than to ericoid mycorrhizae and that ectomycorrhizal specificity is emphasized, as hosts forming arbutoid mycorrhizae are not specific. The role of mycorrhization, *in vitro* and in plants growing in the nursery, on plant development was evaluated in the nursery and in a field trial. Morphological and histological analyses were carried out to monitor mycorrhizae synthesis.

4.3 MATERIAL AND METHODS

4.3.1 Fungal isolates

Fungal isolates of *P. tinctorius* were obtained from sporocarps collected in Central Portugal, Vendas Novas, in a Mediterranean old-growth *Quercus suber* forest stand (38º41'N, 8º29'W). This culture was collected on June 2006, and was established and maintained by the Instituto Nacional dos Recursos Biológicos. Fungal isolates of *L. deliciosus* were from sporocarps collected in Spain (Boñar, León) in an old growth *Pinus sylvestris* stand. This culture, referenced as LEB-2268, was collected on November 2006 and belongs to the Herbario Jaime Andrés (Centro de Recursos Genéticos Forestales "El Serranillo", Universidad de León). In both cases, isolations were carried out through the culture of sporocarps on Modified Melin Norkrans (MMN) agar culture medium (Jacob *et al.*, 2001).

Fungal cultures were cultivated at 30ºC or 25ºC, for *P. tinctorius* and *L. deliciosus*, respectively, and multiplied by transferring five small discs of vigorously growing mycelium to a new fresh media added of glucose 2% (w/v) every 4 weeks. The same culture medium containing a lower glucose concentration (1% w/v) and without agar was used for mycelium growth and afterwards used as substrate vegetative inoculum in nursery conditions. *P. tinctorius* dry sporocarps were also tested in nursery conditions. In this case, the dry sporocarps were collected in Carregal do Sal (40º26'N, 7º59'W), in an old-growth *Eucalyptus globulus* forest stand.

4.3.2 Plant material

Adult trees of *A. unedo* were selected according to their fruit production and quality. The conditions for plant establishment and micropropagation of adult trees through axillary shoot proliferation have been described before on Chapter 2 and have been previously published (Gomes and Canhoto, 2009). In these experiments shoots from clones C1, AL1, AL2 and AL3 were tested.

Inoculation in nursery conditions with *P. tinctorius* was performed with micropropagated plants of a clone named C1. After acclimatization in the greenhouse, C1 plants were transferred to pots (450 cm^3) for plant development and later placed in the nursery before mycorrhizal treatments. Two inoculation treatments (vegetative inocula or dry sporocarps)

were tested and compared to control plants. In the field trial, clonal micropropagated plants inoculated with both *P. tinctorius* treatments were compared to the control (micropropagated plants), to one-year-old seedlings produced in the nursery, as well as with three selected clones (AL1, AL2 and AL3) *in vitro* propagated.

When vessels were used as containers, isolated shoots from selected clones AL1 and AL3 were used to test the inoculation during *in vitro* conditions with *P. tinctorius* and *L. deliciosus*, respectively. Afterwards, only shoots from AL1 clone were assayed in synthesis tubes, either with *P. tinctorius* or *L. deliciosus*.

4.3.3 Mycorrhization with *Pisolithus tinctorius* in the nursery and field tests

To analyse mycorrhization with *P. tinctorius* in nursery conditions, clonal micropropagated plants (C1) were watered with either *P. tinctorius* mycelium (pure cultures in MMN liquid medium) or dry sporocarps and compared with the control. Pure cultures were obtained in MMN liquid medium (glucose 1% w/v) at 30ºC, during 2 months. When the liquid surface was covered with the mycelium the Erlenmeyer flasks were gently shaken, filtered and washed to remove the remains of glucose. The mycelium was diluted in water in a proportion of 1:20 (v/v), and then applied to the substrate of C1 acclimatized plants (treatment referred to as C1M). The dry sporocarps were water mixed by using a mixer and then applied directly to the substrate of C1 acclimatized plants (treatment referred to as C1S). In both treatments, 80 ml were applied (during the growing season, spring) to the substrate (450 cm^3) being distributed by 3 holes previously made in direction of the plant roots. A mix of peat and perlite (7:3; v/v) was used as plant substrate. Fertilizer addition (slow release fertilizer, 20:9:11 NPK + 2Mg) to the substrate (2.6 g/l) was only applied to the control treatment (treatment referred to as C1C). A total of 153 plants were used in the above referred conditions (C1S, C1M and C1C; 45 to 59 per treatment). The effect of the treatments on plant growth was evaluated by measuring the height and increase in plant height ($H_1 - H_0$; cm), two and four months after inoculation treatments in nursery. The last measurement was performed just before the field trial establishment, during the autumn.

In the field trial, clonal micropropagated plants treated in the nursery as described before (C1S, C1M and C1C) were compared to seedlings propagated in the nursery which are commonly used in aforestation programs with strawberry tree. The field trial was established in Estreito (39º57'N, 7º48'W) during the most adequate planting season for a Mediterranean climate (October). Plants from all the mycorrhization treatments (C1S, C1M, C1C) were kept in the field without the addition of any fertilizer and compared in terms of growth with

seedlings and 3 micropropagated clones, locally fertilized with a slow release fertilizer (30g/plant; Nutriforest 9:23:14 (+4; +0.1) N:P:K, + MgO, B, with 8 to 9 months of release period). A total of 7 treatments were tested: 3 inoculation treatments, including the control plants (C1C, neither inoculated nor fertilized), 3 micropropagated adult selected clones and seedlings. Plants were established in the field in rows at a distance of 4 m apart and 5 m between rows (4x5m). The experiment consisted of four blocks with 5 plants per each of the seven treatments in a total of 140 plants (7 treatments x 5 plants x 4 blocks) distributed in an area of 2800 m^2 (140 plants x 20 m^2). Each treatment comprised a total of 20 plants (5 plants x 4 blocks), randomly distributed by the 4 blocks. Survival rate was evaluated 12 months after tree planting. Height was measured immediately after plantation and 20 months later to evaluate the effect of the different treatments tested on plant growth. By this time roots were also checked for the presence of arbutoid mycorrhizae.

4.3.4 Mycorrhizal synthesis *in vitro*

To induce mycorrhizal synthesis different containers were tested: (1) vessels (500 cm^3) or (2) test tubes, from now on called synthesis tubes (3 cm in diameter and 30 cm height). As substrate a peat and vermiculite mixture (1:6.5 v/v) was used. The containers (vessels or synthesis tubes) were filled with the substrate mixture (200 ml or 120 ml, respectively) previously sterilized at 121ºC for 60 minutes. Following this step, the substrate was moistened with MMN liquid medium in a proportion of 30 ml medium for each 100 ml of substrate. To promote mycorrhization, a reduced glucose level (0.5%) was added to MMN liquid media. The containers with the substrate moistened with MMN medium were sterilized again at 121ºC for 20 minutes. To induce mycorrhization three small discs (1cm wide) of vigorously growing mycelium cultured on Petri dishes were added per container. To promote mycelium development containers were incubated at 30ºC or 25ºC (for *P. tinctorius* or *L. deliciosus*, respectively) for a month followed by 15 days in a culture chamber (25/20ºC, on darkness conditions) before transfer of the rooting induced shoots.

The cultures were then maintained in a culture chamber under a photoperiod of 16h light and 8h dark and a temperature of 25ºC during light conditions and 20ºC during dark. The substrate and mycelium were maintained on darkness conditions with the basal part of the tubes covered with aluminum foil. A small amount (3 to 5 ml per container) of liquid medium consisting on Knop macronutrients was added monthly during five months (maximum when tubes were used with substrate inoculated with *P. tinctorius*, due to its superior growth compared to *L. deliciosus*). Again, to promote mycorrhization a small amount of a reduced

carbon source (sucrose 0.2% w/v) was added to the Knop liquid medium. To the control plants (not inoculated, NI treatment) the same liquid medium (Knop), with charcoal (1.5% w/v) and a higher sucrose level (3% w/v) was added, to stimulate plantlet development.

Four sets of experiments were established. On the first and second experiments, vessels (500 cm^3) were used to test the inoculation on selected clones AL1 and AL3, with *P. tinctorius* and *L. deliciosus*, respectively. Two different treatments for mycorrhizal synthesis procedures were tested and compared to control. Five shoots (14-20 mm) per vessel were used (5 shoots x 6 replicas; 30 shoots per treatment; total of 90 plants per inoculum experiment). For mycorrhizal synthesis the following treatments were carried out:

1) the shoots were transferred to the inoculated substrate with the fungi, just after shoot induction period (7 days) on a root induction medium (Knop, Gautheret, 1959) added of 24.6 µM IBA (indol-3-butyric acid), assayed in darkness conditions. This treatment was referred as inoculation after root induction (IRi);
2) after the root induction period, shoots were transferred to the same medium without auxin and containing charcoal (1.5 % w/v) and sucrose (3% w/v) during 2 weeks, for a preliminary root expression, before transferring to the inoculated substrate. In this case the treatment was referred as inoculation after root expression (IRe);
3) both treatments were compared with control plants in which the same substrate not inoculated was used (treatment referred as NI).

On the third and forth experiments for mycorrhizal synthesis, tubes were used to test the inoculation of AL1 shoots with *P. tinctorius* or *L. deliciosus*. Shoots (45/inoculum) were transferred to the inoculated substrate according to the IRe treatment above described.

On the first and second experiments (inoculation in vessels), after six weeks of transfer of induced to root shoots to previously inoculated substrate the acclimatization process was initiated. Plantlets were acclimatized in the greenhouse under controlled conditions of humidity and light, as previously described on Chapter 2. One month after acclimatization the humidity was gradually reduced, and later on plants were transferred to the nursery. A similar procedure was adopted for plantlets from the third and forth experiments (inoculations in test tubes). However, only some plantlets (16) were acclimatized after one month, being all of them acclimatized after 5 months.

Different substrates were used during acclimatization. On the first experiment, in which plantlets were from a substrate inoculated with *P. tinctorius* in vessels, the same mixture used

during *in vitro* conditions was tested, consisting of a peat and vermiculite mixture (1:6.5, v/v). However, this substrate showed a high capacity for water retention a situation that is usually unfavorable for mycorrhizal development. Thus, for plantlets in which the substrate had been inoculated with *L. deliciosus* a perlite and vermiculite mixture was used (1:1, v/v). For those plantlets that had been inoculated with *P. tinctorius* (4th experiment, in synthesis tubes), and due to the great development of the mycelium, the experiments were performed with a vermiculite and perlite mixture (2:1.5; v/v). This substrate was quite different from the first one tested, as perlite improved substrate aeration and drainage through the increasing of macroporosity.

During plantlet acclimatization in substrate inoculated with *L. deliciosus*, and to enhance mycelium development and mycorrhization, the substrates were watered with fungi mycelium (vegetative inocula) just before plantlets transfer. Pure cultures in MMN liquid medium were used, as previously described. Due to the extensive mycelium development observed with *P. tinctorius* this procedure was never applied in *L. deliciosus.*

4.3.5 Data recording *in vitro* conditions

Plantlet development during the first and second experiments (inoculation in vessels) was analysed during the acclimatization process and the following parameters were recorded: rooting rate (%), number of roots (NR), length of the longest root (LLR, mm), length of the shortest root (LSR, mm), and final shoot length (SL, mm). The mycelium development on the substrate was also registered and the root system was examined for mycorrhizae formation. When the substrate was previously inoculated with *L. deliciosus*, the biomass of all plantlets was evaluated to assess the effect of mycorrhizae on secondary and branched roots. Shoot and root biomass was evaluated (dry matter, after 48 hours at 65ºC), as well as the respective ratio. When *P. tinctorius* was tested, two months after acclimatization on nursery, survival rate was evaluated and plant height increment was compared with the control treatment (NI).

On the third and fourth set of experiments, inoculations of AL1 shoots with *P. tinctorius* and *L. deliciosus* (in synthesis tubes) were tested. One month after the establishment of these experiments, 8 plants/per fungi inoculum were examined for mycorrhizal formation and, 4 months later, plantlets were acclimatized. During acclimatization plantlets were evaluated in terms of fungi mycelium and root development. Four months later the survival rate was determined. Nine months after acclimatization (or 14 months after inoculation treatment), roots were examined for arbutoid mycorrhizae formation. Some plants on nursery were

removed from containers and root systems were examined for arbutoid mycorrhizae formation. Root systems from plants previously *in vitro* inoculated with *P. tinctorius* or *L. deliciosus* (in tubes) were compared with control plants.

Twelve months after acclimatization (or 17 months after inoculation treatment), 4 plants on nursery were removed from containers and the root systems examined for arbutoid mycorrhizae formation by using molecular marker techniques. Root systems from plants previously *in vitro* inoculated with *P. tinctorius* or *L. deliciosus* (in tubes) were analysed. Roots were harvested directly from the root system developed in the containers. Young roots were frozen in liquid nitrogen and stored (-80°C). Samples were homogenised in buffer for DNA extraction, by vacuum, using the automatic extractor *ABIPRISM* 6100 Nucleic Acid Prepstation from Applied Biosystems, according to the following protocol: 1) homogenizing 5-50 mg of sample tissue in 800 µl of NucPrep DNA purification solution, followed by tissue lyses for 30 seconds, until material is well homogenized; 2) pre-filtration, loading the wells with 750 µl of digested tissue lyzate plus buffer, followed by vacuum filtration and 3) purification, with addition of buffer (40 µl) to the wells before loading samples already digested, being necessary to cover the unused wells to optimize the vacuum extraction. Following, the PCR (Polymerase Chain Reaction) were accomplished using ITS1F and ITS4 primers to amplify the ITS (Internal Transcribed Spacer) total region (White *et al.*, 1990). PCR were performed in a 25 µl volume containing: 12.5 µl of Jump Start Taq DNA polymerase with $MgCl_2$ (Sigma D 9307), 0.5 µl of each primer (10mM) and 1 µl sample DNA. The initial denaturation step (2 min, 95ºC) was followed by 30 cycles of 60 s at 95 ºC (denaturation), 60 s at 53ºC (annealing) and an extension step of 60 s at 72 ºC, followed by a final extension step at 72ºC for 5 min, to guarantee that all annealed templates were entirely polymerized. The PCR reaction products were then sequenced by the Stabvida company, using an automatic sequencer (ABI Prism 3100 DNA). The nucleotide sequences were run through a software (*Geneious*) and compared to Blast (Basic Length Alignment Search Toll) (Altschul *et al.*, 1997) database from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences identification is then presented according to the degree of genetic similarity.

4.3.6 Morphological and histological studies

Arbutoid mycorrhizae roots and rhizomorphs were carefully examined with the aid of a stereomicroscope. Some observations were carried out in fresh sections obtained with a

freezing microtome. The general terminology follows Agerer and Rambold (2004-2010) and Ingleby *et al.* (1990).

For histological studies root samples were fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer, pH 7.0 for 1.5 h and post-fixed in 1% osmium tetroxide prepared in the same buffer for 1 h at room temperature. Dehydration was accomplished in an ethanol series (20 to 100%) and the samples embedded in Spurr's resin. Sections (1 – 2 µm) were made with glass knives on a LKB Ultratome III and stained with 0.2% toluidine blue.

4.3.7 Experimental design for *in vitro* experiments and statistics

On the first and second experiments, isolated shoots were used, after the root induction period (30 per treatment; total of 90 shoots per inoculum experiment in vessels). The rooting parameters were recorded during the acclimatization process (*in vitro* plantlets transfer to the greenhouse). For statistical analysis an ANOVA was performed, and means were compared using the Duncan test ($P \leq 0.05$) (Duncan, 1955). The software STATISTICA 6 was used. The quantitative data expressed as percentages were first submitted to arcsine transformation and the means corrected for bias before a new conversion of the means and standard error (SE) back into percentages (Zar, 1996).

4.4 RESULTS

4.4.1 Inoculation with *Pisolithus tinctorius* in nursery and field test

Cultures of *P. tinctorius* before being applied to the substrate, were cultivated *in vitro*, mixed, filtered, washed and diluted in water (Figs. 1*A-D*; treatment referred to as C1 M). These plants were compared to the control (C1 C) and to the dry sporocarps water mixed treatment (C1 S).

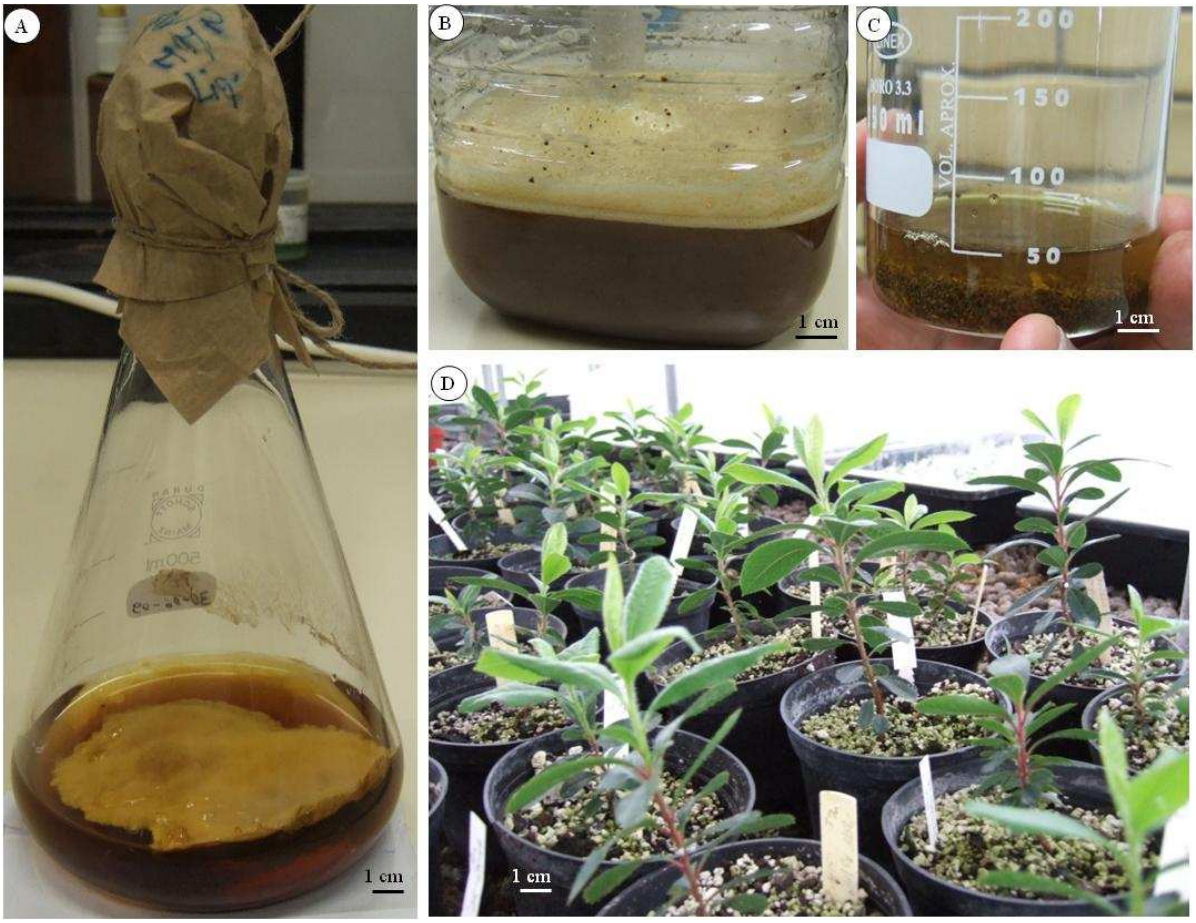


Figure 1 – Inoculation with *P. tinctorius* in nursery. A - *P. tinctorius* growing on MMN liquid medium. B – Following growth, the mycelium and liquid medium were mixed. C - Mycelium after dilution with water and ready to be applied. D - C1 acclimatized plants in the greenhouse just before transference to the nursery and application of the mycorrhization treatments.

Plant height increment (cm) was evaluated 2 and 4 months after *P. tinctorius* inoculation treatments had been applied to the substrate of C1 acclimatized plants in nursery. Two months after inoculation no significant differences were found between treatments and control plants (Appendix Table 1). However, 2 months later, best results were achieved when dry sporocarps water mixed were tested (C1S; Table 1; Appendix Table 1). On these

conditions the control plants showed the lowest height increment value, but not significant as compared to C1M plants (Table 1).

Table 1 - Effect of different inoculation treatments with *P. tinctorius* on plants' height increment (ΔH) evaluated 2 and 4 months after inoculation of the clone C1 in the nursery.

Treatments	Δ H 2 months (cm)	Δ H 4 months (cm)
C1 C - control plants	10.7 ± 0.4 [a]	14.4 ± 0.7 [b]
C1 M - with mycelium *in vitro* produced	11.0 ± 0.4 [a]	16.0 ± 0.6 [b]
C1 S - dry sporocarps water mixed	10.8 ± 0.3 [a]	19.1 ± 0.7 [a]

In each column values (mean ± SE) followed by different letters are significantly different (P≤0.01).

A field trial was established to test all the nursery inoculation treatments and fertilized plants (seedlings and 3 micropropagated clones, AL1 to AL3). The survival rate 12 months after field trial establishment was about 96.8%, without significant differences. Twenty months after field trial establishment, height or height increment did not show significant differences among the treatments (Table 2; Appendix Table 2).

Table 2 – Effect of the different treatments on plant height (H) and height increment (ΔH) evaluated 20 months after field trial establishment.

Mycorrizal treatments and genotype	H 20 months mean ± SE (cm)	Δ H (H20 - H0) mean ± SE (cm)
AL1 - selected clone	76.6 ± 7.2 [a]	58.2 ± 7.7 [a]
AL2 - selected clone	68.6 ± 5.8 [a]	55.1 ± 5.8 [a]
AL3 - selected clone	75.3 ± 5.7 [a]	63.2 ± 6.0 [a]
SE - seedlings	68.6 ± 5.3 [a]	57.7 ± 4.8 [a]
C1 C - control plants	76.2 ± 6.7 [a]	54.8 ± 6.9 [a]
C1 M - mycelium *in vitro* produced	83.0 ± 4.7 [a]	62.0 ± 4.6 [a]
C1 S - dry sporocarps water mixed	84.7 ± 5.1 [a]	60.8 ± 5.0 [a]

In each column values (mean±SE) followed by same letters are not significantly different (P≤0.05).

Seedlings and clone AL2 showed the lowest height average, even though they have been fertilized. Inoculated plants in nursery (C1M and C1S) showed averages of height and height increment higher than the control plants without significant differences. In addition, control plants (C1C) showed the lowest average of height increment after 20 months (Table 2; P>0.05).

Twenty months after the field trial establishment it could be seen that roots of the plants which had been inoculated in nursery (C1M and C1S) showed mycorrhized roots (Figs. 2*A* to *G*). Mycorrhization due to symbiosis with *Cenococcum geophilum* (Figs. 2*A* - *C*),

mycorrhizae displaying a compound structure (Fig. 2*D*) or mycorrhizae possessing a yellow-brown mantle (Fig. 2*E*) were also observed. *Cenococcum geophilum* mycorrhizae are black when young (Figs. 2*A* - *C*), short and may occasionally produce one or two short branches (Fig. 2*B*) but, usually they are single (Fig. 2*C*). Observations performed in material sectioned with a freezing microtome showed a mantle with emanating hyphae with irregularly formed clamp-connections (Fig. 2*F*), elbow-like protrusions (a short and curved hyphae forming a protuberance over the septa) which correspond to typical hyphae from basidiomycete fungi. Mycorrhizae with a typical cruciform appearance were also observed (Fig. 2*G*). However, some roots were also found in which mycorrhization could not be detected (Fig. 2*H*). Young roots without the presence of mycorrhizae (Fig. 2*H*) were characterized by the presence of root hairs morphologically different from the hyphae (Figs. 2*B* - *D*) and that could be easily distinguished.

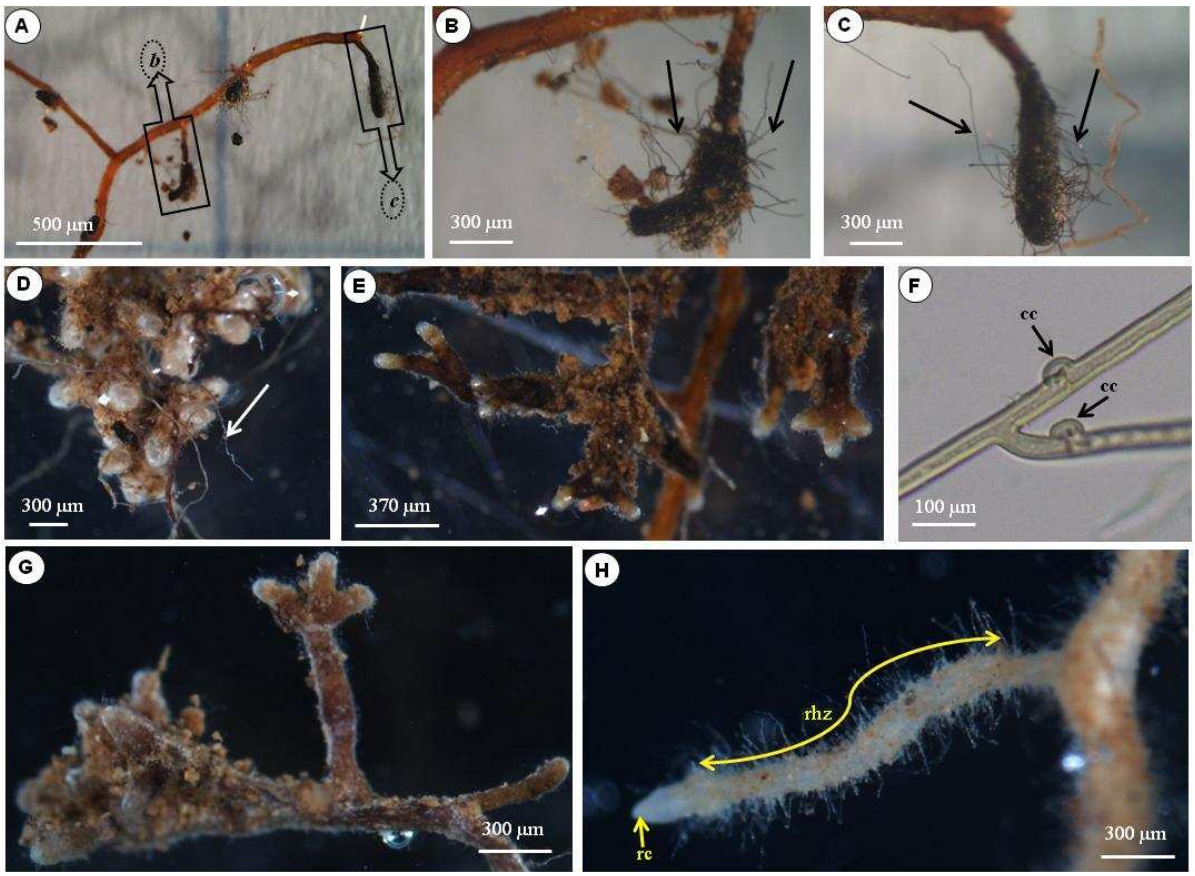


Figure 2 – Root observations 20 months after field trial establishment. A to G - Mycorrhized roots. A to C - *Cenococcum geophilum* mycorrhizae, A - short mycorrhizae with a black mantle; figures details showing a mycorrhiza with two short branches (B) or single branch (C) with a compact black mantle and black hyphae growing from the mantle surface (arrows, B and C). D - Mycorrhiza presenting a compound structure, note the hyphae growing from the mantle (arrows). E - Mycorrhizae showing a yellow-brown mantle. F - Mantle hyphae possessing clamp-connections (cc). G - Mycorrhizae showing a branched architecture and H - a young root without mycorrhizae showing the root hair zone (rhz) and the root cap (rc).

4.4.2 Mycorrhizal synthesis *in vitro* conditions

4.4.2.1 Inoculation with *P. tinctorius* and *L. deliciosus* in vessels

The substrate was inoculated as previously described (Figs. 3*A* to *D*). After transfer of the induced rooted shoots, the containers were kept in a culture chamber (Fig. 3*E*).

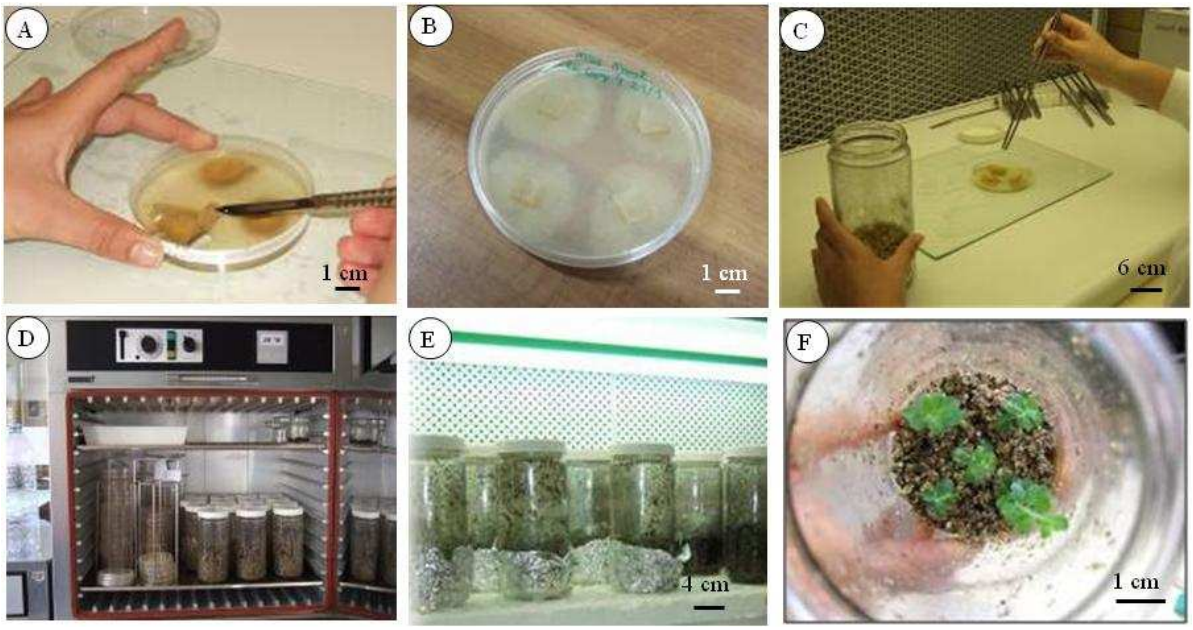


Figure 3 – Substrate inoculation. A – Growing mycelium of *P. tinctorius.* B - Growing mycelium of *L. deliciosus.* C - Disc of mycelium at the time of inoculation in the substrate. D - Incubated containers for mycelium growth. E – Shoots just after inoculation in the containers. F - Plantlets after 6 weeks of culture in the substrate inoculated on culture chamber.

Six weeks after inoculation treatments (Fig. 3*F*), plantlets were acclimatized and root and shoot development was analysed. When *P. tinctorius* was tested the best rooting rate was achieved with IRe treatment (100.0 ± 0 %), but without significant differences (Appendix Table 3). However, for all other parameters, plantlets growing in a substrate which was not inoculated (NI) displayed significantly higher values (Table 3). On the other hand, with *L. deliciosus*, only rooting rate and length of the shortest root showed significant differences according to the treatments tested (Table3). The IRi treatment showed the lowest rooting ($P \leq 0.05$; Table 3).

Table 3 – Effect of *in vitro* mycorrhization with *P. tinctorius* and *L. deliciosus* on the following variables: rooting rate (%), number of roots (NR), length of the longest root (LLR), length of the shortest root (LSR), and final shoot length (SL).

Treatment: substrate inoculated with *P. tinctorius*

	rooting rate %				** NR				** LLR (mm)				** LSR (mm)				** SL (mm)			
IRe	100.0	±	0.0	a	6.8	±	0.9	b	28.8	±	2.3	b	10.8	±	1.7	b	56.1	±	3.3	b
IRi	88.0	±	12.0	a	7.7	±	0.7	b	20.7	±	2.8	c	8.0	±	0.9	b	43.7	±	3.5	c
NI	93.3	±	4.2	a	11.2	±	1.1	a	50.0	±	3.0	a	17.9	±	1.5	a	67.8	±	3.7	a

Treatment: substrate inoculated with L. *deliciosus*

	* rooting rate %				NR				LLR (mm)				* LSR (mm)				SL (mm)			
IRe	80.0	±	13.0	a	7.0	±	0.6	a	23.6	±	2.5	a	5.2	±	0.7	b	43.6	±	2.3	a
IRi	50.0	±	10.0	b	6.3	±	0.6	a	27.5	±	3.1	a	7.0	±	2.1	ab	41.5	±	2.7	a
NI	86.7	±	4.0	a	8.4	±	1.0	a	27.3	±	1.8	a	10.1	±	1.4	a	45.9	±	2.1	a

In each column values (mean ± SE) followed by different letters are significantly different ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$).

The experiments carried out with *P. tinctorius* (Table 3) indicated that the IRe inoculation treatment showed the best rooting rate ($P>0.05$) and both inoculation tested were not significantly different from NI (control) plantlets. The results also pointed out to an increase in shoot length of the IRe when compared to IRi, suggesting that the previous root expression (during 2 weeks, with charcoal) can promote further plantlet development. Moreover, when *L. deliciosus* was tested, no significant differences were found in rooting rate between IRe treatment and control plantlets (NI), contrarily to what happened with the IRi treatment (Table 3). In both experiments, a pathogenic effect on plantlets due to either *P. tinctorius* or *L. deliciosus* presence was never observed. For both treatments the results also showed that roots were neither so long or numerous (NR) compared to the control, but they were branched. This characteristic appeared more pronounced when the substrate was inoculated with *P. tinctorius*. The hyphae presence on substrate, on *L. deliciosus* experiment, could be seen only with the aid of a stereomicroscope. On the other hand, the *P. tinctorius* mycelium on the substrate was visible to the naked eye, showing a better development on the contact surface between the substrate and the glass vessel, probably due to better aeration conditions. When vessels were tested it was difficult to have the perception of substrate moisture content and consequently identify when and how much liquid medium will be needed to add to the substrate. Due to these characteristics synthesis tubes were used instead vessels on the following experiments.

Just before acclimatization, six weeks after inoculation, the root system was checked for mycorrhizae formation. Through the analysis performed neither a mantle nor intracellular

hyphal complexes could be detected. Nevertheless, hyphae were observed covering the roots, suggesting mantle initiation (Fig. 4).

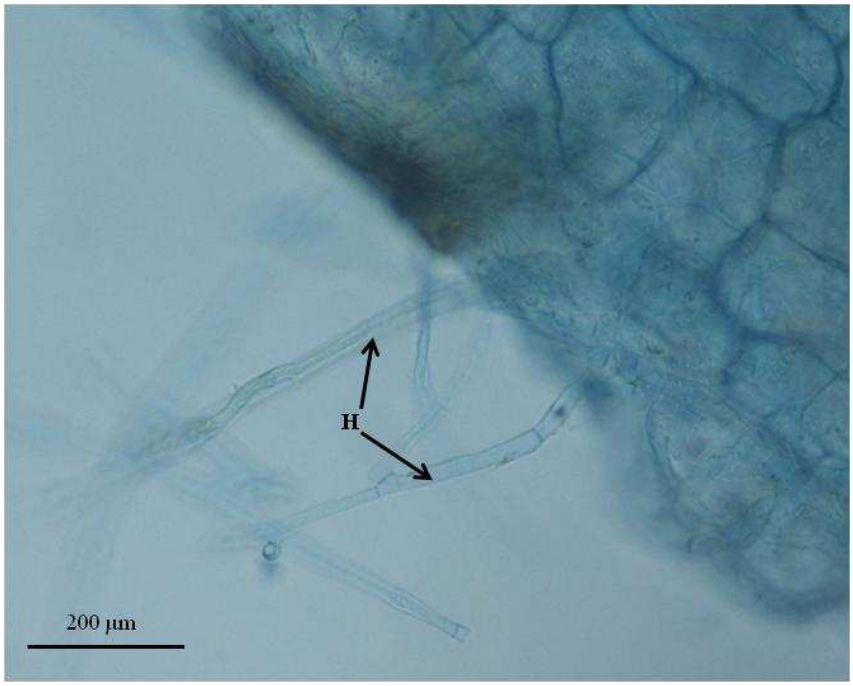


Figure 4 – Superficial part of a root six weeks after *in vitro* inoculation with *P. tinctorius* (in vessels) showing some hyphae (H) associated with the root.

When the biomass of plantlets treated with *L. deliciosus* was analysed, significant differences were found mainly for shoot biomass (Table 4, Appendix Table 4). Control plantlets (NI) showed a shoot biomass higher than in the IRi treatment, but not significantly different from IRe treatment. Regardless of the results obtained in the shoot, no differences were found for root biomass. It should also be referred that the shoot/root biomass ratio points to a positive role of mycorrhization since the plantlets showed to be more balanced, displaying a reduced biomass ratio (Table 4).

Table 4 - – Effect of *in vitro* mycorrhization with *L. deliciosus* on the following variables: shoot and root biomass (dry matter, after 48hours/65ºC), as well as the respective ratio (shoot/root biomass).

	* Shoot biomass (mg)	Root biomass (mg)	Biomass ratio (shoot/root, mg)
IRe - substrate inoculated	16.00 ± 2.60 ^{ab}	1.09 ± 0.14 ^{a}	16.66 ± 4.00 ^{a}
IRi – substrate inoculated	13.44 ± 2.31 ^{b}	1.01 ± 0.10 ^{a}	15.88 ± 4.29 ^{a}
NI – substr. not inoculated	26.00 ± 5.16 ^{a}	1.06 ± 0.20 ^{a}	30.56 ± 6.15 ^{a}

In each column values (mean ± SE) followed by different letters are significantly different ($^{*}P\leq0.05$; $^{**}P\leq0.01$).

Following acclimatization, plants inoculated with *P. tinctorius* in the nursery presented a high survival rate (95.0 ± 2.2 %), without significant differences between treatments (Appendix Table 5; Table 5). Plants from IRe treatment showed the highest average on height increment ($P \leq 0.01$) two months after acclimatization (Table 5), as well as 100% of root and survival rates. In addition, both treatments with mycorrhizae showed higher growth (ΔH) compared to the control treatment (NI; $P \leq 0.01$). These results suggest that mycorrhization was established since the inoculated plants showed a better growth, 2 months after acclimatization in the nursery.

Table 5 - Effect of *in vitro* mycorrhization with *P. tinctorius*, 2 months after acclimatization, on the following variables: survival rate (%) and on plants' height increment.

	Survival rate (%)	**Height increment (ΔH, cm)
IRe - substrate inoculated	100.0 ± 0.0 [a]	7.6 ± 0.4 [a]
IRi – substrate inoculated	92.0 ± 4.9 [a]	6.2 ± 0.5 [b]
NI – substrate not inoculated	93.3 ± 4.2 [a]	4.8 ± 0.3 [c]

In each column values (mean ± SE) followed by different letters are significantly different (*$P \leq 0.05$; **$P \leq 0.01$).

These results indicate that the previous root expression (during 2 weeks, with charcoal; IRe) is required to promote plant development and acclimatization. In this way, this procedure was adopted on the following experiments (inoculations in test tubes).

4.4.2.2 Inoculation with *P. tinctorius* and *L. deliciosus* in synthesis tubes

Shoots induced to root (from AL1 clone) were transferred to the synthesis tubes (Fig. 5*A*) previously inoculated with *P. tinctorius* or *L. deliciosus*. After 1 month 16 plants (8 per fungi inocula) were removed from the test tubes where synthesis occurred and root systems examined for mycorrhizal formation and then acclimatized (100% of survival rate). After 5 months all plantlets were acclimatized (Figs. 5*B* - *E*) and transferred to the greenhouse, under controlled conditions (humidity and light). The root system showed many secondary and branched roots, mainly when *P. tinctorius* was tested (Fig. 5*C*) what can be responsible for the 100% survival rate observed 4 months later. During the acclimatization process, after 5 months on the synthesis tubes, the following features were observed: 1) a well developed root system showing the presence of secondary roots (Fig. 5*C*), 2) an extensive colonization of the substrate by the mycelium of *P. tinctorius* (Figs. 5*D* - *E*), 3) a thick yellow-brown mantle visible to the naked eye (Figs. 5*C* to *E*) and 4) plants displayed a normal pattern of

development indication that colonization of the roots by *P. tinctorius* does not affect plant growth (Figs. 5*C*, *D*).

Figure 5 – Mycorrhizal synthesis with *P. tinctorius* in the synthesis tubes. A - Shoots immediately after transference from the root expression medium to the synthesis tubes containing the inoculated substrate. B to E - Different aspects of plantlet development after 5 months of culture in the substrate (B), showing a well developed root system (C and D) as well as an extensive root and substrate colonization by the *P. tinctorius* mycelium, without any signal of plant stress (C to E).

When *L. deliciosus* was tested, the following aspects were observed: 1) a slight colonization of the substrate by the mycelium with white hyphae (Fig. 6*A*); 2) a branched root system without mantle, and 3) no evidence of pathogenicity on plantlet development (Fig. 6*B*). However, when the root system was examined for mycorrhizae detection, the analyses did not show any trace of arbutoid mycorrhizae development (Fig. 6*C*). According to this, during the acclimatization process, the substrate was moistened with water mixed with *L. deliciosus* mycelium *in vitro* produced on liquid medium (MMN), after being mixed, filtered, washed and diluted with water (Figs. 6*D* to *F*).

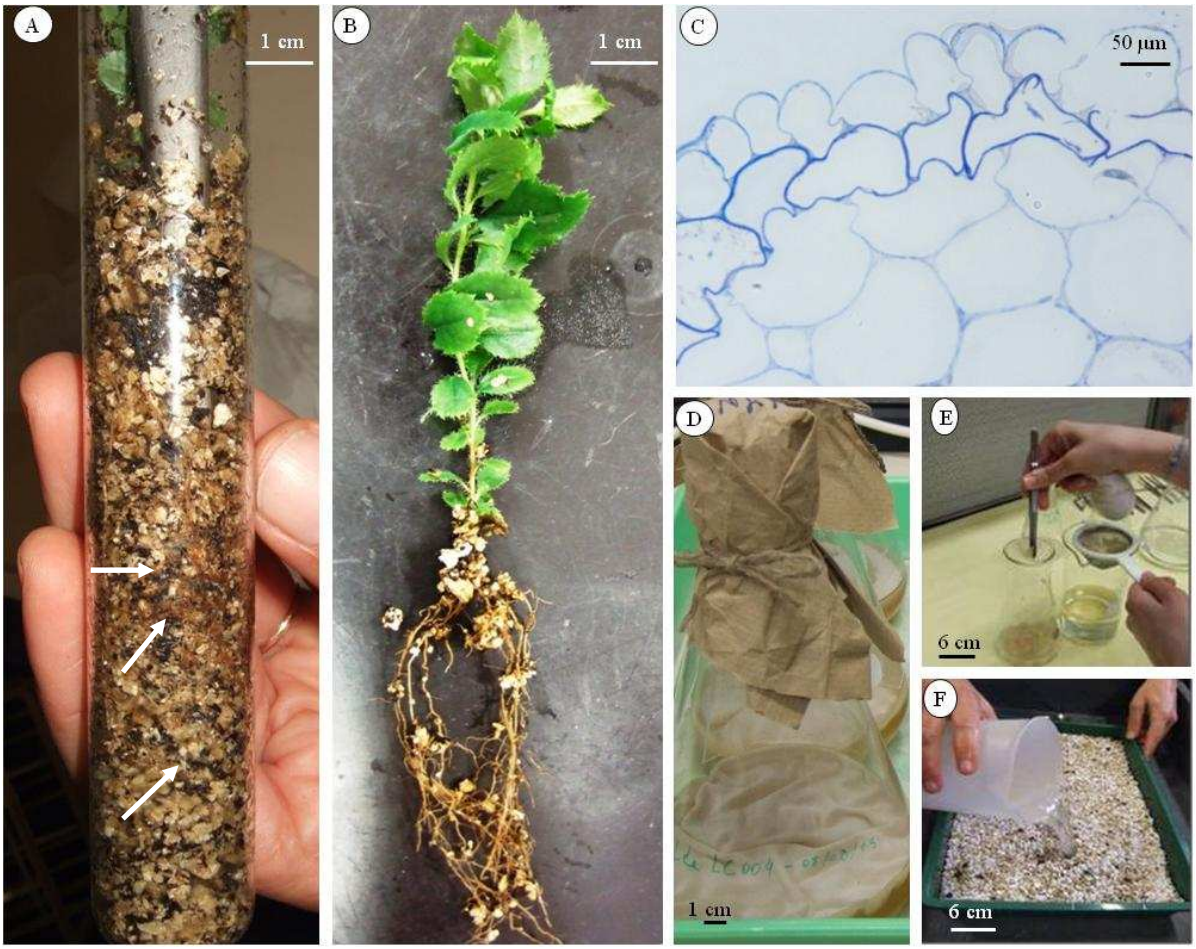


Figure 6 – Aspects of the inoculation of strawberry tree plantlets with *L. deliciosus*. A - Plantlet development after 5 months (note slight substrate mycelium colonization, arrows). B - Plantlet showing a well developed and branched root system. C - Cross section of a root one month after mycorrhization treatment without any evidence of mycorrhizae formation. D - Mycelium of *L. deliciosus* growing in liquid medium and used for watering the plantlets. E and F - Preparation and subsequent application of the inoculum to substrate.

On the contrary, one month after the beginning of the experiments, roots were examined for mycorrhizal formation and the analyses showed that *P. tinctorius* mycorrhizae had already developed, forming a pale yellow to yellow-brown mantle (Figs. *7A*, *B*). The mycorrhizae had a typical cruciform appearance (Fig. 7*A*) and the mantle consisted of loosely interwoven hyphae (Figs. 7*A*, *B*). Histological studies performed in the mycorrhized roots showed that strawberry roots possess two vascular bundles of xylem and phloem (diarch roots) surrounded by 3-5 layers of cortical cells (Fig. 7*C*). Cross sections through these roots showed a typical arbutoid organization with a mantle, the HN, and intracellular hyphal complexes which were confined to the epidermis (Figs. 7*C* - *F*).

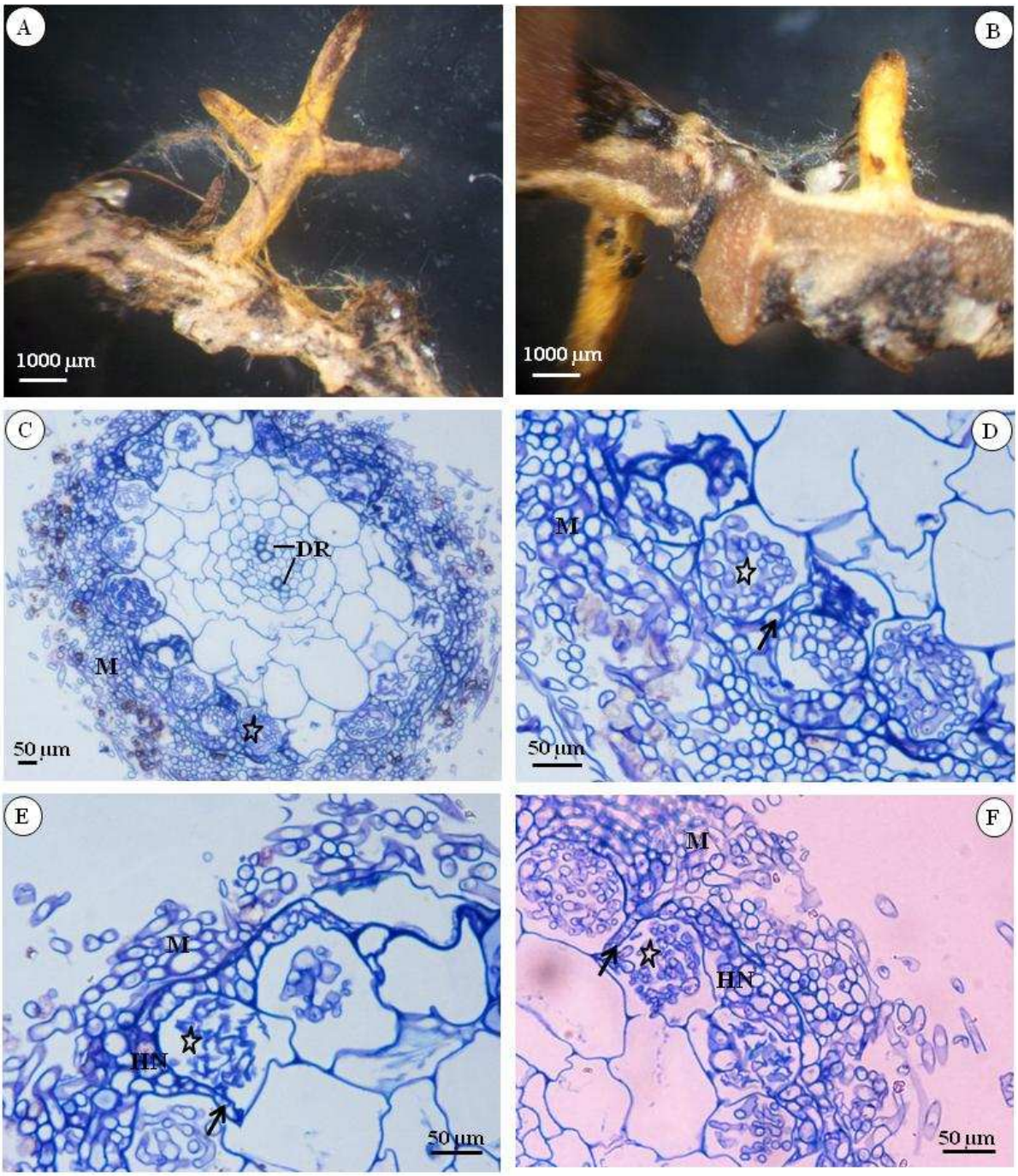


Figure 7 - Arbutoid mycorrhizae observations, after one month, in the synthesis tubes inoculated with *P. tinctorius*. A - Roots showing a cruciform appearance. B - Detail of the thick pale yellow to yellow-brown mantle covering the root surface. C - Cross section of a mycorrhized root showing the diarch root (DR) pattern of vascular bundle organization, the mantle (M), and intracellular hyphal complexes (star) restricted to the epidermis. D to F - Details of the cross sections showing the mantle (M), the Hartig net (HN), the paraepidermal Hartig net hyphae (arrows), and sectioned intracellular hyphal complexes (stars) within epidermal cells.

Nine months after acclimatization (or 14 months after inoculation treatment), plants growing in the nursery were removed from the containers and the root system examined for the presence of arbutoid mycorrhizae and compared to control plants (not inoculated *in vitro*). All plants (inoculated or not) showed arbutoid mycorrhizae formation.

Plants in which the substrate had been inoculated with *L. deliciosus*, showed linear aggregation of hyphae called rhizomorphs (Fig. 8*A*-*C*). The rhizomorphs systems are generally linked to water and nutrient uptake. These were composed of only one type of undifferentiated hyphae, as a linear aggregation of hyphae, loosely formed and showing clamp-connections between them.

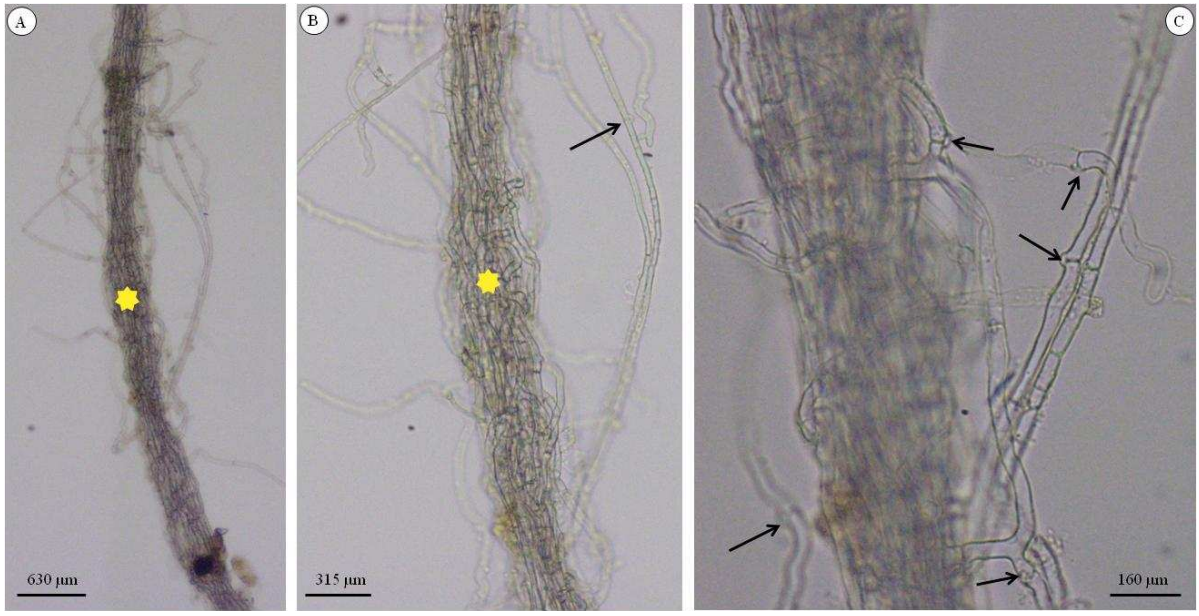


Figure 8 – Aspects of nursery mycorrhized plants inoculated with *L. deliciosus* 9 months after acclimatization. A - Rhizomorph (asterisk). B - Detail of a rhizomorph showing the linear aggregation of hyphae (arrow). C - Detail of a rhizomorph showing clamp-connections (arrows).

Stereomicroscope observations showed roots displaying a cruciform appearance and possessing white hyphae (Figs. 9*A*, *B*), as well as dichotomous mycorrhizae, branching into two, more or less equal arms (Fig. 9*C*), characteristic of *Lactarius* spp. Microscope observations of fresh sections obtained with a freezing microtome showed mycorrhized roots with intracellular hyphae complexes (Figs. 9*D*, *E*) confined to the epidermis (Fig. 9*F*), a feature of arbutoid mycorrhizae, the Hartig net (HN) and clamp-connections (Fig. 9*G*). A multilayered mantle containing a larger type of laticiferous hyphae, characteristic of *Lactarius* spp. could be seen (Fig. 9*E*).

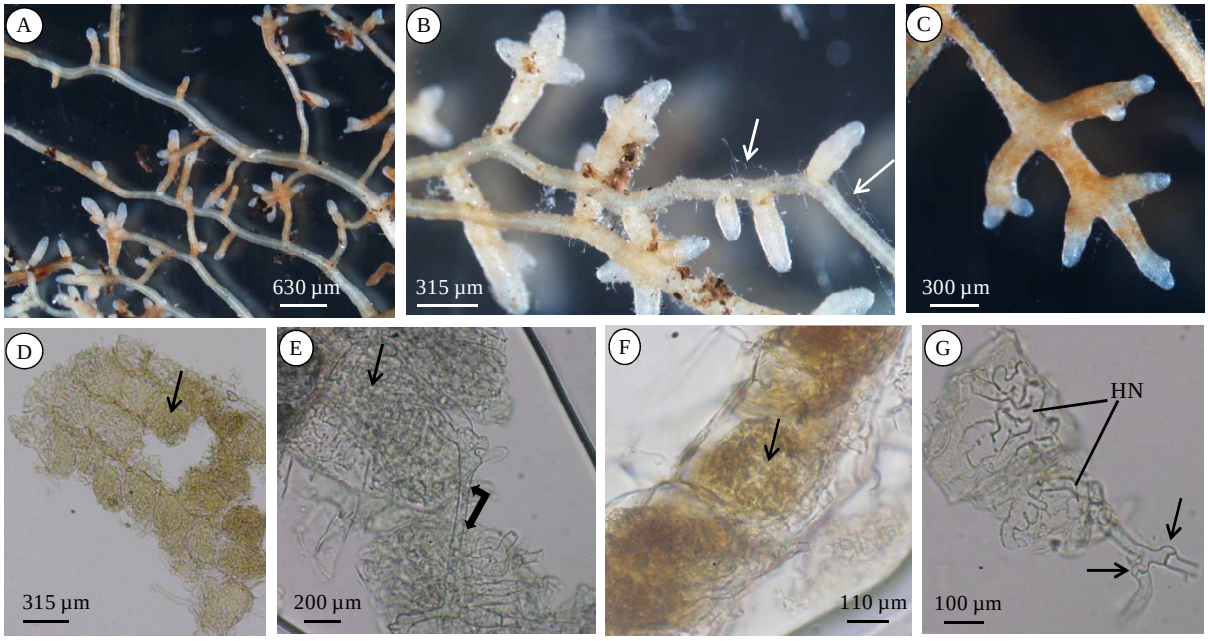


Figure 9 – Arbutoid mycorrhizae observations on plants inoculated with *L. deliciosus* and growing in the nursery 9 months after acclimatization. A and B - Mycorrhizae roots with cruciform morphology and showing white hyphae (arrows). C - Dichotomous mycorrhizae roots, characteristic of *Lactarius* spp. D and E – Sections of fresh roots where the intracellular hyphal complexes can be seen (arrows). E - A mantle layer showing a larger type of laticiferous hyphae (double arrow). F – Hyphae complexes (arrows) confined to the epidermis, a feature of arbutoid mycorrhiza. G - Hartig net (HN) and clamp-connections (arrows) between epidermal cells.

When the substrate was inoculated with *P. tinctorius* the results showed that mycorrhizae had developed, forming a pale yellow to yellow-brown mantle (Fig. 10*A*). The mycorrhizae had a typical cruciform appearance and a compound structure, like a repeated branching (Figs. *10A*, *B*). Observations of fresh root sections made with a freezing microtome indicated the presence of: a mantle which consisted of loosely interwoven hyphae (Fig. 10*C*); the intracellular hyphal complexes confined to the epidermis (Fig. 10*D*); the HN (Fig. 10*E*); clamp-connections (Fig. 10*F*) and a terminal cell found on the surface of the mantle, usually displaying distinctive shape, characteristic of a cystidium (Fig. 10*F*). In both observations, after one month in the synthesis tubes and after nine months in the nursery, mycorrhizae were characterized by a thick mantle with infrequent emanating hyphae.

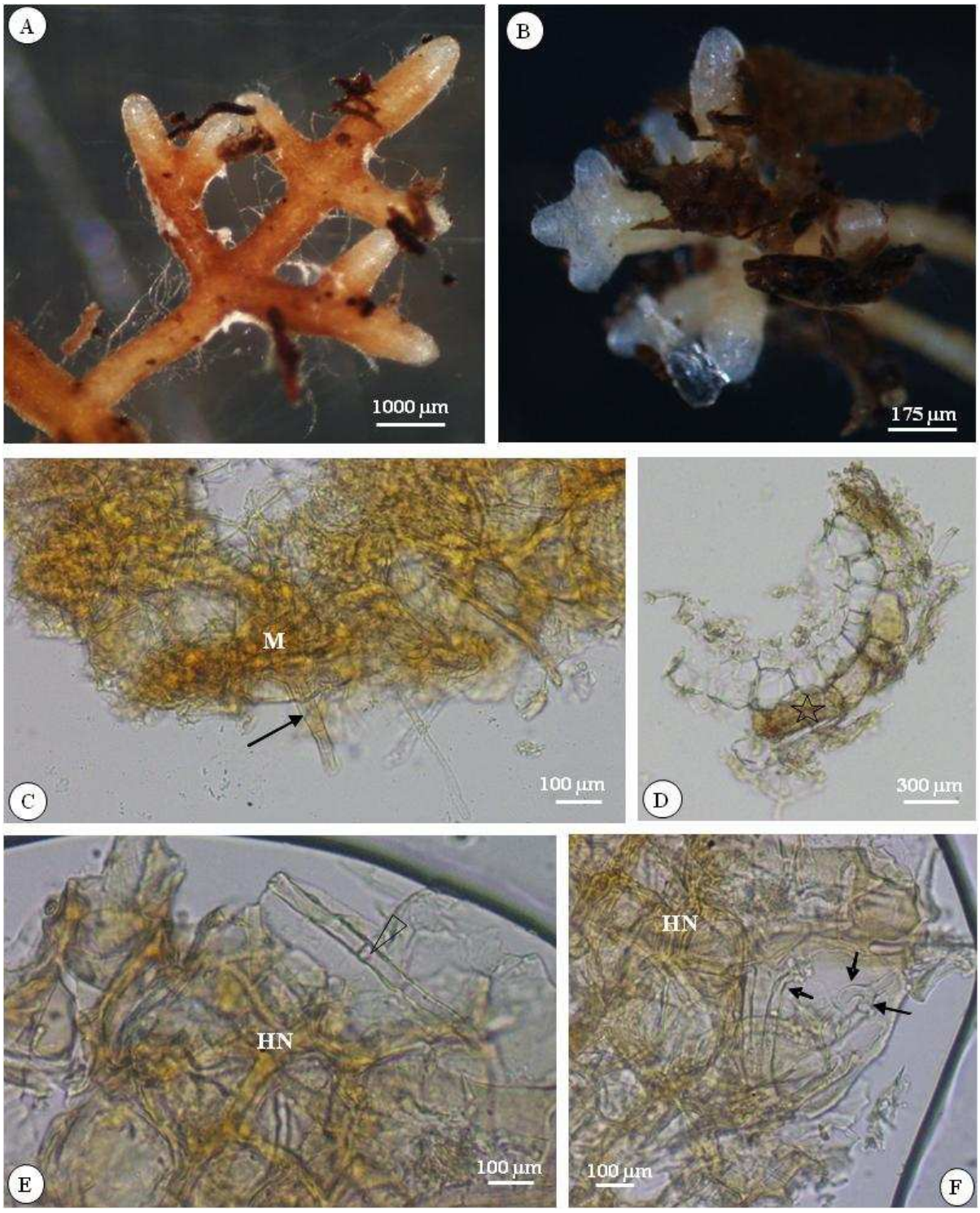


Figure 10 - Arbutoid mycorrhizae observations, nine months after acclimatization of previously mycorrhizal synthesis *in vitro* conditions with *P. tinctorius*. A - Mycorrhized roots showing a cruciform morphology. B - Mycorrhizae showing a branched architecture. C - Tissue section made with a freezing microtome showing the mantle (M) displaying loosely interwoven hyphae with a few emanating hyphae (arrow). D - Intracellular hyphal complexes (star) in epidermal cells. E - Aspect of the Hartig net (HN) and of a clamp-connection (arrow head). F - Another aspect of the Hartig net (HN) showing a cystidium (arrow) and clamps (two arrows).

Nine months after acclimatization, control plants in nursery growing on a substrate which was not previously inoculated showed mycorrhizae development when roots were examined under a stereomicroscope (Fig. 11). Mycorrhizae of various morphologies were found (Figs. 11*A* to *E*), some showing a typical cruciform appearance (Fig. 11*A*), others with a dichotomous branching (Fig. 11*B*), and still others whitish in color (Figs. 11*C*, *D*) or displaying a coralloid type of branching (Fig. 11*E*).

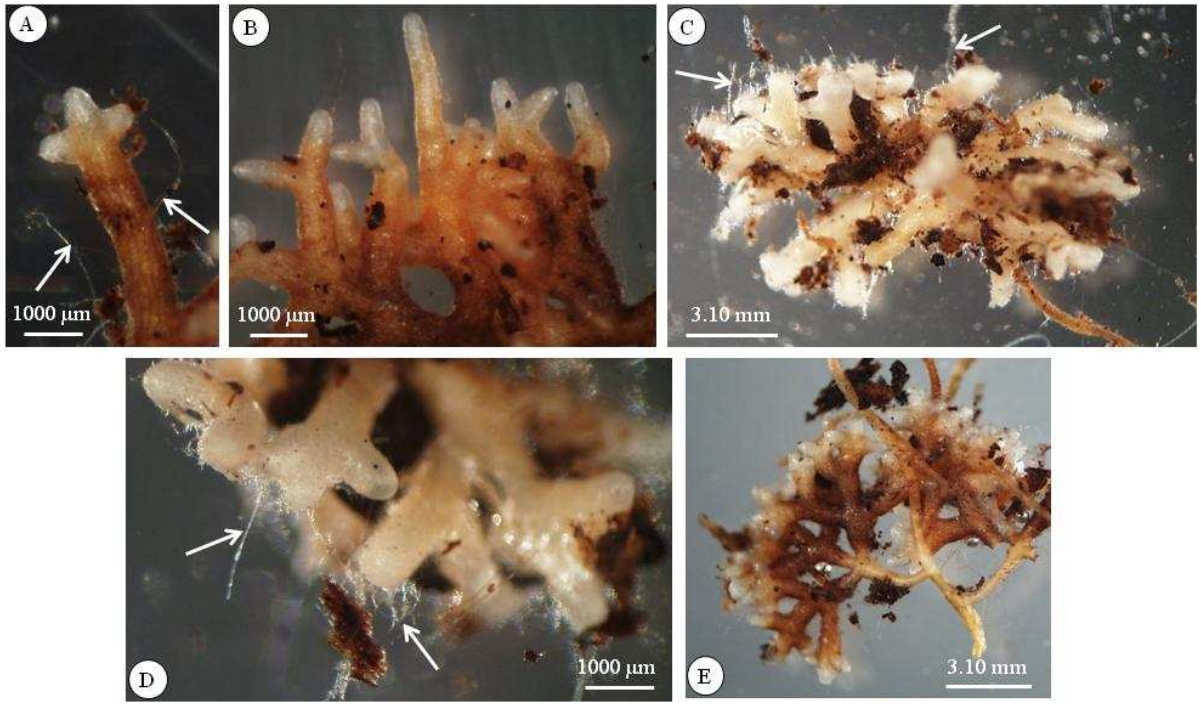


Figure 11 - Mycorrhizae observations on control plants growing in the nursery for nine months after acclimatization. A - Mycorrhiza with a typical cruciform appearance. Note the emanating hyphae (arrows). B - Dichotomous mycorrhizae. C - Mycorrhizae with compound structures (arrows). D - Mycorrhizae with white hyphae (arrows). E - Mycorrhizae showing the coralloid branching morphology.

Twelve months after acclimatization (or 17 months after inoculation treatment), 4 plants growing in the nursery were removed from the containers and the root systems were examined for arbutoid mycorrhizae formation through molecular marker techniques. Root systems from plants previously *in vitro* inoculated with *P. tinctorius* or *L. deliciosus* (in tubes) were analysed. After PCR with ITS1F and ITS4 primers, the reaction products were sequenced. The sequences were compared to Blast database. Sequences identification is reported according to genetic similarity between the nucleotide sequence and the closest match in GeneBank. The results on table 6 show the GeneBank Accession number from the closest sequence; the E-value (probability of another alignment with a higher similarity than that reported) and the similarity percentage (base pairs identification that are identical in the alignment). The best species identification is achieved when there is the highest percentage of

similarity (>95% of the base pairs that are identical in the alignment) and secondly a lower probability (E-value ≈ 0%) of any other alignment.

Table 6 - Closest matches between nucleotide sequences and sequences from the GeneBank data base obtained using the BLAST search tool.

GeneBank's Accession number	E-value	Similarity %	Closest specie
GU998124.1	0.0	99%	*Thelephora*
AY945290.1	0.0	90%	*Thelephora*
AB211272.1	0.0	97%	*Hebeloma*
HQ211740.1	7e-79	94%	*Hebeloma*

The results showed the presence of arbutoid mycorrhizae. However, these were not from *P. tinctorius* or *L. deliciosus*, but from *Thelephora* and *Hebeloma*, also basidiomycetes from Telephoraceae and Cortinariaceae families, respectively.

4.5 DISCUSSION

Inoculation with *P. tinctorius* in nursery

In vitro plants acclimatized in nursery were watered with either *P. tinctorius* mycelium (vegetative inocula) or dry sporocarps and compared with the control. Different kinds of vegetative inocula production can be used for greenhouse or nursery inoculations, such as 1) the inoculum production in a peat-vermiculite substrate, 2) mycelial suspensions on liquid medium, 3) mycelial slurries (agar colonies homogenized in sterile distilled water) and later injected in the rhizosphere zone 4) the alginate-entrapped inoculum and 5) spore inocula (Rincón *et al.*, 2001; Parladé *et al.*, 2004). Parladé *et al.* (2004) report that the percentage of colonized plants and the degree of colonization observed are dependent on the inoculation method and the plant-fungal strain interaction. According to Quinteiro (2005) one of the most interesting practical applications of induced mycorrhization is directly related to the increasing overall growth capacity of the host plants due to an improved water and mineral nutrient uptake and a better tolerance to biotic and abiotic stresses. Our results are consistent with this conclusion since, after 4 months in nursery, inoculated strawberry trees (C1S) grew much better than control plants. However, the positive effect of mycorrhization on plant growth seems not to be a general rule. Thus, Gautry *et al.* (1991) did not find any increase in growth of seven clones of *Pseudotsuga* rooted cuttings inoculated with *L. laccata* or *Hebeloma crustiliniforme*. Rincon *et al.* (2001) tested 7 ectomycorrhizal fungi (vegetative or spore inocula of *Hebeloma crustiliniforme, Laccaria laccata, P. tinctorius, Melanogaster ambiguus, Rhizopogon luteolus, Rhizopogon roseolus* and *Scleroderma verrucosum*) with *P. pinea* seedlings and were also unable to detected any increase in seedling growth. These contradictory results may be ascribed to differences in the physiological conditions of the plant material or to the different types of fungi used. In our experiment, after 4 months, only plants which were treated with spore inocula showed significantly higher growth rates than the control plants. Similar results were obtained by Chen *et al.* (2006) in *E. urophylla* inoculated in nursery with spores of the ectomycorrhizal fungi *Scleroderma*. The substrate used in the containers may also influence the mycorrhization success. Our assays were carried out in a mix of peat and perlite. Perlite helps to maintain a high degree of porosity (macroporosity) thus enhancing substrate aeration, a situation that can facilitate mycorrhizae synthesis and further growth of the fungi (Gautry *et al.*, 1991). Substrates usually used in nurseries, such as composted pine bark and soil mixtures have been associated to the

appearance of pioneer aggressive and highly competitive mycorrhizae, a situation that can impair further colonization by more interesting and less competitive fungi strains (Rincón *et al.*, 2005; Chen *et al.*, 2006). In our assays, the use of composted pine bark was avoided and may have contributed to the success of the mycorrhization process. In other species, different types of substrates have been successfully used such as potting mix (vermiculite, peat and/ or sand) in *P. pinea* (Rincón *et al.*, 2005), and *E. urophylla* (Chen *et al.*, 2006). However, further detailed studies must be conducted to evaluate in a more precise way the interaction between substrate and mycorrhizae establishment.

According to Rincon *et al.* (2001) spore inoculum of *P. tinctorius* has been successfully used in several experiments with different *Pinus* species and also with *Quercus suber* and *Q. rubra*. For other species, such as *Abies* spp. *Pinus ponderosa* and *Pseudotsuga menziesii* the fungus was not effective (Rincón *et al.*, 2001). Our results showed that *P. tinctorius* proved to be useful for *A. unedo* mycorrhization, promoting a significant increase in height increment when dry sporocarps were applied. Moreover, control plants were fertilized, whereas inoculated plants were not, this fact may explain the similar growth results two months after acclimatization. According to Quinteiro (2005) mycorrhization may help to reduce application of fertilizers by increasing nutrient uptake. This seems to occur in *A. unedo* since increased height was higher in mycorrhized plants than in those in which a fertilizer was added to the substrate. In extensive field conditions this may reduce costs and contribute to a cleaner environment through the use of more friendly agricultural practices.

Field trial observations

Following nursery treatments a field trial was established with five types of plant material: inoculated clonal plants (vegetative inocula and dry sporocarps), control plants (not inoculated), seedlings propagated in the nursery and 3 micropropagated selected clones. Only seedlings and the 3 selected clones were fertilized when planted in the field. Results, recorded one year later, showed a survival rate of 97.2%, without significant differences between treatments. Twenty months after the beginning of the experiment, plant height and height increment was higher in both treatments with inoculated plants (vegetative inocula and dry sporocarps; $P>0.05$) than in control plants and seedlings. In addition, the control plants showed the lowest height increment average ($P>0.05$). These results are in accordance with those reported by Kosola *et al.* (2007) showing that mycorrhization (*Rhizoscyphus ericae*) promotes field establishment of *Vaccinium macrocarpon*, stress tolerance and growth, therefore being an useful tool for the management of crop plants. In our experiment no

differences were found between inoculated treatments. Addition of fertilizers is a common agricultural practice in field plantations by using a slow release fertilizer. However, as aforementioned, mycorrhization seems to be a more interesting option since it is cheaper, cleaner and improves the physiological conditions of the plants.

Root analysis indicated that plants of strawberry tree were mycorrhized with different fungi, being one of them *Cenococcum geophilum.* According to Ingleby *et al.* (1990) this fungal species is the most widely described mycorrhiza and easily identified by its black color with characteristic emanating hyphae (thick, straight, and invariably broken because of their fragile nature) and mantle surface (compact and uneven) features. This type of mycorrhizae with emanating hyphae was also observed in *A. unedo* roots. According to Ingleby *et al.* (1990), the emanating hyphae are absent only when *C. geophilum* mycorrhizae get older. In this condition, others mycorrhizae brown/black without hyphae may appear similar (e.g. ITE 5, mycorrhizae identified by Institute of Terrestrial Ecology, Ingleby *et al.*, 1990). Similar conclusions were attained by Richard *et al.* (2005) who have demonstrated, through molecular analysis, that in *A. unedo* plants growing in old Mediterrean forest dominated by *Quercus ilex, C. geophilum* was the dominant species in 30 sampled plants. The same authors were only able to identify three other species besides *C. geophilum*, namely two Thelephoraceae species and *Inocybe tigrina*. The observation of *A. unedo* roots, twenty months after field trial establishment, did not show the presence of *P. tinctorius* previously inoculated under nursery conditions. Fungal persistence is a problem reported by several authors (Parladé *et al.*, 2004; Rincón *et al.*, 2005; Águeda *et al.*, 2008; Parladé *et al.*, 2009). On the other hand, the presence on the roots of mycorrhizae other than the one used for inoculation may explain the high variability of plant growth and hence the lack of significance.

In our experiments, the use of a non-specific strain of *P. tinctorius* to the field trial environment might explain its absence in the roots twenty months after field trial establishment, likely due to the lower competitiveness of the strain used when compared to other mycorrhizal fungi. It is critical to know the threshold colonization level required to ensure fungal persistence, as well as, screening fungal strains for their aggressiveness under nursery and field conditions, which is a prerequisite for the successful commercial application of inoculation techniques (Parladé *et al.*, 2004).

Inoculation with *P. tinctorius* and *L. deliciosus in vitro* conditions

Our experiments showed that after 6 weeks with inoculated substrate in vessels the development of *P. tinctorius* mycelium on the substrate was visible to the naked eye. Moreover, after 5 months on the synthesis tubes, during the acclimatization process of *A. unedo* plants, extensive substrate and root colonization by *P. tinctorius* mycelium was observed. However, similar results were not observed with *L. deliciosus*. Mycorrhizal synthesis experiments are useful to determine fungus-plant host compatibility. According to Quinteiro (2005) there are two relevant applications of mycorrhizae, one directly related to the promotion of plant growth by enhancing their physiological conditions, and other due to the production of edible mushrooms. *L. deliciosus*, a fungus producer of edible mushroom is very well known by its ability to establish mycorrhizae with pine trees (Ingleby *et al.*, 1990). According to Martins (2004), most mycorrhizae fungi used in nurseries are pioneers, but they do not have any economic value since they are unable to develop edible carpophores. However, they perform important ecological functions, as improving plant growth and tolerance to environmental stresses. *Cenoccocum geophillum*, *Hebeloma crustiliniforme Laccaria bicolor*, *L. laccata* and *P. tinctorius* are the most used fungi in nursery assays (Martins, 2004; Oliveira *et al.*, 2010b). The production of edible fungi is less developed and widespread, and has been mainly related to *Terfezia* spp., *Tuber aestivum, T. magnatum* and *T. melanosporum* production due to its high economic value (Quinteiro, 2005; Honrubia, 2009b). Some interesting results have been reported with other species, such as *Boletus aereus* and *B. edulis* with *Castanea sativa* or *Cistus* spp. and *L. deliciosus* with *Pinus* (Martins, 2004; Parladé *et al.*, 2004; Águeda *et al.*, 2008; Honrubia, 2009b). *In vitro* mycorrhization with *L. deliciosus* has been reported for several species namely *Picea abies*, *P. contorta*, *Pinus echinata*, *P. halepensis*, *P. mugo*, *P. nigra*, *P. pinaster*, *P. pinea*, *P. ponderosa*, *P. radiata*, *Pseudotsuga menziesii*, *P. strobus* and *P. sylvestris* (Parladé *et al.*, 2004). However, data on mycorrhizal inoculation of pines with *L. deliciosus* under nursery conditions are reported only for *P. pinea* (Parladé *et al.*, 2004). Mycorrhization with another *Lactarius* species (*L. sanguifluus*) seems to be restricted to *Pseudotsuga menziesii* and *P. sylvestris* (Parladé *et al.*, 2004). Associations between *Betula* and several *Lactarius* species (*L. glyciosmus, L. pubescens* and *L. rufus*) in natural environments has been also referred (Ingleby *et al.*, 1990). Fruitbodies observations suggest that these species may be specific to *Betula* (Ingleby *et al.*, 1990). Under natural conditions the *A. unedo* grows in association with other forest tree species, including *P. pinaster* and *P. pinea* sharing the same forest environment. Therefore, it seems likely that roots of these trees might also share some

mycorrhizae fungi. One purpose of our experiments was to determine fungus-plant host compatibility, between *A. unedo* and *L. deliciosus* or *P. tinctorius, in vitro* conditions. However, arbutoid mycorrhizae, as well as an extensive mycelium development were only observed when *P. tinctorius* was tested. The better *in vitro* culture of *P. tinctorius* compared to *L. deliciosus* may explain its better ability to mycorrhizae formation. *P. tinctorius* shows an efficient development, consequently easily reaches plant roots and establishes the symbiotic association, hence just 30 days after *in vitro* inoculation *Eucalyptus* plantlets showed good mycorrhizal development (Galli *et al.*, 1992). Moreover, *P. tinctorius* has an ubiquitous distribution and tolerates a wide diversity of environmental conditions (Rincón *et al.*, 2001). These data agree with the results we have obtained which also enabled us to conclude that *P. tinctorius* grows faster *in vitro* than *L. deliciosus.*

In our experiments mycorrhization did not cause any detectable loss of vigor in the strawberry tree plants, even when the substrate and roots were totally colonized by *P. tinctorius*. The treated plants showed a well developed and branched root system with the presence of secondary roots. Overall, a thick yellow-brown mantle could be seen with the naked eye when *P. tinctorius* was tested. Experiments performed by Giovannetti *et al.* (1989) in which vesicular arbuscular (VA) mycorrhizal endophytes were tested on *A. unedo* indicated that only *Glomus microcarpum* was able to establish mycorrhizae with roots showing longitudinally running hyphae and many internal vesicles typical of this fungus. However, seedlings rapidly became stunted and died within 2 months. The authors also indicated that the intensive colonization of *A. unedo* roots by *G. microcarpum* and the death of the plants suggest a parasitic more than a symbiotic behaviour of this fungus towards *A. unedo*. The genus *Glomus* is well known by its ability to form VA mycorrhizae with several species such as *Corylus avellana* (Mirabelli *et al.*, 2009), *Juglans regia* (Rai, 2001), *Leucaena leucocephala* (Puthur *et al.*, 1998), *Olea europea* (Cantos *et al.*, 2009), *Panax quinquefolius* (Peterson and Massicotte, 2004), *Prunus avium* (Rai, 2001) and *Sesbania sesban* (Subhan *et al.*, 1998).

During the acclimatization process, we could find that when *L. deliciosus* was tested the IRi treatment (shoot transfer to the inoculated substrate after root induction) showed a rooting rate significantly lower than the control plantlets and IRe treatment (shoot transfer after root expression for 2 weeks). Shoot length of the IRe plantlets inoculated with *P. tinctorius* was significantly higher than that observed with IRi treatments. A similar situation was observed when biomass was evaluated. Moreover, IRe allowed for a 100% of both rooting and survival rate, and a more efficient plant growth ($P \leq 0.01$; height increment). These features suggest

that the root development for 2 weeks promotes further plantlet development on inoculated substrate and further acclimatization. These results are consistent with those reported by Oliveira *et al.* (2003) in *Pinus pinea*. The authors have found by using rooted shoots transferred to a inoculated substrate they could overcome the difficulties encountered in the acclimatization of *P. pinea* micropropagated plantlets (Oliveira *et al.*, 2003).

When biomass analyses were accomplished no differences were found for root biomass. However, during the in *vitro* acclimatization process, it was noticed that roots were neither as long nor as numerous as in control plants. However, root branching and secondary roots were common. Similar results were also reported on ectomycorrhizal fungi of *Castanea sativa* micropropagated plants and seedlings (Martins *et al.*, 1996; Martins, 2004). Similarly, when inoculation with specific fungi strains were tested in vegetative propagation of conifers the mycorrhization enhanced root formation as well as root branching of *in vivo* cuttings and *in vitro* adventitious shoots (Niemi *et al.*, 2004). In our case, the biomass ratio (shoot/root) values suggest that the treatment with mycorrhizae produce more balanced plantlets when compared to the control. Previous experiments have shown that root architecture is strongly related with plant survival during acclimatization of micropropagated plants, with plants possessing a well branched root system displaying higher survival rates (Gonçalves *et al.*, 1998; Gomes and Canhoto, 2003; Gomes *et al.*, 2003).

Arbutoid mycorrhizae were observed *in vitro* 1 month after inoculation indicating compatibility between *A. unedo* and *P. tinctorius*. These characteristics combined with mycorrhizae formation may explain the higher survival rate (100%) observed in our assays. The positive role of mycorrhization on plant development following micropropagation has been reported by a number of researchers. For example, in *Vaccinium angustifolium*, also an Ericaceae, mycorrhization was used to improve plant acclimatization (Ratnaparkhe, 2007). Other species in which mycorrhization helped to achieve higher levels of plant acclimatization were, among others, *Castanea sativa* (Martins, 2010), *Corylus avellana* (Mirabelli *et al.*, 2009), *Juglans regia* (Rai, 2001), *Leucaena leucocephala* (Puthur *et al.*, 1998), *Olea europea* (Cantos *et al.*, 2009), *P. pinaster* and *P. sylvestris* (Parladé *et al.*, 2004), *Pinus pinea* (Oliveira *et al.*, 2003), *Rhododendron* (Eccher and Martinelli, 2010), *Sesbania sesban* (Subhan *et al.*, 1998), *Vaccininium corymbosum* (Eccher and Noé, 2002) and *Vitis vinifera* (Cantos *et al.*, 2009).

In natural conditions, Giovannetti *et al.* (1989) were able to identify two main types of mycorrhizae in *A. unedo*. The first type was characterized by a thick black mantle attributed to *Cenococcum graniforme*. The second type of mycorrhizae was characterized by a thick

mantle of closely interwoven hyphae. Histological studies demonstrated that epidermal cells were completely filled with the mycorrhizal fungus. In our study, plants showed mycorrhizae with a typical arbutoid organization, namely a thick mantle, the HN, intracellular hyphal complexes confined to the epidermis and no evidence of VA formation. Similar observations were made by Münzenberger *et al.* (1992) on arbutoid mycorrhizae of *A. unedo* due to *Laccaria amethystea* where a well developed mantle, the HN, and a intracellular hyphal complex confined to the epidermis were also present. These particularities are quite different from those observed by Fusconi and Bonfante-Fasolo (1984) on the mycorrhizae of *A. unedo* growing in natural conditions and due to ascomycetous fungi where few hyphae occurred and a true mantle could not be found. Concerning the hyphal mantle in arbutoid mycorrhizae development different observations have been reported. Thus, in *Pyrola* sp. mycorrhizae, also classified as arbutoid such as those of *Arbutus* and *Arctostaphylos*, the mature mycorrhizae are characterized by the presence of numerous intracellular hyphae, HN, the absence of sheath and the lack of fungal penetration into the cortex (Robertson and Robertson, 1985). According to Massicotte *et al.* (1993) in *A. menziesii*, the outer row of cortical cells develops a hypodermis showing suberin lamellae in their walls and a Casparian strip in radial walls, therefore confining the development of the HN to the epidermis. The same authors also reported the presence of a variable fungal sheath (Massicotte *et al.*, 1993). In our experiment the presence of thick yellow-brown mantle was clear, and the mycorrhizae had a typical cruciform appearance and a compound structure, like repeated branching. This characteristic was also reported on arbutoid mycorrhizae between *A. menziesii* and the basidiomycetes *P. tinctorius* (Molina and Trappe, 1982; Massicotte *et al.*, 1993) or *Piloderma bicolor* (Smith and Read, 1997). According to Smith and Read (1997) this pattern of branching appears to arise from precocious initiation of individual lateral roots, rather than by dichotomy of the root apical meristem as typically seen in ectomycorrhizae of *Pinus*.

Nine months after acclimatization (or 14 months after *in vitro* inoculation treatment), some plants in the nursery were examined for arbutoid mycorrhizae and compared to control plants. All plants (inoculated or not) showed arbutoid mycorrhizae formation whereas some plants showed rhizomorphs. According to Ingleby *et al.* (1990) the rhizomorphs are an effective support to storage and nutrient transport. The production of extensive rhizomorphs systems and their ability to enhance water supply appears to be related to the ability of mycorrhizae to increase drought tolerance (Cairney and Chambers, 1997). The common explanation of improved water uptake by mycorrhized compared to non-mycorrhized plants is the strongly increased absorbing surface caused by soil growing hyphae combined with the fungal

capability to take up water from soils with a lower (more negative) water potential (Marjanovic´ and Nehls, 2008). Regardless of how successful has been the *in vitro* mycorrhization and plant field behavior it is of the utmost importance to monitor the persistence of the inoculated fungi in the field. Several molecular techniques have been used for intraspecific and specific characterization of the inoculated fungal strains (Parladé *et al.*, 2009). Among them Real-time PCR and Terminal Restriction Fragment Length Polymorphism/T-RFLP have been applied for tracking the dynamics of mycelium in different experimental conditions such as field persistence of the inoculated fungus in experimental plantations and competition of the introduced fungus with native mycorrhizal fungi (Dickie and Fitzjohn, 2007; Parladé *et al.*, 2009). In the current study, the sequences results on the ITS region were compared with those of the Blast database. Twelve months after acclimatization (or 17 months after *in vitro* inoculation treatment) the reaction products, after PCR with ITS1F and ITS4 primers, were sequenced. The sequences identification showed the presence of mycorrhizae fungi on plants growing in the nursery. However, the analysis of the sequences amplified showed that the arbutoid mycorrhizae were not due to *P. tinctorius* or *L. deliciosus* colonization but rather a consequence of *Thelephora* sp. and *Hebeloma* sp. mycorrhization. These are also basidiomycete fungi. According to Ingleby *et al.* (1990), *Hebeloma* mycorrhizae are frequently associated with young trees of different species, and may be considered "early stage" fungi. *Hebeloma* are particularly common on tree nurseries and greenhouse potting composts, and in these conditions tend to dominate root systems impairing colonization by other species of fungi. *Thelephora* mycorrhizae are particularly abundant on seedlings growing in greenhouses or in nurseries. These kind of mycorrhizae is unspecific, are common in a wide range of habitats, and are also considered of the "early stage" (Garbaye, 1990; Ingleby *et al.*, 1990). In addition, *Thelephora* is usually dominant, as fruitbodies are abundant and perennial, spreading spores throughout the year and well adapted to extreme temperatures (Garbaye, 1990). These features may help to explain our results observed 12 months after acclimatization of strawberry tree plants. Results obtained by Parladé *et al.* (2004) in *P. pinaster* and *P. sylvestris* are in line with these data. In fact, they found that one-year old plants growing in the nursery showed levels of colonization with *L. deliciosus* highly reduced (50% and 2% for *P. pinaster* and *P. sylvestris* respectively). Fungal persistence is a common problem following mycorrhization and has been often reported (Parladé *et al.*, 2004; Rincón *et al.*, 2005; Águeda *et al.*, 2008; Parladé *et al.*, 2009).

The results now reported clearly show that *P. tinctorius* can be successfully used to promote arbutoid mycorrhization in *A. unedo* plants. Moreover, it was demonstrated that this

fungus improves the survival rate of micropropagated plantlets ($P>0.05$). These assays of *in vitro* mycorrhization showed that further researches are necessary before delivery of selected mycorrhized strawberry tree plants to the farmers, to increase income and to reduce the inputs necessary for plant growth. Additional assays on the mycorrhization of *A. unedo* plants will be focused on the selection of more competitive fungal strains and on the threshold fungal colonization level required to increase the frequencies of mycorrhization. The optimization of the mycorrhization process to assure mycorrhization persistence during further plant growth must be also subject of analysis. A molecular study to characterize mycorrhizae formation and a time-course analysis of the mycorrhized roots, both at the cytological and molecular levels will be also helpful to better understand the interactions between roots, the mycorrhizae fungi and other fungi that can interfere with the mycorrhization process.

4.6 REFERENCES

Agerer, R., Rambold, G., 2004-2010. DEEMY - An information system for characterization and determination of ectomycorrhizae. München, Germany www.deemy.de Nov./2009.

Águeda, B., Parladé, J.F., Fernández-Toirán, L.M., Cisneros, O., Miguel, A.M., Modrego, M.P., Martínez-Pena, F., Pera, J., 2008. Mycorrhizal synthesis between *Boletus edulis* species complex and rockroses (*Cistus* sp.). Mycorrhiza 18, 443-449.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389-3402.

Cairney, J.W.G., Chambers, S.M., 1997. Interactions between *Pisolithus tinctorius* and its hosts: a review of current knowledge. Mycorrhiza 7, 117-131.

Cantos, M., Liñán, J., Carretero, C.L., Troncoso, A., Azcón, R., 2009. Influence of mycorrhization during the hardening process of micropropagated plants. Acta Hort. 812, 455-460.

Chen, Y.L., Kang, L.H., Dell, B., 2006. Inoculation of *Eucalyptus urophylla* with spores of *Scleroderma* in a nursery in south China: Comparison of field soil and potting mix. Forest Ecol. Manag. 222, 439-449.

Dickie, I.A., Fitzjohn, R.G., 2007. Using terminal restriction fragment length polymorphism (T-RFLP) to identify mycorrhizal fungi: a methods review. Mycorrhiza 17, 259-270.

Duncan, D.B., 1955. Multiple range and multiple F tests. Biometry 11, 1-42.

Eccher, T., Martinelli, M., 2010. Inoculation of *Rhododendron* cultivars *in vitro* with different strains of ericoid endomycorrhizae. Acta Hort. 865, 327-332.

Eccher, T., Noé, N., 2002. Influence of Ericoid endomycorrhizae inoculated *in vitro* on rooting and early growth of micropropagated plants of *Vaccinium corymbosum* L. Acta Hort. 574, 373-378.

Fusconi, A., Bonfante-Fasolo, P., 1984. Ultrastructural aspects of host endophyte relationships in *Arbutus unedo* L. mycorrhizas. New Phytologist 96, 397-410.

Galli, M.A., Krugner, T.L., Gonçalves, A.N., Vieira, J.D., 1992. *In vitro* mycorrhizal synthesis in *Eucalyptus* plantlets inoculated with *Pisolithus tinctorius* in synthetic substrates. Mass Production Technology for Genetically Improved Fast Growing Forest Tree Species. AFOCEL, IUFRO, Tome II, Bordeaux, pp. 227-235.

Garbaye, J., 1990. Pourquoi et comment observer l'état mycorhizien des plants forestiers. Rev. For. Fr. XLII 1, Biologie et Forêt, 35-47.

Gautheret, R.J., 1959. La culture des tissues végétaux, techniques et réalisations. Masson, Paris.

Gautry, J.Y., Jouvenceau, S., Thivolle-Cazat, A., 1991. Mycorrhization artificielle du Douglas et de l'épicéa et interaction clone x champignon. Ann. AFOCEL, 125-158.

Giovannetti, M., Lioi, L., Picci, G., 1989. Host and non-host relationships between *Arbutus unedo* L. and mycorrhizal fungi. Agr. Ecosyst. Environ. 29, 169-172.

Gobert, A., Plassard, C., 2008. The beneficial effect of mycorrhizae on N utilization by host-plant: myth or reality? In: Varma, A. (Ed.), Mycorrhiza Genetics and Molecular Biology, Eco-function, Biotechnology, Eco-physiology, Structure and Systematic. 3rd ed. Springer, Berlin, pp. 209-240.

Gomes, F., Canhoto, J.M., 2003. Micropropagation of *Eucalyptus nitens* Maiden (Shining gum). In Vitro Cell. Dev. Biol.-Plant 39, 316-321.

Gomes, F., Canhoto, J.M., 2009. Micropropagation of strawberry tree (*Arbutus unedo* L.) from adult plants. In Vitro Cell. Dev. Biol.-Plant 45, 72-82.

Gomes, F., Lopes, M.L., Santos, T., Canhoto, J.M., 2009. Micropropagation of selected trees of *Arbutus unedo* L. trough axillary shoot proliferation and somatic embryogenesis. Acta Hort. 839, 111-116.

Gomes, F., Santos, J.M., Canhoto, J., 2003. Efeito de vários nutrientes no crescimento *in vitro* de raízes de *Eucalyptus globulus*. Silva Lusitana 11, 153-163.

Gonçalves, J.C., Diogo, G., Amâncio, S., 1998. *In vitro* propagation of chestnut (*Castanea sativa x Castanea crenata*): effects of rooting treatments on plant survival, peroxidase activity and anatomical changes during adventitious root formation Sci. Hort. 72, 265-275.

Harrison, M.J., 1999. Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 361-389.

Honrubia, M., 2009. Valorización de la investigación en la Región de Murcia: del conocimiento a la empresa de base tecnológica. Producción centífica e innovación. El valor del conocimiento científico-tecnológico en la empresa. Thader Biotecnology, Universidad del Mar, Murcia.

Ingleby, K., Mason, P.A., Last, F.T., Fleming, V., 1990. Identification of ectomycorrhizas, ITE Research Publication nº 5. NE Research Council, HMSO Publ., London.

Jacob, C., Courbot, M., Brun, A., Steinman, H.M., Jacquot, J.P., Botton, B., Chalot, M., 2001. Molecular cloning, characterization and regulation by cadmium of a superoxide

dismutase from the ectomycorrhizal fungus *Paxillus involutus*. Eur. J. Biochem. 268, 3223-3232.

Kosola, K.R., Workmaster, B.A.A., Spada, P.A., 2007. Inoculation of cranberry (*Vaccinium macrocarpon*) with the ericoid mycorrhizal fungus *Rhizoscyphus ericae* increases nitrate influx. New Phytologist 176, 184-196.

Marjanovic´, Ž., Nehls, U., 2008. Ectomycorrhiza and water transport. In: Varma, A. (Ed.), Mycorrhiza Genetics and Molecular Biology, Eco-function, Biotechnology, Eco-physiology, Structure and Systematics. 3rd ed. Springer, Berlin, pp. 149-160.

Martins, A., 2004. Micorrização controlada de *Castanea sativa* Mill.: Aspectos fisiológicos da micorrização *in vitro* e *ex vitro*. PhD Thesis. University of Lisbon.

Martins, A., 2010. Mycorrhizal inoculation of Chestnut seedlings: effect on survival and growth after transplantation. Acta Hort. 866, 325-334.

Martins, A., Barroso, J., Pais, M.S., 1996. Effect of ectomycorrhizal fungi on survival and growth of micropropagated plants and seedlings of *Castanea sativa* Mill. Mycorrhiza 6, 265-270.

Massicotte, H.B., Melville, L.H., Molina, R., Peterson, R.L., 1993. Structure and histochemistry of mycorrhizae synthesized between *Arbutus menziesii* (Ericaceae) and two basidiomycetes *Pisolithus tinctorius* (Pisolithaceae) and *Piloderma bicolor* (Corticiaceae). Mycorrhiza 3, 1-11.

Mirabelli, C., Tullio, M., Pierandrei, F., Rea, E., 2009. Effect of arbuscular mycorrhizal fungi on micropropagated hazelnut (*Corylus avellana* L.) plants. Acta Hort. 812, 467-472.

Molina, R., Trappe, J.M., 1982. Lack of mycorrhizal specificity by the Ericaceous hosts *Arbutus menziesii* and *Arctostaphylos uva-ursi*. New Phytol. 90, 495-509.

Münzenberger, E., Kottke, I., Oberwinkler, F., 1992. Ultrastructural investigations of *Arbutus unedo -Laccaria amethystea* mycorrhiza synthesized *in vitro*. Trees 7, 40-47.

Niemi, K., Scagel, C., Häggman, H., 2004. Application of ectomycorrhizal fungi in vegetative propagation of conifers. Plant Cell Tis. Org. Cult. 78, 83-91.

Noé, N., Eccher, T., Borra, M., 2002. The use of selected mycorrhizae for quality improvement of Highbush Blueberry (*Vaccinium corymbosum* L.) plant production Acta Hort. 574, 387-392.

Oliveira, P., Barriga, J., Cavaleiro, C., Peixe, A., Potes, A.Z., 2003. Sustained *in vitro* root development obtained in *Pinus pinea* L. inoculated with ectomycorrhizal fungi. Forestry 76, 579-587.

Oliveira, R.S., Franco, A.R., Vosátka, M., Castro, P.M.L., 2010. Management of nursery practices for efficient ectomycorrhizal fungi application in the production of *Quercus ilex*. Symbiosis DOI 10.1007/s13199-010-0092-0.

Parladé, J., Pera, J., Luque, J., 2004. Evaluation of mycelial inocula of edible *Lactarius* species for the production of *Pinus pinaster* and *P. sylvestris* mycorrhizal seedlings under greenhouse conditions. Mycorrhiza 14, 171-176.

Parladé, X., Pera, J., De La Varga, H., Hortal, S., 2009. Tracking inoculated ectomycorrhizal fungi by Real-Time PCR. 14th European Congress on Biotechnology. New Biotechnology, Barcelona, Spain, p. *Abst* S375.

Peterson, R.L., Massicotte, H.B., 2004. Exploring structural definitions of mycorrhizas, with emphasis on nutrient-exchange interfaces. Can. J. Bot. 82, 1074-1088.

Piotto, B., Piccini, C., Arcadu, P., 2001. La ripresa della vegetazione dopo gli incendi nella regione mediterrânea. In: Piotto, B., Noi, A. (Eds.), Propagazione per seme di alberi e arbusti della flora mediterranea. Dipart. Prev. Risanam. Ambient., Manuale ANPA (Agenz. Naz. per la Protezi. dell' Ambiente), pp. 32-38.

Puthur, J.T., Prasad, K.V.S.K., Sharmila, P., Saradhi, P.P., 1998. Vesicular arbuscular mycorrhizal fungi improves establishment of micropropagated *Leucaena leucocephala* plantlets. Plant Cell Tis. Org. Cult. 53, 41-47.

Quinteiro, S.L., 2005. Respuesta de siete orígenes ibéricos de *Pinus pinaster* Aiton frente a la inoculación en vivero con *Pisolithus tinctorius* y *Paxillus involutus*. PhD Thesis. University of Santiago de Compostela.

Rai, M.K., 2001. Current advances in mycorrhization in micropropagation. In Vitro Cell. Dev. Biol.-Plant 37, 158-167.

Ratnaparkhe, M.B., 2007. Blueberry. In: Kole, C. (Ed.), Genome mapping and molecular breeding in plants, Fruits and Nuts. Springer-Verlag, Berlin Heidelberg, pp. 217-227.

Richard, F., Millot, S., Gardes, M., Selosse, M.A., 2005. Diversity and specificity of ectomycorrhizal fungi retrieved from an old-growth Mediterranean forest dominated by *Quercus ilex*. New Phytologist 166, 1011-1023.

Rincón, A., Alvarez, I.F., Joan Pera, J., 2001. Inoculation of containerized *Pinus pinea* L. seedlings with seven ectomycorrhizal fungi. Mycorrhiza 11, 265-271.

Rincón, A., Parladé, J., Pera, J., 2005. Effects of ectomycorrhizal inoculation and the type of substrate on mycorrhization, growth and nutrition of containerized *Pinus pinea* L. seedlings produced in a commercial nursery. Ann. For. Sci. 62, 817-822.

Robertson, D.C., Robertson, J.A., 1985. Ultrastructural aspects of *Pyrola* mycorrhizae. Can. J. Bot. 63, 1089-1098.

Smith, S.E., Read, D.J., 1997. Mycorrhizal Symbiosis, 2nd ed. Academic Press, Harcourt Brace and Comp. Pub., San Diego.

Subhan, S., Sharmila, P., Saradhi, P.P., 1998. *Glomus fasciculatum* alleviates transplantation shock of micropropagated *Sesbania sesban*. Plant Cell Rep. 17, 268-272.

White, T.J., Bruns, T.D., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribossomal RNA genes for phylogenetics. PCR Protocols: a guide to methods and amplifications. Academic Press, London, pp. 315-322.

Zar, J.H., 1996. Biostatistical Analysis (4th Ed.). Prentice-Hall, Upper Saddle River.

APPENDIX

Appendix Table 1– ANOVA: effect of different inoculation treatments with *P. tinctorius* plus control on plants' height increment (ΔH) evaluated 2 and 4 months after inoculation under nursery conditions.

Height increment 2 months after treatment application					
Source	SS	d.f.	MS	F	p
Treatment	3.58	2	1.79	0.261	0.770644
Error	1029.37	150	6.86		

Height increment 4 months after treatment application					
Source	SS	d.f.	MS	F	p
Treatment	565.53	2	282.76	12.784	0.000008**
Error	3207.14	145	22.12		

*Significant at P≤0.05; ** Significant at P≤0.01; (Treatments tested: C1C - control plants compared to the C1M - mycelium *in vitro* produced on liquid medium; and C1S - dry sporocarps water mixed).

Appendix Table 2 – ANOVA: effect of the different treatments tested on two variables: plants height and height increment evaluated 20 months after field trial establishment.

Height ($H_{20\ months\ old}$)

Source	SS	d.f.	MS	F	p
Treatment	4512.05	6	752.01	1.153	0.34
Error	83492.09	128	652.28		

Height increment ($H_{20\ months\ old} - H_0$)

Source	SS	d.f.	MS	F	p
Treatment	1285.38	6	214.23	0.320	0.93
Error	85818.72	128	670.46		

Treatments tested: 3 mycorrhizal treatments, 3 selected clones and seedlings.

Appendix Table 3 – ANOVA: effect of the different treatments of mycorrhizal synthesis *in vitro* conditions with *P. tinctorius* or *L. deliciosus* on shoots (in vessels), on different variables: rooting rate (%); number of roots (NR); length of the longest root (LLR); length of the shortest root (LSR) and final shoot length (SL).

Treatments: substrate inoculate with *Pisolithus tinctorius*					
Rooting % (arcsine transformation)					
Source	SS	df	MS	F	p
Mycorrhiz. treat.	311.3	2	155.6	0.6738	0.526715
Error	3002.9	13	231.0		
Number of roots					
Source	SS	df	MS	F	p
Mycorrhiz. treat.	295.813	2	147.907	6.0530	0.0037**
Error	1759.333	72	24.435		
LLR (mm)					
Source	SS	df	MS	F	p
Mycorrhiz. treat.	11501.64	2	5750.82	30.3474	0.0000**
Error	13264.99	70	189.50		
LSR (mm)					
Source	SS	df	MS	F	p
Mycorrhiz. treat.	1298.52	2	649.26	13.1597	0.0000**
Error	3305.57	67	49.34		
SL (mm)					
Source	SS	df	MS	F	p
Mycorrhiz. treat.	7246.3	2	3623.1	11.2743	0.0000**
Error	23138.1	72	321.4		

Treatments: substrate inoculate with *Lactarius deliciosous*					
Rooting % (arcsine transformation)					
Source	SS	df	MS	F	p
Mycorrhiz. treat.	2824.16	2	1412.08	4.3385	0.0342*
Error	4556.64	14	325.47		
Number of roots					
Source	SS	df	MS	F	p
Mycorrhiz. treat.	45.250	2	22.625	1.5550	0.22001
Error	829.333	57	14.550		
LLR (mm)					
Source	SS	df	MS	F	p
Mycorrhiz. treat.	185.33	2	92.66	0.8297	0.4414
Error	6366.20	57	111.69		
LSR (mm)					
Source	SS	df	MS	F	p
Mycorrhiz. treat.	274.443	2	137.222	3.53299	0.0358*
Error	2213.883	57	38.840		
SL (mm)					
Source	SS	df	MS	F	p
Mycorrhiz. treat.	191.1	2	95.6	0.887	0.4175
Error	6141.6	57	107.7		

*Significant at P≤0.05; ** Significant at P≤0.01. Treatments tested: IRe, IRi, NI.

Appendix Table 4 – ANOVA: effect of the different treatments of mycorrhizal synthesis *in vitro* conditions with *L. deliciosus* (in vessels), 6 weeks after shoots transfer to the inoculated substrate on different variables: shoot biomass; root biomass; biomass ratio (shoot/root).

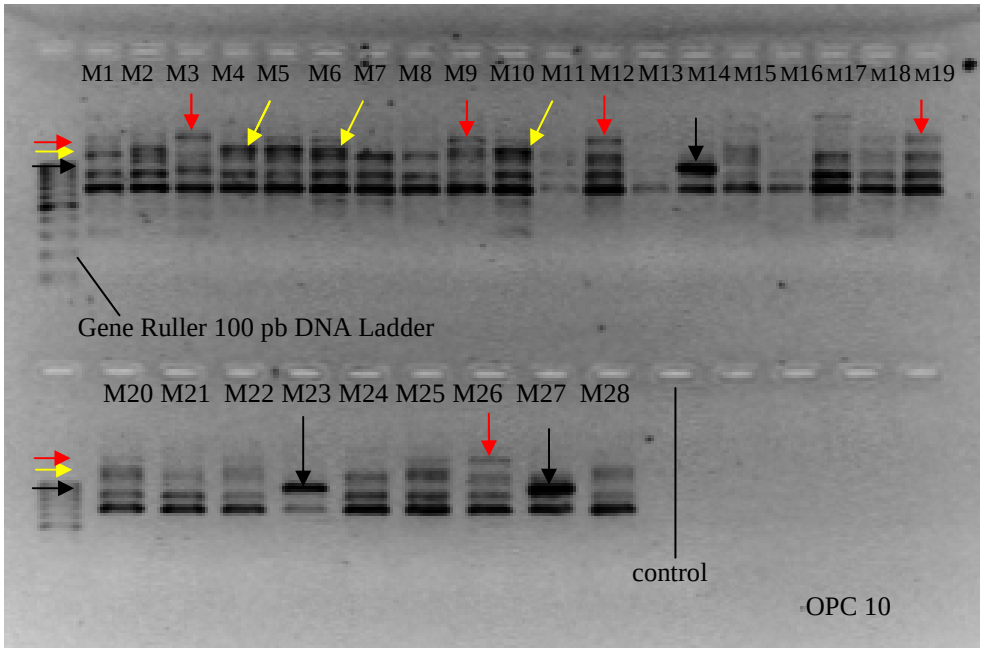
Shoot biomass (dry matter mg)						**Root biomass (dry matter mg)**						**Biomass ratio (shoot / root; dry matter)**					
Source	SS	df	MS	F	p	Source	SS	df	MS	F	p	Source	SS	df	MS	F	p
Mycorrhiz. treat.	792.52	2	396.26	3.413	0.04959*	Factor	0.0274	2	0.0137	0.065	0.937	Factor	1228.67	2	614.33	2.834	0.0786
Error	2786.22	24	116.09			Error	5.0600	24	0.2108			Error	5202.94	24	216.79		

*Significant at P≤0.05; ** Significant at P≤0.01. Treatments tested: IRe, IRi, NI; (Biomass: dry matter, after 48hours/65ºC).

Appendix Table 5 – ANOVA: effect of the different treatments of mycorrhizal synthesis *in vitro* conditions with *P. tinctorius* (in vessels), on shoots 2 months after acclimatization, on different variables: survival rate (%) and plants' height increment.

Survival % (arcsine transformation)						**Plants height increment (cm)**					
Source	SS	df	MS	F	p	Source	SS	df	MS	F	p
Mycorrhiz. treat.	329.450	2	164.7	1.197	0.3332	Mycorrhiz. treat.	94.009	2	47.005	13.1271	0.00002**
Error	1788.44	13	137.6			Error	222.006	62	3.581		

*Significant at P≤0.05; ** Significant at P≤0.01. Treatments tested: IRe, IRi, NI.



5 - Genetic similarity studies in *Arbutus unedo* L. (strawberry tree) using molecular markers

Part of this chapter was submitted to an international journal.

Part of these results was presented in the following congresses:

Oral presentation:

Gomes, F., Costa, R., Ribeiro, M.M., Canhoto, J.M. 2010. Genetic similarity studies in *Arbutus unedo* L. (strawberry tree) using microsatellites. 28th International Horticultural Congress, Science and Horticulture for People (ISHS), Lisboa, p. *Abst* 531.

Poster presentations:

Gomes, F., Costa, R., Ribeiro, M.M., Figueiredo, E., Canhoto, J.M., 2010. DNA markers to study genetic diversity and fingerprint in *Arbutus unedo* L. (strawberry tree). XXXV Jornadas Portuguesas de Genética. SPG, Univ. do Minho, Braga, p. *Abst* 70.

Gomes, F., Costa, R., Ribeiro, M.M., Figueiredo, E., Canhoto, J.M., 2010. Portuguese *Arbutus unedo* L. genotypes - genetic and geographic clustering using RAPD markers. 28th International Horticultural Congress, Science and Horticulture for People (ISHS), Lisboa, p. *Abst* 555.

5 GENETIC SIMILARITY STUDIES IN *ARBUTUS UNEDO* L. (STRAWBERRY TREE) USING MOLECULAR MARKERS

5.1 ABSTRACT

The objective of this study was to assess the genetic diversity in 27 *Arbutus unedo* genotypes from 9 provenances by molecular markers. Random amplified polymorphic DNA (RAPD) and microsatellite markers (SSR) were used. The set of 20 RAPDS primers generated 124 bands, being 71 of them (57.3%) polymorphic. The expected heterozygosity was 0.27±0.014. The Lynch (1990) similarity coefficient was used to make an UPGMA dendrogram, and the tree topology was tested through a Mantel test. This test showed a moderate correlation yet significant (r= 0.64; P<0.001). The cluster analysis revealed a similarity of 83% among trees. In addition, some genotypes shared as much as 95% of the bands. No genotypes could be grouped according to their geographical location. Furthermore, no association was found between genetic similarity and geographical distances matrices after a normalized Mantel computed statistic (r= 0.01; P<0.57).

Some of the microsatellite markers (SSR) from *Vaccinium* (an Ericaceae as *A. unedo*) used by Boches *et al*. (2005) and Bassil *et al*. (2006) were tested. Eleven SSR primers were selected according to the polymorphism level, the number of alleles per *locus*, the number of species amplified, the allele scoring quality and the repeat motif. Nine of these primers produced amplified products, 5 SSR *loci* were polymorphic, with 75% mean expected heterozygosity, 11.6 mean number of alleles, and 71% of average polymorphic information content. A mean homozygote excess was found (Fis=7%) not significant. Estimation of null allele frequency was about 7.6% on average. The Lynch (1990) coefficient showed a degree of allele's similarity between genotypes up to 82%. The Mantel test confirmed the tree topology (r=0.75; P<0.001). No genotypes were grouped according to their geographical origin, in accordance with RAPDs analysis. Mantel test also confirmed lack of correlation between genetic and geographical distances matrices, as well as the absence of correlation between pair-wise markers matrices (Lynch, 1990). Reduced gene flow, due to fragmentary distribution and/or the reproductive system of this species are plausible explanation for these results. These markers proved to be useful for further genetic similarity studies, germplasm fingerprinting and conservation.

Key words: cross amplification; Ericaceae; fingerprint; RAPDs; SSRs.

RESUMO

O objectivo do presente estudo foi avaliar a diversidade genética entre 27 genótipos, de 9 proveniências, de *Arbutus unedo*, com recurso a marcadores moleculares. Como marcadores moleculares foram utilizados RAPD e microsatélites (SSR). Um conjunto de 20 primers RAPDs gerou 124 bandas, sendo 71 polimórficas (57,3%). A heterozigocidade esperada (He) foi de 0,27±0,014. O coeficiente de similaridade de Lynch (1990) foi utilizado para a construção de um dendrograma (UPGMA), cuja topologia foi testada através do teste de Mantel. Este teste mostrou uma moderada correlação, ainda que significativa (r= 0,64; P<0,001). A análise de clusters revelou uma similaridade de 83% entre árvores, chegando alguns genótipos a partilhar 95% das bandas. Nenhum dos genótipos foi agrupado de acordo com a sua origem geográfica. Também, não foi encontrada nenhuma associação entre as distâncias das matrizes genética e geográfica, depois de normalizadas e analisadas pelo teste de Mantel (r= 0,01; P<0,57).

Foram testados microsatélites (SSR) identificados para *Vaccinium* (uma Ericaceae como *A. unedo*) por Boches *et al*. (2005) e Bassil *et al*. (2006). Onze primers foram seleccionados de acordo com o grau de polimorfismo, o número de alelos por *locus* (Na), o número de espécies em que foram observados produtos de amplificação, a qualidade de amplificação do(s) alelo(s) e o tipo de motivo repetitivo. Nove destes primers produziram produtos de amplificação, 5 SSR *loci* apresentaram polimorfismo, com 75% de He, 11,6 média de Na, e 71 % PIC (informação sobre o grau de polimorfismo). O valor médio relativo ao coeficiente de consanguinidade (Fis) foi de 7%, não significativo. A frequência de alelos nulos foi estimada com um valor médio de 7,6%. O coeficiente de Lynch (1990) mostrou um grau de similaridade entre genótipos até 82%. O teste de Mantel confirmou a topologia do dendrograma (r=0,75; P<0,001). Nenhum dos genótipos foi agrupado de acordo com a origem geográfica, confirmando os resultados observados com os marcadores RAPDs. O teste de Mantel também confirmou a falta de correlação entre as duas matrizes genética e geográfica, bem como a não existência de correlação entre as matrizes geradas pelos dois marcadores (Lynch, 1990). O reduzido fluxo genético, devido à distribuição fragmentada da espécie e/ou o tipo de sistema reprodutivo são plausíveis explicações para os resultados observados. Estes marcadores provaram ser úteis para estudos futuros sobre a similaridade genética, *fingerprint* do germoplasma e a conservação da espécie.

Palavras-chave: amplificação cruzada; Ericaceae; fingerprint; RAPDs; SSRs.

5.2 INTRODUCTION

The strawberry tree (*Arbutus unedo* L.) is an evergreen shrub-like or small tree belonging to the Ericaceae family, with a circum-Mediterranean distribution, growing where frost is not very usual (Torres *et al.*, 2002). This species, according to the International Centre for Underutilized Crops (www.icuc-iwmi.org/) (International Centre Underutilized Crops, 2009) and the Global Facilitation Unit for Underutilized Species (www.underutilized-species.org/) (Underutilized-species.org, 2008) falls in the category of NUCs (neglected or underutilized crops) is an undervalued fruit tree, with different possible commercial usages from processed and fresh fruit production to ornamental, pharmaceutical and chemical industrial applications (Celikel *et al.*, 2008). In addition, it is fire resistant and owing to its pioneer status it is valuable for land recovery and desertification avoidance (Piotto *et al.*, 2001).

In Portugal, this species occupies circa 15,500 ha widely distributed across the country (Godinho-Ferreira *et al.*, 2005), but as far as we know its genetic diversity status is unknown. Fruits are usually collected from spontaneous field-growing plants of unknown genotypes which are then forgotten until the next year. The evaluation of germplasm is necessary since the success of plant breeding and conservation depends on the breath of the available genetic diversity. In strawberry tree the variation of different morphological characteristics is substantial according to a study made in one specific region of the country – Algarve (Cardoso, 2004). Nevertheless, genetic variation inferred using the classical quantitative analysis of phenotypic traits is problematic due to the environmental influence, the polygenic character of some traits, and the time and costs to retrieve the information. Therefore, to circumvent those problems DNA-based markers have rapidly overtaken these classical strategies to obtain the requested genetic variability and fingerprinting information (Parker *et al.*, 1998; Joshi *et al.*, 1999; Bell *et al.*, 2008; Chawla, 2009). According to White *et al.* (2007) three PCR-based molecular marker types have been widely used to analyse genetic diversity in forest trees: 1) Random amplified polymorphic DNA (RAPD); 2) Amplified fragment length polymorphisms (AFLPs) and 3) Simple sequence repeats (SSRs).

Random amplified polymorphic DNA (RAPD) markers are based on the use of short primers of arbitrary sequence to generate PCR amplification products, in low stringency conditions, from genomic DNA (Williams *et al.*, 1990). This technique does not require sequence information or laborious cloning, thus the method's speed, sensitivity and versatility make it suitable for a rapid survey of polymorphisms. The advantage of this technique is the simplicity of the system and the relatively low cost (Rafalski, 1991;

Namkoong and Koshy, 2001; Chawla, 2009) features that make them particularly useful to evaluate heterozygosity in species for which the studies of genetic variability are scarce or absent, as in *A. unedo* (Namkoong and Koshy, 2001). However, RAPDs have some limitations such as their inheritance as dominant markers, and the extreme sensitivity to small modifications in PCR protocols making difficult to compare and reproduce results. In spite of these limitations, RAPDs have been used to evaluate genetic diversity in several woody species such as *Argania spinosa* (Majourhat *et al.*, 2008), *Castanea sativa* (Seabra *et al.*, 2001), *Juglans regia* (Nicese *et al.*, 1998), *Morus* sp. (Bhattacharya *et al.*, 2005), *Olea europaea* (Besnard *et al.*, 2001; Figueiredo, 2007), *Prunus* spp. (Martins *et al.*, 2001; Quarta *et al.*, 2001; Ryan *et al.*, 2001) and *Vitis* spp. (Regner *et al.*, 2001) as well as species of the Ericaceae family such as *Calluna vulgaris* (Borchert *et al.*, 2008), *Leucopogon* sp. (Zawko *et al.*, 2001), *Menziesia* spp. (Maki *et al.*, 2002), *Rhododendron* spp. (Jain *et al.*, 2000; Milne and Abbott, 2008) and *Vaccinium* spp. (Levi and Rowland, 1997; Burgher *et al.*, 1998; Vander Kloet and Paterson, 2000; Albert *et al.*, 2003, 2004; Burgher-Maclellan and Mackenzie, 2004; Garkava-Gustavsson, 2004; Albert *et al.*, 2005; Debnath, 2007).

Microsatellites (SSR) are currently widespread markers for fingerprinting, inbreeding and genetic structure studies, among others, because of their high polymorphism, co-dominance, multiallelism and automation analysis (Goldstein and Schlötterer, 1999; Eriksson *et al.*, 2006; White *et al.*, 2007). Moreover, SSRs are quite polymorphic, which makes them useful for identification of single individuals (Eriksson *et al.*, 2006). Their identification is a very expensive and time-consuming process, which generally requires the construction and screening of a genomic library. One strategy to increase the efficiency of the identification of microsatellite regions is to transfer SSR markers across closely related species (Whitton *et al.*, 1997). Those markers have been successfully used to screen polymorphism in members of the Ericaceae family such as *Calluna vulgaris* (Borchert *et al.*, 2008), *Erica coccinea* (Segarra-Moragues *et al.*, 2009), *Monotropa hypopitys* (Klooster *et al.*, 2008), *Phyllodoce* sp. (Kameyama *et al.*, 2006), *Rhododendron* spp. (Kondo *et al.*, 2009; Tan *et al.*, 2009; Wang *et al.*, 2009; Wang *et al.*, 2010) and *Vaccinium* spp. (Levi and Rowland, 1997; Boches *et al.*, 2005; Bassil *et al.*, 2006; Bassil *et al.*, 2010; Debnath, 2010; Hirai *et al.*, 2010).

The objective of this work was to test 20 RAPD primers and to cross-amplify 11 microsatellites from 12 *Vaccinium* species in *A. unedo* genotypes, in order to develop a set of markers to suitably fingerprint and uncover genetic diversity in this specie, for breeding and conservation purposes.

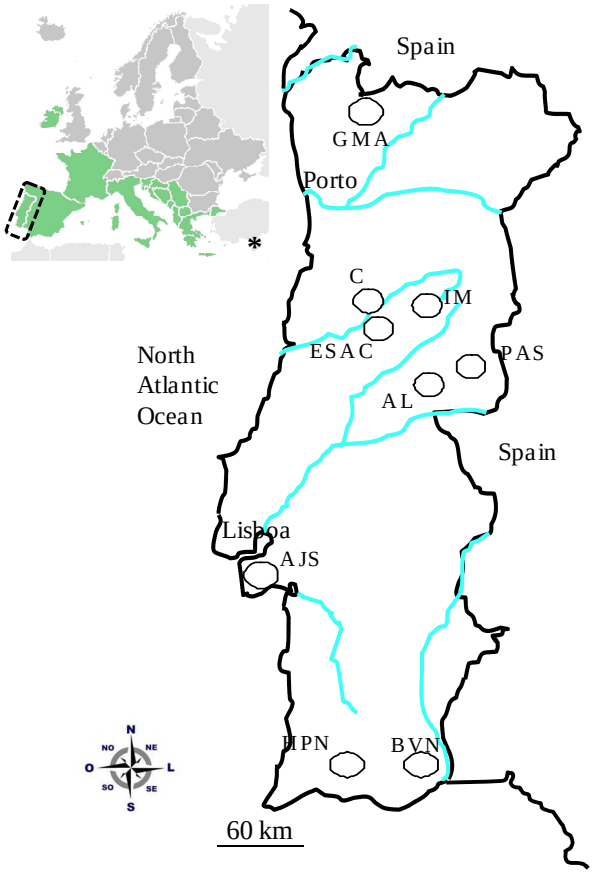
5.3 MATERIAL AND METHODS

5.3.1 Plant material and DNA extraction

We have defined nine *A. unedo* regions in Portugal and 3 trees were sampled from each region (Fig. 1). In two regions (AL and IM, Fig. 1) the plant material was collected from trees selected based on fruit production and quality, in the others the trees were sampled in natural stands were such information was not available.

The twenty-seven trees were sampled as follows (Fig. 1): 3 in the north, growing on granitic soils (Gerês Mata de Albergaria - GMA); 6 in the centre, near Coimbra (C; ESAC growing on sandstone and calcareous derived soils); 6 accessions were from selected trees growing on schist-derived soils (Serra do Açor and Serra de Alvélos - IM and AL, respectively); 3 in the east, growing on schist-derived soils (Serra da Gardunha - PAS); 3 in the west, growing on calcareous soils (Serra da Arrábida - AJS) and 6 in the south (3 from SE and 3 from SW, respectively from Barranco Velho and Herdade da Parra – BVN and HPN), growing also on schist-derived soils. The trees from Serra da Gardunha are routinely used as a source of seeds for plant propagation for a nursery (code PAS; Fig. 1). In the south region where *A. unedo* is widely distributed and with the longest tradition of commercial use, six wild-growing trees were sampled from 2 provenances, from Barlavento and Sotavento, western and eastern Algarve, respectively.

Young leaves were harvested from adult trees and brought to the laboratory, frozen in liquid nitrogen and stored at -80°C. DNA was extracted using the DNeasy Plant Mini kit (Quiagen).



Provenances	Code	Location
GMA	Au 7	Gerês, Mata de Albergaria
	Au 10	
	Au 22	
C	Au 3	Coimbra
	Au 11	
	Au 15	
ESAC	Au 21	Coimbra
	Au 23	
	Au 27	
IM	Au 13	Serra do Açor
	Au 16	
	Au 24	
PAS	Au 6	Serra da Gardunha
	Au 9	
	Au 19	
AL	Au 2	Serra Alvélos
	Au 5	
	Au 17	
AJS	Au 1	Serra da Arrábida
	Au 20	
	Au 25	
HPN	Au 4	Algarve, São Marcos da Serra
	Au 8	
	Au 28	
BVN	Au 12	Algarve, Serra do Caldeirão
	Au 18	
	Au 26	

Figure 1 – The map indicates the provenances of the sampled material from different regions of Portugal whereas the chart gives an indication of the particular locations where the plant material was collected and its respective code (*Distribution in Europe of *A. unedo*, Bot. Sist. http://luirig.altervista.org/gifeurbig2/2724.gif).

5.3.2 Random amplified polymorphic DNA (RAPD)

Twenty arbitrary primers, decamer oligonucleotides from Operon Technologies Kit C (OPC), were tested (Table 1). All PCR reactions were prepared as master mixes for each primer to minimize errors. PCR were performed in a 13.5 µl volume containing: 2.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 2 µM of primer, 0.5 U of Taq DNA polymerase in 1 X reaction buffer and 50 ng of genomic DNA. The initial denaturation step (5 min, 94ºC) was followed by 35 cycles of 30 s at 94 ºC (denaturation), 45 s at 35ºC (annealing) and an extension step of 90 s at 72 ºC. At the end of the cycles, a final extension step at 72ºC for 10 min was performed, to guarantee that all annealed templates were entirely polymerized. The PCR reaction products were separated by electrophoresis in agarose gel (1.5%) with Sybr safe DNA (1.5µl/100 ml). The amplified products from RAPDs primers were electrophoresed and bands were visualized under UV light with the help of fragment size standard.

Table 1 - Sequences of the arbitrary primers from Operon Technologies Kit C (OPC) used in this experiment.

Primer	Sequence 5'-3'	Primer	Sequence 5'-3'	Primer	Sequence 5'-3'	Primer	Sequence 5'-3'
OPC-1	TTCGAGCCAG	OPC-6	GAACGGACTC	OPC-11	AAAGCTGCGG	OPC-16	CACACTCCAG
OPC-2	GTGAGGCGTC	OPC-7	GTCCCGACGA	OPC-12	TGTCATCCCC	OPC-17	TTCCCCCCAG
OPC-3	GGGGGTCTTT	OPC-8	TGGACCGGTG	OPC-13	AAGCCTCGTC	OPC-18	TGAGTGGGTG
OPC-4	CCGCATCTAC	OPC-9	CTCACCGTCC	OPC-14	TGCGTGCTTG	OPC-19	GTTGCCAGCC
OPC-5	GATGACCGCC	OPC-10	TGTCTGGGTG	OPC-15	GACGGATCAG	OPC-20	ACTTCGCCAC

5.3.3 Simple sequence repeat genotyping

Eleven SSRs were selected according to previous studies performed in the genus *Vaccinium* of the same family than *A. unedo*, by Boches *et al*. (2005) and Bassil *et al*. (2006). The selection was based on a series of characteristics found on that studies, namely the amplification and polymorphism detected within 12 *Vaccinium* species, the number of alleles per *locus* of 30 SSR *loci* evaluated in 11 *Vaccinium* cultivars, the allele scoring quality and the repeat motif. These characteristics are summarized on table 2.

Table 2 – Characterization of the eleven primers (M_1 to M_{11}) used in *A. unedo*. This selection was based on the works of Boches (2005), Boches *et al*. (2005) and Bassil *et al.* (2006).

Primers	Repeat motif	Primer Sequence (5' to 3')	*Amplification /Polymorphism	Allele Nb°
M_1 - CA169F	$(GAT)_4$	F- Tag Tgg Agg gTT TTg CTT gg R- gTT TAT CgA AgC gAA ggT CAA AgA	10 / 6	5
M_2 – CA421F	$(CT)_{25}$	F– TCA AAT TCA AAg CTC AAA ATC AA R- gTT TAA ggA TgA TCC cGA AgC TCT	11 / 10	14
M_3 – CA855F	$(GA)_{14}(CGA)_5$	F– CgC gTg AAA AAC gAC CTA AT R- gTT TAC TCg ATC CCT CCA CCT g	12 / 6	10
M_4 – NA800	$(TC)_{13}$	F – CAA TCC ATT CCA AgC ATg Tg R- gTT TCC CTA gAC cAg TgC CAC TTA	12 / 12	31
M_5 – NA398	$(AAAT)_5$	F - TCC TTg CTC CAg TCC TAT Gc R- gTT TCC TTC CAC TCC AAg ATg C	10 / 7	5
M_6 – VCC_K4	$(TC)_{16}(TC)_{12}$	F – CCT CCA CCC CAC TTT CAT TA R- gCA CAC Agg TCC AgT TTT Tg	9 / 8	14
M_7 – CA794F	$(GA)_{12}$	F – Cgg TTg TCC CAC TTC ATC TT R- gTT TgA ATT Tgg CTT Cgg ATTC	12 /10	10
M_8 – NA961	$(TAC)_5$	F – TCA gAC ATg ATT ggg gAg gT R- gTT Tgg AAT AAT AgA ggC ggT ggA	10 / 3	6
M_9 – NA1040	$(TC)_{11}$	F- gCA ACT CCC AgA CTT TCT CC R- gTT TAg TCA gCA ggg TgC ACA A	10 / 8	15
M_{10} – NA741	$(TC)_9$	F- gCC gTC gCC TAg TTg TTg R-gTT TgA TTT Tgg ggg TTA AgT TTg C	10 / 7	14
M_{11} – VCC_I2	$(CT)_{14}$	F- Agg CgT TTT TgA ggC TAA CA R- TAA AAg TTC ggC TCg TTT gC	10 / 8	10

*Amplification and polymorphism within 12 *Vaccinium* species and the number of alleles per *locus* of 30 SSR *loci* evaluated in 11 *Vaccinium* cultivars.

All PCR reactions were prepared as master mixes for each primer to minimize errors. PCR were performed in a 13.5 µl volume and similarly to the previously described RADP markers. PCR reaction conditions previously described by Boches *et al*. (2005) were optimized for *A. unedo* to improve allele scoring quality. The optimum annealing temperature (TaºC) for a primer pair was determined by PCR gradient from 55ºC to 65ºC. After the initial denaturation step at 94ºC for 4 min, DNA was amplified for 35 to 40 cycles in a thermocycler programmed for 45s denaturation step at 94ºC, a 45s annealing step at the optimum annealing temperature of the primer pair, a 45s extension step at 72ºC and a final extension step at 72ºC for 30 min in a total of 6 steps. Since the primers CA169F and NA741 showed multiple peaks a touch-down for these primers was tested in PCR reactions with 9 steps instead of 6. Two TaºC for 30 s were performed (62ºC and 57ºC for 10 and 30 cycles, respectively). The PCR reactions were repeated at least four times in order to confirm the reproducibility of the results. The conditions used for each primer are indicated in table 3.

Table 3 – Conditions of the PCR reactions used for the different SSRs primers in *Vaccinium* and *A. unedo*.

Primers	T_a (ºC) [1, 2]	Allele size range[1] (bp)	Allele size range[2] (bp)	Allele scoring[1, 2]	PCR *A. unedo*
M_1 - CA169F	62	109-130	109-136	G	To 62-57
M_2 – CA421F	60	180-250	153-230	S, P	35 cycles
M_3 – CA855F	64	250-300	225-258	G	40 cycles
M_4 – NA800	60	230-290	180-287	S, M	40 cycles
M_5 – NA398	56	210-240	211-232	G	40 cycles
M_6 – VCC_K4	62	150-300	169-300	S	40 cycles
M_7 – CA794F	60	220-290	141-226	S	40 cycles
M_8 – NA961	60	205-220	176-201	G	35 cycles
M_9 – NA1040	60	180-270	173-263	S	40 cycles
M_{10} – NA741	58	240-290	247-300	S	To 62-57
M_{11} – VCC_I2	62	200-275	205-245	S, P	40 cycles

[1]Boches *et al.* (2005) and [2]Bassil *et al.* (2006) for *Vaccinium* species. T_a(ºC) - Optimum annealing temperature. Allele scoring quality (G- good; S- stutter; P- split peaks; M-multiple *loci*). To - Touch-down PCR reactions. See text for more details.

The success of the PCR reaction was verified in agarose gel and observed under UV light as indicated in the previous section for the RAPD reaction.

For allele scoring and sizing, fluorescently labelled forward primers (FAM, HEX, or NED) and unlabelled reverse primers were used. The PCR reaction's products were diluted with HI-DI formamide for denaturation, and ROX marker was added. The amplified products were denatured and visualized in an automatic sequencer (ABI 310 Applied Biosystems) and the results were scored using Genescan software (Applied Biosystems).

5.3.4 Diversity estimates

5.3.4.1 Random amplified polymorphic DNA (RAPD)

The 20 RAPD (OPC) fragments obtained for all the 27 genotypes were scored in the form of a binary matrix where 1 represented presence and 0 absence of a band. This matrix was used to evaluate pair-wise genetic similarity, calculated with the Lynch similarity coefficient (Lynch, 1990) and an unweighted pair group with arithmetic average method (UPGMA)-based dendrogram was built using the NTSYS-PC software package (Rohlf, 1997), version 2.02i (http://www.exetersoftware.com/). The reliability of the generated dendrogram was tested through a Mantel test by bootstrap analysis with 1000 permutations. The Mantel test statistic was computed to measure the degree of relationship between geographical and genetic distance using the MXCOMP module of the NTSYS-PC software package, version 2.02i.

The genetic diversity analysis was carried out using the AFLP-SURV, version 1.0, software. The diversity parameters comprised the number of polymorphic *loci* and the expected heterozygosity (He) also called Nei's gene diversity (Nei, 1987). The geographical distance matrix between every two individuals was compared with the genetic similarity distance matrix known as the Lynch coefficient (Lynch, 1990).

5.3.4.2 Microsatellites (SSRs)

The diversity parameters and the polymorphic information content (PIC) (Botstein *et al.*, 1980) were computed in the *A. unedo* genotypes per microsatellite polymorphic *loci* using the Cervus 3.0 software (Marshall *et al.*, 1998). The diversity parameters comprised the number of alleles (Na), the observed heterozygosity (Ho), the expected heterozygosity (He) (Nei, 1987), and the fixation index (Fis) (Weir and Cockerham, 1984). The magnitude of inbreeding can be measured by comparing the actual proportion of heterozygous genotypes in a population (Ho) with the proportion that would be produced by random mating in an idealized Hardy-Weinberg population (expected heterozygosity, He). This measure, called the inbreeding coefficient**,** is symbolized by Fis, and defined as Fis=1-(Ho/He). Therefore, Fis measures the reduction in heterozygosity relative to a random mating population with the same allele frequencies. The polymorphism information content value is commonly used in genetics as a measure of polymorphism for a marker *locus*. The PIC values mean *loci*

uninformative if < 0.30, moderately informative if 0.3–0.59 or highly informative if ≥ 0.60 (Mateescu *et al.*, 2005).

Hardy-Weinberg equilibrium (HWE) was tested for each *locus* (Markov-Chain method). The Null allele frequencies (F *Null*) per *loci* were estimated by using a maximum likelihood EM algorithm. Linkage disequilibria (LD) tests were performed for all *loci* combinations. The HWE test, F *Null* and LD were computed with the *Genepop* software. Genetic similarity was accessed using (the same coefficient described above for RAPDs) the Lynch coefficient (Lynch, 1990), computed with the NTSYS-PC software. This coefficient is based on bands or alleles sharing and measures the expected DNA similarity of two unrelated individuals, such as similarity due to chance, by state, not by any class of relatives (Lynch, 1990; Li *et al.*, 1993). Therefore, it is well adapted to our sampling and to the current genetic diversity study based on RAPDs and SSRs markers. A UPGMA-based dendrogram was constructed in which reliability was tested through a Mantel test, as described above. The Mantel test was also used to measure the degree of relationship between geographical and genetic distances and to test the relationships between the two molecular markers matrices.

5.4 RESULTS

5.4.1 Random amplified polymorphic DNA (RAPD)

From 20 RAPDs (OPC) tested 19 showed amplification products and 16 showed polymorphic profiles. As an example, Fig. 2 illustrates the amplification products of the primer OPC-9.

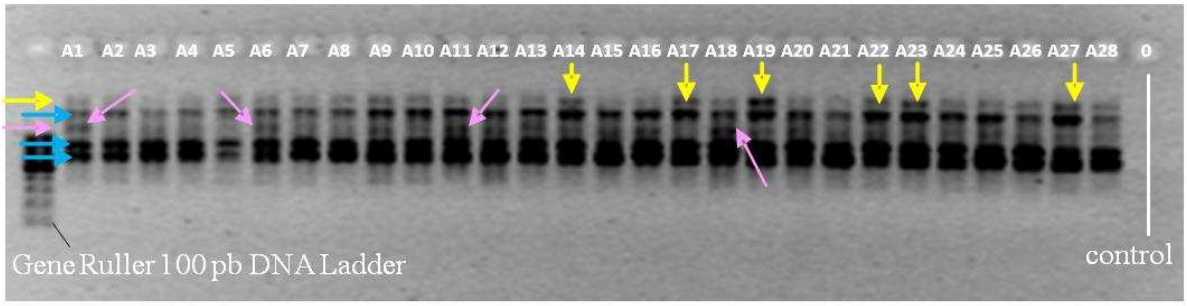


Figure 2 – The agarose gel of the amplified products from OPC-9 RAPD marker after electrophoresis and visualized under UV light. Polymorphic bands are indicated by yellow and rose arrows whereas monomorphic bands are signaled by blue arrows. *A. unedo* genotypes are referred from A1 to A28. A14 and A23 are samples of the same genotype and 0 is the control without DNA).

The group of 19 RAPDs that showed amplification products generated a total of 124 bands, 57.3% of which were polymorphic (71 of the *loci*) as indicated in table 4.

Table 4 – Resume of the results obtained with the 20 OPC primers used in RAPD analysis of *A. unedo*.

Primer OPC	Number of bands	Range size (bp)	Polymorphic bands number (%)
OPC 1	7	500 - 2000	4 (57.1 %)
OPC 2	6	200 - 1200	2 (33.3 %)
OPC 3	8	400 - 1500	5 (62.5 %)
OPC 4	9	400 - 2000	5 (55.6 %)
OPC 5	pe300 -	300 - 1500	3 (50.0 %)
OPC 6	9	300 - 2100	6 (66.7 %)
OPC 7	4	400 - 1450	2 (50.0 %)
OPC 8	4	400 -1200	0 (0.0 %)
OPC 9	5	650 - 1700	2 (40.0 %)
OPC 10	10	300 - 1500	8 (80.0 %)
OPC 11	13	220 - 1600	12 (92.3 %)
OPC 12	8	400 - 2000	6 (75.0 %)
OPC 13	9	500 - 1700	4 (44.4 %)
OPC 14	5	650 - 2100	1 (20.0 %)
OPC 15	0	-	-
OPC 16	7	400 - 1900	5 (71.4 %)
OPC 17	1	1600	0 (0 %)
OPC 18	5	300 - 1800	3 (60.0 %)
OPC 19	7	540 - 1900	3 (42.9 %)
OPC 20	1	400	0 (0 %)
total	124	200 - 2100	71 (57.3 %)

In figure 3 the UPGMA dendrogram built up with genetic similarity pair-wise values calculated with the Lynch similarity coefficient (Lynch, 1990) is displayed. The cluster analysis revealed a similarity of 83% among genotypes. Additionally, some genotypes shared as much as 95% of the bands, however without a consistent geographic pattern as can be seen with the genotypes Au_{12} and Au_{27} (from BVN and ESAC provenances; see Fig. 1 for further details).

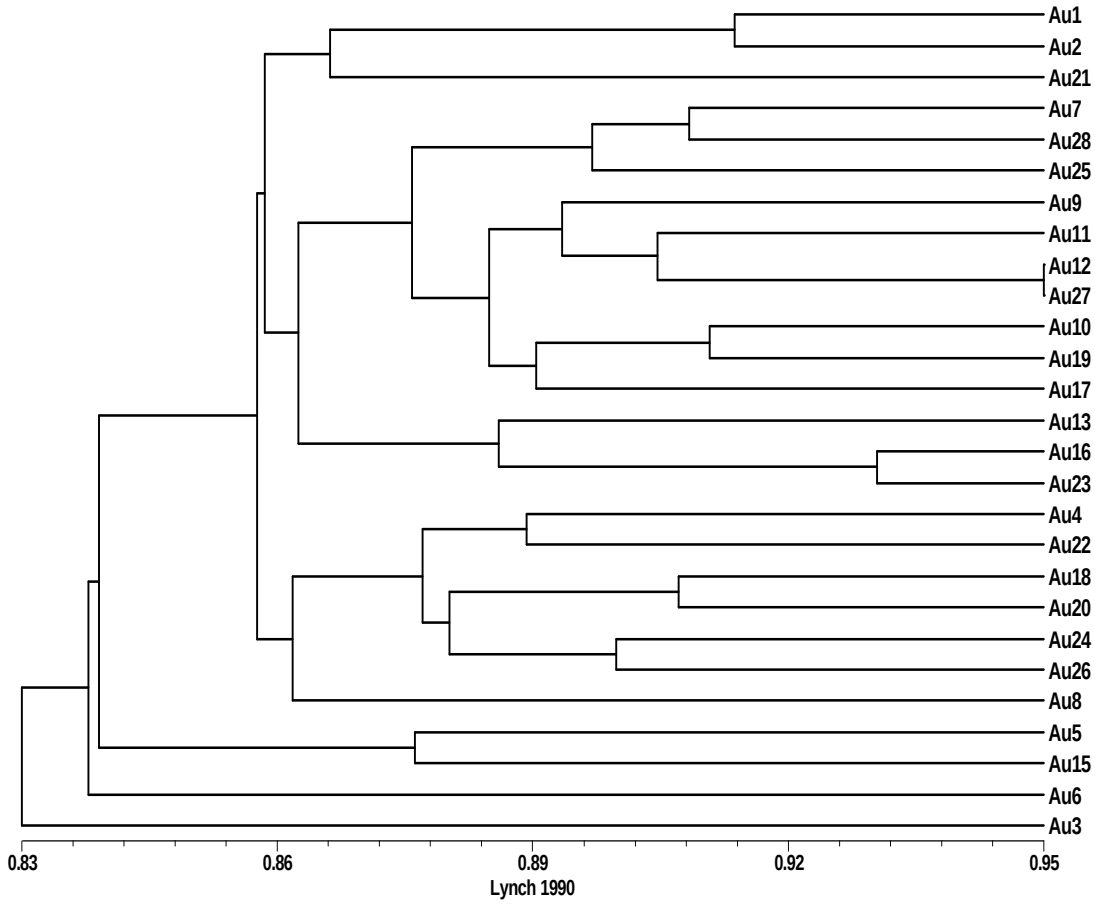


Figure 3- Genetic similarity analysis of 27 *A. unedo* genotypes using the Lynch coefficient (1990) based on 19 of the 20 primers that showed amplified PCR products.

The Mantel test confirmed the UPGMA tree topology, and a moderate correlation yet significant was obtained (matrix correlation: r=0.64; P<0.001). The expected heterozygosity (He) was 0.27±0.014 (SE). No genotypes could be grouped according to their geographical origin. The Mantel test confirmed that there was no correlation between genetic and geographical distances matrices (matrix correlation: r= 0.01; P<0.57).

5.4.2 Microsatellites (SSRs)

The results of SSRs analysis using eleven primers in *A. unedo* are shown on table 5. The primers pairs are characterized based on the repeat motif, the optimum annealing temperature, the existence of amplification products and polymorphism, and the allele size range. Nine out of the eleven microsatellites primers tested produced amplified products in all the screened genotypes. The primers M3/CA855F and M6/VCC_K4 lacked to cross amplify in *A. unedo*, and they had both repeat motif type such as $(GA)_{14}(CGA)_5$ and $(TC)_{16}(TC)_{12}$, respectively, unlike the other primers (Table 5). Six primers had di-nucleotide, two had trinucleotide and one had a tetranucleotide repeats motif. Five SSR *loci* were polymorphic (Table 5) all of them composed of di-nucleotide repeats motif (CT, GA and TC).

Table 5 – The primer pair's characterization (M_1 to M_{11}) with reference to the *A. unedo* optimum annealing temperature (Ta°C), the existence of amplification products and polymorphism (+) and the allele size range (bp).

Primer pair	Repeat motif	T a (°C)	Labeled Forward primers	Amplification products	Polymorphism	Allele size range (bp)
M_1 – CA169F	$(GAT)_4$	62-57	6 FAM - blue	+	-	103-114
M_2 – CA421F	$(CT)_{25}$	60	NED - yellow	+	+	150-211
M_3 – CA855F	$(GA)_{14}(CGA)_5$	X	HEX - green	null alleles	null alleles	---
M_4 – NA800	$(TC)_{13}$	58	NED - yellow	+	+	169-278
M_5 – NA398	$(AAAT)_5$	55	6 FAM - blue	+	-	210
M_6 – VCC_K4	$(TC)_{16}(TC)_{12}$	X	6 FAM - blue	null alleles	null alleles	---
M_7 – CA794F	$(GA)_{12}$	60	HEX - green	+	+	283-294
M_8 – NA961	$(TAC)_5$	60	HEX - green	+	-	186-208
M_9 – NA1040	$(TC)_{11}$	60	6 FAM - blue	+	-	171-239
M_{10} – NA741	$(TC)_9$	62-57	NED - yellow	+	+	165-172
M_{11} – VCC_I2	$(CT)_{14}$	60	HEX - green	+	+	174-274

Partial amplified products, referring to few individuals, from four SSR *loci* (M1, M2, M5 and M8), after visualized and scored in an automatic sequencer and the Genescan software, are displayed in Fig. 4. The *loci* M1, M5 and M8 are monomorphic and the *locus* M2 is polymorphic. They were labeled with the following forward primers: FAM: M_1- CA169F and M_5- NA398; HEX: M_8- NA961; NED: M_2 - CA421F.

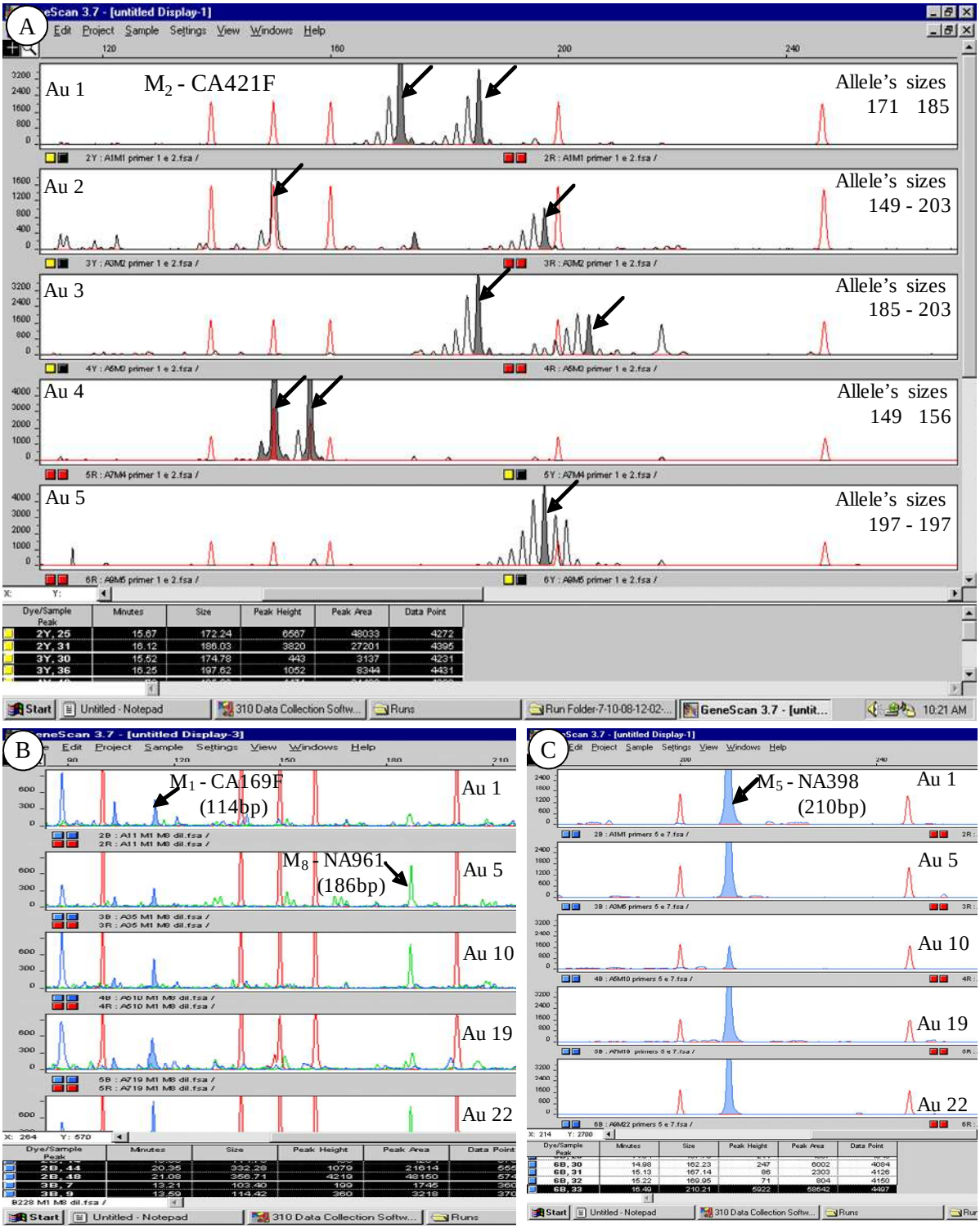


Figure 4 - The amplified products from SSRs: A - Polymorphic primer M_2-CA421. B and C - Monomorphic primers (M_1- CA169F; M_5- NA398 and M_8- NA961) visualized in an automatic sequencer (ABI 310 Applied Biosystems) and scored using Genescan software (Applied Biosystems). The genotype code and the allele's size (bp) are noted. Red peak refers to the ROX marker.

The five polymorphic *loci* showed 75% of mean expected heterozygosity and 70% of mean observed heterozygosity. The mean number of alleles (Na) was 11.6, ranging from 6 to 22 per *locus* (Table 6). The Ho was inferior to He for all *loci* indicating a putative heterozygote

deficit. Indeed a mean homozygote excess was found (7%), albeit not significant (Table 6). The results showed that 4 SSR *loci* were in HWE equilibrium (after Bonferroni correction). Additionally, the estimation of null allele frequency (F *Null*) was about 8% on average (Table 6) what could explain, at least partially, the heterozygote deficiency observed.

Table 6 - Diversity parameters obtained for the 5 SSR polymorphic *loci* found in *A. unedo*. Na refers to the number of alleles per *locus*, He to the expected heterozygosity, Ho is the observed heterozygosity, Fis represents the fixation index, and PIC the polymorphic information content.

Locus	Na	He	Ho	Fis	P-value	Sig.	F (*Null*)	PIC
M2-CA421F	22	0.90	0.85	0.05	0.2018	NS	0.0546	0.88
M4-NA800	9	0.79	0.78	0.02	0.0146	NS	0.0534	0.75
M7-CA794F	7	0.78	0.69	0.11	0.0159	NS	0.0985	0.73
M10-NA741	6	0.57	0.56	0.03	0.004	NS	0.0868	0.51
M11-VCC_I2	14	0.73	0.62	0.16	0.0007	*	0.0887	0.70
Mean	11.6	0.75	0.70	0.07	0.0474		0.0764	0.71

Sig. refers to the significance resulting from the HWE test (after Bonferroni correction) NS - not significant and *- significant. *Null* refers to null allele frequency estimates.

The mean polymorphism information content (PIC) was 0.71. Four *loci* showed PIC values higher than 0.60 which indicates their usefulness as highly putative indicators of diversity (Table 6). The linkage *disequilibrium* test showed that all the *loci* are independent. The *locus* M2- CA421F displayed values higher than the average: 22 alleles, He=0.90 and a high PIC value (88%). By contrast, *locus* M10-NA741 showed the lowest PIC value (51%), number of alleles (6), and expected diversity (0.57).

Pair-wise genetic similarity analysis using the Lynch coefficient showed that the level of alleles sharing between genotypes ranged from 21% to 82% (Fig. 5). The Mantel test confirmed the tree topology ($r=0.75$; $P<0.001$). According to the Lynch coefficient, the genotypes Au_4 and Au_{25} (from HPN and AJS provenances), and Au_5 and Au_{12} (from AL and BVN provenances) shared as much as 82% alleles. However, these genotypes did not show a consistent geographic pattern clustering (see Fig. 1 for further details).

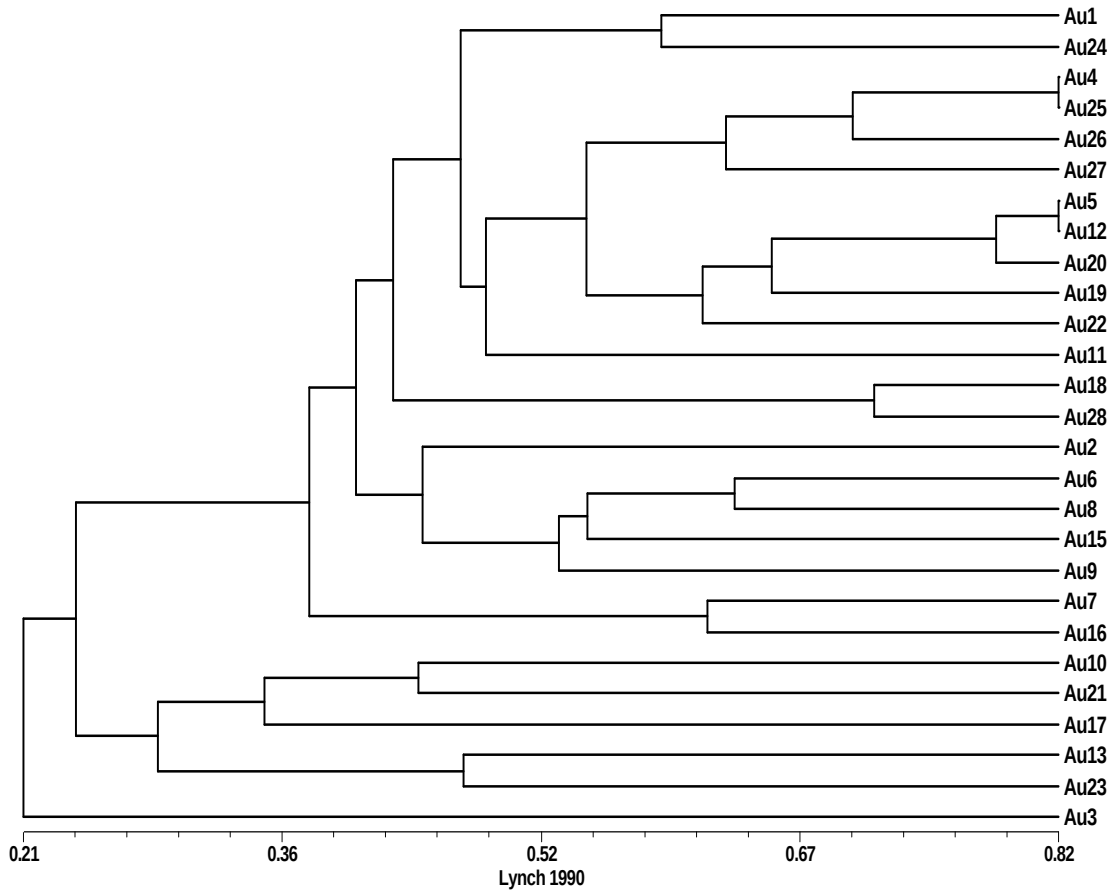


Figure 5- Genetic similarity analysis of 27 *A. unedo* genotypes using the Lynch (1990) coefficient based on 5 SSRs polymorphic *loci*.

No genotypes could be grouped according to their geographical origin, which was confirmed by the Mantel test (matrix correlation: r=0.09; P<0.17). The lack of consistence between genetic and geographic distances is consistent with the RAPDs results. Mantel test also confirmed the absence of correlation between pair-wise molecular markers (RAPDs and SSRs) matrices (matrix correlation: r=0.05; P<0.69).

5.5 DISCUSSION

RAPDs have been used to analyse genetic diversity in several species. Different studies have reported the use of RAPDs for single genotypes identification (Eriksson *et al.*, 2006), cross species amplification, mainly within species (Glaubitz and Moran, 2000) and for linkage mapping (Cervera *et al.*, 2000). For the first application referred, we used a set of 20 RAPDs markers to the molecular characterization of 27 *A. unedo* genotypes. The same approach has been applied to different species by several authors. Majourhat *et al* (2008) used 19 RAPDs markers (OP) for the characterization of 38 *Argania spinosa* tree accessions (from 3 fruit types). Likewise, Albert *et al*. (2003) identified 32 *Vaccinium myrtillus* clones using RAPDs and AFLP markers and Kapteyn and Simon (2002) used 22 RAPDs for the characterization of 19 accessions of *Echinacea* (Asteraceae). In our experiment the OPC primers generated a total of 124 bands, ranging from 200 to 2100 bp. Nineteen RAPDs markers tested out of 20, generated 42.7% of monomorphic bands, polymorphism on about 71 of the *loci* (57.3%) and the average for the expected heterozygosity (He) was about 0.27±0.014. Similar results are referred by Agrama and Tuinstra (2003) when carrying out the analysis in 22 sorghum genotypes using 32 RAPDs, they found 40% of monomorphic bands. While, Majourhat *et al* (2008) working in *Argania spinosa* tree found an higher level of genetic diversity, in a total of 146 RAPD bands, 140 (95.8%) were polymorphic. An higher level of genetic diversity was also found by Debnath (2007) working in 48 *Vaccinium macrocarpon* genotypes, 14 primers generated 161 polymorphic RAPD bands. On the contrary, Schneller *et al*. (1998) on their studies in *Dryopteris remota* found a reduced level of polymorphism, in 22 genotypes using 19 RAPDs. From the 19 primers tested only 12 generated amplification PCR products and from these only 4 showed polymorphism. A total of 67 bands were scored, being only 12 polymorphic bands (18%), which revealed a low genetic diversity among *D. remota* genotypes from different locations (18%) compared to our experiment (57.3%). Figueiredo (2007) studied the genetic variation in79 *Olea europea* genotypes using 20 RAPDs (OP) and reported that in a total of 114 bands generated only 46 of them showed polymorphism (31.9%) and the average of the expected heterozygosity (He) was about 0.10±0.012, lower than we observed in our experiment (0.27±0.014). According to the author this low polymorphism was probably due to the samples' origin, as they were closely located. Another study refers that with a set of 5 RAPDs (OP) markers (in a total of 16 tested) 61 bands were generated, being 24 polymorphic (39%), in 66 *Prunus* species

(Quarta *et al.*, 2001). Even though, the polymorphism level (39%) was lower than we obtained in our experiment (57.3%), they report that it was possible to discriminate about 39 *Prunus* genotypes. Other studies indicated that RAPDs markers were suitable to characterize genotypes. Seabra *et al.* (2001) using 8 RAPDs (OP) showed that the *Castanea sativa* genotypes of fruit orchards (40 trees) were less differenciated than genotypes (41 trees) collected in high forest and coppice stands. Kapteyn and Simon (2002) using 22 RAPDs for the characterization of 19 accessions of *Echinacea,* were able to obtain a total of 17 RAPD markers which distinguish among commercially *Echinacea* species. Additionally, their results allowed identifying the presence of specific adulterants in botanical samples used for medical purposes. Martins *et al.* (2001) using RAPDs to characterize the genetic variability of 40 *Prunus amygdalus* cultivars, found out high levels of polymorphism which revealed the inter-varietal differences.

The results from the current study point out an important polymorphism level found among *A. unedo* genotypes with the RAPDS primers tested (57.3% polymorphic *loci* and 27% of He). Additionally, the cluster analysis revealed a similarity (Lynch coefficient) of 83% among individuals, which tree topology was supported by a moderate correlation yet significant ($r=0.64$; $P<0.001$). Some genotypes shared as much as 95% of bands. However, the genotypes were not grouped according to their matrix distance. A geographical consistent pattern was not found as confirmed by the Mantel test ($r=0.014$; $P<0.57$). Similar results were achieved by Agrama and Tuinstra (2003) when screening 22 sorghum genotypes using 32 RAPDs. These authors reported a value of 0.61 of genetic similarity between accessions, and some genotypes shared even 95% of the bands. The correlation of pair-wise genetic distances with geographical and race data was also low ($r=0.43$). Similarly, the genetic distances between pairs of 32 *Vaccinium myrtillus* clones were not related to the spatial distances between them (Albert *et al.*, 2003). Likewise, Schneller *et al.* (1998) also found no geographical pattern of genetic variation in a UPGMA analysis when analysing the genetic variation in 22 individuals of *Dryopteris remota* from different locations using RAPDs. On the other hand, other studies have reported a good correlation between genetic and geographical matrices distances. For instance, Burgher *et al.* (1998) screened 26 wild *Vaccinium angustifolium* clones using RAPDs. According to this study clustering of genotypes correlated fairly well with the geographic origin of the clones. Similarly, Besnard *et al.* (2001) could distinguish between 102 *Olea europaea* accessions (of 113 genotypes, 90%) from different collections and orchards around the Mediterranean Basin with 3 RAPDs primers. Many of the RAPD profiles were consistent with the geographical distribution

pattern of the species since they were correlated with the country or region of origin of the accessions. The genetic diversity studies by RAPD analysis in 48 *V. macrocarpon* genotypes reported by Debnath (2007) referred that UPGMA analysis separated the wild clones and three cultivars into five main clusters. Furthermore, the geographical distribution explained 10% of total variation as revealed by analysis of molecular variance. Likewise, Escaravage *et al*. (1998) found that the genetic distance obtained between clone pairs of *Rhododendron ferrugineum* was related to the geographical distance.

Lopes *et al.* (2010) also used RAPDs to characterize 38 *A. unedo* genotypes from two provenances in NE and centre east of Portugal. Among a total of 20 RAPDs, only 7 produced a polymorphic profile. In our study a higher degree of polymorphism was found since from the 20 RAPDs tested, 19 showed amplification products and 16 showed polymorphic profiles (57.3% polymorphic bands). These differences may be explained by the largest area of distribution of the plant material used in our experiments. A much lower diversity was observed in *A. unedo* populations in Tunisia (He = 0.216), in this case due to deforestation followed by species' fragmentation and consequent bottleneck and genetic drift (Takrouni and Boussaid, 2010), with the lowest diversity levels found in the populations with the highest habitat destruction.

For single genotype characterization more than a molecular marker must be used to strongly support the data obtained. SSRs are particularly attractive for distinguishing among cultivars as they are co-dominant and the level of polymorphism detected by *loci* is higher than that detected with other molecular markers (Muchugi *et al.*, 2007). The molecular characterization of a species for the first time is a laborious task, especially if we want to identify SSRs in the genome. Cross-species amplification among species from the same genera is a relevant alternative. For instance, Chandra and Tiwari (2009) developed 15 polymorphic SSR *loci* in *Panicum maximum*, which amplified in 5 other species from the same genera and then after were used in interspecific breeding programme. Similarly cross amplification approaches have been reported in species from Ericaceae. Eleven polymorphic SSRs were developed and revealed polymorphism in *Monotropa hypopitys.* A subset of those primers amplified in the congener *Monotropa uniflora* and in 5 other closely related genera (Klooster *et al.*, 2008). Kameyama *et al.* (2006) refer that 1 of the 13 SSR primers developed for *Rhododendron metternichii* cross amplified *Phyllodoce aleutica* and *Phyllodoce caerulea* (also Ericaceae). However, according to Gupta and Varshney (2000), low amplifying capacity of genomic SSRs in related genera has been reported in many crops. Still, several authors report successful studies in Ericaceae, even in crop species, as it is *Vaccinium*. Bassil

et al. (2010) used SSR isolated from domestic *Vaccinium* in *V. calycinum, V. myrtillus* and *V. reticulatum* other endemic species, 18 SSRs cross-amplified and appeared polymorphic in most of the genotypes evaluated. In the case of *A. unedo* the RAPD analysis was complemented by a SSR study in which nine primers developed for *Vaccinium* were used (Boches *et al.*, 2005; Bassil *et al.*, 2006). From these primers, five showed to be highly polymorphic *loci* (PIC > 0.60), with a mean number of alleles (Na) of 11.6, ranging from 6 to 22 per *locus*. Boches *et al.* (2005) found a similar polymorphism level with 24 single *locus* SSRs in 12 *Vaccinium* accessions, with 8.16 average allele number, ranging from 2 to 15 per *locus*. This suggests that cross amplification is a useful approach to evaluate genetic diversity even when species from different genera (*Arbutus* and *Vaccinium*) were used. Otherwise, Tan *et al.* (2009) tested 11 SSRs for the characterization of 20 *Rhododendron simsii* (Ericaceae) genotypes. Of the 11 SSR markers, 8 displayed polymorphic products. The average allele number was 7.1 per *locus* (ranging from 6 to 9). In their experiments the expected heterozygosity varied from 0.28 to 0.94 (with an average of 0.75) whereas the observed heterozygosity ranged from 0.55 to 0.87 (with an average of 0.60) and consequently with higher values (ranging from -17% to 63%) of the fixation index (Fis). In the case of *A. unedo* we have found that the expected heterozygosity (He=0.75) was identical but within narrow limits (0.57 to 0.90). Besides, the observed heterozygosity (Ho=0.70) was higher reflecting an inferior homozygote excess measured by the fixation index (Fis=7%) although not significant. High Fis values reflect the differences between observed and expected heterozygosity due to its loss of heterozygosity as a result of non-random mating of parents. Cipriani *et al.* (2001) report high polymorphism level in fruit crops species using 26 SSRs, with the following number of alleles/*locus*: 9 to 17 in *Actinidia*; 5 to 15 in *Vitis* and with a lower polymorphism level, 2 to 8 in *Prunus persica*. Similarly result is referred by Kenis *et al.*(2001). The authors used 15 SSRs in 28 *Malus x domestica* cultivars. The total number of alleles/*locus* ranged from 4 to 13 (with an average of 8.7).

It must be emphasized that the strawberry trees used in our experiments were collected in different stands, producing an assembly unlikely to fit in the Hardy-Weinberg equilibrium (HWE) even though only one *locus* (VCC_I2) displayed deviation from what the Hardy-Weinberg equilibrium would anticipate. The presence of null alleles is another factor that may contribute to the deviation from HWE. Indeed, the null allele frequency estimates (F *Null*) was about 7.6%, on average. Fernandes *et al.* (2008) screened 60 genotypes with three SSRs in a *Pinus pinaster* clonal seed orchard, and found the following diversity parameters (mean values): 11 of Na; 0.71 and 0.79 for Ho and He respectively, and 9.1% for Fis. Almost

all genotypes (58) were identified with the 3 SSR markers. This latter study reported a similar high genetic diversity compared to our experiment since 5 polymorphic SSRs showed the following mean values: Na=11.6; Ho=0.70; He=0.75 and consequently an inferior value for Fis about 7%. Tan *et al.* (2009) using 8 SSRs for characterization of 20 *R. simsii* genotypes, report that 3 of 8 *loci* showed significant deviation from HWE after applying Bonferroni correction, as well as null alleles (F *Null*), which frequencies, for those 3 *loci*, ranged from 15% to 44%. These high frequencies of null alleles can contribute to the deviation from HWE and explain the high range values of the fixation index (Fis) even the negative (-17%, related to the higher Ho values than He). In our experiment the F *Null* estimate was inferior 7.6% (ranging from 5.3% to 9.9%), as well as Fis index (7%).

Our results indicated that the mean PIC showed that all analysed *loci* may be considered as informative (PIC≥ 0.30). Moreover, 4 SSR *loci* were found to be highly informative (PIC>0.60). Similarly results have been reported by several authors. In the study of Agrama and Tuinstra (2003) with 22 sorghum genotypes, the PIC content of 28 SSR markers ranged from 0.23 up to 0.81 with an average of 0.62. According to the authors 75% of the SSR *loci* were highly informative (PIC>0.60). In Fernandes *et al.* (2008) the PIC content of *P. pinaster* genotypes was also higher than 60% being considered highly informative for the 3 SSR *loci* used, and directly correlated with He, but not with Na. The results obtained in *A. unedo*, showed that the lowest PIC value (0.51) obtained with the NA741 primer is directly correlated with the lowest values of Na (6), Ho (0.56) and He (0.57). On the other hand, the *locus* (CA421F) with the highest PIC value (88%), the most informative *locus*, also displayed the highest values for genetic diversity parameters: Na=22, Ho= 0.85, He=0.90 and PIC=0.88. These results suggest that the former primer (NA741) could be discarded for further studies of genetic diversity analysis in strawberry tree.

The Lynch (1990) coefficient (band sharing) for SSRs showed allelic similarities between genotypes until a maximum of 82%. The Mantel test supported the tree topology (r=0.75; P<0.001). However, no genotypes could be grouped according to their geographical origin suggesting that physical distance is not the main factor contributing to the genetic diversity observed in *A. unedo*. These data are in line with the results obtained by RAPD analysis. The Mantel test also confirmed a lack of correlation between genetic and geographical distances matrices, for both RAPD and SSR markers. Levi and Rowland (1997) also reported that genetic similarity among genotypes (for both RAPD and SSR markers) did not group according to the *V. corymbosum* geographical distance. Similar results were reported by Cipriani *et al.* (2001) in *Prunus persica* analysed with 26 SSRs, since the genotypes could not

be grouped according to their geographical origin. However, a good correlation between genetic and geographical matrices distances has been found for other species. For example, in *Vitis vinifera*, Filippetti *et al.* (2001) were able to characterized the genetic diversity in 53 *Vitis vinifera* accessions using 6 SSRs *loci* and the genetic cultivar dendrogram matched the geographical distribution of the varieties. In strawberry tree, the lack of correlation between genetic and geographical distances matrices, for both markers (RAPDs and SSRs) may be related to a reduced gene flow between plants due to the species mating system as well as to the fragmentary distribution of *A. unedo* in Portugal.

The results so far obtained in strawberry suggest that SSRs are more effective than RAPDs to evaluate genetic diversity. In fact, the He value (75%) for SSRs is considerable higher than the same value found for RAPDs (27%). This observation is consistent with the higher levels of polymorphism usually displayed by SSRs though monomorphic bands were used in the RAPDs analysis. These results support the data obtained by Agrama and Tuinstra (2003) in sorghum who reported that RAPD primers were less polymorphic (with 40% of monomorphic bands) than SSR markers (with an average of 4.5 alleles per primer and 0.62 of PIC value).

The Lynch similarity coefficient (Lynch, 1990) was also used to evaluate which of the two markers was more effective to assess genetic diversity. According to this coefficient, the genetic diversity found with SSRs was also much higher than with RAPDs. In fact, similarity values for SSRs varied between 21 and 82% whereas for RAPDs the variation was between 83 and 95%. Also, the genotype differences reached a maximum of 79% with SSRs being much lower when the evaluation was based on RAPDs (17%). Similar results were published by Agrama and Tuinstra (2003), who report that the average genetic diversity between sorghum genotypes was higher when it was estimated using SSR markers (similarity of 0.44) compared to RAPD (similarity of 0.61).

Attempts to find a correlation, between the SSR and the RAPD pair-wise similarity matrices were unsuccessful. This absence of correlation was also verified by Majourhat *et al.* (2008) in *Argania spinosa*. However, the results reported by Agrama and Tuinstra (2003), in 22 sorghum genotypes, pointed out to a high correlation (r=0.79) between the SSR and the RAPD pair-wise similarity matrices. Similar results are referred by Hurtado *et al.*(2001) since high correlations were found between RAPDs and AFLPs in *Prunus armeniaca* genetic analysis.

Equally important are the correlations between pair-wise genetic matrix and relevant quantitative traits with economic, environmental or adaptive value. For instance, Ryan *et al.*

(2001) used RAPDs in 26 heritage *Prunus amygdalus* accessions. They found a mismatch between selection traits (kernel or nut weights) and genetic clustering. According to the authors, this mismatch was probably due to the polygenic nature of the interest traits. The establishment of a marker-assisted selection system for the economically important crops or ornamental phenotypes is of great interest to breeding programs. For instance, two candidate RAPD markers in coupling of the trait bud-flowering' phenotype in the ornamental *Calluna vulgaris* were identified by Borchert and Hohe (2009). Molecular markers can be applied in other studies in plant breeding. Debnath (2010) and Testolin and Cipriani (2010) reported the employment of molecular markers in micropropagated plants for the assessment of genetic fidelity, uniformity, stability, and true-to-typeness among donor plants and tissue culture regenerates. The germplasm genetic diversity is crucial information, due to its potential in strategic breeding program. Zawko *et al*. (2001) used markers in a rare and endangered species *Leucopogon obtectus* (Ericaceae), they referred that germplasm management and conservation should concentrate on maintaining the high levels of genetic variability through mixing genotypes and promoting outcrossing. The opposite decision can lead to a genetic diversity reduction. Borchert *et al*. (2008) reported that the narrow gene pool detected in *Calluna vulgaris* and *Erica* spp. using RAPD and SSR may be related to juridical conflicts between breeders. In the last few years there are many reports of combination of classical breeding and modern biotechnological approaches which have unlimited scope in agriculture and forestry (Milne and Abbott, 2008). Marcucci Poltri *et al*. (2003) used molecular data information (AFLP and SSR) to generate information about genetic diversity of *Eucalyptus dunnii* accessions to design a clonal seed orchard. Similar approaches are reported for *V. angustifolium* (Burgher-Maclellan and Mackenzie, 2004), *V. macrocarpon* (Debnath, 2007) and *Vitis* species (Regner *et al.*, 2001). The prior knowledge of the geographical distribution of genetic diversity level is needed to preserve the species genetic diversity and to plan a conservation strategy (Derory *et al.*, 2002). Molecular markers contribute to define conservation strategies in the species, as well as may develop tests for seed origin identification and breeding programs (Volk *et al.*, 2010).

The results so far obtained in *A. unedo* have shown that 16 RAPDs and 5 SSRs primers displayed a high degree of polymorphism; being 4 SSRs highly informative for a marker *locus*. In conclusion, these markers proved to be useful, which results and further studies can be applied for genetic diversity germplasm fingerprinting, to evaluate the genetic uniformity of the *in vitro* propagated plants, to evaluate germplasm variability for breeding programs and conservation purposes and to develop marker-assisted selection system.

5.6 REFERENCES

Agrama, H.A., Tuinstra, M.R., 2003. Phylogenetic diversity and relationships among sorghum accessions using SSRs and RAPDs. Afr. J. Biotechnol. 2, 334-340.

Albert, T., Raspe, O., Jacquemart, A.L., 2003. Clonal structure in *Vaccinium myrtillus* L. revealed by RAPD and AFLP markers. Int. J. Plant Sci. 164, 649-655.

Albert, T., Raspe, O., Jacquemart, A.L., 2004. Clonal diversity and genetic structure in *Vaccinium myrtillus* populations from different habitats. Belgian J. Bot. 137, 155-162.

Albert, T., Raspe, O., Jacquemart, A.L., 2005. Diversity and spatial structure of clones in *Vaccinium uliginosum* populations. Can. J. Bot. 83, 211-218.

Bassil, N.V., Boches, P., Rowland, L., 2006. Genetic diversity in the highbush blueberry evaluated with microsatellite markers. J. Amer. Soc. Hort. Sci. 131, 674-686.

Bassil, N.V., Bunch, T., Nyberg, A., Hummer, K., Zee, F.T., 2010. Microsatellite markers distinguish Hawailan ohelo from other *Vaccinium* L. section *Myrtillus* species Acta Hort. 859, 81-88.

Bell, D.J., Rowland, L.J., Polashock, J.J., Drummond, A.A., 2008. Suitability of EST-PCR markers developed in highbush blueberry for genetic fingerprinting and relationship studies in lowbush blueberry and related species. J. Amer. Soc. Hort. Sci. 133, 701-707.

Besnard, G., Baradat, P., Bervillé, A., 2001. Olive cultivar identification using nuclear RAPDs and mitochondrial RFLP Acta Hort. 546, 317-324.

Bhattacharya, E., Dandin, S.B., Ranade, A.S., 2005. Single primer amplification reaction methods reveal exotic and indigenous mulberry varieties are similarly diverse. J. Biosci. 30, 669–677.

Boches, P., 2005. Microsatellite marker development and molecular characterization in Highbush Blueberry (*Vaccinium corymbosum* L.) and *Vaccinium* species. Thesis of Master of Science in Horticulture. Oregon State Univ.

Boches, P.S., Bassil, N.V., Rowland, L.J., 2005. Microsatellite markers for *Vaccinium* from EST and genomic library. Mol. Ecol. 5, 657-660.

Borchert, T., Hohe, A., 2009. Identification of molecular markers for the flower type in the ornamental crop *Calluna vulgaris*. Euphytica 170, 203-213.

Borchert, T., Krueger, J., Hohe, A., 2008. Implementation of a model for identifying essentially derived varieties in vegetatively propagated *Calluna vulgaris* varieties. BioMed. Central Genetics doi:10.1186/1471-2156-9-56 9.

Botstein, W.R.L., Skolnick, K., Davis, R.W., 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphism. Am. J. Hum. Genet. 32, 314–331.

Burgher-Maclellan, K., Mackenzie, K., 2004. An overview of RAPD analysis to estimate genetic relationships in Lowbush blueberry. Small Fruits Rev. 3, 295-305.

Burgher, K., Jamieson, A., Lu, X., McRae, K., 1998. Genetic relationship of 26 wild blueberry clones (*Vaccinium angustifolium*), as determined by random amplified polymorphic DNA analysis. Proc. Atlantic. Plant Tissue Culture Assoc. Ann. Mtg., New Brunswick, p. *Abst.* 7.

Cardoso, A.V.R., 2004. Historial recente da propagação vegetativa do medronheiro no Algarve, contribuição para a criação de um futuro parque de pés-mãe. Tese de Licenciatura. ISA - UTL, Lisboa.

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

Cervera, M.T., Plomion, C., Malpica, C., 2000. Molecular markers and genome mapping in woodyplants. In: Jain, S.M., Minocha, S.C. (Eds.), Molecular biology of woody plants. Forestry Sciences. Kluwer Academic Pub., Dordrecht, pp. 375-394.

Chandra, A., Tiwari, K.K., 2009. Isolation and characterization of microsatellite markers from guineagrass (*Panicum maximum*) for genetic diversity estimate and cross-species amplification. Plant Breeding 129, 120-124

Chawla, H.S., 2009. Introduction to plant biotechnology, 3rd ed. Science Publis., Inc., New Hampshire.

Cipriani, G., Marrazzo, M.T., Di Gaspero, G., Testolin, R., 2001. DNA microsatellite in fruit crops: isolation, length polymorphism, inheritance, somatic stability and cross-species conservation. Acta Hort. 546, 145-150.

Debnath, S.C., 2007. An assessment of the genetic diversity within a collection of wild Cranberry (V*accinium macrocarpon* Ait.) clones with RAPD-PCR. Genet. Resour. Crop. Evol. 54, 509-517.

Debnath, S.C., 2010. Berry bioreactor micropropagation and molecular analysis for genetic fidelity. Acta Hort. 865, 43-51.

Derory, J., Mariette, S., Gonzaléz-Martínez, S.C., Chagné, D., Madur, D., Gerber, S., Brach, J., Persyn, F., Ribeiro, M.M., Plomion, C., 2002. What can nuclear microsatellites tell us about maritime pine genetic resources conservation and provenance certification strategies? Ann. For. Sci. 59, 699-708.

Eriksson, G., Ekberg, I., Clapham, D., 2006. An introduction to forest genetics. SLU. http://vaxt.vbsg.slu.se/forgen/, Uppsala.

Escaravage, N., Questiau, S., Pornon, A., Doche, B., Taberlet, P., 1998. Clonal diversity in a *Rhododendron ferrugineum* L. (Ericaceae) population inferred from AFLP markers. Mol. Ecol. 7, 975-982.

Fernandes, L., Rocheta, M., Cordeiro, J., Pereira, S., Gerber, S., Oliveira, M.M., Ribeiro, M.M., 2008. Genetic variation, matting patterns and gene flow in a *Pinus pinaster* Aiton clonal seed orchard. Ann. For. Sci. 65, 1-9.

Figueiredo, E., 2007. Avaliação e discriminação de cultivares de *Olea europea* L. através de marcadores RAPDS. Tese de Mestrado. FCTUC, Coimbra, p. 65.

Filippetti, I., Silvestroni, O., Thomas, M.R., Intrieri, C., 2001. Genetic characterisation of Italian wine grape cultivars by microsatellite DNA analysis. Acta Hort. 546, 395-399.

Garkava-Gustavsson, L., 2004. Genetic diversity in lingonberry (Ericaceae) revealed by RAPD analysis. Proceed. XIth Eucarpia Symp. Fruit Breeding Genetics pp. 621-624.

Glaubitz, C.J., Moran, G.F., 2000. Genetic tools: the use of biochemical and molecular markers. In: Young, A., Bosheir, D., Boyle, T. (Eds.), Forest conservation genetics: principles and practice. CABI Publishing, London, pp. 39-59.

Godinho-Ferreira, P.G., Azevedo, A.M., Rego, F., 2005. Carta da tipologia florestal de Portugal Continental. Silva Lusitana 13, 1-34.

Goldstein, D.B., Schlötterer, C., 1999. Microsatellites, evolutions and apllications. Oxford Univ. Press, N. York.

Gupta, P.K., Varshney, R.K., 2000. The development and use of microsatellite markers for genetic analysis and plant breeding with special emphasis on bread wheat. Euphytica 113, 163-185.

Hirai, M., Yoshimura, S., Ohsako, T., Kubo, N., 2010. Genetic diversity and phylogenetic relationships of the endangered species *Vaccinium sieboldii* and *Vaccinium ciliatum* (Ericaceae). Plant Syst. Evol. 287, 75-84.

Hurtado, M.A., Badenes, M.L., Llácer, G., Westman, A., Beck, E., Abbott, G.A., 2001. Contribution to apricot genetic analysis with RFLP, RAPD and AFLP markers. Acta Hort. 546, 417-420.

International Centre Underutilized Crops, 2009. web site of the International Centre for Underutilized Crops, http://www.icuc-iwmi.org/ February/2009.

Jain, A., Pandit, M.K., Elahi, S., Bhaskar, A., Kumar, V., 2000. Reproductive behaviour and genetic variability in geographically isolated populations of *Rhododendron arboreum* (Ericaceae). Curr. Sci. 79, 1377-1381.

Joshi, S.P., Ranjekar, P.K., Gupta, V., 1999. Molecular markers in plant genome analysis Curr. Sci. 77, 230-240.

Kameyama, Y., Kasagi, T., Kudo, G., 2006. Eight microsatellite markers for sympatric alpine shrubs, *Phyllodoce aleutica* and *P. caerulea* (Ericaceae). Mol. Ecol. 6, 402-404.

Kapteyn, J., Simon, J.E., 2002. The use of RAPDs for assessment of identity, diversity, and quality of *Echinacea*. In: Janick, J., Whipkey, A. (Eds.), Trends in new crops and new uses. ASHS Press, Alexandria, pp. 509-513.

Kenis, K., Pauwels, E., Van Houtvinck, N., Keulemans, J., 2001. The use of microsatellites to establish unique fingerprints for apple cultivars and some of their descendants Acta Hort. 546, 427-431.

Klooster, M.R., Hoenle, A.W., Culley, T.M., 2008. Characterization of microsatellite *loci* in the myco-heterotrophic plant *Monotropa hypopitys* (Ericaceae) and amplification in related taxa. Mol. Ecol. Resources 9, 219-221.

Kondo, T., Nakagoshi, N., Isagi, Y., 2009. Shaping of genetic structure along pleistogene and modern river systems in the hydrochorous riparian azalea, *Rhododendron ripense* (Ericaceae). Amer. J. Bot. 96, 1532-1543.

Levi, A., Rowland, L.J., 1997. Identifying blueberry cultivar and evaluating their genetic relationships using randomly amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR)-anchored primers. J. Amer. Soc. Hort. Sci. 122, 74–78.

Li, C.C., Weeks, D.E., Chakravarti, A., 1993. Similarity of DNA fingerprinters due to chance and relatedness. Hum. Hered. 43, 45-52.

Lopes, L., Sá, O., Baltazar, J.L., Pereira, J.A., Baptista, P., 2010. Analysis of molecular and morphological diversity of *Arbutus unedo* in the interior North and Center of Portugal. In: SPG (Ed.), XXXV Jornadas Portuguesas de Genética. Univ. do Minho, Braga, p. *Abst* 76.

Lynch, M., 1990. The similarity index and DNA fingerprinting. Mol. Biol. Evol. 7, 478-484.

Majourhat, K., Jabba, Y., Hafidi, A., Martínez-Gómez, P., 2008. Molecular characterization and genetic relationships among most common identified morphotypes of critically endangered rare Moroccan species *Argania spinosa* (Sapotaceae) using RAPD and SSR markers. Ann. For. Sci. 65, 1-6.

Maki, M., Horie, S., Yokoyama, J., 2002. Comparison of genetic diversity between narrowly endemic shrub *Menziesia goyozanensis* and its widespread congener *M-pentandra* (Ericaceae). Conserv. Genet. 3, 421-425.

Marcucci Poltri, S.N., Zelener, N., Rodriguez Traverso, J., Gelid, P., Hopp, H.E., 2003. Selection of a seed orchard of *Eucalyptus dunnii* based on genetic diversity criteria calculated using molecular markers. Tree Physiology 23, 625-632.

Marshall, T.C., Slate, J., Kruuk, L., Pemberton, J.M., 1998. Statistical confidence for likelihood-based paternity inference in natural populations. Mol. Ecol. 7, 639–655.

Martins, M., Farinha, A., Ferreira, E., Cordeiro, V., Monteiro, A., Tenreiro, R., Oliveira, M., 2001. Molecular analysis of the genetic variability of Portuguese almond collections Acta Hort. 546, 449-452.

Mateescu, R.G., Zhang, Z., Tsai, K., Phavaphutanon, J., Burton-Wurster, N.I., Lust, G., Quaas, R., Murphy, K., Acland, G.M., Todhunter, R.J., 2005. Analysis of allele fidelity, polymorphic information content, and density of microsatellites in a genome-wide screening for Hip Dysplasia in a Crossbreed Pedigree. J. Heredity 96, 847-853.

Milne, R.I., Abbott, R.J., 2008. Reproductive isolation among two interfertile *Rhododendron* species: low frequency of post-F-1 hybrid genotypes in alpine hybrid zones. Mol. Ecol. 17, 1108-1121.

Muchugi, A., Kadu, C., Kindt, R., Kipruto, H., Lemurt, S., Olale, K., Nyadoi, P., Dawson, I., Jamnadass, R., 2007. Molecular markers for tropical trees – a practical guide to principles and procedures. World Agroforestry Centre, Technical Manuel, nº 9, Nairobi.

Namkoong, G., Koshy, M.P., 2001. Application of genetic markers to forest tree species. Report to IPGRI of the project Developing Decision-making Strategies on Priorities for Conservation and Use of Forest Genetic Resources. Univ. of British Columbia.

Nei, M., 1987. Molecular evolutionary genetics. Columbia Univ. Press, N. York.

Nicese, F.P., Hormaza, J.I., McGranahan, G.H., 1998. Molecular characterization and genetic relatedness among walnut (*Juglans regia* L.) genotypes based on RAPD markers. Euphytica 101, 199-206.

Parker, P.G., Snow, A.A., Schug, M.D., Booton, G.C., Fuerst, P.A., 1998. What molecules can tell us about populations: choosing and using a molecular marker. Ecology 79, 361-382.

Piotto, B., Piccini, C., Arcadu, P., 2001. La ripresa della vegetazione dopo gli incendi nella regione mediterrânea. In: Piotto, B., Noi, A. (Eds.), Propagazione per seme di alberi e

arbusti della flora mediterranea. Dipart. Prev. Risanam. Ambient., Manuale ANPA (Agenz. Naz. per la Protezi. dell' Ambiente), pp. 32-38.

Quarta, R., Dettori, M.T., Verde, I., Marchesi, U., Palombi, M.A., 2001. Characterization and evaluation of genetic diversity in peach germplasm using RAPD and RFLP markers. Acta Hort. 546, 489-496.

Rafalski, J.A., 1991. RAPD markers- a new technology for genetic mapping and plant breeding. Ag. Biotech. News and Information 3, 645-648.

Regner, F., Stadlbauer, A., Eisenheld, C., 2001. Molecular markers for genotyping grapevine and for identifying clones of traditional varieties. Acta Hort. 546, 331-341.

Rohlf, F.J., 1997. NTSYS-pc. Numerical taxonomy and multivariate analysis system version 2.01. Dep. of Ecology and Evolution, State Univ. of N. York, N. York.

Ryan, F.J., Ledbetter, C.A., Ramming, D.W., Palmquist, D.E., Bell, D.E., Peterson, S.J., 2001. Challenges in developing molecular markers for almond (*Prunus amygdalus*) and grape (*Vitis species*). Acta Hort. 546, 629-639.

Schneller, J., Holderegger, R., Gugerli, F., Eichenberger, K., Lutz, E., 1998. Patterns of genetic variation detected by RAPDs suggest a single origin with subsequent mutations and long-distance dispersal in the apomictic fern *Dryopteris remota* (Dryopteridaceae). Amer. J. Bot. 85, 1038-1042.

Seabra, R.C., Simões, A.M., Baeta, J., Salomé Pais, M., 2001. Evaluation of Portuguese chestnut stands by RAPDs. For. Snow Landsc. Res. 76, 435-438.

Segarra-Moragues, J.G., Donat-Caerols, S., Ojeda, F., 2009. Isolation and characterization of microsatellite *loci* in the cape fynbos heath *Erica coccinea* (Ericaceae). Conserv. Genet. 10, 1815-1819.

Takrouni, M.M., Boussaid, M., 2010. Genetic diversity and population's structure in Tunisian strawberry tree (*Arbutus unedo* L.). Scientia Hortic. 126, 330-337.

Tan, X.-X., Li, Y., Ge, X.-J., 2009. Development and characterization of eight polymorphic microsatellites for *Rhododendron simsii* Planch (Ericaceae) Conserv. Genet. 10, 1553-1555.

Testolin, R., Cipriani, G., 2010. Molecular markers for germplasm identification and characterization Acta Hort. 859, 59-72.

Torres, J.A., Valle, F., Pinto, C., Garcia-Fuentes, A., Salazar, C., Cano, E., 2002. *Arbutus unedo* communities in southern Iberian Peninsula mountains. Plant Ecol. 160, 207-223.

Underutilized-species, O., 2008. Global Facilitation Unit for Underutilized Species, www.underutilized-species.org/ Nov./2009.

Vander Kloet, S.P., Paterson, I.G., 2000. RAPD assessment of novelties resulting in a new species of *Vaccinium* L. (Ericaceae) from Vietnam. Bot. J. Linnean Society 134, 575-586.

Volk, G.M., Richards, C.M., Forsline, P.L., 2010. A comprehensive approach toward conserving *Malus* germplasm. Acta Hort. 859, 177-182.

Wang, N., Qin, Z.C., Yang, J.B., Zhang, J.L., 2010. Development and characterization of 15 microsatellite *loci* for *Rhododendron delavayi* Franch. (Ericaceae). Hortscience 45, 457-459.

Wang, X.Q., Huang, Y., Long, C.L., 2009. Isolation and characterization of twenty-four microsatellite *loci* for *Rhododendron decorum* Franch. (Ericaceae). Hortscience 44, 2028-2030.

Weir, C.C., Cockerham, B.S., 1984. Estimating F-statistics for the analysis of population structure. Evolution 38, 1358–1370.

White, T.L., Adams, W.T., Neale, D.B., 2007. Forest Genetics. CAB Internacional Oxfordshire.

Whitton, J., Rieseberg, L.H., Ungerer, M.C., 1997. Microsatellite *loci* are not conserved across the *Asteraceae*. Mol. Biol. Evolution 14, 204-209.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18, 6531-6535.

Zawko, G., Krauss, S.L., Dixon, K.W., Sivasithamparam, K., 2001. Conservation genetics of the rare and endangered *Leucopogon obtectus* (Ericaceae). Mol. Ecol. 10, 2389 - 2396.

6 - General Discussion

6 GENERAL DISCUSSION

6.1 DISCUSSION

As stated in the previous chapters, *A. unedo* is an under-exploited crop in Portugal and other Mediterranean countries and in Ireland where it grows spontaneously as a bush or small tree. The plant has no tradition of breeding and its use by local populations is based rather in traditional habits than in agricultural practices supported by the selection and culture of the best producing trees (Celikel *et al.*, 2008). However, in some regions, farmers and local population are becoming increasingly interested in this culture as a fruit crop or ornamental tree with the consequent demand of high-quality plant material (Celikel *et al.*, 2008; Zizzo *et al.*, 2010). Moreover, this species is quite important in the centre region of Portugal where it is well adapted to the environmental conditions being also a fire resistant plant that may help to reduce the impact of the common fires occurring during the dry season. This forested area is densely populated with pines (*Pinus pinaster)* and eucalypts (*Eucalyptus globulus)* a situation favoring fire propagation thus reducing both farmer incoming and genetic diversity (S. Silva and Harrison, 2010). Considering this situation, forestry associations have began to use *A. unedo* in forestation programs as a broadleaf for protection and firebreak to slow or stop propagating fires, to improve ecosystem quality, and for hunting projects. To support the increasing interest of farmers for this species is necessary to select and propagate the most promising plants in order to be able to deliver high quality plant material that can increase profits of the land owners interested in investing in this species. During the last couple of years, selected *A. unedo* plants obtained through micropropagation in our lab have been delivered to several farmers and are now in the field. Besides, local authorities and farmers associations have became increasingly aware of the potential of this tree and have started to pay more attention to its potential both as an important ecological species and as a fruit crop. In fact, is nowadays common in the centre region of Portugal the organization of meetings and seminars related with the culture and production of *A. unedo*. Attempts to isolate and characterize secondary metabolites are another way of research in this plant and some data seem to indicate that the plant has a great potential for extracts production with antioxidant activity, such as total phenolic and flavonoid contents (Kivçak and Mert, 2001; Pabuccuoglu *et al.*, 2003; Andrade *et al.*, 2009; Oliveira *et al.*, 2009; Sá *et al.*, 2010). These extracts show high values of interesting compounds when compared with other species described in the

literature (Andrade *et al.*, 2009). The antiaggregant properties of *A. unedo* extracts in human platelets have been reported and might be used for the treatment and/or prevention of cardiovascular diseases (El Haouari *et al.*, 2007). The experiments aiming to evaluate food quality have also been performed. Fruits show high amounts of sugars (fructose, glucose, sucrose and maltose), minerals, vitamins (C and E) and a large variety of compounds with antioxidant ability, such as phenolic acids including gallic, gentisic, protocatechuic, p-hydroxybenzoic, vanillic and m–anisic acids (Ayaz *et al.*, 2000; Pawlowska *et al.*, 2006; Demirsoy *et al.*, 2007; Özcan and HacIseferogullarI, 2007; Pallauf *et al.*, 2008; Oliveira *et al.*, 2010a). Fructose and glucose among the sugars, fumaric and malic acids among the non-volatile acids and gallic acid among the phenolic acids were found to be major compounds contributing to the taste of the fruits (Ayaz *et al.*, 2000).

Studies presented in Chapter II and III have provided important insights concerning the establishment of an effective *in vitro* propagation system for *A. unedo* through axillary shoot proliferation from adult trees selected according to their fruit production and quality. In a first set of experiments the different phases of the micropropagation process were optimized. Later on, both the role of the genotype and the effect of different growth regulators were evaluated.

The *in vitro* establishment of adult explants from woody plants has proven to be a difficult task in many species (George and Debergh, 2008; Preece, 2008). However, propagation from adult trees is the only way to assure the genetic quality of selected trees. Although juvenile explants, such as those obtained from seedlings or young plants are easier to establish and propagate *in vitro*, their genotypes are unknown thus impairing the effectiveness of the micropropagation process. The correct choice of the explant is another factor that can affect the success of plant tissue culture experiments (George, 2008). Plants growing in field conditions are usually contaminated with different types of microorganisms a major drawback for *in vitro* establishment. To overcome this difficulty, a common practice is to maintain a stock of plants growing in controlled conditions to avoid extensive contamination during the *in vitro* establishment assays. It is also known that explants taken from stock plants at different times of the year may not give reproducible results in tissue culture experiments a situation that can be related to the variations in the amount and diversity of microorganisms present in the tissues or to seasonal changes in the levels of endogenous growth regulators in the stock plants (Preece, 2008). To overcome these limitations, epicormic shoots from adult selected trees were used as donor explants for the establishment phase (Chapter II). The main

advantage of using these epicormic shoots is related to the possibility of using them all over the year since shoots can easily sprout in a culture chamber under controlled conditions. Moreover, disinfection of this type of plant material is easier than using field growing explants (George, 2008).

The results so far obtained indicate that the epicormic shoots developed on branches of selected trees, are a good source of establishment explants, and shoot tips are more efficient than nodal segments (Chapter II).

The success of plant tissue culture for plant propagation is greatly influenced by the type of the culture medium used (George and De Klerk, 2008). In our assays the FS medium combined with the micronutrients of the MS medium gave the highest multiplication rates (Chapter II) when compared with Anderson or MS reduced at half-strength (1/2 MS). Besides the basal medium, the PGRs have also a crucial importance during the multiplication stage. Cytokinins are generally used to promote axillary shoot formation (Chawla, 2009). The developing shoots can be further used to initiate new cycles of multiplication thus contributing to the high number of plantlets obtained through this technique. This positive role of cytokinins on shoot proliferation has been also confirmed in several Ericaceae species (Gonçalves and Roseiro, 1994; Jaakola, 2001; Mereti *et al.*, 2002; Almeida *et al.*, 2005). To optimize the micropropagation process in *A. unedo* several cytokinins in combination with an auxin (NAA) were tested (Chapter III). The inclusion of auxins on the multiplication media must be used with precaution since this type of hormone can promote callus formation from which adventitious shoots can develop. Contrarily to the shoots arising from axillary meristems, adventitious shoots may display some kind of genetic or epigenetic modifications that can be responsible for the appearance of somaclones among the regenerated plants (Machakova *et al.*, 2008). The assays performed with *A. unedo* indicated that 8.9 µM kinetin gave the best rates of multiplication. However, the results were not significantly different from those obtained with BA or zeatin. When TDZ was used or NAA was tested in combination with a cytokinin callus growth was usually observed and its formation increased with the number of subcultures (Chapter III). Similar results have been reported when BA and TDZ were tested for shoot proliferation of *Tilia platyphyllos* (Chalupa, 2003). In our experiments these calli seem to have a low morphogenic potential since, in the conditions tested, adventitious shoot formation was seldom observed through the appearance of underdeveloped shoots showing abnormal leaf formation (Chapter III). Organogenic callus formation has been used to the propagation of several species and, once proved that the plantlets obtained are genetically uniform, could be an alternative for *in vitro* strawberry tree

propagation. According to Preece (2008) shoot proliferation is sometimes difficult to achieve and often the explants tend to growth without considerable branching. In these conditions, propagation can be achieved by segmentation of the shoots and culture of the nodal segments. This situation can be the result of the strong apical dominance occurring in the tissues or of unbalanced combinations and/or concentrations of growth regulators, mainly cytokinins. The way how micropropagated cultures behave *in vitro* can be also the result of the type of explants used. Thus, proliferation occurs more readily when explants from juvenile material are used whereas elongation seems to be more common in adult-derived explants. This aspect was frequently observed in some selected adult genotypes of *A. unedo*. Therefore, shoot length was one of the variables used to evaluate the proliferation rate (Chapter II and III).

Several works have indicated that the genotype of the donor plants is of utmost importance for micropropagation and other aspects on *in vitro* cultures (Chalupa, 2003; Gajdošová *et al.*, 2007; Ostrolucká *et al.*, 2007; Gahan and George, 2008). The results obtained with different lines of *A. unedo* also showed that the genotype influences not only shoot proliferation but also the rooting ability of the proliferating shoots (Chapter III). As these factors were also extensively dependent on the PGRs present in the culture media it is plausible to assume that different genotypes possess different levels of endogenous auxins and/or cytokinins or display different sensibility to PGSs that influence their behavior *in vitro*. Further research on *A. unedo* micropropagation is necessary to better understand the interaction between genotype and PGRs on *in vitro* morphogenesis. In particular it would be interesting to follow the endogenous levels of auxins and cytokines during shoot proliferation and rooting.

Rooting is a crucial step to the micropropagation success. Root formation is a complex process that is influenced by a large number of factors, such as genotype, type and concentration of PGRs and culture conditions (Mylona and Dolan, 2002; Van Staden *et al.*, 2008). The results obtained in Chapter II and III showed that the highest rooting rate was achieved when shoots were treated with IBA for a short period followed by subculture on an auxin-free medium containing charcoal. Treatments with auxin have been used to stimulate adventitious root formation although shoots of some species can root on media without auxins (Machakova *et al.*, 2008; Preece, 2008). In our experiments a positive interaction between IBA and rooting ability was found (Chapter II). A ten day period of contact to IBA showed to be more appropriate for rooting, and plantlet acclimatization should not occur before 35-40 days on the root development medium (without auxin). Several authors have pointed out that the cytokinins used during the multiplication stage may negatively influence root primordia induction and further development (Van Staden *et al.*, 2008). Our results are

not in line with these data since it was observed that the number of roots formed per shoot showed a positive influence with BA concentration (Chapter III). A promoting effect of the cytokinins used on the multiplication media on further rooting has been occasionally referred with *Eucalyptus* (Bennett *et al.*, 1994) and in fruit-tree rootstocks (Nemeth, 1979). Machakova *et al*. (2008) suggested that the induction of rhizogenesis usually requires an adjustment in the endogenous levels of auxins and cytokinins.

The success of acclimatization of rooted shoots, following hardening and transfer to field conditions is a necessary condition for the implementation of any reliable micropropagation method (George and Debergh, 2008). Our results indicate that for plant acclimatization in greenhouse, perlite (100%) without fertilizer is the best substrate to obtain a large percentage of acclimatized plants (Chapter II). After 5 weeks on root development medium, shoots were healthy, rarely showed callus formation at the shoot base or apical necrosis, and consequently were successfully acclimatized (Chapter II, III and IV). Histological studies indicated that adventitious roots of strawberry tree had a deep origin, near the vascular tissues probably from the secondary phloem and/or from the cambial zone. These two features (callus absence and root origin) are probably linked to the acclimatization success. Roots originated from more peripheral tissues were never found (Chapter II). An origin from or near the vascular tissues is required since the adventitious roots are in close association with the vascular tissues of the stem, which means that a good vascular connection shoot/root is present, contributing to the acclimatization success and field survival (Smith *et al.*, 1991; Smith *et al.*, 1992; Ziv and Chen, 2008). According to this, the field trial established showed a survival rate of 96.8% one year after plant transfer to field conditions (Chapter IV).

The data presented in Chapter IV provide information about the compatibility between *A. unedo* clones and *P. tinctorius* or *L. deliciosus* during *in vitro* conditions. Inoculation treatments with *P. tinctorius* in nursery conditions followed by a field trial establishment were also evaluated.

A review of the literature about mycorrhizae clearly points out to a positive and critical component in crop systems because these symbiotic fungi may increase plant growth, plant reproductive capacity, plant water stress tolerance and plant health through antagonistic and competitive effects on pests and pathogens. They may also enhance the plant's resistance to other abiotic stresses as salinity and soil texture (Goltapeh *et al.*, 2008). Thus, the mycorrhizae can improve not only the field plant survival but also their nutrition (by nutrients and water uptake) and consequently production. Several factors may affect this type of

symbiosis such as the host genotype, the structure and morphology of roots, the type of fungi, the specific cultivar–fungus response and the interaction with other microorganisms (Goltapeh *et al.*, 2008). Our results revealed a reduced *in vitro* growth of *L. deliciosus* compared to *P. tinctorius*. Any pathogenic or harmful effect, for both fungi, was observed in plantlets. The presence of arbutoid mycorrhizae one month after inoculation *in vitro* was observed only when *P. tinctorius* was tested. These showed a tick mantle, Hartig net and intracellular hyphal complexes, both confined to the epidermis (Chapter IV). These features are similar to other descriptions of arbutoid mycorrhizae found in the literature (Smith and Read, 1997; Peterson and Massicotte, 2004). Mycorrhization has been used in several species to facilitate acclimatization of micropropagated plantlets (Oliveira *et al.*, 2003; Parladé *et al.*, 2004; Ratnaparkhe, 2007). The results obtained in the assays of *A. unedo* mycorrhization showed that a preliminary phase of root expression of induced shoots, before shoot transfer to inoculated substrate enhances mycorrhizal synthesis. *L. deliciosus* compared to *P. tinctorius* showed a reduced *in vitro* growth, therefore periods for both substrate inoculation and mycorrhiza synthesis must be according to *in vitro* fungi growth. It was also observed that a system in which test tubes instead of vessels were used is more suitable for root development and mycorrhization, probably due to substrate deepness. Finally, it was clear that *in vitro* inoculated plantlets displayed a more branched root system, a feature which may explain the higher rates of acclimatization occurred in the assays with these plants. However, arbutoid mycorrhizae were never observed with *L. deliciosus*, the edible fungi we tested (Chapter IV). *L. deliciosus* is known by its natural symbiotic association with *Pinus* (Ingleby *et al.*, 1990). When forest species share the same natural environment there is a chance they share some ectomycorrhizal fungi (Richard *et al.*, 2005). As *A. unedo* usually grows in association with other forest tree species, such as pines and oaks, we decided to test mycorrhizal synthesis either with *L. deliciosus* or *P. tinctorius in vitro* conditions.

All plantlets were acclimatized in greenhouse, including those inoculated with *P. tinctorius*, which had shown the presence of arbutoid mycorrhizae. To confirm mycorrhizae formation on *A. unedo* plants it was necessary to test the persistence of the inoculated fungi in field growing plants. Molecular techniques as single-strain conformation polymorphism (SSCP) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) have been used for the identification of mycorrhizal fungi (Dickie and Fitzjohn, 2007). Real-time PCR has been applied for tracking the dynamics of the mycelium in different experimental conditions: field persistence of the inoculated fungus in experimental plantations and competition of the introduced fungus with native mycorrhizae (Parladé *et al.*, 2009). Twelve months after

acclimatization (or 17 months after *in vitro* inoculation treatment with *P. tinctorius*), *Thelephora* and *Hebeloma* were characterized by molecular markers techniques in inoculated plants (Chapter IV). Both mycorrhizae are particularly aggressive and well-known on nurseries (Garbaye, 1990). For further research, it is essential to perform more frequently root cuts to check for arbutoid mycorrhizae and use molecular markers to identify the types of fungi occurring after acclimatization procedures. Fungal persistence is a severe problem reported by several authors (Parladé *et al.*, 2004; Rincón *et al.*, 2005; Águeda *et al.*, 2008; Parladé *et al.*, 2009). According to Parladé *et al*. (2004) it is essential to evaluate fungal strains for their aggressiveness under nursery conditions in order to achieve a successful mycorrhizal synthesis.

In nursery two inoculation treatments with *P. tinctorius* (vegetative inocula produced in liquid medium and dry sporocarps) were tested and compared to control plants. After 4 months, plants from both inoculation treatments showed increased growth than control plants. Twenty months after a field trial establishment both mycorrhizae inocula treatments improved plant growth compared to control plants and fertilized seedlings (Chapter IV). Our results agree with those observed in *V. macrocarpon* by Kosola *et al.* (2007). The mycorrhization contributes to reduce the application of fertilizers and biocides a situation with positive impacts , on reducing inputs and environmental pollution (Quinteiro, 2005). Parladé *et al.* (2004) report that the inoculation method and the plant-fungal strain interaction are of significant importance for the percentage of colonized plants and the degree of colonization observed. Still, it is critical to know the threshold colonization level required to ensure fungal persistence. Further studies are needed to select competitive fungal strains, to evaluate plant-fungal strain interaction and the colonization level required to ensure fungal persistence.

As a whole our studies represent a first step for mycorrhizal synthesis in *A. unedo* with edible fungi. One of our goals is to increase the economic value to the species, considering the interest of the fungi for culinary uses. This income source may encourage the culture of *A. unedo* and enlarge the area occupied by this species. As a result, the continuous area occupied by *P. pinaster* or *E. globulus* monocultures, which are quite fire proneness, can be considerably reduced. Additionally, unlike the above referred monocultures *A. unedo* can contribute to create local employment and to reduce the abandon of rural areas a situation. The people presence in the country associated with the requirement to keep the orchards properly for fruit harvesting contributes to the reduction of helophyte vegetation, usually associated with forest fires.

Studies presented in Chapter V provide information about the genetic diversity of 27 *A. unedo* genotypes from 9 provenances analysed through molecular markers (RAPDs and SSRs). The set of 20 RAPDs primers (OPC) showed 71 polymorphic bands (57.3%). Eleven microsatellite markers were selected according to the results reported by Boches *et al*. (2005) and Bassil *et al*. (2006) in *Vaccinium*. The expected heterozygosity for SSR was much higher (75%) than the same value found for RAPDs (27%). This observation is consistent with the higher levels of polymorphism usually displayed by SSRs (Agrama and Tuinstra, 2003; Eriksson *et al.*, 2006). Five SSR markers showed polymorphism. The reproducibility was confirmed in 4 PCR reactions. From all *loci* tested the most robust and polymorphic was the *locus* CA421F which displayed values higher than the average: 22 alleles, He=0.90 and a high PIC value (88%). By the contrary, *locus* NA741 showed the lowest PIC, number of alleles, and expected diversity. According to the results 4 primers showed to be highly informative (PIC>0.60) and can be selected for further studies. Our results point out to an important polymorphism level found among the 27 *A. unedo* genotypes. The knowledge acquired represents a first step for future research, which should include a larger number of genotypes and provenances. The knowledge of germplasm diversity is crucial information due to its potential in strategic planning of future breeding towards species biodiversity and sustainability (Zawko *et al.*, 2001; Eriksson *et al.*, 2006; Borchert and Hohe, 2009). For instance, Zawko *et al*. (2001) refer that conservation and management of *Leucopogon obtectus* (Ericaceae) should concentrate on maintaining the high levels of genetic variability through mixing genotypes and promoting outcrossing. The opposite decision can lead to the reduction of genetic diversity.

The cluster analysis for RAPDs revealed a similarity Lynch coefficient (Lynch, 1990) from 83% of similarity among genotypes up to 95%, which tree topology was supported by a moderate correlation yet significant ($r=0.64$; $P<0.001$). The same Lynch coefficient (Lynch, 1990) revealed for SSRs a similarity among trees, from 21% up to 82%, as well supported by a superior correlation ($r=0.75$; $P<0.001$). These results agree with previous information about heterozygosity, because expected and observed heterozygosity were higher with SSR markers (75% and 70%, respectively) and consequently their similarity was inferior to RAPD values. However, for both markers no genotypes could be grouped according to their geographical origin suggesting that physical distance is not the main factor contributing to the genetic diversity observed in *A. unedo*. Several factors may explain these observations. The low gene flow is probably associated with the matting system by insects and the species fragmentary

distribution. On the other hand, the seed dispersal by animals, particularly birds and the human intervention could explain the similarity found between some genotypes far apart geographically. Similar results are referred by Gerlach and Musolf (2000) as the landscape fragmentation was a cause for genetic diversity in *Clethrionomys glareolus*. According to the same authors not only the old geographic barriers (rivers) but also more recent fragmentation of landscape (highways) have a relevant effect on gene flow and consequently on genetic diversity and genetic substructuring of populations. On the other hand, a high level of gene flow due to seed dispersal by animals, birds and humans (El-Mousadik and Petit, 1996; Ribeiro *et al.*, 2001; Jordano *et al.*, 2007) may also contribute to the high genetic diversity observed.

6.2 FINAL REMARKS

Over the course of the past five years a lot of effort has been put in the selection, characterization and *in vitro* propagation of strawberry tree. In spite of the considerable amount of data so far obtained, and as it happens in any scientific project, the work is never finished and new approaches can be taken to improve *A. unedo* breeding and to explore is potential as a crop. As G. Bernard Shaw once stated "Science never solves a problem without creating ten more". Thus, further studies on this species must be pursued trying to develop new strategies of *in vitro* propagation such as organogenesis and somatic embryogenesis. Preliminary studies have shown that some tissues have the potential to produce organogenic callus from which shoots can be produced. Attempts to better understand how *A. unedo* cells can embark into an organogenic pathway of regeneration must be carried out. In a similar way, the ability of strawberry tree tissues to undergo somatic embryogenesis is an aspect that might open new perspectives in terms of cloning and breeding. The results so far obtained have indicated that young leaves from micropropagated shoots of seedling or mature origin can be induced to form somatic embryos which mean that adult selected plants can be vegetative propagated. As it is well known, several woody plants have been propagated through somatic embryogenesis. However, in most of the cases, the multiplication process is effective only when juvenile material of unknown genotypes, such as zygotic embryos or cotyledons have been used. The ability of adult-deriver tissues of *A. unedo* to embark into an embryogenic pathway is being explored.

Large-scale propagation is absolutely required to produce a great number of propagules that can be distributed to those interested in the culture of *A. unedo*. Moreover, the cost of the propagated material must be competitive *vis a vis* other types of clonal propagation. Thus, an evaluation of the costs of the process must be undertaken and attempts to reduce costs must be pursued. For example, more effective rooting methods (one-step rooting) that can avoid the use of two culture media would be of particular interest.

The analysis of a group of *A. unedo* individuals by molecular markers showed a wide genetic diversity. These studies are important to evaluate the degree of diversity of a species but more analyses must be carried out with an increased number of trees to correctly determine the extent of diversity. Moreover, these studies must be complemented by experiments trying to better understand the mating system in strawberry tree. The biology of pollination as well as the development of seeds and fruits and their dispersal are poorly characterized and further studies must focus on these subjects. Crossing and selection of new

traits are absolutely required for the development of cultivars but this can only be achieved if the biology of reproduction of a given species is well known. Besides their potential to evaluate genetic diversity, molecular markers are useful tools in plant breeding. Marker-assisted selection has been increasingly applied to the selection of particular traits, such as drought-tolerance, fruit quality and productivity. Although trees are much more difficult to manage than annual crops, due to their long juvenile periods and heterozygosity, one can envisage the application of molecular techniques for the selection of traits related with fruit quality. Molecular markers are useful to apprise the individuals that should be a focus for conservation purposes and to estimate those for a base-breeding population. Finally, molecular analyses can also be used to evaluate the genetic uniformity of plants obtained through axillary shoot proliferation or any other *in vitro* cloning method.

Field analysis of the micropropagated plants is another point that deserves further attention. In our experiments we have produced a large amount of plants; some of them are now growing and will produce fruits sooner. Analysis of fruit productivity and quality of these trees must be carried and compared with the original trees in order to determine if the selection was effective or not. Preliminary results seem to indicate that micropropagated plants displaying precocity when compared with plants propagated by other ways.

Mycorrhization is an interesting approach in many aspects. It can increases the rates of plant acclimatization following *in vitro* propagation, can reduces costs in terms of fertilizers and pesticide application and may represent an important income through the production of edible mushrooms. Further studies on *A. unedo* mycorrhization must be centered in particular aspects in order to make the process more effective. Thus, it will be necessary to optimize the inoculation methods by dosage experiments to obtain maximized root colonization. Experiments aiming to ensure fungal persistence by screening fungal strains and/or other fungi for their competitiveness under nursery and field conditions are also required. To confirm mycorrhizae formation on *A. unedo* plants it is necessary to test the persistence of the inoculated fungi in field growing plants. Thus, molecular markers should be useful for the identification of mycorrhizal fungi.

The northern and centre interior regions of Portugal can hardly survive for much longer based exclusively on the monocultures of pine and eucalypts. We must be able to increase the biodiversity and plant biomass discontinuity, factors that can contribute to a healthier forest, improve the conservation of natural resources and prevent forest fires. This would be more relevant if simultaneously it would be possible to increase employment and profits of local populations. The use of strawberry tree is not certainly the only solution, but could help in the

midst of other opportunities such as beekeeping, tourism and leisure and landscape fragmentation through agricultural practices.

Plants are an interesting source of bioactive compounds that have been used in the pharmaceutical, cosmetic and food industries. The potential of *A. unedo* and of other species considered as underutilized crops only know stars to be explored on this perspective. *In vitro* culture systems can be effective ways to produce interesting chemicals and the insights already obtained with *A. unedo in vitro* culture can be useful to explore this species as a source of bioactive compounds. Preliminary assays have indicated that callus cultures of *A. unedo* have the ability to convert hydroquinone into arbutin, a compound with interesting pharmaceutical applications.

6.3 REFERENCES

Agrama, H.A., Tuinstra, M.R., 2003. Phylogenetic diversity and relationships among sorghum accessions using SSRs and RAPDs. Afr. J. Biotechnol. 2, 334-340.

Águeda, B., Parladé, J.F., Fernández-Toirán, L.M., Cisneros, O., Miguel, A.M., Modrego, M.P., Martínez-Pena, F., Pera, J., 2008. Mycorrhizal synthesis between *Boletus edulis* species complex and rockroses (*Cistus* sp.). Mycorrhiza 18, 443-449.

Almeida, R., Gonçalves, S., Romano, A., 2005. *In vitro* propagation of endangered *Rhododendron ponticum* L. subsp. *baeticum* (Boissier & Reuter) Handel-Mazzetti. Biodiv. Conservation 14, 1059-1069.

Andrade, D., Gil, C., Breitenfeld, L., Domingues, F., Duarte, A.P., 2009. Bioactive extracts from *Cistus ladanifer* and *Arbutus unedo* L. Industrial Crops Products 30, 165-167.

Ayaz, F.A., Kucukislamoglu, M., Reunanen, M., 2000. Sugar, non-volatile and phenolic acids composition of strawberry tree (*Arbutus unedo* L. var.*ellipsoidea*) fruits. J. Food Compos. Anal. 13, 171-177.

Bassil, N.V., Boches, P., Rowland, L., 2006. Genetic diversity in the highbush blueberry evaluated with microsatellite markers. J. Amer. Soc. Hort. Sci. 131, 674-686.

Bennett, I.J., McComb, J.A., Tonkin, C.M., Mcdavid, D.A.J., 1994. Alternating cytokinins in multiplication media stimulates *in vitro* shoot growth and rooting of *Eucalyptus globulus* Labill. Ann. Bot. 74, 53-58.

Boches, P.S., Bassil, N.V., Rowland, L.J., 2005. Microsatellite markers for *Vaccinium* from EST and genomic library. Mol. Ecol. 5, 657-660.

Borchert, T., Hohe, A., 2009. Identification of molecular markers for the flower type in the ornamental crop *Calluna vulgaris*. Euphytica 170, 203-213.

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

Chalupa, V., 2003. *In vitro* propagation of *Tilia platyphyllos* by axillary shoot proliferation and somatic embryogenesis. J. For. Science 49, 537-543.

Chawla, H.S., 2009. Introduction to plant biotechnology, 3rd ed. Science Publis., Inc., New Hampshire.

Demirsoy, H., Demirsoy, L., Celikel, G., Koyuncu, T., 2007. Effects of dried on some properties of strawberry tree fruits. Asian J. Chemistry 19, 1777-1782.

Dickie, I.A., Fitzjohn, R.G., 2007. Using terminal restriction fragment length polymorphism (T-RFLP) to identify mycorrhizal fungi: a methods review. Mycorrhiza 17, 259-270.

El-Mousadik, A., Petit, R.J., 1996. Chloroplast DNA phylogeography of the argan tree of Morocco. Mol. Ecol. 5, 547-555.

El Haouari, M., López, J.J., Mekhfi, H., Rosado, J.A., Salido, G.M., 2007. Antiaggregant effects of *Arbutus unedo* extracts in human platelets. J. Ethnopharmacology 113, 325-331.

Eriksson, G., Ekberg, I., Clapham, D., 2006. An introduction to forest genetics. SLU. http://vaxt.vbsg.slu.se/forgen/, Uppsala.

Gahan, P.B., George, E.F., 2008. Adventitious regeneration. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 355-401.

Gajdošová, A., Ostrolucká, M.G., Libiaková, G., Ondrušková, E., 2007. Protocol for micropropagation of *Vaccinium vitis-idaea* L. In: Jain, S., Häggman, H. (Eds.), Protocols for Micropropagation of Woody Trees and Fruits. Springer, Berlin, pp. 457-464.

Garbaye, J., 1990. Pourquoi et comment observer l'état mycorhizien des plants forestiers. Rev. For. Fr. XLII 1, Biologie et Forêt, 35-47.

George, E.F., 2008. Plant tissue culture procedure - background 1. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 1-28.

George, E.F., De Klerk, G.-J., 2008. The components of plant tissue culture media I : macro and micro-nutrients. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 65-114.

George, E.F., Debergh, P.C., 2008. Micropropagation: uses and methods. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 29-64.

Gerlach, G., Musolf, K., 2000. Fragmentation of landscape as a cause for genetic subdivision in Bank Voles. Conservation Biology 14, 1066-1074.

Goltapeh, E.M., Danesh, Y.R., Prasad, R., Varma, A., 2008. Mycorrhizal fungi: what we know and what should we know? In: Varma, A. (Ed.), Mycorrhiza Genetics and Molecular Biology, Eco-function, Biotechnology, Eco-physiology, Structure and Systematics. 3rd ed. Springer, Berlin, pp. 3-27.

Gonçalves, J.C., Roseiro, R.J., 1994. Establishment and *in vitro* multiplication of *Arbutus unedo* L. seedlings. VIII International Congress of Plant Tissue and Cell Culture, Firenze, Italy, p. *Abst* 20.

Ingleby, K., Mason, P.A., Last, F.T., Fleming, V., 1990. Identification of ectomycorrhizas, ITE Research Publication nº 5. NE Research Council, HMSO Publ., London.

Jaakola, L., 2001. Effect of N^6-isopentenyladenine concentration on growth initiation *in vitro* and rooting of bilberry and lingonberry microshoots. Plant Cell Tis. Org. Cult. 66, 73-77.

Jordano, P., García, C., Godoy, J.A., García-Castaño, J.L., 2007. Differential contribution of frugivores to complex seed dispersal patterns. Proc. Nat. Acad. Sci. USA 104, 3278-3282.

Kivçak, B., Mert, T., 2001. Quantitative determination of [alpha]-tocopherol in *Arbutus unedo* by TLC-densitometry and colorimetry. Fitoterapia 72, 656-661.

Kosola, K.R., Workmaster, B.A.A., Spada, P.A., 2007. Inoculation of cranberry (*Vaccinium macrocarpon*) with the ericoid mycorrhizal fungus *Rhizoscyphus ericae* increases nitrate influx. New Phytologist 176, 184-196.

Lynch, M., 1990. The similarity index and DNA fingerprinting. Mol. Biol. Evol. 7, 478-484.

Machakova, I., Zazimalova, E., George, E.F., 2008. Plant growth regulators I: Introduction; auxins, their analogues and inhibitors. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 175-204.

Mereti, M., Grigoriadou, K., Nanos, G.D., 2002. Micropropagation of the strawberry tree, *Arbutus unedo* L. Sci. Hort. 93, 143-148.

Mylona, P., Dolan, L., 2002. The root meristem. In: McManus, M.T., Weit, B.E. (Eds.), Meristematic Tissues in Plant Growth and Development. Sheffield Academic Press, Sheffield, pp. 279-292.

Nemeth, G., 1979. Benzyladenine-stimulated rooting in fruit-tree rootstocks cultured *in vitro*. Z. Pflanzenphysiol. 95, 389-396.

Oliveira, I., Coelho, V., Baltasar, R., Pereira, J.A., Baptista, P., 2009. Scavenging capacity of strawberry tree (*Arbutus unedo* L.) leaves on free radicals. Food and Chemical Toxicology 47, 1507-1511.

Oliveira, I., Pereira, J.A., Bento, A., Baptista, P., 2010. Actividade sequestrante de radicais livres do medronho (*Arbutus unedo* L.). Actas Portuguesas Hortic. 16, 173-178.

Oliveira, P., Barriga, J., Cavaleiro, C., Peixe, A., Potes, A.Z., 2003. Sustained *in vitro* root development obtained in *Pinus pinea* L. inoculated with ectomycorrhizal fungi. Forestry 76, 579-587.

Ostrolucká, M.G., Gajdošová, A., Libiaková, G., Hrubíková, K., Bezo, M., 2007. Protocol for micropropagation of selected *Vaccinium*. In: Jain, S.M., Häggman, H. (Eds.), Protocols for Micropropagation of Woody Trees and Fruits. Springer, Berlin, pp. 445-455.

Özcan, M.M., HacIseferogullarI, H., 2007. The Strawberry (*Arbutus unedo* L.) fruits: Chemical composition, physical properties and mineral contents. J. Food Engineering 78, 1022-1028.

Pabuccuoglu, A., Kivcak, B., Bas, M., Mert, T., 2003. Antioxidant activity of *Arbutus unedo* leaves. Fitoterapia 74, 597-599.

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

Parladé, J., Pera, J., Luque, J., 2004. Evaluation of mycelial inocula of edible *Lactarius* species for the production of *Pinus pinaster* and *P. sylvestris* mycorrhizal seedlings under greenhouse conditions. Mycorrhiza 14, 171-176.

Parladé, X., Pera, J., De La Varga, H., Hortal, S., 2009. Tracking inoculated ectomycorrhizal fungi by Real-Time PCR. 14th European Congress on Biotechnology. New Biotechnology, Barcelona, Spain, p. *Abst* S375.

Pawlowska, A.M., Marinella, D.L., Braca, A., 2006. Phenolics of *Arbutus unedo* L. (Ericaceae) fruits: identification of anthocyanins and gallic acid derivatives J. Agric. Food Chem. 54, 10234-10238.

Peterson, R.L., Massicotte, H.B., 2004. Exploring structural definitions of mycorrhizas, with emphasis on nutrient-exchange interfaces. Can. J. Bot. 82, 1074-1088.

Preece, J., 2008. Stock plant physiological factors affecting growth and morphogenesis. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 403-422.

Quinteiro, S.L., 2005. Respuesta de siete orígenes ibéricos de *Pinus pinaster* Aiton frente a la inoculación en vivero con *Pisolithus tinctorius* y *Paxillus involutus*. PhD Thesis. University of Santiago de Compostela.

Ratnaparkhe, M.B., 2007. Blueberry. In: Kole, C. (Ed.), Genome mapping and molecular breeding in plants, Fruits and Nuts. Springer-Verlag, Berlin Heidelberg, pp. 217-227.

Ribeiro, M.M., Plomion, C., Vendramin, G.G., Szmidt, A.E., 2001. Variation in chloroplast single-sequence repeats in Portuguese maritime pine (*Pinus pinaster* Ait.) Theor. Appl. Genet. 102, 97-103.

Richard, F., Millot, S., Gardes, M., Selosse, M.A., 2005. Diversity and specificity of ectomycorrhizal fungi retrieved from an old-growth Mediterrean forest dominated by *Quercus ilex*. New Phytologist 166, 1011-1023.

Rincón, A., Parladé, J., Pera, J., 2005. Effects of ectomycorrhizal inoculation and the type of substrate on mycorrhization, growth and nutrition of containerized *Pinus pinea* L. seedlings produced in a commercial nursery. Ann. For. Sci. 62, 817-822.

S. Silva, J., Harrison, S.P., 2010. Humans, climate and land cover as controls on European fire regimes. In: S. Silva, J., Rego, F., Fernandes, P., Rigolot, E. (Eds.), Towards integrated fire management – outcomes of the European project fire paradox. European Forest Institute, Joensuu, pp. 49-59.

Sá, O., Malheiro, R., Baltasar, J.L., Pereira, J.A., Baptista, P., 2010. Actividade antioxidante de folhas de medronheiro (*Arbutus unedo* L.) de 19 genótipos provenientes da região de Bragança. Actas Portuguesas Hortic. 16, 179-183.

Smith, M.A.L., Eichorst, S.M., Rogers, R.B., 1992. Rhizogenesis pretreatments and effects on microcuttings during transition. Acta Hort. 319, 77-82.

Smith, M.A.L., Mcclell, M.T., Timerman, R., 1991. Anomalous root structure on woody plants *in vitro*. J. Environ. Hortic. 9, 61-64.

Smith, S.E., Read, D.J., 1997. Mycorrhizal Symbiosis, 2nd ed. Academic Press, Harcourt Brace and Comp. Pub., San Diego.

Van Staden, J., Zazimalova, E., George, E.F., 2008. Plant growth regulators II: cytokinins, their analogues and inhibitors. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 205-226.

Zawko, G., Krauss, S.L., Dixon, K.W., Sivasithamparam, K., 2001. Conservation genetics of the rare and endangered *Leucopogon obtectus* (Ericaceae). Mol. Ecol. 10, 2389 - 2396.

Ziv, M., Chen, J., 2008. The anatomy and morphology of tissue cultured plants. In: George, E.F., Hall, M.A., De Klerk, G.J. (Eds.), Plant propagation by tissue culture, 3rd ed. Springer, Dordrecht, pp. 465-477.

Zizzo, G., Aprile, S., La Mantia, A., S., L., 2010. The biodiversity of *Arbutus unedo* L. (Ericaceae Fam.) to develop new floricultural crops for the temperate areas. 28th International Horticultural Congress, Science and Horticulture for People, ISHS, Lisboa, p. *Abst* 417.