



## **UNIVERSIDADE DO ALGARVE**

### **Cryopreservation as a tool for preserving genetic variability of endangered species endemic from Algarve region**

Natacha Rodrigues Coelho

Tese para obtenção do grau de Doutor em Ciências Biológicas  
(Especialidade em Biotecnologia)

Trabalho efetuado sob a orientação de Professora Doutora Anabela Romano e  
coorientação de Professora Doutora María Elena González-Benito

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## **OUTLINE OF THE THESIS**

This thesis is divided in five chapters. Chapter 1 refers to a general literature review of the main subjects of the thesis. Chapters 2 to 4 describe the experimental work and the main results accomplished. Chapter 5 comprises a general discussion of the results and conclusions. This thesis was designed to be based on a number of papers that have been published, accepted or submitted for publication, and that will provide an account of the research that underpins this thesis. All the publications presented were written to stand alone and, therefore, the reader may find some repetition in parts of the manuscript.



## ABSTRACT

This thesis aims the development of complementary conservation strategies based on plant biotechnology to support the preservation of three rare endemic species of the Algarve, Portugal. The species studied were *Thymus lotocephalus* G. López & R. Morales, *Plantago algarbiensis* Samp. and *Tuberaria major* (Willk.) P. Silva & Rozeira. An *in vitro* propagation protocol was firstly developed for *T. lotocephalus* using seedlings as explants (Chapter 2.1). The germination conditions and feasibility of cryopreservation was also studied for *T. lotocephalus* seeds (Chapter 2.2). In Chapter 2.3 a shoot tip cryopreservation protocol was developed for this species and the genetic stability of the cryopreserved material was assessed. For *P. algarbiensis*, the work was initiated with the cryopreservation of seeds and nodal segments (Chapter 3.1), followed by the study of the genetic diversity of three wild populations (Chapter 3.2). Chapter 4.1 was dedicated to the improvement of the germination and cryopreservation of *T. major* seeds and the development of a shoot tip cryopreservation protocol.

An efficient micropropagation protocol was established for the mass production of *T. lotocephalus* (Chapter 2.1) and the seeds germination requirements were determined (Chapter 2.2). From the shoots produced *in vitro*, apices were excised and used in the development of a shoot tip cryopreservation protocol (Chapter 2.2). *P. algarbiensis* nodal segments were successfully cryopreserved using two different methods (Chapter 3.1). The structure of *P. algarbiensis* populations was effectively evaluated using molecular markers, revealing high levels of genetic diversity within populations (Chapter 3.2). Cryopreservation proved to be a suitable method for the conservation of seeds from both species (Chapters 2.2 and 3.1). Finally, the germination conditions and cryopreservation of *T. major* seeds were considerably improved and the cryopreservation of *T. major* shoot tips was accomplished without major optimizations of the different procedures (Chapter 4.1).

In conclusion, during the course of this thesis different *ex situ* conservation strategies were developed giving a major contribution to the preservation of *T. lotocephalus*, *P. algarbiensis* and *T. major*.

**Keywords:** *Ex situ* conservation, *in vitro* techniques, molecular markers, *Plantago algarbiensis*, *Thymus lotocephalus*, *Tuberaria major*.



## RESUMO

A biodiversidade está em risco um pouco por todo o planeta e as plantas não são exceção. As plantas são um elemento fundamental em qualquer ecossistema e os recursos fitogenéticos são a base da alimentação, para além de serem muito importantes em diversas atividades humanas. Vários fatores têm contribuído para a erosão das populações naturais e conseqüentemente dos recursos fitogenéticos, mas a principal causa é o desenvolvimento levado a cabo pelo Homem.

Recentemente tem aumentado a preocupação e a promoção para a implementação de medidas que contribuam para a conservação dos recursos fitogenéticos. Contudo, ainda existe muito trabalho a fazer e todos os esforços são essenciais.

De forma a manter o maior nível possível de diversidade genética de uma dada espécie, é importante existirem várias medidas de conservação que se complementem entre si. A conservação de biodiversidade pode ser dividida em duas grandes estratégias: *in situ* e *ex situ*. A conservação *in situ* refere-se à conservação da biodiversidade no seu habitat natural; enquanto a conservação *ex situ* consiste na conservação de biodiversidade fora do seu habitat natural, o que implica a colheita e armazenamento de material. Dentro destas estratégias, é importante referir a importância da biotecnologia no desenvolvimento de novas abordagens de conservação. A biotecnologia tem permitido desenvolver técnicas que em muito contribuem para a preservação de espécies: propagação *in vitro*, criopreservação, análises moleculares e marcadores moleculares.

Neste estudo foram escolhidas três espécies endémicas do Algarve, Portugal, para desenvolver estratégias de conservação *ex situ*, devido à sua vulnerabilidade e risco de extinção: *Thymus lotocephalus* G. López & R. Morales, *Plantago algarbiensis* Samp. e *Tuberaria major* (Willk.) P. Silva & Rozeira. Estas espécies estão legalmente protegidas por leis portuguesas e comunitárias, no entanto medidas adicionais são necessárias para sustentar a preservação destas três espécies raras. O principal objetivo deste trabalho é o desenvolvimento de estratégias complementares para a preservação destas espécies baseadas em técnicas de biotecnologia vegetal.

O primeiro passo foi o desenvolvimento de um protocolo de propagação *in vitro* para *T. lotocephalus* usando sementes como explante inicial (Capítulo 2.1). Para testar a capacidade de proliferação dos rebentos, foram testadas diferentes concentrações de meio Murashige and Skoog (MS), diferentes tipos e concentrações de citocininas e

diferentes combinações de citocininas/auxinas. A capacidade de proliferação dos rebentos foi de 100% em todos os meios testados. Embora os melhores resultados de proliferação tenham sido obtidos em meios suplementados com 6-benziladenina (BA), também se observaram elevadas percentagens de rebentos hiperhídricos e estes eram significativamente mais pequenos do que os produzidos em meio sem citocininas. Desta forma, o meio escolhido para a proliferação foi MS sem reguladores de crescimento, no qual se obteve elevada proliferação de rebentos saudáveis e com um comprimento considerável. No que respeita ao enraizamento, foi necessária uma fase suplementar para o induzir. Os melhores resultados foram obtidos em meio MS sem auxinas ( $92,00 \pm 6,11\%$ ,  $6,54 \pm 0,52$  e  $1,60 \pm 0,10$  cm para frequência de enraizamento, número de raízes por rebento e raiz mais comprida, respetivamente) ou suplementado com  $0,5 \text{ mg l}^{-1}$  3-indole ácido acético ( $98,00 \pm 2,11\%$ ,  $11,14 \pm 0,75$  e  $2,40 \pm 0,24$  cm, respetivamente). As plântulas obtidas foram aclimatizadas com sucesso às condições *ex vitro* com uma percentagem de sobrevivência de 93,33%.

Seguiu-se o estudo dos requisitos de germinação e da tolerância à criopreservação de sementes de *T. lotocephalus* colhidas de quatro populações (Algoz, Tunes, Belharucas e Gambelas) (Capítulo 2.2). As sementes foram colocadas a germinar a 15 e 25°C com um fotoperíodo de 16/8 h luz/escuro ou escuro constante. Verificou-se que a melhor temperatura de germinação foi 15°C, tanto na presença como na ausência de luz, com percentagens de germinação acima dos 80% e tempos médios de germinação inferiores a 10 dias. As sementes foram por isso consideradas não dormentes. Também se observaram poucas diferenças nos resultados entre sementes de diferentes populações. Para avaliar a sua tolerância à criopreservação, as sementes foram imersas diretamente em azoto líquido durante 30 dias, tendo-se concluído que as baixas temperaturas do azoto líquido não prejudicaram a germinação de sementes de *T. lotocephalus*.

Também foi desenvolvido um protocolo de criopreservação de ápices de *T. lotocephalus* no Capítulo 2.3. Foram testados três métodos diferentes, vitrificação-droplet, vitrificação e encapsulação-desidratação, sendo o primeiro aquele que apresentou os melhores resultados. As diferentes fases e condições de cada método foram posteriormente otimizadas, obtendo-se uma percentagem de recuperação de ápices criopreservados de 67%. Essas condições foram: quatro semanas de subcultura das plantas dadoras *in vitro* a 25°C, um dia de pré-cultura em meio MS suplementado com 0,3 M de sacarose dos ápices excisados, tratamento de 60 min em PVS2 (“plant

vitrification solution 2”) e meio MS suplementado com 0,2 de mg l<sup>-1</sup> de zeatina como meio de recuperação. A estabilidade genética dos ápices criopreservados foi também avaliada usando marcadores RAPD (“random amplified polymorphic DNA”). Não se observaram variações significantes apresentando os rebentos regenerados a partir de ápices criopreservados um desenvolvimento normal quando comparados com os rebentos *in vitro* que não sujeitos à criopreservação.

No caso de *P. algarbiensis*, como já existia um protocolo de propagação *in vitro* e já havia sido realizado o estudo dos requisitos de germinação, o trabalho incidiu na criopreservação de sementes e segmentos nodais (Capítulo 3.1). Verificou-se que a criopreservação de sementes não afetou negativamente a sua germinação e que este é um método viável para a conservação de material vegetal desta espécie. Os métodos testados na criopreservação de segmentos nodais, vitrificação-droplet e encapsulação-desidratação, apresentaram percentagens de recuperação de 60,0 ± 15,2% e de 63,3 ± 9,6%, respetivamente. Estes resultados foram obtidos após 120 min de exposição à PVS2, com o método vitrificação-droplet, e após 3 h de dessecação, com o método encapsulação-desidratação. Para ambos os casos, os segmentos nodais foram pré-cultivados em meio MS e, depois de criopreservados, foram cultivados em meio MS suplementado com 0,2 mg l<sup>-1</sup> BA para recuperar.

No capítulo 3.2, foi avaliada a diversidade genética de três populações selvagens de *P. algarbiensis* (Algoz, Tunes and Gambelas), usando marcadores RAPD. As amostras foram amplificadas com o recurso a 10 primers que geraram 145 marcadores, dos quais 80% eram polimórficos. Foram detetados elevados níveis de polimorfismo e a população de Tunes foi a que apresentou a percentagem de polimorfismo mais elevada (73,68%). Na análise de cluster, os indivíduos das populações de Tunes e Algoz agruparam-se juntos, formando um grupo, e os de Gambelas agruparam-se num grupo separado, o que está de acordo com as suas localizações geográficas. De acordo com os valores estimados tanto pelo índice de diversidade genética de Nei como pela medida de informação de Shannon, os níveis mais elevados de diversidade genética foram encontrados na população de Tunes e os mais baixos na população de Algoz. Valores semelhantes foram obtidos a partir do índice de Shannon e da análise AMOVA: a proporção de diversidade dentro das populações foi mais elevada do que entre populações, aproximadamente 86 e 14%, respetivamente. Foi estabelecida uma correlação entre distâncias geográficas e genéticas entre populações e o nível de fluxo

genético entre populações foi elevado e inversamente proporcional à distância entre populações.

Por fim, para *T. major*, como também já havia sido estabelecido um protocolo de propagação *in vitro*, o estudo focou-se no desenvolvimento de um protocolo de criopreservação de ápices, tendo-se testado os métodos vitrificação-droplet, vitrificação e encapsulação-desidratação (Capítulo 4.1). As percentagens de recuperação mais elevadas, 60 e 65%, foram obtidas usando os métodos vitrificação, após 60 min de exposição à PVS2, e encapsulação-desidratação, após 3 h de dessecação, respetivamente. Com o método vitrificação-droplet, obtiveram-se percentagens de recuperação abaixo dos 40%.

Os requisitos de germinação e a criopreservação de sementes de *T. major* também já tinham sido anteriormente estudados, embora as percentagens finais de germinação tenham sido relativamente baixas. Por esta razão, neste trabalho foi também investigado no Capítulo 4.1 como melhorar as condições de germinação e conseqüentemente a criopreservação de sementes. Verificou-se que a percentagem de germinação melhorou consideravelmente ao efetuar um pré-tratamento de escarificação das sementes (aproximadamente 95%), quando comparado com o pré-tratamento previamente usado, calor seco (aproximadamente 65%). Por conseguinte, a percentagem de germinação de sementes após criopreservação também foi melhorada, não sofrendo qualquer variação em relação à de sementes não criopreservadas.

Durante o presente trabalho foram conseguidas diversas abordagens viáveis que irão apoiar a conservação *ex situ* de *T. lotocephalus*, *P. algarbiensis* e *T. major*. Os protocolos desenvolvidos neste estudo, assim como outros anteriormente descritos, irão complementar a proteção legal de que todas estas espécies já possuem. Todas estas estratégias juntamente com uma apropriada gestão poderão prevenir e evitar a extinção destas espécies. Este trabalho é um contributo importante para a conservação de espécies endémicas em perigo.

**Palavras-chave:** Conservação *ex situ*, marcadores moleculares, *Plantago algarbiensis*, técnicas *in vitro*, *Thymus lotocephalus*, *Tuberaria major*.

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## ABBREVIATIONS

2,4-D – 2,4-dichlorophenoxyacetic;  
AFLP – amplified fragment length polymorphism;  
AMOVA – analysis of molecular variance;  
AMP – amplified DNA methylation polymorphism;  
ANOVA – analysis of variance;  
BA – 6-benzyladenine;  
bp – base pairs;  
CBD – Convention on Biological Diversity;  
CTAB – cetyl trimethylammonium bromide;  
DMSO – dimethyl sulphoxide;  
DNA – deoxyribonucleic acid;  
EDTA – ethylenediaminetetraacetic acid;  
FAO – Food and Agriculture Organization of the United Nations;  
GA<sub>3</sub> – gibberellic acid solution;  
GSPC – Global Strategy for Plant Conservation;  
IAA – indole-3-acetic acid;  
IBA – indole-3-butyric acid;  
ISSR – inter-simple sequence repeat;  
IUCN – International Union for Conservation of Nature;  
KIN – kinetin;  
LN – liquid nitrogen;  
LN+ – immersion in LN;  
+LN – cryopreserved plant material;  
-LN – non-cryopreserved plant material;  
LSD – least significant difference;  
MGT – mean germination time;  
MS – Murashige and Skoog medium (1962);  
NAA – 1-naphthalene-acetic acid;  
PA – Algoz population;  
PCR – polymerase chain reaction;  
PG – Gambelas population;  
PGR – plant genetic resources;  
PGRs – plant growth regulators;  
PT – Tunes population;  
PVP – polyvinylpyrrolidone;  
PVS – plant vitrification solution;  
PVS2 – plant vitrification solution 2;  
RAPD – random amplified polymorphic DNA;  
RAF – randomly amplified DNA fingerprinting;  
RFLP – restriction fragment length polymorphism;  
RNA – ribonucleic acid;  
SE – standard error;  
SIC – “Sítios de Interesse Comunitário” (Sites of Community Importance);  
SRAP – sequence-related amplified polymorphism;  
SSR – simple sequence repeat;  
TBE – tris-borate-EDTA buffer;  
TE – tris-EDTA buffer;  
Tween-20 – polyoxyethylenesorbitan monolaurate;  
UPGMA – unweighted pair group method analysis;

ZEA – zeatin;

ZPE – “Zonas de Proteção Especial” (Special Protection Areas).

# CHAPTER 1



**General introduction**



### **1.1. Conservation of plant genetic resources**

Plant genetic resources (PGR) represent the genetic material which determines the characteristics of plants and thus their ability to adapt and survive. According to the Food and Agriculture Organization of the United Nations (FAO) PGR are classified as the reproductive or vegetative propagation material of cultivated varieties (cultivars) in current use and newly developed varieties; obsolete cultivars; primitive cultivars (landraces); wild and weed species, near relatives of cultivated varieties; and special genetic stocks (FAO 2013).

Life on earth is dependent on plants, which are a crucial component of all ecosystems. Mankind is no exception and directly or indirectly we are dependent on PGR. Not only they are the basis of world food, but also can provide us fuel, clothes and medicine and play a major role in atmosphere and water purification and prevention of soil erosion. They also have cultural relevance in most societies. Besides the living, economical and social interest, plants are part of our natural heritage and it is our responsibility to preserve and protect them for future generations.

Worldwide, many plant species are threatened with extinction especially due to various human activities. Since the settlement of human populations, with the beginning of agriculture approximately 10,000 years ago, the domestication of animals and plants began to change the natural habitats, but the process was slow enough to create a new natural balance. However, with the rising of the population in the last century, the demand for food and other goods increased dramatically and a massive urban expansion and industrial development have been taken place since then. The outcome is the drastic reduction of the genetic diversity, which is the basis for natural selection and for plant improvement, all over the globe. This genetic erosion is thus a major concern and the loss is sometimes irreversible.

Several factors contribute to the genetic erosion of natural populations. The developmental human activities, such as urbanization, hydroelectric projects, road laying and clearing of wild vegetation for agriculture, contribute to habitat degradation and deforestation. Other concerns come from the spread of invasive alien species, unsustainable harvesting, hunting and fishing and more recently pollution and climate changes. The traditional local cultivars and landraces are also threatened by modern agriculture and introduction of new and uniform varieties (Cuttelod et al. 2008, Reed et al. 2011).

During the last decades, there has been a growing concern about the importance in preserving biodiversity and its sustainable use and development. An important asset in the conservation of PGR is the Red List of Threatened Plants, created by the International Union for Conservation of Nature (IUCN) and annually updated. According to this list, in 2013 there are more than 9,800 threatened plant species in the world, representing an increase in more than 80% since 1998, when the list was first created (IUCN 2013).

In June 1992, the Convention on Biological Diversity (CBD) was held for the first time in Rio de Janeiro, Brazil, and the basis for discussion and policy implementation were formed. The main goals of this convention were: conservation of biodiversity, sustainable use of its components and fair and equitable sharing of benefits arising from genetic resources (Glowka 1994, Ramanatha Rao and Hodgkin 2002). Following these achievements, the Global Plan of Action for the Conservation and Sustainable Utilization of PGR for Food and Agriculture was formally adopted by representatives of 150 countries during the Fourth International Technical Conference organized by FAO in Leipzig, Germany, in June 1996. The first report of the State of the World's PGR for Food and Agriculture was elaborated during this conference (FAO 1997) and recently updated in 2010 (FAO 2010).

More recently, in 2010, the conference to the CBD adopted the updated program Global Strategy for Plant Conservation (GSPC) 2011-2020, firstly endorsed in 2002 and signed by more than 180 countries. The initiative intends to reduce the loss of plant diversity by setting targets for understanding and conserving plant diversity, promoting their sustainable use, providing education and building capacity to support plant conservation (CBD 2010, Reed et al. 2011). Therefore, the GSPC five main objectives are: I) Plant diversity is well understood documented and recognised; II) Plant diversity is urgently and effectively conserved; III) Plant diversity is used in a sustainable and equitable manner; IV) Education and awareness about plant diversity, its role in sustainable livelihoods and importance to all life on Earth is promoted; and V) The capacities and public engagement necessary to implement the Strategy have been developed (CBD 2010).

The conservation of PGR is being promoted worldwide and many initiatives are being developed by international organizations. However there is still too much to be done and all efforts are important and essential.

## 1.2. Conservation strategies

Conservation of PGR requires the use of different strategies, supported by efficient technologies. Conservation measures are important and needed in the protection of natural habitats, endangered species and ecosystems, and in the sustainable utilization of biological resources for the benefit of mankind.

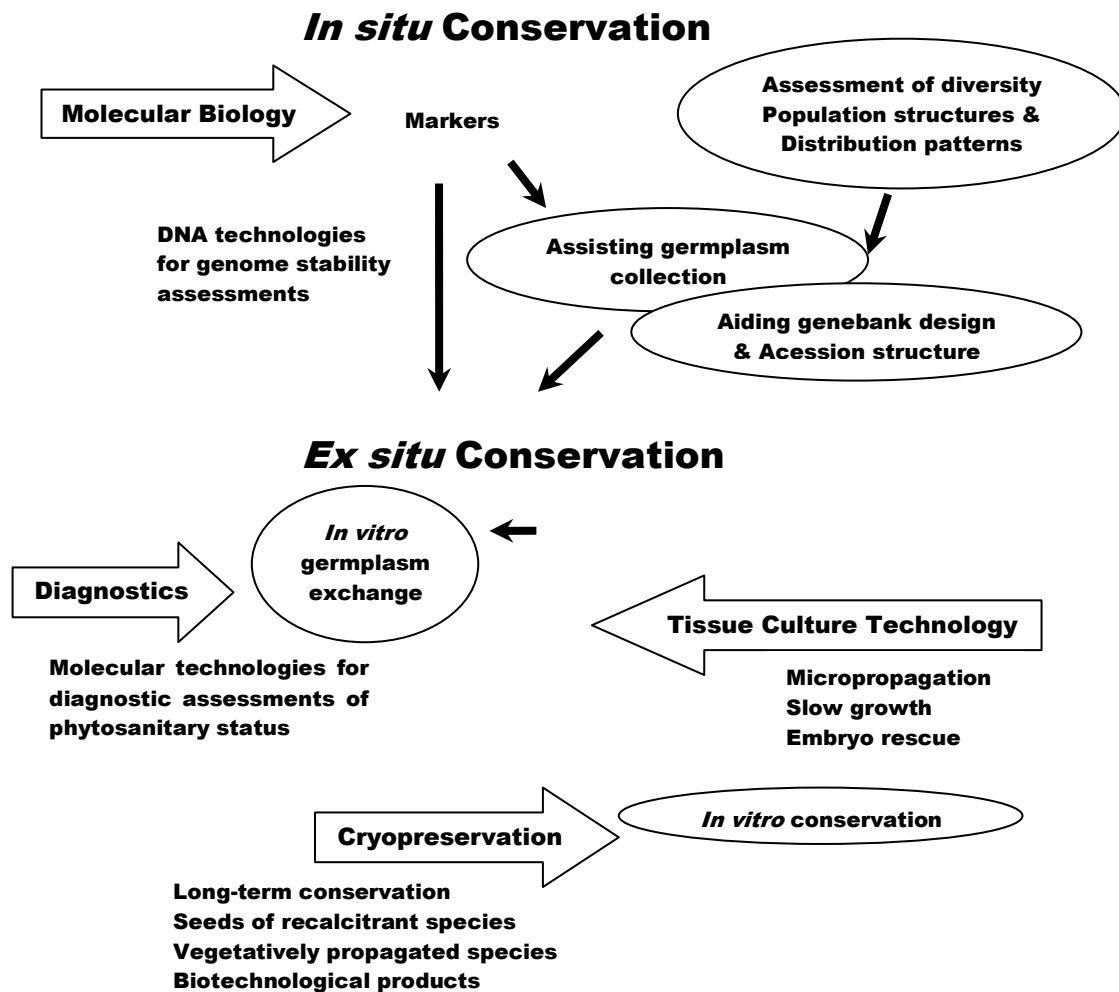
The two main conservation strategies are *in situ* and *ex situ* conservation, each including several techniques and approaches. *In situ* conservation is the conservation of ecosystems and biodiversity in their natural habitats; also includes cultivars in the locations where they developed their unique characteristics. *Ex situ* conservation refers to the conservation of biodiversity outside its natural habitat.

The choice of the conservation technique is dependent on the species distribution and ecology, the available resources, and the aims and potential utilization of the germplasm. However, all strategies have advantages and disadvantages and the use of a combination of techniques available, rather than only one, in the conservation programme of a given species is more favourable since the different methodologies complement and support each other (Maxted et al. 1997). Whatever the technique employed, an effective scientific and technical knowledge is needed. It is important to understand the species structure and distribution in nature, the material to be conserved, the techniques that can be used and the ways in which they can be applied (Ramanatha Rao and Hodgkin 2002).

The application of new conservation approaches based on biotechnology has been a major contribution to plant conservation. The purpose is not the replacement of traditional conservation methods but to complement and improve the means available (Benson 1999, Engelmann 2011). Biotechnology can provide tools not only for PGR conservation, but also in its management and utilization. The methods are employed in the collection, characterization, disease indexing, propagation, patenting, storage, documentation and exchange of PGR. Besides, the range of application goes from the conservation of endangered species to the storage of economically important crop plants or to genetically transformed material (Benson 1999).

The different applications of biotechnology in plant conservation are schematized in Figure 1.1. The main areas of biotechnology that contribute to plant conservation are: molecular diagnosis, molecular markers technology, *in vitro* culture and

cryopreservation (Benson 1999). The latter three techniques will be further illustrated in the text in separate sub chapters.



**Figure 1.1.** – Schematic representation of the use of biotechnological techniques in plant conservation (adapted from Benson 1999).

### 1.2.1. *In situ* conservation

*In situ* conservation involves the designation, management and monitoring of PGR in their own ecosystem. It allows the conservation of a high quantity of genotypes, while the evolution process in relation to environmental changes, pests and diseases continues in the natural habitat (Maxted et al. 1997).

The traditional and most appropriate approach to preserve wild species, including the ancestors of economically important plants, is in their genetic and geographical centres of origin (Maxted et al. 1997, Engelmann 2011). This is usually undertaken by national governments through nature conservancy and protection of wilderness. Sites declared as Natural or National Parks, World Heritage or Rede Natura 2000, are some examples of this conservation approach. The advantages are the conservation of a diverse range of wild plants and their ecosystems and the easy access for evolutionary and genetic studies. The drawbacks are the high level of active supervision and monitoring needed and the vulnerability to natural (floods, natural fires) and man induced (fire, vandalism, urban development, pollution) disasters (Maxted et al. 1997).

The maintenance of local varieties and their cultivation within traditional systems by farmers, in the region of origin, is also an *in situ* conservation technique and is known as on-farm conservation. This conservation approach allows the conservation of traditional landraces of field crops and of weedy crop relatives and ancestral forms. However, it is restricted to field crops, is vulnerable to changes in farming practices, requires maintenance of traditional farming systems, and the diversity maintained on each farm is limited (Maxted et al. 1997).

### **1.2.2. *Ex situ* conservation**

*Ex situ* conservation involves collecting whole organisms or their germplasm from their natural locations and maintaining them outside their original site in a growing and reproductive state or under storage conditions that suspend their growth. The main objective is to obtain a limited number of samples with the highest genetic diversity possible. However that will depend upon the genetic range of organisms or samples collected from the original *in situ* population. Some examples of *ex situ* conservation are: botanic gardens, field collections, seed storage and *in vitro* conservation (Maxted et al. 1997).

The advantages common to every *ex situ* conservation technique are the simple access to germplasm and the possibility to recover threatened or even extinct species in the wild. However, only the germplasm is preserved and not the ecosystem and the genetic diversity conserved is restricted (Maxted et al. 1997).

Seed storage is the most efficient, easy and economical method for the *ex situ* preservation of PGR and, because each seed is genetically different, it allows the regeneration of whole plants from genetically diverse material. For these reasons seed storage is the most widely used form of *ex situ* PGR conservation. The conditions for conventional seed bank storage, for long-term storage, are drying at 10-25°C and 10-15% relative humidity to 3-7% moisture content, followed by storage at about -18°C (FAO 1994, Pritchard 2007, Pritchard and Nadarajan 2008).

However, not all plant species can be preserved by this technique. Species with recalcitrant (desiccation-sensitive) and intermediate (relatively desiccation-tolerant) seeds cannot stand the desiccation conditions and cold storage without losing viability; only orthodox (desiccation-tolerant) seeds are able to do so. Besides, vegetatively propagated species are usually highly heterozygous and, in some cases, do not produce seeds (González-Benito et al. 2004, Engelmann 2011, Reed et al. 2011).

The traditional *ex situ* conservation method for these plant species is in the form of field collections (González-Benito et al. 2004, Engelmann 2011). Field collections preserve living plant material that was transferred and planted there, but collected in another location (Maxted et al. 1997). It usually requires large areas of land and is labour intensive; besides, collections are exposed to losses due to plague attacks or natural disasters, which limits its effectiveness (González-Benito et al. 2004).

*In vitro* conservation is therefore a feasible alternative for the conservation of plants when they cannot be stored using conventional seed-banking technologies (González-Benito et al. 2004, Reed et al. 2011). This approach consists in the maintenance of plant germplasm in culture collections using tissue culture technologies. *In vitro* conservation techniques can be divided according to the storage duration in: short-term storage, using standard tissue culture (*in vitro* propagation); medium-term storage, achieved by reducing growth, thus increasing intervals between subcultures; and long-term storage, by cryopreservation in liquid nitrogen (LN) (González-Benito et al. 2004, Engelmann 2011). *In vitro* conservation provides easy access for evaluation and utilization and safe exchange of plant material. The main disadvantage is the need to develop individual maintenance protocols for the majority of species (Maxted et al. 1997, González-Benito et al. 2004, Reed et al. 2011).

Besides field collections, living plant material is also kept worldwide in botanic gardens. For centuries botanic gardens have been exhibiting plants more out of curiosity

rather than for conservation purposes. Nevertheless, the increasing concern with environmental and conservation matters in the last decades is leading to the creation of conservation divisions within botanic gardens.

In genebanks, plant germplasm is preserved in an active or inactive state under the form of seeds, pollen, embryos, vegetative propagules, buds and others. There are several categories of genebanks, depending on the conservation technique, the category of plant material preserved and the type of facility. The most common types of conservation facilities are: seed banks, *in vitro* genebanks, cryobanks, field genebanks, cultivation in controlled environments, pollen storage and DNA banks (Maunder et al. 2004).

### **1.3. *In vitro* propagation**

*In vitro* propagation or micropropagation can be defined as the vegetative propagation of plants from cells or organs in a controlled environment, through the use of aseptic techniques and proper containers, and in a defined medium. The technique is based on the totipotency theory, which states the capacity of a single cell to regenerate the phenotype and genotype of the original organism, and originates a new plant.

There are three different micropropagation techniques, meristem culture, organogenesis and somatic embryogenesis.

An *in vitro* propagation protocol can be divided into five stages (Debergh and Read 1991):

- Stage 0 – the preparative stage;
- Stage 1 – initiation of cultures;
- Stage 2 – multiplication;
- Stage 3 – elongation, root induction and development;
- Stage 4 – acclimatisation.

Micropropagation begins with the selection and preparation of plant material to be propagated – stage 0. Mother plants, or their parts, are raised under more hygienic conditions, by the use of sterilization agents and growing them in greenhouses, to fight possible contaminations before culture initiation. During this phase it is also important to manipulate certain parameters, such as temperature, light, photoperiod and growth regulators, to make an explant more reactive to *in vitro* growth (Debergh and Read 1991).

On stage 1 the main objectives are to establish cultures in aseptic conditions without contaminations and to produce a considerable amount of explants with high proliferation ability. The development, physiological age and size of the initial explant are very important in this stage and can determine the success of the *in vitro* cultures (Debergh and Read 1991). The explants are initially surface disinfected with different agents to minimize contaminations before inoculation on a sterile basal medium. The medium should include a support material consisting of a semisolid or liquid medium; a mineral salt mixture with essential major and minor elements; an energy source, normally sucrose; and vitamin supplements (Hartmann et al. 2011). The most often used growth regulators in this phase are cytokinins, as they promote the formation and development of axillary shoots (Debergh and Read 1991).

After several weeks in culture, depending upon the plant, the mass culture is divided and subcultured on fresh medium. This process is repeated until a uniform, well-growing culture is produced. The explants are then ready to be transplanted to stage 2 (Hartmann et al. 2011).

The second stage is characterized by the fragmentation and distribution of the plant material in new media to exponentially increase the number of shoots with high proliferation capacity, keeping their genetic patrimony. The medium used is essentially the same as that used in stage 1, but often the growth regulators supplement is increased (Hartmann et al. 2011). The concentration and ratio of cytokinin to auxin are especially important during this stage; their adjustment will promote the development of axillary shoots, reduce terminal shoot elongation and diminish rooting potential (Debergh and Read 1991, Hartmann et al. 2011). Multiplication may be repeated several times to increase the supply of material to a predetermined level for subsequent rooting and transplanting (Hartmann et al. 2011). However, successive subcultures can reduce the multiplication capacity of the culture.

The third stage consists in the elongation of the produced shoots and their rooting. Frequently, elongation can be obtained by transfer of isolated shoots from the multiplication medium to an appropriate elongation medium, devoid of cytokinins (Debergh and Read 1991). Rooting can be attained *in vitro*, whether combined with elongation or not, or *ex vitro*. Usually rooting involves a medium in which the auxin level is increased and the cytokinin is decreased or suppressed (Hartmann et al. 2011).

Plantlets growing *in vitro* are largely heterotrophic since they obtain their energy from sucrose present in the medium and therefore their photosynthetic activity is very low. These plantlets are also exposed to a very high relative humidity. All together, these aspects make the *in vitro* plantlets very sensitive to *ex vitro* transplantation. In the final stage of plant micropropagation, the plantlets should be kept in very high humidity conditions and gradually exposed to a natural environment (Hartmann et al. 2011).

As an alternative to the use of explants as starting material, micropropagation can be initiated from seeds. The activation of the metabolic machinery of the seed embryo leading to a new seedling plant is known as germination. Seeds are the ideal starting material for *in vitro* propagation when the species conservation is a concern. For germination to be initiated the seeds must be viable and subjected to the appropriate environmental conditions. Seeds are often under a dormancy state which must be overcome during this process. Dormancy prevents immediate germination and regulates the time, conditions and place that germination will occur (Hartmann et al. 2011). The use of specific treatments, like stratification, temperature or chemical treatment, will break this state and consequently germination takes place.

One of the major drawbacks of *in vitro* propagation is the risk of somaclonal variation. Genetic changes may occur when organized plant cells are introduced into culture and forced into a phase of cell division by growth hormones (Larkin and Scowcroft 1981, Scowcroft 1985). Those variations are expressed in changes in plant morphology, number of chromosomes, accumulation of gene mutations, levels of gene expression in ribonucleic acid, protein profiles and molecular changes in DNA sequences (Harding 2004).

Nevertheless, *in vitro* propagation is a fast process; from a single explant it is possible to obtain thousands of plants in a short period of time. This feature is extremely important for endangered plants, because it avoids their collection from the natural environment for investigation and allows the production of plants to replenish natural populations. Plants free from contamination, the possibility to propagate species impossible to clone *in vivo* and the use of a reduced storing space, comparing to field collections, are other advantages of micropropagation.

#### 1.4. Cryopreservation

Cryopreservation is a type of long-term *ex situ* conservation in which viable biological resources, cells, tissues, organs and whole organisms (e.g. microorganisms and algae), are stored at ultra low temperatures in LN (-196°C) and/or its vapour phase (-150°C) (Benson 2008a, Engelmann 2011). The extreme low temperatures slow down significantly metabolic processes and as a result, living material can be stored without deterioration, in theory, for an unlimited period of time. In addition, the material is stored in a small space, protected from contamination and requiring limited maintenance (Engelmann 2011).

The long-term storage of living cells at low temperatures became a practical possibility when Polge and co-workers (1949) discovered that fowl spermatozoa could survive freezing if glycerol was included in the suspending medium (Fuller 2004, Benson 2008a). In the following years, several investigations were carried out to evaluate the role of the cryoprotectants using different animal cells and tissues. Another important breakthrough was the application of dimethyl sulphoxide (DMSO) as a cryoprotectant by Lovelock and Bishop (1959) (Benson 2008a, Heine-Dobbernack 2008, Reed and Uchendu 2008).

Plant cryopreservation procedures were firstly developed following the success of animal cell cryopreservation. The first report on plant cryopreservation was published by Sakai in 1960 and the first successful *in vitro* cryopreservation experiment was carried out by Quatrano in 1968 (Engelmann et al. 2008, Heine-Dobbernack 2008). In the 1980s, protocols that included pretreatment with cryoprotectants followed by controlled rate cooling were developed and applied for storing cultured cells and apical meristems from several species of temperate origin. However, for some species, such as those of tropical origin, the controlled rate cooling method could not be successfully applied. As a result vitrification-based protocols, which are relatively simpler to apply and less expensive, were later developed in the beginning of the 1990s (Engelmann et al. 2008, Sakai et al. 2008). Also in 1990, Sakai and co-workers (1990) developed the most widely used solution for plant cryopreservation, plant vitrification solution 2 (PVS2).

Nowadays, different types of plant cell, tissues and organs can be cryopreserved. Shoot tips excised from *in vitro*-grown plants are the most commonly used for vegetatively propagated species (González-Benito et al. 2004).

### **1.4.1. Cryopreservation techniques**

An efficient cryopreservation protocol is accomplished when cells can be cooled and recovered from ultra low temperatures, after a long period, without suffering damages or changes in their metabolism and structure.

Because of their cellular constitution and high water content, most plant cells/structures, such as cell suspensions, calli, shoot tips, and embryos, are extremely vulnerable to freezing injury and therefore more difficult to cryopreserve (Heine-Dobbernack 2008, Engelmann 2011). The main cause of cellular damage is the transition of water to ice during the cooling process (Fuller 2004). To prevent the formation of ice crystals, cells have to be dehydrated and exposed to cryoprotectant solutions. The exceptions are, for instance, orthodox seeds and dormant buds that possess natural dehydration processes and can be directly cryopreserved without pretreatment (Engelmann 2011).

To improve the efficiency of cryoprotectants and consequent survival of cryopreserved germplasm, also several preconditioning strategies can be used to prepare plant material before cryoprotection. Some examples are cold acclimation or hardening, sucrose-simulated acclimation and osmotic adjustments. Furthermore, for *in vitro*-grown material it is extremely important the standardization of culture conditions (age, morphogenetic status, subculture period, light and nutritional requirements) and the size of the explants or cell culture samples (Benson 2008a, Reed and Uchendu 2008).

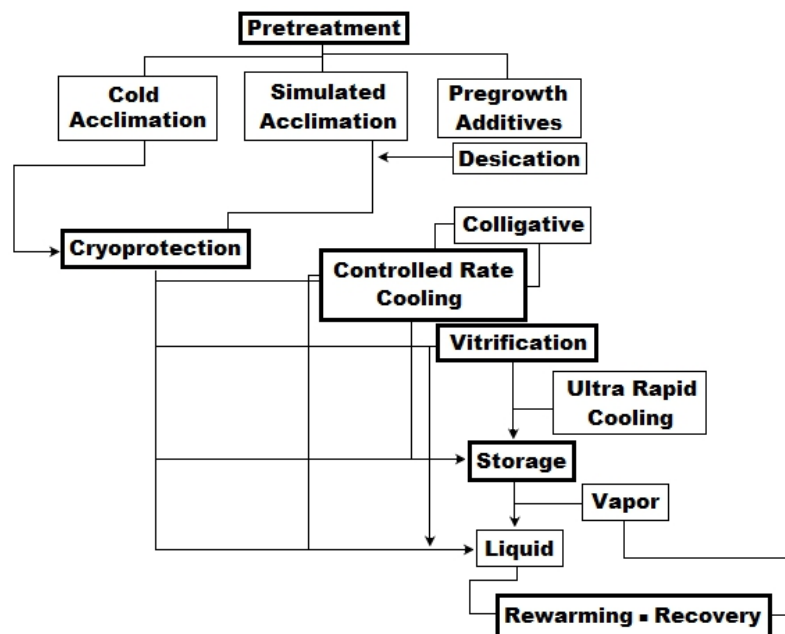
Cryopreservation techniques can be divided in two: classical techniques, based on the use of cryoprotectants combined with freeze-induced dehydration, followed by immersion in LN; and new techniques, based on vitrification, where samples are usually plunged directly in LN (González-Benito 2004, Benson 2008a, Engelmann 2011).

Rewarming of cryopreserved samples can be done slowly at room temperature or by a rapid system. For recovery, it is usual the employment of growth regulators to improve survival and regrowth (Benson 2008a). Figure 1.2 shows the basic steps in the development of a cryopreservation protocol.

#### **1.4.1.1. Classical cryopreservation techniques**

The classical cryopreservation techniques can be named controlled rate cooling, slow cooling or two step cooling (Reed 2008). These methods combine chemical cryoprotection, to regulate cell water content, and slow cooling, to induce cell

dehydration, followed by immersion in LN. After pretreated with cryoprotectant solutions, samples are slowly cooled at a standard rate to a defined prefreezing temperature. At the freezing point of the cryoprotectant solution, ice is formed in the external medium and intercellular spaces. The cell wall prevents ice crystals from damaging the cell interior and the cytoplasm remains unfrozen due to solute concentration. As the temperature decreases, the formation of ice in the extracellular solution increases and cells gradually lose water to the exterior, leading to a cytoplasm further concentrated and free from ice. Once samples are plunged into LN, most of the freezable water was removed from the cell interior, thus avoiding the formation of ice and its damages (Reed and Uchendu 2008, Engelmann 2011). The different stages of the controlled rate cooling method are: pregrowth of samples, cryoprotection, slow cooling (for instance at a rate of  $0.5\text{-}2.0^{\circ}\text{C min}^{-1}$ ) to a determined prefreezing temperature (normally  $-40^{\circ}\text{C}$ ), rapid immersion of samples in LN, storage, rapid thawing and recovery (Engelmann 2011).



**Figure 1.2.** – Schematic for the different cryopreservation routes (adapted from Benson 2008a). Vitrification is a general term referring to the new techniques.

The main advantages of the controlled rate cooling method, once the protocol is optimized and programmable freezers are used, are the use of standardized procedures, the cryopreservation of large batches of samples at a time and the effective use of

technician time (Benson 2008a, Reed and Uchendu 2008). The programmable freezers are though sophisticated and very expensive, requiring a high initial investment in equipment. Another disadvantage of this method is that many types of plant material, mainly larger structures that comprise different cell types, are not effectively cryopreserved. Controlled rate cooling method is usually more efficient in the cryopreservation of dedifferentiated cultures, such as cell suspensions and callus cultures (González-Benito et al. 2004, Benson 2008a, Engelmann 2011).

#### **1.4.1.2. New cryopreservation techniques**

The new cryopreservation techniques are based in vitrification. Vitrification is a physical process in which liquids solidify without crystallization: the solution becomes an amorphous glass that possesses the mechanical and physical properties of a solid but without the same structure. This “glassy state” is achieved by increasing cell viscosity, i.e., increasing cell solution concentration. Dehydration of plant material is thus performed before freezing by exposure to highly concentrated cryoprotective solutions and/or air desiccation, followed by rapid cooling, generally by direct immersion in LN. This will enable water to solidify into a meta-stable glass, without ice crystal formation (Benson 2008b, Sakai et al. 2008, Engelmann 2011).

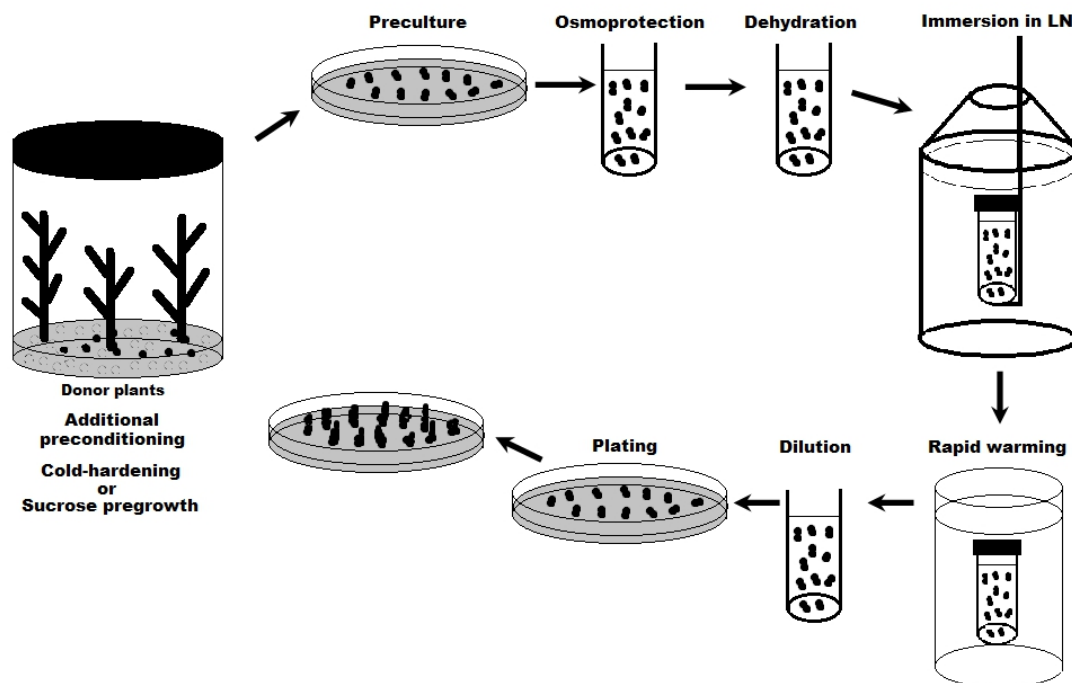
Cryopreservation techniques based on vitrification have several advantages comparing to the controlled rate cooling method. No special equipment is needed to control the slow cooling process and therefore costs are much lower and the process is less complex, a standard *in vitro* culture laboratory is sufficient to perform the experiments. Besides, these new techniques can be applied to more complex plant material with different cell types, such as shoot tips (González-Benito et al. 2004, Benson 2008b, Engelmann 2011).

The new cryopreservation techniques can be divided in two major types: vitrification (*sensu stricto*) and encapsulation-dehydration. Combinations and derivations of both procedures have also been developed, such as droplet-vitrification and encapsulation-vitrification.

**a) Vitrification (*sensu stricto*)** – The vitrification technique consists in the exposure of plant material to highly concentrated cryoprotectant solutions for short periods. The

most widely used solutions are the glycerol-based vitrification solutions developed by Sakai and co-workers (1990) in a series known as plant vitrification solution (PVS), from which PVS2 is the most widely used (Sakai and Engelmann 2007, Sakai et al. 2008).

The complete vitrification procedure comprises several steps, schematically represented in Figure 1.3 for *in vitro*-grown shoot tips. Initially, tissues are cultured on medium with high concentrations of sucrose or other osmotic agents to induce desiccation tolerance and then transferred to a glycerol-sucrose solution, the loading solution, for osmoprotection. Dehydration is achieved by exposing the tissues to highly concentrated cryoprotectant solutions, such as PVS2. Samples (inside cryovials) are then rapidly transferred to LN. After a rapid rewarming, essential to avoid recrystallization of cellular solutes and usually in a water bath at 40°C, the vitrification solution is replaced by an unloading solution for dilution. Samples are finally transferred to fresh recovery medium (Sakai and Engelmann 2007, Sakai et al. 2008).



**Figure 1.3.** – Schematic representation of a vitrification procedure for shoot tips (adapted from Sakai et al. 2008).

In the development of a cryopreservation protocol by vitrification, all steps of the process (preculture, loading, exposure to the vitrification solution, unloading) have to be

optimized. The optimal equilibrium is achieved when dehydration by the vitrification solution able cells to vitrify upon rapid cooling in LN but without damages, due to the high toxicity of some vitrification solutions (Sakai and Engelmann 2007).

The vitrification technique is a fast procedure since the time needed to dehydrate samples is reduced, and the levels of recovery are high (Sakai et al. 1990, Reed 2004). It has been used in the cryopreservation of a great number of tropical plants, which normally are not easily cryopreserved using the classical methods (Sakai and Engelmann 2007). On the other hand, this technique requires careful timing of solution changes making difficult to handle simultaneously a large number of samples, besides the difficulty in manipulating the small explants. Some cryoprotectant solutions can be toxic to certain plants (Reed 2004, Benson 2008a).

**b) Droplet-vitrification** – The droplet-vitrification technique is the most recent cryopreservation procedure (Sakai and Engelmann 2007). This technique can be considered as a combination of the previously vitrification procedure and the droplet-freezing technique, developed by Kartha et al. (1982), in which plant material is placed in droplets of cryoprotective solution and cooled slowly in a programmable freezer. After loading and pretreatment with a vitrification solution, usually PVS2, like in the vitrification procedure, the droplet-vitrification protocol consists in the transfer of plant material to droplets of vitrification solution placed on small aluminium foil strips, before rapid immersion in LN. Rewarming takes place at room temperature in unloading solution, followed by transfer to fresh medium for recovery (Sakai and Engelmann 2007, Engelmann 2011).

The main advantage of this technique comes from the fact that a minimum volume of cryoprotective solution containing the plant material is used, allowing ultra rapid cooling and warming rates (Sakai and Engelmann 2007, Benson 2008a). However, this technique requires the direct exposure of the set droplet/plant material to LN, which can carry a contamination risk (Benson 2008a).

Droplet-vitrification is currently one of the most used cryopreservation procedures and has a great success in the cryopreservation of shoot tips from crop plants (Benson 2008a).

**c) Encapsulation-dehydration** – The encapsulation-dehydration technique was developed by Fabre and Dereuddre (1990) and is based on the artificial seed technology. Plant material is encapsulated in calcium alginate beads and cultured in a highly concentrated sucrose solution. Before rapid exposure to LN, beads are partially dehydrated either in the air flow of the laminar flow cabinet or with silica gel to a water content around 20% (fresh weight basis). Rewarming can be slow, in the laminar flow cabinet, or rapid, in a warm water bath. After culture in fresh recovery medium, regenerating explants can be excised or grow out of the beads (González-Benito et al. 2004, Engelmann et al. 2008, Engelmann 2011).

This method is an appropriate choice to cryopreserve germplasm sensitive to the toxicity of some cryoprotectant solutions used in the vitrification procedures. Another advantage is the simplification of the rewarming step, because alginate beads containing plant material once properly dehydrated form a stable structure which prevents devitrification during the process and therefore the formation of ice crystals. In addition, due to the larger size of the alginate beads, encapsulated explants are very easy to manipulate. However, this technique has several steps and is demanding in technician time, each bead needs to be handled several times and can take up to two days to carry out the entire protocol (Reed 2004, Benson 2008a).

The encapsulation-dehydration technique has been applied to numerous plant species from temperate and of tropical origin, especially in the cryopreservation of shoot tips, but also to cell suspensions and somatic embryos of several species (Gonzalez-Arno and Engelmann 2006).

**d) Encapsulation-vitrification** – The encapsulation-vitrification technique is a combination of encapsulation-dehydration and vitrification procedures, in which samples are encapsulated in alginate beads and then subjected to dehydration using vitrification solutions, merging advantages of both procedures. This technique was developed by Sakai and co-workers (2008) and the general protocol comprises pregrowth of explants with sucrose, followed by alginate encapsulation, exposure to loading solution and dehydration with PVS2. Beads (inside cryovials) are then rapidly plunged in LN. For rewarming, cryovials are immersed in a water bath at 35-45°C and the PVS2 solution is then replaced by an unloading solution. After unloading, beads are

transferred to fresh recovery medium. This method has been successfully applied to a wide range of plant species (Sakai and Engelmann 2007).

#### **1.4.2. Seed cryopreservation**

As previously stated, seed storage is the easiest, most economical and widely used *ex situ* conservation method, but it cannot be applied to recalcitrant or intermediate seeds. Cryopreservation can provide an alternative, viable and, most likely, the only option for long-term storage of non-orthodox seeds. In addition, cryogenic storage prolongs dry seed longevity compared to conventional freezer storage as theoretically seed deterioration is greatly slowed down at cryopreservation temperatures, and, therefore, can greatly contribute to the conservation of orthodox seeds (Pritchard 2007, Normah and Makeen 2008, Pritchard and Nadarajan 2008).

The cryopreservation of orthodox seeds is relatively simple. After dehydration to water contents below the high moisture freezing limit ( $\approx 70$ -80% relative humidity), dry seeds can be directly transferred to LN. Dried seeds do not contain water that can be converted into ice crystals and injure the cells. Rewarming can be performed at ambient laboratory conditions until seeds equilibrate to room temperature, before sowing. Only some lipid-rich seeds may require slow cooling and warming (Pritchard 2007).

As for recalcitrant seeds, the cryopreservation procedure can be much more complex and difficult, and is dependent on the seeds capacity to survive cryogenic stress and dehydration. The same basic principles of cryoprotection to prevent ice crystals formation previously described for other plant material, applies to recalcitrant seeds (Benson 2008a, Walters et al. 2008). Research in this field is still limited and there is a great number of wild species with recalcitrant seeds that little or no information is available on their storage behaviour (Engelmann 2011). An important approach, often employed in recalcitrant large seeds, that cannot be frozen directly, is the use of desiccated embryos or embryonic axes (Normah and Makeen 2008, Engelmann 2011). In all cases, the optimization of the desiccation and cooling conditions must be highly controlled to succeed in the cryopreservation of recalcitrant seeds and their derivate germplasm.

### 1.4.3. Genetic stability of cryopreserved plant material

During the cryopreservation process plant cells are exposed to a series of physical, chemical and physiological stresses, which can cause cryoinjury and result in the loss of the genetic integrity of the cryopreserved material. Therefore it is very important to assess the genetic stability of cryopreserved plant material comparing to the same material before storage (Harding 2004, Engelmann 2011).

Presently, there is no clear evidence that morphological, cytological or genetic alterations are a consequence of cryopreservation itself (Harding 2004). The whole cryopreservation process includes several stages, from the *in vitro* culture until the regeneration of complete plants, and in any of these stages plant cells may suffer variations. The somaclonal variation phenomenon (Larkin and Scowcroft 1981) which can occur during *in vitro* culture (mentioned above), before cryopreservation or after, during regeneration, may lead to a number of variations at the cytological, phenotypic, biochemical and molecular levels (Scowcroft 1985, Harding 2004). As for the cryopreservation technique, which comprises cryoprotection, freezing and thawing, plant cells can suffer from mechanical damages, severe dehydration and cellular disruption caused by the osmotic agents, toxic cryoprotectants, extreme temperatures and/or others steps of the procedure (Harding 2004).

There are several techniques, phenotypic, cytological, biochemical and molecular, that can be employed to assess the genetic stability of cryopreserved plant material. Phenotypic variation can be evaluated by examining different morphological characters per plant and using biometric analysis. Cytological techniques include several procedures to detect chromosomal instability. Biochemical techniques compare the products of gene expression from cryopreserved and original plants in metabolite and protein/enzyme analysis. The molecular analysis comprises several hybridization and polymerase chain reaction (PCR) techniques, including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and restriction fragment length polymorphism (RFLP) analysis (Harding 2004).

The various studies so far reporting the assessment of genetic stability after cryopreservation show that there are no differences or very few variations in the cryopreserved plant material compared to the original material before storage (Harding 2004, Engelmann 2011).

### 1.5. Molecular markers

Molecular markers techniques are an important asset in the conservation of PGR. The understanding of the amount and distribution of plant genetic variation can greatly help in the design of effective conservation programmes and in their improvement and management. *In situ* conservation benefits from data concerning population structures and gene distribution patterns within ecosystems. The molecular knowledge of genetic diversity can also assist in germplasm collection and genebank management of *ex situ* conservation approaches (Benson 1999, Harris 1999). In addition, as previously stated, molecular markers techniques are used in the assessment of genetic stability of preserved plant germplasm.

Molecular markers can be divided in two groups: markers based on DNA-DNA hybridization techniques and markers based on PCR (Harding 2004, Poczai 2013).

RFLP analysis, included in the first group, uses restriction enzymes to digest the DNA combined with a hybridisation probe, detecting variations in primary DNA structure. Previous to hybridisation, the DNA probe is labelled either radioactively or with a non-radioactive chemical. The detection of homologous genomic DNA sequences after DNA probe hybridisation generates an informative DNA fragment profile (Harris 1999, Harding 2004).

Within the PCR-based group, there are the arbitrarily amplified markers techniques, such as RAPD and AFLP, and the specific amplified markers techniques, such as SSR.

RAPD analysis utilizes single, arbitrary primers of around ten nucleotides to amplify regions of the genome using PCR. Priming sites are thought to be randomly distributed throughout the genome and polymorphism results in different amplification products (Harris 1999). This technique does not require a previous knowledge of the species genome, which is particularly advantageous for wild species. In addition, it generates markers randomly over the whole genome, sampling multi loci, and a large quantity per sample (Harris 1999, Poczai 2013). It is a simple and fast technique which has been widely used in the characterization and analysis of populations.

AFLP combines RFLP and RAPD characteristics. Arbitrary subsets of restriction fragments, generated by restriction enzymes digestion of DNA, are selectively amplified using primers complementary to sequences that have been ligated to each end.

Polymorphic DNA fragments are obtained after two consecutive amplifications (Harris 1999).

SSR or microsatellite markers are short tandem repeats of DNA of one to four nucleotides, which are assumed to be randomly and abundantly distributed throughout the genome. Characteristically they are robust PCR markers, co-dominant, single locus, simple and highly polymorphic (Harris 1999, Harding 2004).

### 1.6. The Algarve

The Algarve is the most southern region of mainland Portugal, located southwest in the Iberian Peninsula, with an area of approximately 5,000 km<sup>2</sup>. It is delimited by the Alentejo region in the north, the Spanish Huelva province in the east and the Atlantic Ocean in the south and west. The coast line is approximately 320 km and the region is divided from the rest of the Portuguese territory by groups of small mountains, granting a natural isolation with particular conditions. The permanent population is around 450,000 inhabitants, value that may triplicate during the high touristic season. The weather is Mediterranean type with dry summers, short periods of rainfall and soft winters.

The Algarve is located within the Mediterranean region, which was recognized as one of the first 25 Global Biodiversity Hotspots. In the Mediterranean there are about 25,000 native species of plants, from which more than half are endemic (Cuttelod et al. 2008). The peripheral geographical location of the Algarve in the extreme west of the Mediterranean region bounded by the Atlantic Ocean and close to the north of Africa gave the region exceptional conditions for the development of a rich biodiversity. The flora present in the region results from the combination of species originated from the different biogeographical surrounding locations. There is a considerable number of species and habitats that are nationally and globally restricted to the Algarve. Besides, there are important populations of rare and threatened species whose global conservation is dependent upon the conservation in the Algarve (CCDR Algarve 2004). Some examples of endemic plant species are: *Thymus lotocephalus*, *Plantago algarbiensis*, *Tuberaria major*, *Centaurea occasus*, *Narcissus willkommii* and *Sideritis arborescens subsp. lusitanica* (Gomes and Ferreira 2005). The first three examples are also rare species.

The major threat to conservation of biodiversity in the Algarve is industrial and urban expansion, with a bigger impact in the southern coast. Usually urban expansion implies the complete and irreversible destruction of habitats and populations, leading to their disappearance. The desertion of traditional agriculture in some areas along with intensive agriculture in more productive areas and the intensive forestation with alien species, such as eucalyptus, also contribute for the destruction of biodiversity. Fires, which are characteristic to Mediterranean ecosystems, have increased in the last decades, devastating several important wild life locations. The development of infrastructures, such as dams, dikes and roads, and mining extraction in the interior also affect and may destroy natural habitats. In recent years, climate change is also becoming a major concern in the conservation of biodiversity worldwide. Whether the cause is natural or anthropogenic, the weather is changing and the consequences to the environment are still unknown (CCDR Algarve 2004).

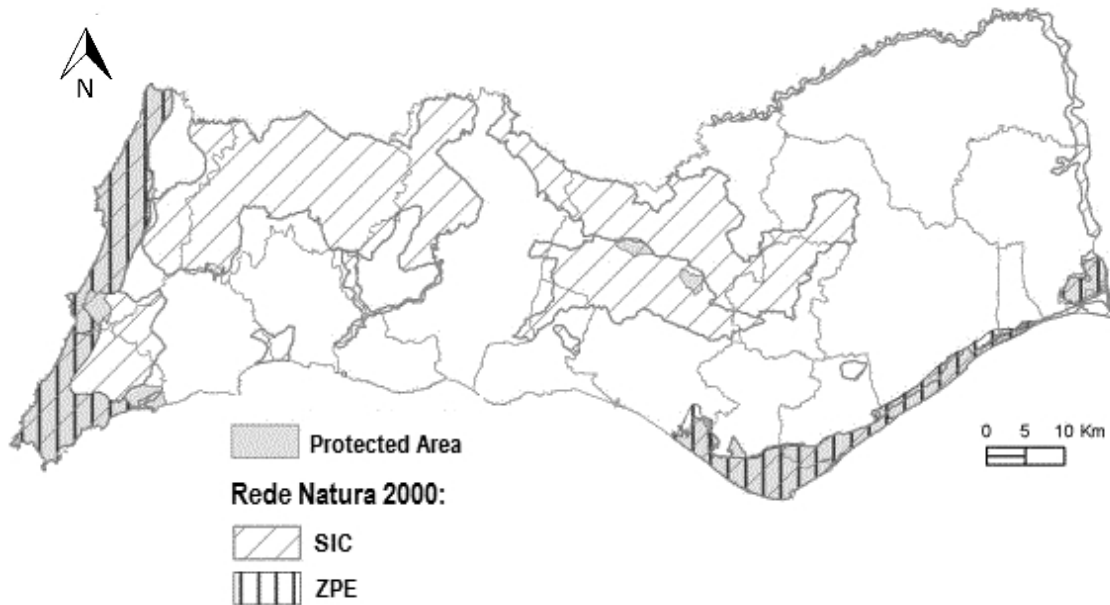
In the Algarve, the National Network of Protected Areas (“Rede Nacional de Áreas Protegidas”) officially recognised by the Portuguese State are (Figure 1.4) (CCDR 2012):

- Sudoeste Alentejano e Costa Vicentina Natural Park;
- Ria Formosa Natural Park;
- Sapal de Castro Marim e Vila Real de Santo António Natural Reserve;
- “Sítio Classificado da Rocha da Pena”;
- “Sítio Classificado da Fonte Benémola”.

In addition, as part of the Rede Natura 2000, fourteen Sites of Community Importance (“Sítios de Interesse Comunitário”, SIC) and Special Protection Areas (“Zonas de Proteção Especial”, ZPE) are under protection (Figure 1.4) (CCDR 2012):

- Costa Sudoeste (ZPE and SIC);
- Leixão da Gaivota (ZPE);
- Arade / Odelouca (SIC);
- Ria de Alvor (SIC);
- Ria Formosa – Castro Marim (SIC);
- Ribeira de Quarteira (SIC);
- Ria Formosa (ZPE);
- Sapais de Castro Marim (ZPE);

- Monchique (SIC);
- Cerro da Cabeça (SIC);
- Barrocal (SIC);
- Caldeirão (SIC);
- Guadiana (SIC);
- Vale do Guadiana (ZPE).



**Figure 1.4.** – Protected areas and Rede Natura 2000 SIC and ZPE in the Algarve (adapted from CCDR 2012).

### 1.7. Species studied

The Campus of Gambelas of the University of Algarve, where most of this work was developed, is located in the borders of the Ria Formosa Natural Park, one of the National Protected Areas in the Algarve, which encompasses unique habitats and many species, some in risk of extinction. In the present work, the focus was to study the conservation of local species. With this in view, the species studied were chosen due to their identical status: rare and endemic species of the Algarve region. These species are composed by few populations with a low number of individuals and restricted to a small area, in a specific regional habitat. The species studied were *Thymus lotocephalus*, *Plantago algarbiensis* and *Tuberaria major*.

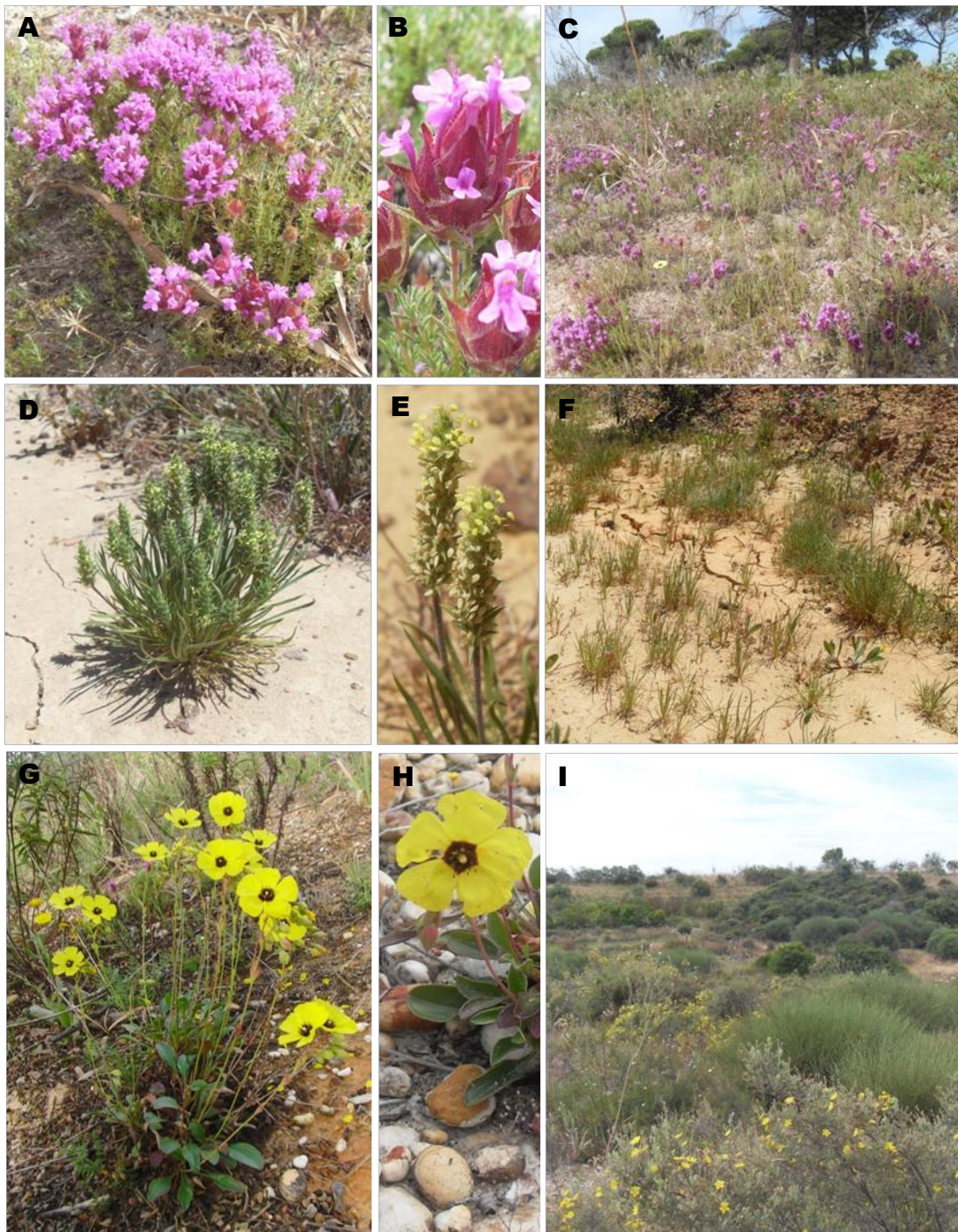
### 1.7.1. *Thymus lotocephalus*

*Thymus lotocephalus* G. López & R. Morales, commonly known as “erva-ursa” or “tomilho-cabeçudo”, is an undershrub endemic to the eastern Algarve, belonging to the Lamiaceae family (Figure 1.5 A, B, C). This small shrub can be found in open spaces of pine woods and xerophile shrubland on calcareous soils with clayey loam or on sandy acid soils (ICN 2006b, Barreto Caldas 2011).

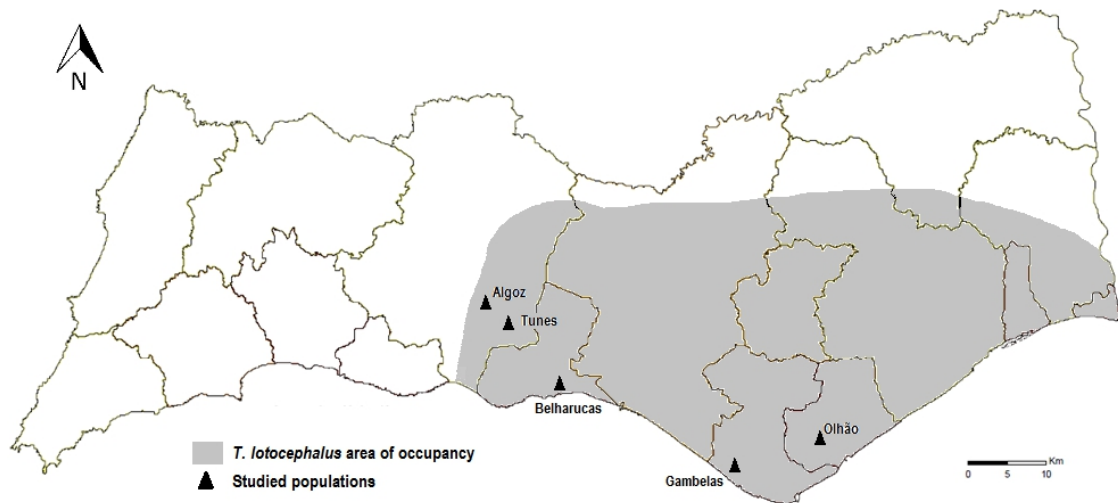
*T. lotocephalus* is an erect plant that can grow up to 30 cm. Possesses holotrichous woody stems and linear ciliate small leaves, usually hairy and with cilia at the base. The purple flowers, with a long corolla, are grouped in large capituliform inflorescences with broad bracts (Castroviejo 1986, Morales 2002). The blossom period goes from April to June. Each capsule may produce more than one seed, which are rounded and very small, approximately 1 mm in diameter.

The species is confined to an area of occupancy of approximately 1,600 km<sup>2</sup> and distributed into a low number of populations (Figure 1.6). There are some large and continuous populations in the area of Pontal and in the Ria Formosa Natural Park, close to the coast, but most of the populations are scattered and fragmented. In the country side area, Barrocal SIC, a few small isolated populations can also be found. The increasing human pressure in the region caused by urbanization, tourism, including the creation of new golf courses, and agriculture expansion is reducing and destroying the populations that still exist. Other concerns are the intense garbage disposal that further degrades the habitat and natural succession, which increases the competition for the species (ICN 2006b, Barreto Caldas 2011).

*T. lotocephalus* is legally protected in Portugal as priority species on Annex B of the law reference 140/99 of April 24. At the European level, it is listed as priority species on Annex II of the European Habitats Directive 92/43/CEE and under Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention). According to the IUCN Red List, *T. lotocephalus* is considered near threatened (IUCN 2013). The management strategies consist in the control of urban and agricultural expansion, as well as site management to control natural succession, and reinforce supervision over garbage disposal (ICN 2006b). Besides the legal protection, up to date no complementary conservation measures, in particular *ex situ* conservation approaches, have been developed to support the preservation of *T. lotocephalus*.



**Figure 1.5.** – Several aspects of *Thymus lotocephalus* (A, B and C), *Plantago algarbiensis* (D, E and F) and *Tuberaria major* (G, H and I): flowering plant (A, D and G), flower detail (B, E and H) and population general aspect (C, F and I).



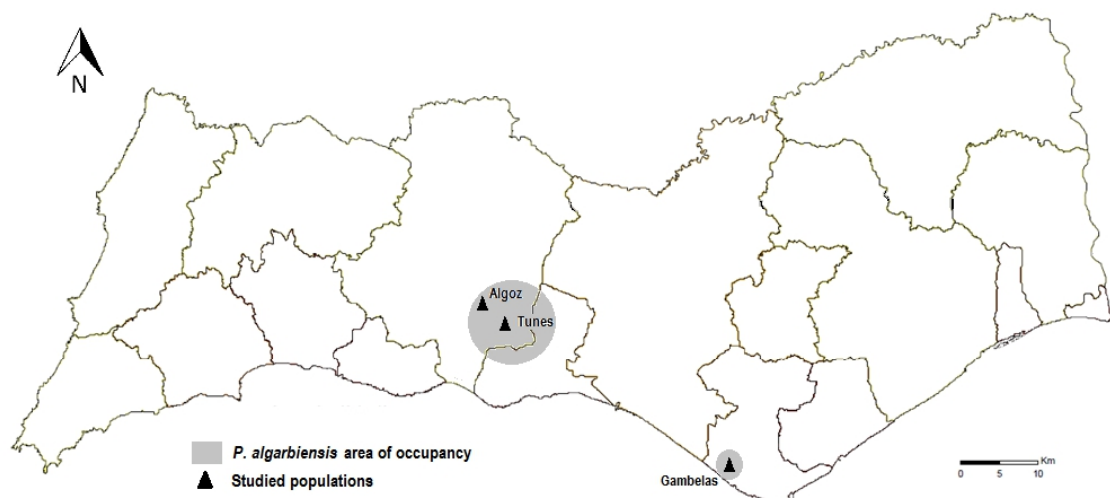
**Figure 1.6.** – Distribution of *Thymus lotocephalus* in the Algarve (adapted from IUCN 2013) and location of studied populations.

The genus *Thymus* has a wide distribution in the Mediterranean area and its species are known and used worldwide for their aromatic and medicinal properties. Some are commercially very important, such as *T. vulgaris*, and the products with major interest are the fresh or dried herb, plant extracts and as ornamental plants. Their essential oils are also used in the manufacture of perfumes and cosmetics (Stahl-Biskup and Saéz 2002). *T. lotocephalus* is traditionally used to condiment food in some areas of the Algarve, but its aromatic properties indicate its great potential as a source of extracts and essential oils. Several studies have already reported the different properties of *T. lotocephalus*. Salgueiro and colleagues (2000) described the composition of the essential oil of the aerial parts at the flowering stage of four populations of *T. lotocephalus* and the main constituents found were linalool, geranyl acetate, 1,8-cineol and intermedeol. Another group compared the essential oil composition of the aerial parts at the flowering and vegetative stages and all oils presented high amounts of 1,8-cineole, linalol and linalyl acetate (Figueiredo et al. 2001). The antimicrobial activity of *T. lotocephalus* essential oil was also reported (Faleiro et al. 2003). More recently, the anti-cholinesterase activity, the phenolic content and antioxidant activity of the essential oil and different extracts were studied, revealing that this species is a good source of antioxidant and anti-cholinesterase compounds (Costa et al. 2012a,b).

### 1.7.2. *Plantago algarbiensis*

*Plantago algarbiensis* Samp., commonly known as “diabelha do Algarve”, is an endemic species to the central-west Algarve, belonging to the Plantaginaceae family (Figure 1.5 D, E, F). This species can be found in clay-rich soils that are temporarily flooded in winter and spring and dry out with the opening of cracks in summer. It also occurs in shrubland clearings, in low acid soils (ICN 2006a, 2007; Bilz 2011a). *P. algarbiensis* is a perennial plant 7-30 cm high with linear leaves 30-90 mm long grouped in a basal rosette. The cylindrical stalks possess prominent bracts where small yellow flowers bloom from May to August. Seeds are approximately 1.5-2 mm in length (Castroviejo 1986).

This species has a very small area of occupancy, approximately 50 ha and the populations are scarce and small, located close to the villages of Tunes and Algoz in the interior of the Algarve. The populations located within the Barrocal SIC have a very restricted effective population that is estimated to be below 10,000 individuals and occupies around 0.05 ha. A more abundant population is located outside the protected site, south of Algoz, and includes several thousand individuals, occupying an area of about 29 ha, though in recent years the construction of a residential project destroyed part of this population (ICN 2006a, 2007). Another small population is known in Gambelas, right next to the Ria Formosa Natural Park, close to the coast (Figure 1.7).



**Figure 1.7.** Distribution of *Plantago algarbiensis* in the Algarve (adapted from IUCN 2013) and location of studied populations.

The main threats are mining of clay soils for the production of construction materials, which may lead to topographic changes that disrupt the natural drainage function of soils, urbanization, trampling and grazing by livestock. However, it is not clear how mining affects the species, though populations can be destroyed during the process, *P. algarbiensis* can also benefit from the deposition of inert material and from the exclusion of agriculture in the area (ICN 2006a, Bilz 2011a).

*P. algarbiensis* is legally protected in Portugal on Annex B of the law reference 140/99 of April 24. At the European level, it is listed on Annexes II and IV of the European Habitats Directive 92/43/CEE. According to the IUCN Red List, *P. algarbiensis* is considered endangered (IUCN 2013). As management guidelines it has been recommended to study the effects of mining on the species and discuss the potential threats with mining companies, to establish micro-reserves, to recover the habitat quality close to the populations, and establish possible new populations (ICN 2006a).

In 2009, an *in vitro* propagation protocol was developed from seeds for *P. algarbiensis* to support its conservation and for the large-scale production of plants, to replenish natural populations and to be used in various studies (Gonçalves et al. 2009b). The effects of temperature, light, collection year and seed size on the germination behaviour of the species were also investigated and the data obtained can as well contribute and support other conservation measures (Martins et al. 2012).

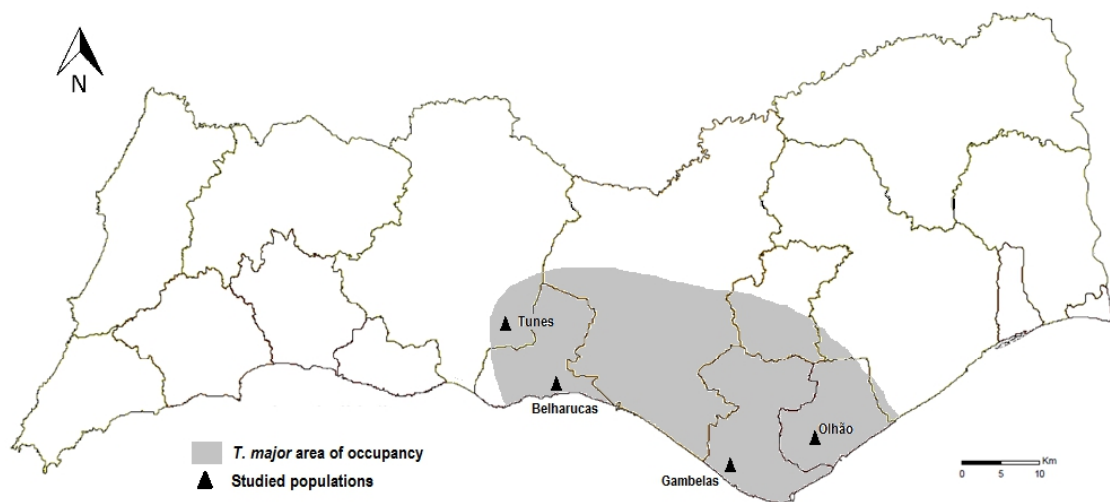
*In vitro*-grown plantlets and seedlings of *P. algarbiensis* were recently used to assess the capacity of this species to accumulate aluminium and therefore as a possible source of aluminium/low pH tolerance genes. The impact of low pH and aluminium on the metabolism (Martins et al. 2013c), organic acid secretion (Martins et al. 2013a), biochemical parameters (Martins et al. 2011), physiological responses (Martins et al. 2013d) and oxidative stress (Martins et al. 2013e) of shoots, leaves and roots were studied. The effect of Al on germination and seedling development was also evaluated (Martins et al. 2013b). Based on these studies, it was proved that *P. algarbiensis* accumulates considerable amounts of aluminium.

### **1.7.3. *Tuberaria major***

*Tuberaria major* (Willk.) P. Silva & Rozeira, commonly known as “alcár do Algarve”, is an endemic species to the centre-eastern Algarve, belonging to the Cistaceae family

(Figure 1.5 G, H, I). It occurs in sandy soils or acid gravel, especially in the clearings of xerophilous shrubland. After fires, common in the region, grows abundantly, even in places where it is scarce (ICN 2006c, Bilz 2011b). *T. major* is a small perennial plant with a woody ramified base and leaves placed around the stalk, which can grow up to 40 cm. Yellow flowers bloom from February to May in a terminal inflorescence. Seeds are very small with less than 1 mm diameter (Castroviejo 1986).

The species has an area of occupancy of 800 km<sup>2</sup>, but with a very limited and fragmented distribution area. In the past, it was extended over much of the Algarve coast but its distribution is now reduced to small clusters. Closer to the coast are located the most dense and extensive populations, up to 10,000 individuals, in sandy soils of the west border of the Ria Formosa, within the Ria Formosa Natural Park. In the interior, in the Barrocal SIC and outside the protected area, there are few populations with dozens of individuals (Figure 1.8). The main causes of habitat degradation and fragmentation are the increasing urban and touristic pressure, since most of the populations are located in or close to construction sites. The disposal of garbage and natural succession are also concerning treats (ICN 2006c, 2007; Bilz 2011b).



**Figure 1.8.** Distribution of *Tuberaria major* in the Algarve (adapted from IUCN 2013) and location of studied populations.

*T. major* is legally protected in Portugal as priority species on Annex B of the law reference 140/99 of April 24. At the European level, it is listed as priority species on Annex II of the European Habitats Directive 92/43/CEE and under Appendix I of the

Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention). According to the IUCN Red List, *T. major* is considered endangered (IUCN 2013). It is recommended, as management strategies, to control urban expansion, reinforce supervision over garbage disposal and control natural succession (ICN 2006c).

Several *ex situ* conservation measures have already been established for *T. major*. The study of the germination requirements and the cryopreservation tolerance of seeds proved that a heat pretreatment significantly enhances germination and that cryopreservation is a useful method for long-term storage of *T. major* seeds (Gonçalves et al. 2009a). An effective and reproducible *in vitro* propagation protocol was also developed achieving high proliferation rates and easy rooting and acclimatisation from small amounts of starting plant material (Gonçalves et al. 2010). With this protocol it is possible to produce large quantities of plant material that can later be used to replenish natural populations. Having this in mind, a study to evaluate the capacity of *T. major* micropropagated plants to acclimatise in the field was performed. The results proved that *in vitro* propagation combined with the acclimatisation procedure described is a suitable method for conservation and restoration of this species (Osório et al. 2013a). In addition, the study on the genetic diversity of *T. major* wild populations was recently carried out (Trindade et al. 2012) and the information acquired will provide additional and complementary knowledge for the protection and restoration of natural populations and sampling strategies.

*T. major in vitro*-propagated plants were used in a study to assess the physiological response under high temperature and drought, considering the predicted climatic changes for the Mediterranean region and how it will affect endangered species. The results suggest that *T. major* is prepared to deal with these changes, revealing an adequate capacity for protection against the development of oxidative stress during drought (Osório et al 2013b).

## 1.8. Objectives

*T. lotocephalus*, *P. algarbiensis* and *T. major* are three rare endemic species of the Algarve region, legally protected by Portuguese and European legislation. The distribution of these species is consistent with a particular habitat at a regional comparison scale level, is restricted to a very small area in the world wide context and

the number of individuals in each scattered population is low. Besides legal protection, additional measures must be employed to preserve the genetic diversity of these species and avoid their extinction. Therefore, the main objective of this study is to develop complementary conservation strategies based on plant biotechnology to support the preservation of these three rare species.

The specific objectives of this study are:

- To develop an *in vitro* propagation protocol for *T. lotocephalus* using seedlings as explants;
- To investigate the germination behaviour and the feasibility of cryopreservation for *T. lotocephalus* and *P. algarbiensis* seeds;
- To improve the germination conditions and therefore cryopreservation of *T. major* seeds;
- To develop a shoot tip cryopreservation protocol for *T. lotocephalus* and assess the genetic stability;
- To develop a cryopreservation protocol for *P. algarbiensis* using nodal segments;
- To investigate different methods for the cryopreservation of *T. major* shoot tips;
- To study the genetic diversity of three wild populations of *P. algarbiensis*.

The final purpose of this work is to avoid the extinction of *T. lotocephalus*, *P. algarbiensis* and *T. major*. It is expected that the methods developed in this thesis will contribute for their preservation and supply information for the implementation and management of conservation strategies for these species.

## 1.9. References

- Barreto Caldas F (2011) *Thymus lotocephalus*. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.1 <[www.iucnredlist.org](http://www.iucnredlist.org)> Accessed 31 August 2013;
- Benson EE (1999) An Introduction to Plant Conservation Biotechnology. In: Benson EE (ed) Plant Conservation Biotechnology (pp 3-10) Taylor & Francis, London;
- Benson EE (2008a) Cryopreservation of Phytodiversity: A Critical Appraisal of Theory & Practice. *Critical Reviews in Plant Sciences* 27: 141-219;
- Benson EE (2008b) Cryopreservation Theory. In: Reed BM (ed) Plant Cryopreservation: A Practical Guide (pp 15-32) Springer, New York;
- Bilz M (2011a) *Plantago algarbiensis*. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.1 <[www.iucnredlist.org](http://www.iucnredlist.org)> Accessed 1 September 2013;
- Bilz M (2011b) *Tuberaria major*. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.1 <[www.iucnredlist.org](http://www.iucnredlist.org)> Accessed 3 September 2013;
- Castroviejo S (coord. gen.) (1986-2012) Flora iberica 1-8, 10-15, 17-18, 21. Real Jardín Botánico, CSIC, Madrid;
- CBD (2010) COP 10 Decision X/17 Consolidated update of the Global Strategy for Plant Conservation 2011-2020 <<http://www.cbd.int/>> Accessed 23 August 2013;
- CCDR Algarve (2004) ProtAlgarve Volume II – Caracterização e Diagnóstico. Comissão de Coordenação e Desenvolvimento Regional do Algarve, Portugal;
- CCDR Algarve (2012) Comissão de Coordenação e Desenvolvimento Regional do Algarve, Portugal <<http://www.ccdr-alg.pt/ccdr/index.php>> Accessed 30 August 2013;
- Costa P, Gonçalves S, Grosso C, Andrade PB, Valentão P, Bernardo-Gil MG and Romano A (2012a) Chemical profiling and biological screening of *Thymus lotocephalus* extracts obtained by supercritical fluid extraction and hydrodistillation. *Industrial Crops and Products* 36: 246-256;
- Costa P, Gonçalves S, Grosso C, Valentão P, Andrade PB, Coelho N and Romano A (2012b) *Thymus lotocephalus* wild plants and *in vitro* cultures produce different profiles of phenolic compounds with antioxidant activity. *Food Chemistry* 135: 1253-1260;
- Cuttelod A, García N, Abdul Malak D, Temple H and Katariya V (2008) The Mediterranean: a biodiversity hotspot under threat. In: Vié J-C, Hilton-Taylor C and Stuart SN (eds) The 2008 Review of The IUCN Red List of Threatened Species. IUCN, Gland;
- Debergh PC and Read PE (1991) Micropropagation. In: Debergh PC and Zimmerman RH (eds) Micropropagation: Technology and Application (pp 1-14) Kluwer Academic Publishers, Dordrecht;
- Engelmann F, Gonzalez-Arno MT, Wu Y and Escobar R (2008) The Development of Encapsulation Dehydration. In: Reed BM (ed) Plant Cryopreservation: A Practical Guide (pp 59-76) Springer, New York;
- Engelmann F (2011) Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cellular & Developmental Biology – Plant* 47: 5-16;

- Fabre J, Dereuddre J (1990) Encapsulation-dehydration: a new approach to cryopreservation of *Solanum* shoot tips. *CryoLetters* 11: 413-426.4;
- Faleiro ML, Miguel MG, Ladeiro F, Venâncio F, Tavares R, Brito JC, Figueiredo AC, Barroso JG and Pedro LG (2003) Antimicrobial activity of essential oils isolated from Portuguese endemic species of *Thymus*. *Letters in Applied Microbiology* 36: 35-40;
- FAO (1994) Genebank Standards. Food and Agriculture Organization of the United Nations, Rome, International Plant Genetic Resources Institute, Rome;
- FAO (1997) The State of the World's Plant Genetic Resources for Food and Agriculture <<http://www.fao.org>> Accessed 23 August 2013;
- FAO (2010) The Second Report on The State of the World's Plant Genetic Resources for Food and Agriculture <<http://www.fao.org>> Accessed 23 August 2013;
- FAO (2013) FAO Glossary of Biotechnology for Food and Agriculture <<http://www.fao.org>> Accessed 21 August 2013;
- Figueiredo AC, Miguel MG, Duarte AMF, Barroso JG and Pedro LG (2001) Essential oil composition of *Thymus lotocephalus* G. López & R. Morales, collected during flowering and vegetative phases. *Flavour and Fragrance Journal* 16: 417-421;
- Fuller BJ (2004) Cryoprotectants: the essential antifreezes to protect life in the frozen state. *CryoLetters* 25: 375-388;
- Glowka L, Burhenne-Guilmin F, Synge H, McNelly JA and Gündling L (1994) A Guide to the Convention on Biological Diversity. IUCN, Gland and Cambridge;
- Gomes CJP and Ferreira RJPP (2005) Flora e Vegetação do Barrocal Algarvio (Tavira – Portimão). Comissão de Coordenação e Desenvolvimento Regional do Algarve;
- Gonçalves S, Fernandes L, Pérez-García F, González-Benito ME and Romano A (2009a) Germination requirements and cryopreservation tolerance of seeds of the endangered species *Tuberaria major*. *Seed Science & Technology* 37: 480-484;
- Gonçalves S, Martins N and Romano A (2009b) Micropropagation and conservation of endangered species *Plantago algarbiensis* and *P. almogravensis*. *Biologia Plantarum* 53: 774-778;
- Gonçalves S, Fernandes L and Romano A (2010) High-frequency *in vitro* propagation of the endangered species *Tuberaria major*. *Plant Cell, Tissue and Organ Culture* 101: 359-363;
- Gonzalez-Arno MT and Engelmann F (2006) Cryopreservation of plant germplasm using the encapsulation–dehydration technique: review and case study on sugarcane. *CryoLetters* 27: 155-168;
- González-Benito ME, Clavero-Ramírez I and López-Aranda JM (2004) Review. The use of cryopreservation for germplasm conservation of vegetatively propagated crops. *Spanish Journal of Agricultural Research* 2: 341-351;
- Harding K (2004) Genetic integrity of cryopreserved plant cells: a review. *CryoLetters* 25: 3-22;
- Harris SA (1999) Molecular Approaches to Assessing Plant Diversity. In: Benson EE (ed) *Plant Conservation Biotechnology* (pp 11-24) Taylor & Francis, London;

- Hartmann HT, Kester DE, Davies Jr FT and Geneve RL (2011) Hartmann and Kester's Plant Propagation: Principles and Practices (8<sup>th</sup> edition). Prentice Hall, Upper Saddle River;
- Heine-Dobbernack E, Kiesecker H and Schumacher HM (2008) Cryopreservation of Dedifferentiated Cell Cultures. In: Reed BM (ed) Plant Cryopreservation: A Practical Guide (pp 141-176) Springer, New York;
- ICN (Instituto da Conservação da Natureza) (2006a) Plano Sectorial da Rede Natura. Flora: *Plantago algarbiensis* Samp. <[http://www.icn.pt/psrn2000/caracterizacao\\_valores\\_naturais/flora/Plantago%20algarbiensis.pdf](http://www.icn.pt/psrn2000/caracterizacao_valores_naturais/flora/Plantago%20algarbiensis.pdf)> Accessed 1 September 2013;
- ICN (Instituto da Conservação da Natureza) (2006b) Plano Sectorial da Rede Natura. Flora: *Thymus lotocephalus* G. López & R. Morales <[http://www.icn.pt/psrn2000/caracterizacao\\_valores\\_naturais/flora/Thymus%20lotocephalus.pdf](http://www.icn.pt/psrn2000/caracterizacao_valores_naturais/flora/Thymus%20lotocephalus.pdf)> Accessed 31 August 2013;
- ICN (Instituto da Conservação da Natureza) (2006c) Plano Sectorial da Rede Natura. Flora: *Tuberaria major* (Willk.) P. Silva & Rozeira <[http://www.icn.pt/psrn2000/caracterizacao\\_valores\\_naturais/flora/Tuberaria%20major.pdf](http://www.icn.pt/psrn2000/caracterizacao_valores_naturais/flora/Tuberaria%20major.pdf)> Accessed 3 September 2013;
- ICN (Instituto de Conservação da Natureza) (2007) Plano Nacional de Conservação da Flora em Perigo (1<sup>a</sup> fase). Relatório final do Projecto Life – Natureza III P/8480. Volume IV. Instituto da Conservação da Natureza, Lisboa;
- IUCN (2013) The IUCN Red List of Threatened Species. Version 2013.1 <<http://www.iucnredlist.org>> Accessed 23 August 2013;
- Kartha KK, Leung NL and Mroginski LA (1982) *In vitro* growth responses and plant regeneration from cryopreserved meristems of Cassava (*Manihot esculenta* Crantz). Zeitschrift für Pflanzenphysiologie 107: 133-140
- Larkin PJ and Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theoretical and Applied Genetics 60: 197-214;
- Lovelock JE, Bishop MWH (1959) Prevention of freezing damage to living cells by dimethyl sulphoxide. Nature 183: 1394-1395;
- Martins N, Gonçalves S, Palma T and Romano A (2011) The influence of low pH on *in vitro* growth and biochemical parameters of *Plantago almogravensis* and *P. algarbiensis*. Plant Cell, Tissue and Organ Culture 107: 113-121;
- Martins N, Gonçalves S, Palma T and Romano A (2012) Seed germination of two critically endangered plantain species, *Plantago algarbiensis* and *P. almogravensis* (Plantaginaceae). Seed Science & Technology 40: 144-149;
- Martins N, Gonçalves S, Andrade PB, Valentão P and Romano A (2013a) Changes on organic acid secretion and accumulation in *Plantago almogravensis* Franco and *Plantago algarbiensis* Samp. under aluminum stress. Plant Science 198: 1-6;
- Martins N, Gonçalves S and Romano A (2013b) Aluminum inhibits root growth and induces hydrogen peroxide accumulation in *Plantago algarbiensis* and *P. almogravensis* seedlings. Protoplasma 250: 1295-1302;

- Martins N, Gonçalves S and Romano A (2013c) Metabolism and aluminum accumulation in *Plantago almogravensis* and *P. algarbiensis* in response to low pH and aluminum stress. *Biologia Plantarum* 57: 325-331;
- Martins N, Osório ML, Gonçalves S, Osório J, Palma T and Romano A (2013d) Physiological responses of *Plantago algarbiensis* and *P. almogravensis* shoots and plantlets to low pH and aluminum stress. *Acta Physiologiae Plantarum* 35: 615-625;
- Martins N, Osório ML, Gonçalves S, Osório J and Romano A (2013e) Differences in Al tolerance between *Plantago algarbiensis* and *P. almogravensis* reflect their ability to respond to oxidative stress. *Biometals* 26: 427-437;
- Maunder M, Havens K, Guerrant EO and Falk DA (2004) *Ex situ* methods: a vital but underused set of conservation resources. In: Guerrant EO, Havens K, Maunder M (eds) *Ex situ* Plant Conservation: Supporting Species Survival in the Wild (pp 3-20) Island press, Washington;
- Maxted N, Ford-Lloyd BV and Hawkes JG (1997) Complementary conservation strategies. In: Maxted N, Ford-Lloyd BV and Hawkes JG (eds) *Plant Genetic Conservation: The In Situ Approach* (pp 15-40) Chapman and Hall, London;
- Morales R (2002) The history, botany and taxonomy of the genus *Thymus*. In: Stahl-Biskup E and Saéz F (eds) *Thyme: The genus Thymus, Medicinal and Aromatic Plants - Industrial Profiles* (pp. 1-43) Taylor & Francis, London;
- Normah MN and Makeen AM (2008) Cryopreservation of Excised Embryos and Embryonic Axes. In: Reed BM (ed) *Plant Cryopreservation: A Practical Guide*. (pp 211-240) Springer, New York;
- Osório ML, Gonçalves S, Coelho N, Osório J and A Romano (2013a) Morphological, physiological and oxidative stress markers during acclimatization and field transfer of micropropagated *Tuberaria major* plants. *Plant Cell, Tissue and Organ Culture* 115: 85-97;
- Osório ML, Osório J and Romano A (2013b) Photosynthesis, energy partitioning, and metabolic adjustments of the endangered Cistaceae species *Tuberaria major* under high temperature and drought. *Photosynthetica* 51: 75-84;
- Poczai P, Varga I, Laos M, Cseh A, Bell N, Valkonen JPT and Hyvönen J (2013) Advances in plant gene-targeted and functional markers: a review. *Plant Methods* 9: 6;
- Polge C, Smith AU and Parkes AS (1949) Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* 164: 666;
- Pritchard HW (2007) Cryopreservation of desiccation tolerant seeds. In: Day JG and Stacey GN (eds) *Cryopreservation and freeze-drying protocols* (pp 183-199) Humana Press Inc, Totowa;
- Pritchard HW and Nadarajan J (2008) Cryopreservation of Orthodox (Desiccation Tolerant) Seeds. In: Reed BM (ed) *Plant Cryopreservation: A Practical Guide*. (pp. 485-502) Springer, New York;
- Quatrano RS (1968) Freeze-preservation of cultured flax cells utilizing dimethyl sulfoxide. *Plant Physiology* 43: 2057-2061;
- Ramanatha Rao V and Hodgkin T (2002) Genetic diversity and conservation and utilization of plant genetic resources. *Plant Cell, Tissue and Organ Culture* 68: 1-19;

- Reed BM (2004) Shoo-tip cryopreservation manual. USDA-ARS National Clonal Germplasm Repository, Corvallis;
- Reed BM (2008) Plant Cryopreservation: A Practical Guide. Springer, New York;
- Reed BM and Uchendu E (2008) Controlled Rate Cooling. In: Reed BM (ed) Plant Cryopreservation: A Practical Guide (pp 77-92) Springer, New York;
- Reed BM, Sarasan V, Kane M, Bunn E and Pence VC (2011) Biodiversity conservation and conservation biotechnology tools. In Vitro Cellular & Developmental Biology – Plant 47: 1-4;
- Sakai A (1960) Survival of a twig of woody plants at  $-196^{\circ}\text{C}$ . Nature 185: 393-394;
- Sakai A, Kobayashi S, Oiyama I (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. Plant Cell Reports 9: 30-33;
- Sakai A and Engelmann F (2007) Vitrification, encapsulation-vitrification and droplet-vitrification: a review. CryoLetters 28: 151-172;
- Sakai A, Hirai D and Niino T (2008) Development of PVS-Based Vitrification and Encapsulation-Vitrification Protocols. In: Reed BM (ed) Plant Cryopreservation: A Practical Guide (pp 33-58) Springer, New York;
- Salgueiro LR, Vila R, Tomás X, Cañigüeral S, Paiva J, Proença da Cunha A and Adzet T (2000) Essential oil composition and variability of *Thymus lotocephalus* and *Thymus × mourae*. Biochemical Systematics and Ecology 28: 457-470;
- Scowcroft WR (1985) Somaclonal variation: the myth of clonal uniformity. In: Hohn B and Dennis ES (eds) Genetic flux in plants (pp 217-243) Springer-Verlag, New York;
- Stahl-Biskup E and Saéz F (2002) Thyme: The genus *Thymus*, Medicinal and Aromatic Plants – Industrial Profiles. Taylor & Francis, London;
- Trindade H, Sena I, Gonçalves S and Romano A (2012) Genetic diversity of wild populations of *Tuberaria major* (Cistaceae), an endangered species endemic to the Algarve region (Portugal), using ISSR markers. Biochemical Systematics and Ecology 45: 49-56;
- Walters C, Wesley-Smith J, Crane J, Hill LM, Chmielarz P, Pammenter NW and Berjak P (2008) Cryopreservation of Recalcitrant (i.e. Desiccation-Sensitive) Seeds. In: Reed BM (ed) Plant Cryopreservation: A Practical Guide (pp 465-484) Springer, New York.











### 2.1.1. Abstract

The aim of this work was to develop an *in vitro* propagation protocol for the endangered species *Thymus lotocephalus* using seedlings as explants. Several macronutrient concentrations of Murashige and Skoog medium (MS), cytokinin types and concentrations, and cytokinin/auxin combinations were tested to assess the shoots' proliferation capacity. Although the best proliferation results were obtained with 6-benzyladenine, high percentages of hyperhydric shoots were observed. Because high proliferation of healthy shoots was observed in MS medium that was free of plant growth regulators, this medium was chosen for proliferation studies. The best rooting results were achieved in  $\frac{1}{4}$ MS medium without auxins ( $92.00 \pm 6.11\%$ ,  $6.54 \pm 0.52$  and  $1.60 \pm 0.10$  cm regarding rooting frequency, number of roots per shoot and longest roots, respectively) or supplemented with  $0.5 \text{ mg l}^{-1}$  IAA (indole-3-acetic acid) ( $98.00 \pm 2.11\%$ ,  $11.14 \pm 0.75$  and  $2.40 \pm 0.24$  cm, respectively). Plantlets were successfully acclimatised to *ex vitro* conditions with a survival rate of 93.33%. With the development of this micropropagation protocol, an important contribution has been made to the conservation of the endangered species *T. lotocephalus*.

### 2.1.2. Introduction

*Thymus lotocephalus* G. López & R. Morales (Lamiaceae), commonly known as “tomilho-cabeçudo” or “erva-ursa”, is an undershrub endemic from the Algarve region south of Portugal (Figure 2.1 A). This species belongs to a genus with wide distribution in the Mediterranean area and can be found in dry open areas and scrubs. Though it is approximately 30 cm high, this species is easily recognised in the fields during the blossom period from April to June because of its characteristic small purple flowers.



**Figure 2.1.** – Micropropagation of *Thymus lotocephalus*. Wild plant (bar 1.6 cm) (A). Seeds collected from the field (B). Seedlings after 45 days of germination at 25°C (bar 5.2 cm) (C). Shoots at the end of the proliferation stage in MS medium (bar 2.2 cm) (D). Shoot detail at the end of the proliferation stage in MS medium supplemented with 0.5 mg l<sup>-1</sup> BA (E). Rooted shoot detail at the end of the rooting stage in ¼MS medium (F). Acclimatised plants after 6 weeks (bar 4.2 cm) (G).

The leaves from *Thymus* spp., fresh or dried, are used throughout the world as aromatic herbs for flavouring a wide variety of foods, beverages and confectionery products. The essential oils produced by *Thymus* spp. are important for the cosmetic industry, and due to their antiseptic, antispasmodic and antimicrobial properties, they are also used for medicinal purposes (Cosentino et al. 1999, Figueiredo et al. 2001, Stahl-Biskup and Saéz 2002, Ozudogru et al. 2011). The chemical composition and the antimicrobial activity of *T. lotocephalus* essential oils have already been reported (Salgueiro et al. 2000, Figueiredo et al. 2001, Faleiro et al. 2003).

The distribution of *T. lotocephalus* is confined to a few populations in the Algarve region. Furthermore, the constant human pressure in the region, mainly due to tourism, agriculture and construction, is reducing and destroying populations, making this species extremely vulnerable. Legally, this species is protected by both European Habitats Directive 92/43/CEE and by Portuguese law (reference 140/99 of April 24)

(ICN 2006). The development of both *in situ* and *ex situ* strategies to preserve this species is necessary to ensure its long-term conservation.

Biotechnological techniques and procedures play an essential role in assisting the conservation of plant biodiversity. Plant propagation by tissue culture is an important biotechnological tool for the *ex situ* propagation of endangered plant species because it is possible to obtain thousands of plants from a single explant in a short period of time. The *in vitro*-cultured plants can subsequently be used for numerous different studies, avoiding collection from their natural environment. *In vitro* propagation protocols have already been established for *Thymus* spp., such as *T. vulgaris* (Lê 1989, Furmanowa and Olszowska 1992, Ozudogru et al. 2011), *T. piperella* (Saéz et al. 1994), *T. mastichina* (Mendes and Romano 1999) and *T. longicaulis* (Ozudogru et al. 2011). However, to date, the *in vitro* propagation of *T. lotocephalus* has not been reported. Therefore, with the aim of conservation of *T. lotocephalus*, an *in vitro* propagation protocol using seedlings as explants was developed during this research. This study will be the starting point for the development of other *ex situ* conservation techniques, such as cryopreservation.

### **2.1.3. Materials and Methods**

#### **2.1.3.1. Seed germination**

*Thymus lotocephalus* seeds were collected from plants growing in a natural population in Algoz (Algarve region, Portugal) in June 2009. A specimen voucher has been preserved at the herbarium of the University of Algarve under the number ALGU 8081. Collection was made from several plants to ensure the capture of a broad range of genetic variation. After extracting the small seeds from the capsules (Figure 2.1 B), these seeds were stored dry under laboratory conditions at  $25 \pm 2^\circ\text{C}$  under darkness until the beginning of the experiments in January 2010. Prior to germination, all seeds were surface-sterilised with 20% (v/v) commercial bleach (5% sodium hypochlorite) and a few drops of Tween-20 for 15 min followed by three rinses in sterile distilled water. Seeds were aseptically sown in glass Petri dishes (11-cm inner diameter) on 1% (w/v) agar (Iberagar, Portugal) and incubated under a 16-h light photoperiod provided by cool-white fluorescent lamps at a photon flux density of  $40 \mu\text{mol m}^{-2} \text{s}^{-1}$  at  $25^\circ\text{C}$ . Forty-five days after sowing, nodal segments isolated from one seedling (one genotype) were

cultured in full-strength MS medium (Murashige and Skoog 1962) to obtain enough shoots for the subsequent proliferation assays.

### **2.1.3.2. Shoot proliferation**

Initially, three concentrations of MS macronutrients (full-strength MS, ½MS and ¼MS) without plant growth regulators (PGRs) were tested. In order to assess the effect of PGRs on the proliferation of *T. lotocephalus* shoots, MS medium was supplemented with several cytokinins at different concentrations [6-benzyladenine, BA (0.2, 0.5 or 1.0 mg l<sup>-1</sup>); zeatin, ZEA (0.2 or 0.5 mg l<sup>-1</sup>); or kinetin, KIN (0.2 or 0.5 mg l<sup>-1</sup>)] and cytokinin/auxin combinations (0.2 mg l<sup>-1</sup> ZEA/0.2 mg l<sup>-1</sup> indole-3-acetic acid, IAA; 0.5 or 1.0 mg l<sup>-1</sup> BA/0.2 mg l<sup>-1</sup> IAA). For each medium, 10 replicates of 5 explants (nodal segments) each were used. Explants were cultured in 500-ml Erlenmeyer flasks that contained 90 ml of medium and were capped with aluminium foil. All media were supplemented with 2% (w/v) sucrose, solidified by adding 1% (w/v) agar, and the pH was adjusted to 5.75 before autoclaving at 121°C and 1.1 kg cm<sup>-2</sup> for 20 min. The growth conditions were the same as those described during the germination stage. After 6 weeks, shoot proliferation was assessed based on the proliferation frequency (percentage of shoots showing proliferation capacity), the total number of shoots produced per culture, and the length of the longest shoot produced.

### **2.1.3.3. Rooting**

Shoots with three nodes (about 2.5 cm long), harvested at the end of the proliferation stage on MS medium were used in all of the rooting assays. The rooting capacity of the shoots was evaluated in PGRs-free MS medium at three different macronutrient concentrations (full-strength MS, ½MS or ¼MS) and in ¼MS media supplemented with the auxins IAA, NAA (1-naphthalene-acetic acid) or IBA (indole-3-butyric acid) at 0.5 mg l<sup>-1</sup>. Rooting was also induced by dipping the basal end of the shoots in a concentrated auxin solution (1.0 g l<sup>-1</sup> IAA, NAA or IBA) for 2 min followed by culture in MS, ½MS or ¼MS auxin-free medium. Cultures were incubated for 6 weeks in the same growth conditions described above, and 10 replicates of 5 shoots each were used per medium. Following this period, rooting frequency, root number and longest root length per plantlet were measured.

#### **2.1.3.4. Acclimatisation**

Plantlets with well-developed roots that were originally grown in auxin-free ¼MS or from ¼MS with 0.5 mg l<sup>-1</sup> IAA were selected for acclimatisation and transplanted to 350-ml plastic pots containing a mixture of peat and vermiculite (3:1, v/v). Next, the pots were either placed in a plant growth chamber or into transparent polyethylene boxes. The chamber was set at 25 ± 2°C during the day and 22 ± 1°C at night. Light was provided by Grow-Lux F18 W/GRO lamps placed at the top of the chamber; and light intensity during the 16-h light photoperiod was 100 µmol m<sup>-2</sup> s<sup>-1</sup>. Relative humidity was programmed using an ultrasonic fog system controlled by a hygrometer; which was initially set at 98% and was decreased to 70% during the acclimatisation period. The polyethylene boxes were kept under the same growth conditions as the *in vitro* cultures and were gradually opened in order to expose the plants to a progressively reduced relative humidity. All plantlets were watered weekly with a nutritive solution (12:4:6 NPK and essential micronutrients). The number of surviving plants was recorded at the end of the acclimatisation period.

#### **2.1.3.5. Statistical analysis**

The data were subjected to one-way analysis of variance (ANOVA) to assess treatment differences using the SPSS statistical package for Windows (release 18.0; SPSS Inc., Chicago, IL, USA). Significant differences between means were determined using Duncan's New Multiple Range Test ( $P = 0.05$ ). Before analysis of rooting percentage data, arcsin square root transformation was used. Data are presented as means ± standard error.

#### **2.1.4. Results and Discussion**

As the ultimate objective of this study is the preservation of *T. lotocephalus*, seeds are the ideal starting material to initiate *in vitro* cultures because they allow the maintenance of a wider genetic base (Fay 1992, Benson et al. 2000, Gonçalves et al. 2010). Forty-four percent of the *T. lotocephalus* seeds (Figure 2.1 B) germinated after 45 days at 25°C without any pretreatment (Figure 2.1 C). The nodal segments isolated from one selected seedling were cultured in MS medium, originating shoots with a high proliferation capacity (Figure 2.1 D). During the proliferation stage, 100% of the shoots

showed proliferation capacity in all of the tested media (Figure 2.1 D). The mean number of shoots and shoot length were influenced by MS medium strength ( $P < 0.05$ ) (Table 2.1). The best results were obtained with full-strength MS, although no significant differences were observed between MS and  $\frac{1}{2}$ MS for the mean number of shoots (Table 2.1). Following these results, MS medium supplemented with different cytokinins at several concentrations was tested. The highest mean number of shoots was obtained with BA. This parameter was significantly improved by increasing BA in the medium up to  $0.5 \text{ mg l}^{-1}$ , decreasing thereafter (Table 2.1). These results are in agreement with those obtained by Saéz et al. (1994) for *T. piperella*, who found that the shoot proliferation capacity of BA was greater than that of KIN. The same authors also observed an enhancement in the number of shoots with the increase of BA concentration in the medium up to  $1.0 \text{ mg l}^{-1}$ , lowering thereafter. As for the shoot length, the longest shoots were achieved in MS and MS supplemented with KIN (Table 2.1). Ozudogru et al. (2011) also proved that KIN was the best cytokinin in promoting shoot elongation in *T. vulgaris*.

Although the highest number of shoots was obtained in BA-containing media, the percentage of hyperhydric shoots was high (Table 2.1, Figure 2.1 E), and the shoots were significantly smaller than the ones produced in cytokinin-free media. Hyperhydricity is a morphological and physiological disorder that may occur in plants propagated *in vitro* and it is characterised by thick, rigid and easily breakable stems and leaves. A number of factors can contribute to this condition, such as water availability, micronutrient content and hormonal imbalance in the media (Hazarika 2006). Several authors reported a higher occurrence of these symptoms when using BA containing media (Cuenca and Amo-Marco 2000, Paek and Hahn 2000, Gonçalves and Romano 2005).

As during the micropropagation of *T. mastichina* (Mendes and Romano 1999) and *T. vulgaris* (Ozudogru et al. 2011), the addition of auxins stimulated shoot elongation, additional media supplemented with the auxin IAA were tested. The addition of auxin to MS medium with  $1.0 \text{ mg l}^{-1}$  BA significantly improved the number of shoots (Table 2.1), however, shoot length was only improved in MS medium with  $0.2 \text{ mg l}^{-1}$  ZEA or  $1.0 \text{ mg l}^{-1}$  BA (Table 2.1). Moreover, the percentage of hyperhydric shoots in media supplemented with BA and IAA was even higher than without auxin. Of all of the tested media, MS supplemented with  $0.2 \text{ mg l}^{-1}$  ZEA and  $0.2 \text{ mg l}^{-1}$  IAA resulted in the longest

shoots (Table 2.1). Considering these results and the fact that PGRs-free MS medium presented high shoot proliferation with a considerable shoot length and without hyperhydricity symptoms, this medium was used further in subsequent experiments to multiply *T. lotocephalus* shoots *in vitro*.

**Table 2.1.** – Effect of MS medium concentration, cytokinin type and concentration and cytokinin/auxin combinations on the mean number of shoots, shoot length and percentage of hyperhydric shoots of *Thymus lotocephalus in vitro* propagated shoots.

Basal medium	Growth regulators (mg l <sup>-1</sup> )	No. new shoots per explant	Shoot length (cm)	Hyperhydric shoots (%)
MS	-	33.42 ± 3.29 A d	3.70 ± 0.15 A ab	-
½MS	-	30.42 ± 3.02 A	2.50 ± 0.09 B	-
¼MS	-	8.02 ± 0.63 B	2.20 ± 0.05 C	-
MS	BA (0.2)	61.96 ± 4.54 b	2.80 ± 0.12 c	69.15 ± 6.32 a
	BA (0.5)	76.24 ± 6.38 a	2.30 ± 0.08 d	66.60 ± 2.97 a
	BA (1)	48.56 ± 5.24 c	1.90 ± 0.05 e	51.88 ± 7.95 a
	ZEA (0.2)	39.66 ± 3.96 cd	3.10 ± 0.17 c	-
	ZEA (0.5)	37.18 ± 2.56 cd	3.20 ± 0.17 bc	-
	KIN (0.2)	33.18 ± 2.82 d	3.50 ± 0.16 ab	-
	KIN (0.5)	33.44 ± 2.91 d	3.80 ± 0.20 a	-
	BA (0.5) + IAA (0.2)	90.80 ± 5.53 ns	2.00 ± 0.07 *	84.72 ± 2.52 *
	BA (1) + IAA (0.2)	111.32 ± 4.44 *	2.10 ± 0.07 *	79.11 ± 4.15 *
	ZEA (0.2) + IAA (0.2)	31.18 ± 1.94 ns	4.50 ± 0.14 *	-

Values represent means ± SE of 10 replicates of 5 shoots each. For each variable, values followed by the same letter are not significantly different at  $P < 0.05$  according to Duncan's Multiple Range Test. Capital and lower-case letters should be considered separately: capital letters refer only to basal media without PGRs (MS, ½MS and ¼MS) and lower-case letters refer only to MS media, with or without PGRs. \* and ns: Comparison between media with and without IAA for each cytokinin concentration studied (0.2 and 0.5 mg l<sup>-1</sup> BA; 0.2 mg l<sup>-1</sup> ZEA).

Shoots from MS medium were then induced to root and the rooting frequency was significantly higher in the medium with the lowest macronutrient concentration, ¼MS,

although the number of roots and the root length were not significantly different between the three media (Table 2.2, Figure 2.1 F). Experiments with *T. piperella* demonstrate that a reduction of 25–50% of macronutrients promoted root formation (Stahl-Biskup and Saéz 2002). Moreover, in other species belonging to the Lamiaceae family, such as *Lavandula vera* (Andrade et al. 1999), *Lavandula viridis* (Dias et al. 2002) and *Teucrium stocksianum* (Bouhouche and Ksiksi 2007), it was observed that lowering the macronutrient concentration improved root formation. These results differ from those observed by Lê (1989) and Ozudogru et al. (2011) with *T. vulgaris*, who obtained 100% rooting on MS medium.

**Table 2.2.** - Effect of MS medium concentration and auxin type on the rooting frequency, mean number of roots per plantlet and root length of *Thymus lotocephalus* *in vitro* propagated shoots.

Basal medium	Auxin (0.5 mg l <sup>-1</sup> )	Rooting frequency (%)	No. roots	Root length (cm)
MS	-	4.00 ± 2.67 C	3.00 ± 2.00 A	0.80 ± 0.45 A
½MS	-	62.00 ± 8.67 B	3.59 ± 0.69 A	1.00 ± 0.22 A
¼MS	-	92.00 ± 6.11 A a	6.54 ± 0.52 A b	1.60 ± 0.10 A b
¼MS	IAA	98.00 ± 2.11 a	11.14 ± 0.75 a	2.40 ± 0.24 a
	NAA	34.00 ± 5.48 b	1.82 ± 0.30 c	0.70 ± 0.06 c
	IBA	88.00 ± 7.07 a	3.25 ± 0.26 c	1.00 ± 0.08 c

Values represent means ± SE of 10 replicates of 5 shoots each. For each variable, values followed by the same letter are not significantly different at  $P < 0.05$  according to Duncan's Multiple Range Test. Capital and lower-case letters should be considered separately: capital letters refer only to basal media with no auxins (MS, ½MS and ¼MS) and lower-case letters refer only to ¼MS media, with or without auxin.

To assess the influence of auxins in rooting, ¼MS media was supplemented with IAA, NAA or IBA. The addition of IAA to ¼MS improved the rooting frequency, though not significantly, and the number of roots and root length (Table 2.2). Moreover, considering all of the parameters, results obtained with NAA or IBA were lower than with IAA (Table 2.2). Conversely, *T. mastichina* rooted better in media containing these two auxins (Mendes and Romano 1999), and *T. vulgaris* rooted easily in medium with IBA (Furmanowa and Olszowska 1992). Ozudogru et al. (2011) tested the effect of

several auxins [IAA, IBA, NAA and 2,4-dichlorophenoxyacetic (2,4-D)] on *in vitro* rooting of *T. vulgaris* and observed the best rooting results with 2,4-D. Adventitious rooting is a complex process which is largely genotype-dependent (Ioio et al. 2008) and regulated by both environmental and endogenous factors (Sorin et al. 2005). The differences in rooting response between *T. lotocephalus* and other *Thymus* species could be related to multiple factors, namely, the endogenous cytokinin/auxin ratio, the influence of shoot multiplication medium, the sensitivity of tissues to absorb or utilise the exogenous auxin, and the type of initial explants (mature or juvenile), among others (de Klerk et al. 1999, de Klerk 2002).

Shoots were also induced to root by basal immersion in an auxin-rich solution prior to culture in auxin-free medium. Despite the auxin used, full-strength MS medium presented the lowest results for all of the studied parameters (Table 2.3).

**Table 2.3.** - Effect of MS medium concentration and auxin type, after dipping, on the rooting frequency, mean number of roots and root length of *Thymus lotocephalus in vitro* propagated shoots. The basal ends of the shoots were dipped in 1 g l<sup>-1</sup> auxin for 2 min, followed by culturing on auxin-free medium.

Basal medium	Auxin	Rooting frequency (%)	No. Roots	Root length (cm)
MS	IAA	8.00 ± 4.42 e	2.00 ± 0.71 d	0.40 ± 0.08 c
	NAA	34.00 ± 7.33 d	2.18 ± 0.46 cd	0.50 ± 0.08 c
	IBA	40.00 ± 6.89 d	2.00 ± 0.26 d	0.40 ± 0.07 c
½MS	IAA	64.00 ± 9.33 c	4.09 ± 0.35 ab	0.90 ± 0.11 b
	NAA	92.00 ± 3.27 a	5.43 ± 0.36 ab	1.40 ± 0.10 a
	IBA	84.00 ± 6.53 abc	4.36 ± 0.41 ab	1.00 ± 0.10 ab
¼MS	IAA	70.00 ± 8.03 bc	4.40 ± 0.73 ab	1.00 ± 0.12 ab
	NAA	82.00 ± 6.29 abc	5.88 ± 0.49 a	1.30 ± 0.11 a
	IBA	90.00 ± 3.33 ab	3.60 ± 0.37 bcd	0.80 ± 0.06 bc

Values represent means ± SE of 10 replicates of 5 shoots each. For each variable, values followed by the same letter are not significantly different at  $P < 0.05$  according to Duncan's Multiple Range Test.

The rooting frequencies were above 60% in the media with the lowest macronutrient concentration (Table 2.3). The highest rooting frequency (92%) was obtained from

shoots dipped in NAA and cultured on ½ MS medium (Table 2.3). The highest number of roots and the longest roots were also observed with NAA (Table 2.3). In spite of the good results observed for the rooting frequency, there was no improvement in rooting using the dipping method in comparison with ¼MS without auxins or supplemented with 0.5 mg l<sup>-1</sup> IAA.

Healthy plantlets rooted in ¼ MS and ¼ MS with 0.5 mg l<sup>-1</sup> IAA were transferred to *ex vitro* conditions and acclimatised. After 6 weeks, the transplanted plantlets were successfully acclimatised to *ex vitro* conditions with survival rates of 93.33 and 73.33% for plantlets originated from ¼MS and ¼MS with 0.5 mg l<sup>-1</sup> IAA, respectively, and acclimatised in a plant growth chamber (Figure 2.1 G). The newly produced plants presented no apparent morphological variation, and the number of new leaves and shoot length increased considerably.

#### **2.1.5. Conclusion**

Shoot cultures of *T. lotocephalus* were established from seeds collected in the wild. Shoots were easily multiplied and presented a high proliferative capacity. Growth regulator-free MS medium was the most effective medium tested, considering all of the evaluated parameters. Shoots needed a subsequent rooting stage to induce root formation, which was achieved with a high frequency in ¼MS or ¼MS supplemented with 0.5 mg l<sup>-1</sup> IAA. Plantlets were successfully acclimatised to *ex vitro* conditions.

For the first time, the present study reports the establishment of an effective micropropagation protocol from seeds for the mass production of *T. lotocephalus* plants contributing to the conservation of this endangered species in the wild. This protocol will be applied to several genotypes, and the plants produced in this study can be used for further biochemical or other studies or to develop long-term conservation of vegetative material using cryopreservation.

### 2.1.6. References

- Andrade LB, Echeverrigaray S, Fracaro F, Pauletti GF and Rota L (1999) The effect of growth regulators on shoot propagation and rooting of common lavender (*Lavandula vera* DC). *Plant Cell, Tissue and Organ Culture* 56: 79-83;
- Benson EE, Danaher JE, Pimbley IM, Anderson CT, Wake JE, Daley S and Adams LK (2000) *In vitro* micropropagation of *Primula scotica*: a rare Scottish plant. *Biodiversity and Conservation* 9: 711-726;
- Bouhouche N and Ksiksi T (2007) An efficient *in vitro* plant regeneration system for the medicinal plant *Teucrium stocksianum* Boiss. *Plant Biotechnology Report* 1: 179-184;
- Cosentino S, Tuberoso CIG, Pisano B, Satta M, Mascia V, Arzedi E and Palmas F (1999) *In vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. *Letters in Applied Microbiology* 29: 130-135;
- Cuenca S and Amo-Marco JB (2000) *In vitro* propagation of two Spanish endemic species of *Salvia* through bud multiplication. *In Vitro Cellular & Developmental Biology – Plant* 36: 225-229;
- de Klerk G-J, van der Krieken W and de Jong JC (1999) The formation of adventitious roots: new concepts, new possibilities. *In Vitro Cellular & Developmental Biology – Plant* 35: 189-199;
- de Klerk G-J (2002) Rooting of microcuttings: theory and practice. *In Vitro Cellular & Developmental Biology – Plant* 38: 415-422;
- Dias MC, Almeida R and Romano A (2002) Rapid clonal multiplication of *Lavandula viridis* L'He'r through *in vitro* axillary shoot proliferation. *Plant Cell, Tissue and Organ Culture* 68: 99-102;
- Faleiro ML, Miguel MG, Ladeiro F, Venâncio F, Tavares R, Brito JC, Figueiredo AC, Barroso JG and Pedro LG (2003) Antimicrobial activity of essential oils isolated from Portuguese endemic species of *Thymus*. *Letters in Applied Microbiology* 36: 35-40;
- Fay M (1992) Conservation of rare and endangered plants using *in vitro* methods. *In Vitro Cellular & Developmental Biology – Plant* 28: 1-4;
- Figueiredo AC, Miguel MG, Duarte AMF, Barroso JG and Pedro LG (2001) Essential oil composition of *Thymus lotocephalus* G. López & R. Morales, collected during flowering and vegetative phases. *Flavour and Fragrance Journal* 16: 417-421;
- Furmanowa M and Olszowska O (1992) Micropropagation of Thyme (*Thymus vulgaris* L.). In: Stahl-Biskup E and Saéz F (eds) *Thyme: the genus Thymus, medicinal and aromatic plants – industrial profiles* (pp 177-196) Taylor & Francis, London;
- Gonçalves S and Romano A (2005) Micropropagation of *Drosophyllum lusitanicum* (L.) Link. an endangered West Mediterranean endemic insectivorous plant. *Biodiversity and Conservation* 14: 1071-1081;
- Gonçalves S, Fernandes L and Romano A (2010) High-frequency *in vitro* propagation of the endangered species *Tuberaria major*. *Plant Cell, Tissue and Organ Culture* 101: 359-363;
- Hazarika BN (2006) Morpho-physiological disorders in *in vitro* culture of plants. *Scientia Horticulturae* 108: 105-120;

- ICN (Instituto da Conservação da Natureza) (2006) Plano Sectorial da Rede Natura. Flora: *Thymus lotocephalus* G. López & R. Morales. [http://www.icn.pt/psrn2000/caracterizacao\\_valores\\_naturais/flora/Thymus%20lotocephalus.pdf](http://www.icn.pt/psrn2000/caracterizacao_valores_naturais/flora/Thymus%20lotocephalus.pdf). Accessed 4 April 2011;
- Ioio RD, Nakamura K, Moubayidin L, Perilli S, Taniguchi M, Morita MT, Aoyama T, Costantino P and Sabatini S (2008) Root system architecture: opportunities and constraints for genetic improvement of crops. *Science* 322: 1380-1384;
- Lê CL (1989) Microbouturage *in vitro* du thym (*Thymus vulgaris* L.). In: Stahl-Biskup E and Saéz F (eds) Thyme: the genus *Thymus*, medicinal and aromatic plants – industrial profiles (pp 177-196) Taylor & Francis, London;
- Mendes ML and Romano A (1999) *In vitro* cloning of *Thymus mastichina* L. field-grown plants. *Acta Horticulturae* 502: 303-306;
- Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497;
- Ozudogru EA, Kaya E, Kirdok E and Issever-Ozturk S (2011) *In vitro* propagation from young and mature explants of thyme (*Thymus vulgaris* and *T. longicaulis*) resulting in genetically stable shoots. *In Vitro Cellular & Developmental Biology – Plant* 47: 309-320;
- Paek K-Y and Hahn E-J (2000) Cytokinins, auxins and activated charcoal affected organogenesis and anatomical characteristics of shoottip cultures of Lisianthus [*Eustoma grandiflorum* (raf.) Shinn]. *In Vitro Cellular & Developmental Biology – Plant* 36: 128-132;
- Saéz F, Sánchez P and Piqueras A (1994) Micropropagation of *Thymus piperella*. *Plant Cell, Tissue and Organ Culture* 39: 269-272;
- Salgueiro LR, Vila R, Tomàs X, Cañigüeral S, Paiva J, Cunha A-P and Adzet T (2000) Essential oil composition and variability of *Thymus lotocephalus* and *Thymus × mouroi*. *Biochemical Systematics and Ecology* 28: 457-470;
- Sorin C, Bussell JD, Camus I, Ljung K, Kowalczyk M, Geiss G, McKhann H, Garcion C, Vaucheret H, Sandberg G and Bellinia C (2005) Auxin and light control of adventitious rooting in *Arabidopsis* require ARGONAUTE1. *Plant Cell* 17:1-17;
- Stahl-Biskup E and Saéz F (2002) Thyme: the genus *Thymus*, medicinal and aromatic plants – industrial profiles. Taylor & Francis, London.

## 2.2

### **Germination and cryopreservation tolerance of seeds from the rare aromatic species *Thymus lotocephalus***



### 2.2.1. Abstract

*Thymus lotocephalus* G. López and R. Morales is a rare aromatic species endemic from the south region of Portugal. We investigated the germination behaviour and the cryopreservation tolerance of *T. lotocephalus* seeds collected from four populations. Seeds were set to germinate at 15 or 25°C under light or darkness. The best germination temperature tested was 15°C, under either light or darkness, with final germination percentages above 80% and mean germination time (MGT) below 10 days. The results showed that *T. lotocephalus* has non-dormant seeds. Seeds from different populations presented few differences on the final germination percentage and MGT. To assess seed tolerance to cryopreservation, they were directly immersed in liquid nitrogen (LN) for 30 days. Overall, we found that cryopreservation was non-detrimental to germination of *T. lotocephalus* seeds, thus being a reliable method for germplasm conservation of this endangered species.

### 2.2.2. Introduction

The genus *Thymus* (Lamiaceae) is a group of aromatic plants traditionally used for flavouring a wide variety of foods, beverages and confectionary products and their essential oils are used in the manufacture of perfumes and cosmetics. Commercial thyme products include the fresh or dried herb, plant extracts and ornamental plants (Stahl-Biskup and Saéz 2002, Figueiredo et al. 2008). *Thymus lotocephalus* G. López and R. Morales is an endemic species from the south region of Portugal, Algarve. Besides its aromatic characteristics, it also has a great potential to be used as an ornamental in gardens. The low number of populations (less than ten) left, together with the intense human activity in the region, is leading to a drastic reduction of individuals, making this species extremely vulnerable. Though it is legally protected under the European Habitats Directive (92/43/CEE) and by Portuguese law (Reference 140/99, April 24, 1999), *in situ* conservation may not always be sufficient to ensure the survival of this endangered species.

Recently, our group has developed an *in vitro* propagation protocol for *T. lotocephalus* aiming its conservation (Coelho et al. 2012). To complement this strategy, different methods could be studied for its long term germplasm conservation. Seed storage coupled with cryopreservation is a simple, efficient and reliable method for long-term conservation. Cryopreservation storage is particularly important in the preservation of endangered germplasm because extremely low temperatures reduce metabolic processes (Walters et al. 2004, Gonçalves and Romano 2009). Studies concerning seed cryopreservation have been reported in several wild species (Pérez-García and González- Benito 2008, Gonçalves and Romano 2009, Zaidi et al. 2010). Prior to the study of seed cryopreservation tolerance, it is important to assess seed germination behaviour (Kadis and Georghiou 2010). The germination requirements of several *Thymus* spp. have been previously reported (Thanos et al. 1995, Pérez-García et al. 2003, González-Benito et al. 2004). However, there is no data available concerning the germination requirements of *T. lotocephalus* seeds.

We therefore set out to evaluate the germination behaviour of *T. lotocephalus* seeds from several populations at different incubation conditions, and the feasibility of seed cryopreservation for the conservation of *T. lotocephalus* germplasm.

### **2.2.3. Materials and Methods**

#### **2.2.3.1. Seed collection**

Seed samples were collected from four wild populations in the Algarve region, Portugal, in 2010 (Algoz, Tunes, Gambelas and Olhão). Collection was made from 20 to 30 individuals randomly selected from each population. After being extracted from the capsules, seeds were placed in paper bags and stored inside an hermetic sealed glass jar with silica gel and kept under laboratory conditions at  $25 \pm 2^\circ\text{C}$  in the dark for two months before the beginning of the trials. Seed water content was  $3.7 \pm 0.06\%$  fresh weight basis.

#### **2.2.3.2. Germination and cryopreservation trials**

Seed germination was tested at 15 or  $25^\circ\text{C}$  with a 16-h photoperiod (cool white fluorescent lamps,  $69 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) or continuous darkness, on 0.6% (w/v) agar (Duchefa, The Netherlands). Seeds from the populations Algoz, Tunes and Olhão were placed in 2-ml cryovials and directly immersed in liquid nitrogen (LN) for 30 days. Subsequently, rewarming took place at room temperature for approximately 18 h. Seeds were then sown and incubated at  $15^\circ\text{C}$  with a 16-h photoperiod. A control treatment, without immersion in LN, was performed under the same conditions.

#### **2.2.3.3. Statistical analysis**

In all trials, four replicates of 25 seeds each were tested. Germinating seeds were counted every two days over a total 30-day incubation period. At the end of this period, final germination percentage (%) and mean germination time (MGT, in days) (Ellis and Roberts 1981) were calculated. The values obtained were expressed as means  $\pm$  standard error. The data were subjected to analysis of variance (ANOVA) using the SPSS statistical package for Windows (release 18.0; SPSS Inc., Chicago, IL, USA). Final germination percentages were arcsine transformed prior to statistical analysis.

#### 2.2.4. Results and Discussion

The final germination percentage of *T. lotocephalus* seeds was significantly affected by temperature ( $P < 0.001$ ), whereas light conditions and the interaction between temperature and light had no significant effect ( $P \geq 0.05$ ) (Table 2.4).

The final germination percentages were significantly higher at 15°C, presenting values above 80%, and MGT below 10 days (Table 2.4), resulting in faster germination. According to Thanos et al. (1995) the optimal germination temperature of *Thymus capitatus* was 15°C, which agrees with the obtained results. More recently, other studies reported high germination percentages at the same germination temperature for several *Thymus* spp. (Pérez-García et al. 2003, González-Benito et al. 2004).

Considering the present results, with final germination percentages above 80% at 15°C without pretreatments, and according to Baskin and Baskin (2004) definition of seed dormancy, we conclude that *T. lotocephalus* seeds can be considered non-dormant.

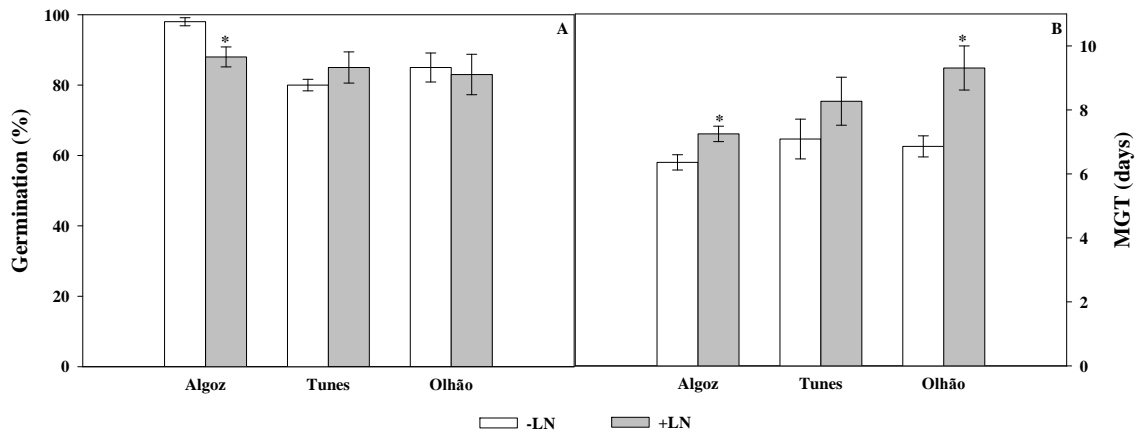
The geographic origin of the seeds can have an important effect on their germination (Pérez-García et al. 2003). Therefore, studies of germination behaviour should comprise several populations from the same species. The populations of *T. lotocephalus* that showed the best results varied according to the germination conditions assayed. For instance, under light at 15°C the best germination percentages and shorter MGT were obtained in the populations from Algoz and Gambelas ( $P < 0.05$ ) (Table 2.4). Pérez-García et al. (2003) obtained highly significant intraspecific population differences in the final germination percentages of Mediterranean Lamiaceae species, including *Thymus* spp., which could reflect local adaptation to a particular environment. In this study, the absence of major variations between populations might suggest that the origin sites of seed samples have similar environmental characteristics.

Cryopreserved seeds (+LN) presented final germination percentages above 80% for all the populations studied (Figure 2.2). The final germination percentage of the cryopreserved seeds significantly decreased only when compared to the control (-LN), in the Algoz population ( $P < 0.05$ ). Control seeds germinated faster than the cryopreserved in all the populations, with significant differences for Algoz and Olhão ( $P < 0.05$ ), MGT values being always below 10 days. Among populations, no significant differences were observed in the final germination percentage and MGT for cryopreserved seeds ( $P \geq 0.05$ ).

**Table 2.4.** – Final germination percentage (%) and MGT (days) of *Thymus lotocephalus* seeds from different populations at different germination temperatures, under light or dark, after 30 days of incubation.

			Population			
			Algoz	Tunes	Gambelas	Olhão
<b>Germination (%)</b>	15°C	Light	98.0 ± 2.00 a A	83.0 ± 5.00 b B	100.0 ± 0.00 a A	82.0 ± 6.22 a B
		Dark	97.0 ± 1.00 a A	98.0 ± 1.15 a A	97.0 ± 1.91 a A	82.0 ± 4.16 a B
	25°C	Light	60.0 ± 2.83 b A	48.0 ± 5.66 c AB	-	30.0 ± 9.31 c B
		Dark	48.0 ± 7.30 b A	44.0 ± 5.89 c A	-	56.0 ± 9.38 bc A
<b>MGT (days)</b>	15°C	Light	6.7 ± 0.53 b AB	7.6 ± 0.62 b A	4.7 ± 0.16 a B	7.6 ± 1.08 a A
		Dark	4.8 ± 0.65 b A	5.9 ± 0.36 b A	5.0 ± 0.15 a A	6.0 ± 0.31 b A
	25°C	Light	13.9 ± 1.54 a A	10.7 ± 1.18 a A	-	6.3 ± 1.21 b B
		Dark	6.8 ± 1.34 b B	10.3 ± 0.87 a A	-	9.2 ± 0.57 a AB

Values represent means ± SE of 4 replicates of 25 seeds each. Lower-case letters: for each population and parameter, values followed by the same letter are not significantly different at  $P < 0.05$  according to Duncan's Multiple Range Test. Capital letters: for each germination condition and parameter, values followed by the same letter are not significantly different at  $P < 0.05$  according to Duncan's Multiple Range Test. Trials for Gambelas population were only performed at 15°C.



**Figure 2.2.** – Final germination percentage (A) and MGT (B) of *Thymus lotocephalus* seeds from different populations after storage for 30 days in LN. –LN: control seeds; +LN: cryopreserved seeds. Bars represent means  $\pm$  SE of 4 replicates of 25 seeds each. \*: indicates significant differences ( $P < 0.05$ ) between cryopreserved and control seeds.

In most of the populations, the exposure of *T. lotocephalus* seeds to ultra low temperatures of LN was non-detrimental to seed germination, and germination percentages were always higher than 80%. Different studies have already reported the absence of negative effects of cryopreservation on seed germination of several wild species (Pérez-García and González-Benito 2008, Gonçalves and Romano 2009, Zaidi et al. 2010). The results obtained also indicate that gradual cooling rate is not required because the direct immersion of cryovials with seeds into LN did not decrease considerably the germination percentages of the different populations. This fact greatly simplifies the cryopreservation process and significantly reduces the costs (Gonçalves and Romano 2009, Zaidi et al. 2010). Hence, cryopreservation is a reliable and economical method for seed conservation of *T. lotocephalus*.

### 2.2.5. References

- Baskin JM and Baskin CC (2004) A classification system for seed dormancy. *Seed Science Research* 14: 1-16;
- Coelho N, Gonçalves S, González-Benito ME and Romano A (2012) Establishment of an *in vitro* propagation protocol for *Thymus lotocephalus* a rare aromatic species of the Algarve (Portugal). *Plant Growth Regulation* 66: 69-74;
- Ellis RH and Roberts EH (1981) The quantification of ageing and survival in orthodox seeds. *Seed Science and Technology* 9: 373-409;
- Figueiredo AC, Barroso JG, Pedro LG, Salgueiro L, Miguel MG and Faleiro ML (2008) Portuguese *Thymbra* and *Thymus* species volatiles: chemical composition and biological activities. *Current Pharmaceutical Design* 14: 3120-3140;
- Gonçalves S and Romano A (2009) Cryopreservation of seeds from the endangered insectivorous plant *Drosophyllum lusitanicum*. *Seed Science and Technology* 37: 485-490;
- González-Benito ME, Albert MJ, Iriando JM, Varela F and Pérez-García F (2004) Seed germination of four thyme species after short-term storage at low temperatures at several moisture contents. *Seed Science and Technology* 32: 247-254;
- Kadis C and Georghiou K (2010) Seed dispersal and germination behavior of three threatened endemic labiates of Cyprus. *Plant Species Biology* 25: 77-84;
- Pérez-García F, Hornero J and González-Benito ME (2003) Interpopulation variation in seed germination of five Mediterranean Labiatae shrubby species. *Israel Journal of Plant Sciences* 51: 117-124;
- Pérez-García F and González-Benito ME (2008) Seed cryopreservation of *Halimium* and *Helianthemum* species. *CryoLetters* 29: 271-276;
- Stahl-Biskup E and Saéz F (2002) *Thyme: The Genus Thymus, Medicinal and Aromatic Plants – Industrial Profiles*. Taylor & Francis, London;
- Thanos CA, Kadis CC and Skarou F (1995) Ecophysiology of germination in the aromatic plants thyme, savory and oregano (Labiatae). *Seed Science Research* 5: 161-170;
- Walters C, Wheeler L and Stanwood PC (2004) Longevity of cryogenically stored seeds. *Criobiology* 48: 229-244;
- Zaidi CA, González-Benito ME and Pérez-García F (2010) Morphology germination and cryopreservation of seeds of the Mediterranean annual plant *Tuberaria macrosepala* (Cistaceae). *Plant Species Biology* 25: 149-157.







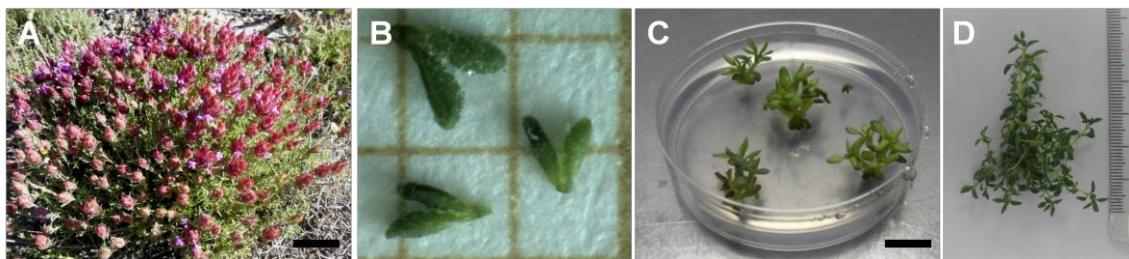
### 2.3.1. Abstract

*Thymus lotocephalus* is a rare endemic species from the Algarve, Portugal, and is legally protected by Portuguese and European legislation. The aim is to develop a cryopreservation protocol for *T. lotocephalus* shoot tips, as an alternative approach for the long-term conservation of this species. Several methods (droplet-vitrification, vitrification and encapsulation-dehydration) were tested. Conditions regarding the subculture period, cold-hardening and preculture were optimized. Cryopreserved shoot tips were also assessed for their genetic stability using random amplified polymorphic DNA (RAPD) markers. Droplet-vitrification presented the best results. The best regrowth of cryopreserved shoot tips obtained eight weeks after rewarming was 67%. This was accomplished with four weeks subculture period of *in vitro*-donor plants at 25°C, preculture of excised shoot tips for one day on Murashige and Skoog (MS) medium containing 0.3 M sucrose, treatment in PVS2 (plant vitrification solution 2) for 60 min, and MS supplemented with 0.2 mg l<sup>-1</sup> zeatin as recovery medium. The assessment using RAPD markers observed variation at a low frequency and shoots regenerated from cryopreserved apices showed normal development compared to the regular *in vitro*-grown shoots. Droplet-vitrification is thus a viable method for the cryopreservation of *T. lotocephalus* shoot tips.

### 2.3.2. Introduction

The genus *Thymus* (Lamiaceae) is a group of aromatic plants widely distributed in the Mediterranean region, but used all over the world for flavouring foods and beverages. Essential oils obtained from the plants are very important in the cosmetic industry and due to their antiseptic, antispasmodic and antimicrobial properties they are also used for medicinal purposes (Figueiredo et al. 2001, Stahl-Biskup and Saéz 2002, Figueiredo et al. 2008).

*Thymus lotocephalus* G. López & R. Morales (Figure 2.3A) is a rare endemic species from the Algarve, in the south of Portugal. It is considered vulnerable and is legally protected by Portuguese and European legislation [Portuguese law (Reference 140/99, April 24 1999) and the European Habitats Directive (92/43/CEE)] (ICN 2006). This undershrub is confined to a few populations in the centre and eastern part of the region, which are being drastically reduced because of the increasing urbanization during the last few decades. The preservation of *T. lotocephalus* is thus important as a way of keeping the genetic diversity, but also due to its value as a source of natural products. Several studies revealed that this species produces phytochemicals with medicinal properties, such as antimicrobial (Faleiro et al. 2003), antioxidant and anti-cholinesterase (Costa et al. 2012). This species also has great potential to be used as an ornamental in gardens. The plant requires low maintenance and produces small purple flowers that have, like other thyme species, a pleasant scent.



**Figure 2.3.** – Cryopreservation of *Thymus lotocephalus* shoot tips. Wild plant (bar: 7 cm approximately) (A). Excised shoot tips 1 mm long (B). Cryopreserved shoot tips by the droplet-vitrification method, four weeks after culture (bar: 1 cm) (C). Plantlet from cryopreserved shoot tip by the droplet-vitrification method, eight weeks after culture (D).

In order to fight the extinction of this species and contribute to its conservation, our group has been developing *ex situ* strategies, such as *in vitro* propagation (Coelho et al.

2012a) and seed cryopreservation (Coelho et al. 2012b). To complete the previous works, the next step is to implement a cryopreservation protocol for *T. lotocephalus* shoot tips, as another alternative for the long-term conservation of germplasm of this species. Cryopreservation of shoot tips has been recently reported for other *Thymus* species: *T. moroderi* (Marco-Medina et al. 2010a,b), *T. vulgaris* and *T. cariensis* (Ozudogru and Kaya 2012).

Cryopreservation storage (in or above liquid nitrogen, LN, at  $-196^{\circ}\text{C}$  to c.  $-170^{\circ}\text{C}$ ) is particularly useful for the preservation of germplasm because extremely low temperatures slow down metabolic processes. As a result, living plant material can be stored without deterioration, in theory, for an unlimited period of time. In addition, the material is stored in a small space, protected from contamination and requiring limited maintenance (Walters et al 2004, Gonçalves and Romano 2009, Engelmann 2011). Recent cryopreservation techniques allow cells and shoot tips to be cryopreserved by direct transfer to LN, avoiding the use of expensive devices to control the slow cooling process that reduces cell water and therefore crystallization. With the new techniques, cells are dehydrated before cooling. In vitrification procedures cells are exposed to a concentrated cryoprotectant solution that, at ultralow temperatures, enables water to solidify into a metastable glass, without ice formation. In encapsulation-dehydration procedures cells are exposed to air-drying and water is removed by evaporation (Gupta and Reed 2006, Sakai and Engelmann 2007, Ozudogru and Kaya 2012).

However, the extreme physical conditions and toxic compounds in the cryoprotectant solutions that cells are exposed to throughout cryopreservation process can reduce genetic integrity of cryopreserved plant materials. The assessment of genetic stability of cryopreserved plant material is thus crucial and can be achieved by cytological, histological, morphological, biochemical and molecular techniques (Engelmann 2004, Harding 2004). Within the latter, random amplified polymorphic DNA (RAPD) markers are a useful tool for the detection of variations in primary DNA structure because they are randomly and widely distributed throughout the genome; besides, it is a very interesting technique when working with wild species, without available genome information. In addition, RAPDs are easy and fast to assess (Martín and González-Benito 2005,) Several studies have reported the use of these markers to assess genetic stability after cryopreservation, for instance chrysanthemum (Martín et al. 2011), mint (Martín et al. 2013) and black pine (Salaj et al. 2011).

The aim of this study was to develop a cryopreservation protocol for *T. lotocephalus* shoot tips, testing encapsulation-dehydration and vitrification-based methods, and to assess their genetic stability after cryopreservation.

### **2.3.3. Materials and Methods**

#### **2.3.3.1. Plant material**

Experiments were conducted with shoot tips (Figure 2.3B) excised under a binocular microscope from *in vitro*-grown plantlets of *T. lotocephalus*, clone TL1. *In vitro* cultures, established from *T. lotocephalus* seedlings, were maintained on MS medium (Murashige and Skoog 1962), supplemented with 2% (w/v) sucrose and solidified by adding 1% (w/v) agar, in a culture chamber at 25°C under a 16 h light/ 8 h dark photoperiod provided by cool-white fluorescent lamps at a photon flux density of 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , with a subculture period of 4-6 weeks (Coelho et al. 2012a).

#### **2.3.3.2. Recovery medium**

Prior to the cryopreservation experiments, 1 and 2 mm long *T. lotocephalus* shoot tips were excised and cultured on two different media, MS and MS supplement with 0.2 mg l<sup>-1</sup> zeatin (ZEA) to assess their recovery.

#### **2.3.3.3. Cryopreservation by droplet-vitrification**

Experiments were conducted following the droplet-vitrification protocol by Panis et al. (2005). Shoot tips were excised and placed on semisolid preculture medium; preculture media and/or preculture time varied according to experiments.

After the different pretreatments, shoot tips were transferred onto two layers of sterile filter paper in 6 cm Petri dishes (ten shoot tips per dish) containing 2 ml of loading solution (0.4 M sucrose and 2 M glycerol in MS medium, pH adjusted to 5.8 before autoclaving) for 20 min at room temperature. After loading, shoot tips were transferred along with the filter paper to new Petri dishes containing 2 ml of ice-cooled plant vitrification solution 2 (PVS2). The PVS2 solution consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO (dimethyl sulphoxide) dissolved in MS medium that contained 0.4 M sucrose. The pH was adjusted to 5.8 and solution was

freshly prepared each time and filter sterilized (Sakai et al. 1990). Petri dishes were maintained on ice during exposure to PVS2. The dehydration times tested varied according to the experiments.

After PVS2 treatment, five shoot tips were placed on a sterile aluminium foil strip (25×5×0.03 mm), with each shoot tip in a 2 µl drop of PVS2. Strips were previously placed in Petri dishes on the top of a frozen cooling block, to keep the temperature of the strip close to 0°C. With a sterile forceps, each aluminium strip was placed inside a cryotube previously filled with LN and kept inside a polystyrene box containing LN. Strips remained in LN for at least 10 min. For rewarming, strips were rapidly transferred from the cryotube to a 6 cm Petri dish containing 4 ml of unloading solution (1.2 M sucrose in MS medium, adjusted to pH 5.8 and autoclaved), and kept there for 20 min at room temperature. Shoot tips were cultured on semisolid MS medium for one night at 25°C in the dark. Then they were transferred to recovery medium (semisolid MS supplement with 0.2 mg l<sup>-1</sup> ZEA) and maintained one week in the dark at 25°C before being transferred to light conditions (16 h light/ 8 h dark photoperiod provided by cool-white fluorescent lamps at a photon flux density of 40 µmol m<sup>-2</sup> s<sup>-1</sup>). Four weeks after rewarming, shoot tips were transferred to multiplication medium, MS without growth regulators. Control treatments refer to replicates carried out in the same conditions but without immersion in LN. For the treatment 0 min in PVS2, shoot tips were transferred from the loading solution directly to LN (except for the control, which was directly transferred to unloading solution).

Shoot tip size and PVS2 exposure time – In the first droplet-vitrification experiment, the influence of shoot tip size and PVS2 exposure time in the cryopreservation of *T. lotocephalus* apices was tested, adjusting the studied periods to the shoot apex size. Shoot tips 1 and 2 mm in length were precultured on semi-solid MS medium for one day and subsequently exposed to PVS2 for 0, 15, 30, 60 and 90 min prior to cryopreservation. The additional steps of the protocol were performed as described above.

Shoot tip preculture time – Shoot tips of 1 mm were used in a second experiment, to test different preculture periods (0, 1 or 2 days) on liquid MS medium containing 0.3 M sucrose (0 day refers to shoot tips precultured in semi-solid MS medium for 1 day). PVS2 exposure time was 30 and 60 min. The additional steps of the protocol were performed as described above.

Cold-hardening of *in vitro*-donor plants – In a third experiment, apices (1 mm long) were excised from *in vitro*-grown shoots of *T. lotocephalus* maintained at a constant temperature of 25°C during the first two weeks after the last subculture and then transferred to constant (10°C) or alternating (25/-1°C) temperature for a further two weeks. As a control, apices were also excised from shoots maintained at a constant temperature of 25°C for four weeks. After excision, shoot tips were precultured on liquid MS medium containing 0.3 M sucrose, for one day. The PVS2 exposure time was 60 min. The additional steps of the protocol were performed as described above.

Subculture period and culture age – In the last droplet-vitrification experiment, apices (1 mm long) excised from shoots of two different *in vitro*-grown clones (TL1 established *in vitro* 6 months earlier than TL11) with different intervals of subculture (3 to 8 weeks) were precultured on liquid MS medium containing 0.3 M sucrose, for one day. The PVS2 exposure time was 60 min. The additional steps of the protocol were performed as described above.

#### **2.3.3.4. Cryopreservation by vitrification**

Shoot tips (1 mm long) were excised and precultured on liquid MS medium containing 0.3 M sucrose, in 6 cm Petri dishes for one day. Following the pretreatment, shoot tips were immersed in 1 ml of loading solution for 20 min at room temperature in 2 ml cryotubes (five shoot tips per tube). The loading solution was removed with a hypodermic needle and replaced by ice-cooled PVS2. Cryotubes were maintained on ice during the period of exposure to PVS2. Dehydration times tested were 0, 30, 45, 60, 90 and 120 min. After PVS2 treatment, cryotubes were directly plunged in LN for at least 1 h. For rewarming, cryotubes were immersed in a water bath at 40°C for 2 min. PVS2 was removed and 1 ml of unloading solution was added to each cryotube and maintained for 20 min at room temperature. Shoot tips were cultured under the same conditions as described above.

#### **2.3.3.5. Cryopreservation by encapsulation-dehydration**

Shoot tips (2 mm long) were excised and placed on semi-solid MS medium or liquid MS medium containing 0.3 M sucrose, in 6 cm Petri dishes, for one day. Following the pretreatment, shoot tips were immersed in a 3% (w/v) sodium alginate solution

(prepared in MS medium containing 0.35 M sucrose, and the pH adjusted to 5.8 before autoclaving). With the help of a Pasteur pipette, drops, each containing one shoot tip, were dispensed on a 100 mM CaCl<sub>2</sub> solution (prepared in MS medium containing 0.35 M sucrose, with the pH adjusted to 5.8 before autoclaving). The beads formed were kept in the CaCl<sub>2</sub> solution for 30 min at room temperature and then transferred to liquid MS medium containing 0.75 M sucrose and kept there for 18-20 h at 25°C with continuous shaking, 120 rpm. Beads were removed from the previous solution, dried and placed in Petri dishes inside a laminar air flow cabinet for different periods of time (0, 1, 2, 3, 4, 5 and 6 h) at room temperature. Desiccated beads were placed in cryotubes (five beads per tube) and immersed in LN for at least 1 h. For rewarming, cryotubes were immersed in a water bath at 40°C for 2 min and beads were cultured under the same conditions as described for shoot tips.

#### **2.3.3.6. Assessment of survival and statistical analysis**

For each cryopreservation treatment, 15 to 60 shoot tips were used. Shoot apex regrowth (shoot tips developing into normal shoots) was recorded after eight weeks in culture, and expressed as the percentage over the number of tips cultured (excluding a very small number of contaminated apices). Data were subjected to a logistic regression analysis with binomial distribution to assess treatment differences using the SPSS statistical package (SPSS Inc., Chicago, IL). Significant differences between means were determined using Least Significant Difference (LSD,  $P = 0.05$ ).

#### **2.3.3.7. DNA isolation and RAPD amplification**

Samples for DNA analysis were obtained from *in vitro*-grown shoots of *T. lotocephalus* and from shoots recovered from cryopreserved shoot tips. The latter were obtained from the three different treatments (different subculture temperatures: constant 10 and 25°C and alternating 25/-1°C) of the droplet-vitrification cold hardening experiment, eight weeks after recovery. DNA was extracted according to a slightly modified method described by Doyle and Doyle (1987) and modified by Trindade et al. (2008). In short, approximately 20 mg of plant material was powdered with the help of a tissue lyser (Qiagen, Germany) and incubated with CTAB (cetyl trimethylammonium bromide) buffer [2% CTAB, 1.4 M NaCl, 20 mM EDTA (ethylenediaminetetraacetic acid), 50

mM TrisHCl, 2% (w/v) PVP-40 (polyvinylpyrrolidone) and 1%  $\beta$ -mercaptoethanol] at 60°C for 30 min. Two chloroform/isoamyl alcohol (24:1) extractions were performed and 2.5 M NaCl was added to remove polysaccharides. DNA was precipitated by adding cold isopropanol (-20°C). The precipitate was washed first with 0.2 M sodium acetate in 76% ethanol and then with 10 mM ammonium acetate in 76% ethanol. The pellet was dissolved in TE (Tris-EDTA) buffer and RNA was removed by digestion with DNase-free RNase A.

Twelve arbitrary primers from Operon Technology (Alameda/CA, USA) were tested, from them 10 were selected to obtain the RAPD amplifications: OPO-5 (5' CCCAGTCACT 3'), OPO-7 (5' CAGCACTGAC 3'), OPO-10 (5' TCAGAGCGCC 3'), OPO-20 (5' ACACACGCTG 3'), OPF-1 (5' ACGGATCCTG 3'), OPF-3 (5' CCTGATCACC 3'), OPF-4 (5' GGTGATCAGG 3'), OPF-10 (5' GGAAGCTTGG 3'), OPE-19 (5' ACGGCGTATG 3') and OPA-11 (5' CAATCGCCGT 3'). DNA amplification reactions were performed in 25  $\mu$ l reaction volume, containing approximately 10 ng template DNA, 1 or 2  $\mu$ l 20  $\mu$ M primer, 5  $\mu$ l 5 $\times$  MyTaq reaction buffer and 1 U MyTaq DNA polymerase (Bioline, UK). Amplifications were performed in a thermal cycler (Eppendorf, Germany) with the following program: one cycle of 1 min at 95°C, 35 cycles of 15 s at 95°C, 15 s at 37°C and 10 s at 72°C, and a final cycle of 10 min at 72°C. Amplification products were separated in a 1.5% (w/v) agarose gel in 1 $\times$  TBE (Tris-borate-EDTA) buffer, followed by staining with ethidium bromide. Molecular weights were estimated by reference to a 100 Base-Pair Ladder (GE Healthcare, UK). All the amplifications were repeated at least twice and only bands reproducible in both PCR (polymerase chain reaction) runs were considered for analysis.

## **2.3.4. Results**

### **2.3.4.1. Recovery medium**

Prior to cryopreservation experiments, it was necessary to determine the best medium to regenerate shoot tips, i.e., the recovery medium. For *T. lotocephalus*, two media were tested, based on the results obtained by Coelho et al. (2012a) during the development of *in vitro* propagation protocol for this species. The MS medium supplemented with 0.2 mg l<sup>-1</sup> ZEA presented a regrowth of 100%, for both shoot tip sizes, while on MS

medium the average was 69%, for shoot tips with 1 and 2 mm long (without significant difference between them). For the following cryopreservation experiments, the medium MS supplemented with 0.2 mg l<sup>-1</sup> ZEA was used as recovery medium.

#### **2.3.4.2. Shoot tip size and PVS2 exposure time**

The optimization of PVS2 exposure time is important to produce a high level of shoot tip regeneration after cryopreservation. Therefore in the first droplet-vitrification experiment we evaluated the effect of different exposure periods to PVS2 with shoot tips 1 and 2 mm long. Eight weeks after rewarming the results demonstrate that exposure to PVS2 during the time lapses tested had no negative effect on the growth of *T. lotocephalus* shoot tips (Table 2.5). Non-cryopreserved (-LN) shoot tips presented high, above 90%, regrowth percentages, with no significant differences ( $P \geq 0.05$ ) between the different conditions tested (Table 2.5). However, the percentages decreased for cryopreserved shoot tips (+LN). The highest regrowth were obtained for 1 mm shoot tips after 60 min PVS2 dehydration ( $P < 0.05$ ) (Table 2.5).

**Table 2.5.** – Effect of shoot tip size and PVS2 exposure time on the regrowth percentages of *Thymus lotocephalus* cryopreserved shoot tips by the droplet-vitrification method, eight weeks after culture.

<b>Shoot tip size (mm)</b>	<b>PVS2 exposure time (min)</b>	<b>-LN Regrowth (%)</b>	<b>+LN Regrowth (%)</b>
1	0	100.0 a	-
	15	100.0 a	10.7 b
	30	100.0 a	14.3 b
	60	93.3 a	40.0 a
2	0	100.0 a	-
	30	100.0 a	16.0 b
	60	93.3 a	20.0 ab
	90	100.0 a	4.0 b

In each column, values followed by the same letter are not significantly different at  $P < 0.05$  according to LSD. -LN: non-cryopreserved shoot tips (control); +LN: cryopreserved shoot tips; -: treatment not carried out.

### 2.3.4.3. Effect of shoot tip preculture period

Before cryopreservation, shoot tips are usually precultured on medium with high sugar concentrations to improve their survival and regrowth after freezing. In a second droplet-vitrification experiment, different preculture periods on MS medium supplemented with 0.3 M sucrose were tested. Since on the previous experiment the highest regrowth percentages were obtained with 1 mm shoot tips, in this experiment only this size of apices was used. The results obtained eight weeks after rewarming for non-cryopreserved (-LN) shoot tips again showed that the PVS2 had no negative effect on the growth of *T. lotocephalus* shoot tips, with regrowth percentages above 90% (Table 2.6). Cryopreserved (-LN) shoot tips had low regrowth percentages, except for the treatment of one day on 0.3 M sucrose followed by 60 min dehydration in PVS2 (Figure 2.3C,D), with regrowth percentages of 67% (Table 2.6). There was an improvement of more than 25% on the regrowth of cryopreserved *T. lotocephalus* shoot tips, compared to the second best result, zero day followed by 60 min dehydration in PVS2. Thus far, the optimal conditions for cryopreservation of *T. lotocephalus* shoot tips were one day on MS medium containing 0.3 M sucrose, followed by dehydration in PVS2 solution for 60 min, and using 1 mm long shoot tips.

**Table 2.6.** – Effect of preculture period (0, 1 and 2 days) in MS supplemented with 0.3 M sucrose on the regrowth percentages of *Thymus lotocephalus* cryopreserved shoot tips by the droplet-vitrification method, eight weeks after culture.

Preculture time (days)	PVS2 exposure time (min)	-LN Regrowth (%)	+LN Regrowth (%)
0*	30	95.0 a	15.0 c
	60	95.0 a	40.0 b
1	30	100.0 a	16.7c
	60	100.0 a	66.7 a
2	30	95.0 a	6.7 c
	60	80.0 a	3.3 c

In each column, values followed by the same letter are not significantly different at  $P < 0.05$  according to LSD. -LN: non-cryopreserved shoot tips (control); +LN: cryopreserved shoot tips; \*: shoot tips precultured on semi-solid MS medium for 1 day;

#### 2.3.4.4. Cold-hardening of *in vitro*-donor plants

Before excision of the shoot tips *in vitro*-donor plants are also commonly exposed to lower temperatures, to improve the regeneration response after cryopreservation. *T. lotocephalus* shoot cultures were exposed to constant (10°C) or alternating (25/-1°C) temperature for two weeks before excision of the apices. As a control, apices were also excised from shoots maintained at a constant temperature of 25°C. The droplet-vitrification method was conducted according to conditions described in the previous experiment as being optimal. Non-cryopreserved (-LN) shoot tips from the treatments 25 and 25/-1°C had high regrowth percentages (Table 2.7), and significantly higher than the treatment 10°C. Regarding the cryopreserved (+LN) shoot tips, there were no significant differences between treatments (Table 2.7). According to the results, cold-hardening of *in vitro*-donor plants did not affect the regrowth of *T. lotocephalus* cryopreserved shoot tips by droplet-vitrification, when compared to the previously described optimal conditions.

**Table 2.7.** Effect of cold-hardening of *in vitro*-donor plants on the regrowth percentages of *Thymus lotocephalus* cryopreserved shoot tips by the droplet-vitrification method, eight weeks after culture.

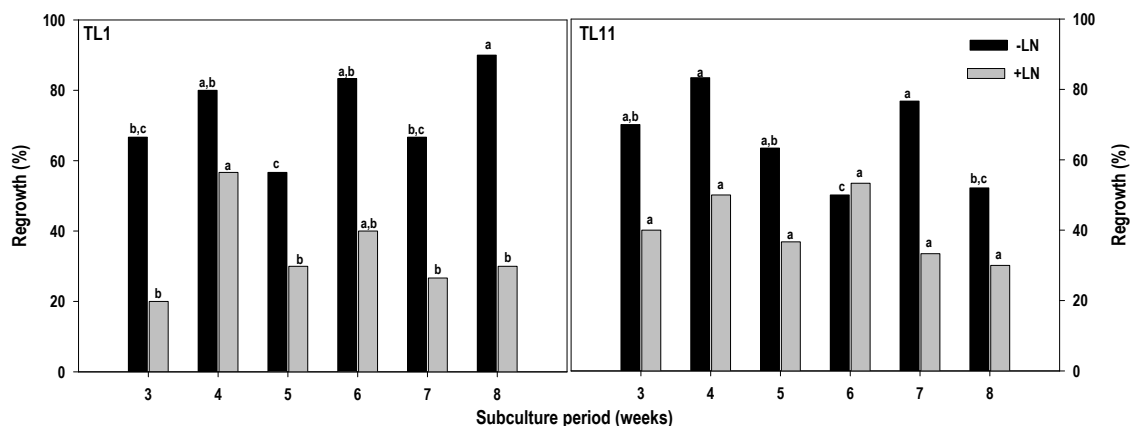
Temperature (°C)	-LN Regrowth (%)	+LN Regrowth (%)
10	58.6 b	53.4 a
25/-1	85.0 a	61.7 a
Control	78.0 a	56.7 a

In each column, values followed by the same letter are not significantly different at  $P < 0.05$  according to LSD test. Control refers to apices excised from *in vitro*-grown shoots of *T. lotocephalus* maintained at a constant temperature of 25°C for four weeks. -LN: non-cryopreserved shoot tips (control); +LN: cryopreserved shoot tips

#### 2.3.4.5. Subculture period and age of donor plants

The last experiment was conducted to test if the subculture period and age of *in vitro*-donor plants may affect the response of cryopreserved shoot tips. The conditions applied in the experiment were those previously described as optimal, without cold-hardening of *in vitro*-donor plants. The subculture period significantly affected regrowth of cryopreserved (+LN) shoot tips from clone TL1 ( $P = 0.043$ ; Figure 2.4), and the best

result, 57%, was obtained after four weeks in culture. As for the clone TL11, percentages above 50% were obtained in apices excised from six-week old shoots, but with no significant differences when compared to other subculture periods (Figure 2.4). Considering the age of the cultures (time maintained *in vitro*), clone TL11 (younger) presented higher regrowth percentages for the majority of subculture periods, however, this superiority was only significant ( $P < 0.05$ ) for three-weeks old shoots.



**Figure 2.4.** – Effect of subculture period on the regrowth percentages of *Thymus lotocephalus* (genotypes TL1 and TL11) cryopreserved shoot tips by the droplet-vitrification method, eight weeks after culture. In each graph, for the same LN condition, bars with different letters are significantly different at  $P < 0.05$  according to LSD test. -LN: non-cryopreserved shoot tips (control); +LN: cryopreserved shoot tips.

#### 2.3.4.6. Cryopreservation by vitrification

*T. lotocephalus* shoot tips were also cryopreserved using the vitrification method. Cryopreserved shoot tips showed very low survival, 10% and below, and no regrowth was observed for all PVS2 treatments tested with this method (data not shown). According to the results, it can be concluded that the vitrification method is not a suitable method for the cryopreservation of *T. lotocephalus* shoot tips.

#### 2.3.4.7. Cryopreservation by encapsulation-dehydration

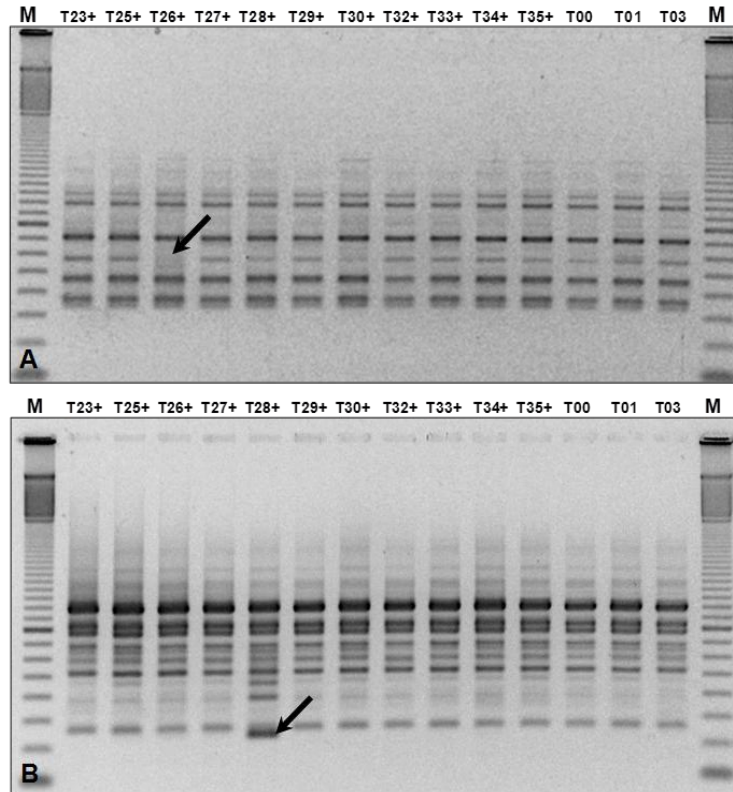
The response of cryopreserved shoot tips exposed to different dehydration periods was evaluated. For shoot tips precultured on MS medium, there was no regeneration after cryopreservation for beads desiccated for 0, 1 and 2 h. The highest regrowth percentage, 44%, was obtained after 4 h desiccation, decreasing with lower and higher desiccation

periods. As for shoot tips precultured on MS medium containing 0.3 M sucrose, regrowth was observed only after 5 h desiccation, however, with a very low percentage of 16%.

#### **2.3.4.8. RAPD analysis**

From the droplet-vitrification cold hardening experiment, five samples regenerated non-cryopreserved shoot tips (-LN) from each treatment (different subculture temperatures: constant 10 and 25°C, and alternating 25/-1°C), and ten samples from the corresponding regenerated cryopreserved shoot tips (+LN), plus three samples from *in vitro*-grown shoots, all of them corresponding to genotype TL1, were used for analysis of the genetic stability of cryopreserved shoot tips. The ten primers used in this study yielded 97 scorable bands, ranging from 200 to 2000 base pairs (bp). The total number of RAPD fragments considering all the material studied was 4656. Seven out of the ten RAPD primers tested showed an identical pattern in all the samples, and no variation was detected between regenerated shoots from cryopreserved and non-cryopreserved shoot tips and between *in vitro*-grown shoots. However, amplifications with primers OPO-5, OPO-7 and OPO-10 showed differences. In sample T26+ (+LN, 10°C treatment) is lacking the RAPD fragment of 580 bp present in all the other samples, obtained with OPO-10 (Figure 2.5).

The band pattern observed in the sample T28+ (+LN, 10°C treatment) with OPO-5 had a new RAPD fragment (270 bp) (Figure 2.5) when compared to all the other samples. Amplifications with OPO-7 revealed two new RAPD fragments, one of 690 bp in sample T1+ (+LN, 25/-1°C treatment) and another of 620 bp in sample T22+ (+LN, 25°C treatment). The frequency of variation was 0.06%. After several weeks in culture, regenerated shoots developed normally compared to the *in vitro*-grown shoots.



**Figure 2.5.** – RAPD profiles of DNA samples from cryopreserved material (T23+ to T35+) and *in vitro*-grown shoots (T00, T01, T03) of *Thymus lotocephalus*. Amplification products were generated by primers OPO-10 (A) and OPO-5 (B). M: 100 Base-Pair Ladder Marker. Arrow shows lack of band at 580 bp (A) and a new band at 270 bp (B).

### 2.3.5. Discussion

In the present study, three different methods (droplet-vitrification, vitrification and encapsulation-dehydration) were tested in order to develop a cryopreservation protocol for apices from *in vitro*-grown shoots of *T. lotocephalus*. Cryopreservation protocols have been developed recently for other *Thymus* spp (Marco-Medina 2010a,b; Ozudogru and Kaya 2012). In addition, several cryopreservation reports are available for other members of the Lamiaceae family, including *Mentha* spp. (e.g., Senula et al. 2007, Uchendu and Reed 2008) and *Lavandula* spp. (Tanaka and Takahashi 1995). From the results obtained, the best method tested was droplet-vitrification, presenting regrowth percentages above 60%. For the encapsulation-dehydration method the highest percentage was around 40% and for the vitrification method no regeneration was achieved for cryopreserved *T. lotocephalus* shoot tips. Similarly, Ozudogru and Kaya

(2012) obtained the best results with the droplet-vitrification method for *T. vulgaris* shoot tips, with a recovery percentage of 80%. The method was also applied to *T. cariensis*, but with lower results, 25% recovery (Ozudogru and Kaya 2012). *T. moroderi* was cryopreserved using the same three methods applied in this study (Marco-Medina 2010a,b). The reports showed that the highest survival percentages, above 80%, were obtained once again with the droplet-vitrification method (Marco-Medina 2010b). However, contrary to the present study, the survival percentages of *T. moroderi* using the vitrification method were much higher, around 70%. As for the encapsulation-dehydration method, survival percentages of 50% were achieved for *T. moroderi* shoot tips (Marco-Medina 2010a). The droplet-vitrification method was also successfully applied to *Mentha* spp. by Senula et al. (2007) with 89% recovery.

Droplet-vitrification is a method derived from the droplet-freezing technique, developed by Kartha et al. (1982), and the vitrification method, because of the plant vitrification solutions used developed by Sakai et al. (1990). Though droplet-vitrification is a vitrification-based procedure, the results obtained in this study were quite different between the two methods. In droplet-vitrification explants are placed in a very small volume of cryoprotective solution, compared to the conventional vitrification method, thus allowing faster cooling/warming rates and avoiding the possible formation of ice crystals during the process (Panis et al. 2005, Sakai and Engelmann 2007).

The recovery medium is important in the regeneration of cryopreserved shoot tips, as proved by Wang et al. (2003), for grapevine and citrus, and Pennycooke and Towill (2001), for sweet potato. In the present study it was defined that the medium to be used as recovery medium for the regeneration of *T. lotocephalus* shoot tips was MS supplemented with 0.2 mg l<sup>-1</sup> ZEA.

In vitrification-based methods the vitrification solution, such as PVS2, plays a major role in the cryopreservation success. This solution allows the dehydration of cells without causing damage, so that these are able to vitrify upon cooling in LN, without crystal formation. However, for successful cryopreservation, the time that samples are exposed to the vitrification solution needs to be optimized for each plant species and/or genotype. In an optimal treatment, cells are dehydrated long enough to reduce their water content, but without inducing injury due to the vitrification solution toxicity (Panis et al. 2005, Sakai and Engelmann 2007). Likewise, the size of the shoot tips influences their regeneration after cryopreservation. The smaller ones usually survive

cryopreservation better, but can also be more easily damaged by solution toxicity (Panis et al. 2005). For *T. lotocephalus*, the PVS2 treatment was non-detrimental for non-cryopreserved shoot tips of both sizes tested. After cryopreservation, the best regrowth percentages were obtained with a 60 min exposure to PVS2 with 1 mm shoot tips. Marco-Medina et al. (2010a) also obtained the highest survival percentages after 60 min PVS2 treatment for cryopreserved *T. moroderi* shoot tips, using the vitrification method. But with the droplet-vitrification method, the highest result was observed after 30 min PVS2, with the smaller (0.5-1 mm) shoot tips (Marco-Medina 2010b). As for *T. vulgaris* shoot tips, 90 min PVS2 treatment was the most effective using the droplet-vitrification method (Ozudogru and Kaya 2012).

To improve the dehydration tolerance of samples to the vitrification solution, and therefore the regeneration of cryopreserved shoot tips, donor plants can be preconditioned to cold acclimation (cold-hardening), to induce freezing tolerance, and/or apices can be precultured on medium with high levels of sugar, to reduce moisture content before cryopreservation (Panis et al. 2005, Sakai and Engelmann 2007, Ozudogru and Kaya 2012). The pretreatment of *T. lotocephalus* shoot tips in MS medium supplement with 0.3 M sucrose for one day, considerably improved the survival and regrowth percentage after cryopreservation. However, no differences were observed between shoot tips excised from cold-hardened and non-cold-hardened *in vitro*-donor plants and therefore the precondition to cold acclimation did not improve the survival and regrowth of *T. lotocephalus*. Cryopreservation of *T. vulgaris* shoot tips was also improved using sucrose pretreatment prior to cryopreservation (Ozudogru and Kaya 2012). The cold-hardening treatment was beneficial not only for *T. vulgaris* but also for *T. moroderi* (Marco-Medina 2010a,b; Ozudogru and Kaya 2012).

Yoon et al. (2006) demonstrated for potato shoot tips that the subculture conditions of *in vitro*-donor plants can influence the regeneration of cryopreserved shoot tips. In the present study the subculture period of *in vitro*-donor plants influenced the regrowth percentage of *T. lotocephalus* shoot tips from clone TL1. Besides it was verified that shoot tips excised from younger cultures (clone TL11) presented better results than the ones excised from older ones (clone TL1). This fact was also verified by Marco-Medina et al. (2010b) in *T. moroderi* shoot tips.

During the cryopreservation process plant tissues are exposed to physical, chemical and physiological stresses that may cause injuries to the cells and as a result lead to changes

in the genome. Therefore it is important to assess the genetic integrity of plants resulting from cryopreservation storage (Engelmann 2004, Harding 2004). In this study, genetic stability of cryopreserved *T. lotocephalus* shoot tips by droplet-vitrification was evaluated using RAPD markers. The RAPD analysis produced 97 scorable bands and a variation frequency of 0.06%. Similarly, in *T. moroderi* a 0.34% variation in cryopreserved apices was observed with RAPD markers (Marco-Medina and Casas 2013). The few variations observed had no influence on the morphological characteristics of the plants recovered from *T. lotocephalus* cryopreserved shoot tips. Kaity et al. (2013) also detected some variations by RAF (randomly amplified DNA fingerprinting) and AMP (amplified DNA methylation polymorphism) analysis on acclimatised papaya plants regenerated from cryopreserved shoot tips, with no morphological differences. There are other recent studies reporting variations at a low frequency using RAPD (Martín and González-Benito 2005), RAPD and AFLP (amplified fragment length polymorphism) (Martín et al. 2011) and SRAP (sequence-related amplified polymorphism) markers (Ai et al. 2012).

Most published reports concerning genetic stability find no variation (Haggman et al. 1998, DeVerno et al. 1999) or few variations (Martín and González-Benito 2005, Martín et al. 2011, Ai et al. 2012, Marco-Medina and Casas 2013) in plant material after cryopreservation. However there could be genetic changes that are not detected by the molecular techniques, such as RAPD markers, due to the fact that these techniques screen a low fraction of the genome (Harding 2004, Martín and González-Benito 2005). Moreover, the variations observed may be induced during the whole *in vitro* process and not just during the cooling stage (Harding 2004, Martín et al. 2011). The assessment of the genetic stability of chrysanthemum throughout all stages of an encapsulation-dehydration cryopreservation protocol proved that most of the variations observed occurred after the preculture stage (Martín et al. 2011). This preliminary study on the genetic stability of cryopreserved *T. lotocephalus* shoot tips demonstrated that variations occur at a low frequency, but these do not change the feasibility of the cryopreservation protocol. However further studies are required focused on the different stages of the cryopreservation procedure and using different genotypes.

In conclusion, *T. lotocephalus* shoot tips can be cryopreserved using the droplet-vitrification method. The best conditions tested in this study were: four weeks subculture of *in vitro*-donor plants at 25°C, one day preculture of excised shoot tips on

MS medium containing 0.3 M sucrose, 60 min treatment in PVS2 and recovery medium MS supplemented with 0.2 mg l<sup>-1</sup> ZEA. In addition, in the preliminary study on the genetic stability of *T. lotocephalus* shoot tips no major variations were observed. This protocol provides an important long-term conservation option for this species. Together with the *in vitro* propagation protocol and seed cryopreservation previously developed by the group, three different *ex situ* strategies will now contribute for the long-term conservation of *T. lotocephalus*.

### 2.3.6. References

- Ai P-F, Lu L-P and Song J-J (2012) Cryopreservation of *in vitro*-grown shoot-tips of *Rabdosia rubescens* by encapsulation-dehydration and evaluation of their genetic stability. *Plant Cell, Tissue and Organ Culture* 108: 381-387;
- Coelho N, Gonçalves S, González-Benito ME and Romano A (2012a) Establishment of an *in vitro* propagation protocol for *Thymus lotocephalus*, a rare aromatic species of the Algarve (Portugal). *Plant Growth Regulation* 66: 69-74;
- Coelho N, Gonçalves S, González-Benito ME and Romano A (2012b) Germination and cryopreservation tolerance of seeds from the rare aromatic species *Thymus lotocephalus*. *Scientia Horticulturae* 145: 84-86;
- Costa P, Gonçalves S, Grosso C, Andrade PB, Valentão P, Bernardo-Gil G and Romano A (2012) Chemical profiling and biological screening of *Thymus lotocephalus* extracts obtained by supercritical fluid extraction and hydrodistillation. *Industrial Crops and Products* 36: 246-256;
- DeVerno LL, Park YS, Bonga JM and Barret JD (1999) Somaclonal variation in cryopreserved embryogenic clones of white spruce [*Picea glauca* (Moench) Voss.]. *Plant Cell, Reports* 18: 948-953;
- Doyle JJ and Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11-15;
- Engelmann F (2004) Plant cryopreservation: progress and prospects. *In Vitro Cellular & Developmental Biology – Plant* 40: 427-433;
- Engelmann F (2011) Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cellular & Developmental Biology – Plant* 47: 5-16;
- Faleiro ML, Miguel MG, Ladeiro F, Venâncio F, Tavares R, Brito JC, Figueiredo AC, Barroso JG and Pedro LG (2003) Antimicrobial activity of essential oils isolated from Portuguese endemic species of *Thymus*. *Letters in Applied Microbiology* 36: 35-40;
- Figueiredo AC, Miguel MG, Duarte AMF, Barroso JG and Pedro LG (2001) Essential oil composition of *Thymus lotocephalus* G. López & R. Morales, collected during flowering and vegetative phases. *Flavour and Fragrance Journal* 16: 417-421;
- Figueiredo AC, Barroso JG, Pedro LG, Salgueiro L, Miguel MG and Faleiro ML (2008) Portuguese *Thymbra* and *Thymus* species volatiles: chemical composition and biological activities. *Current Pharmaceutical Design* 14: 3120-3140;
- Gonçalves S and Romano A (2009) Cryopreservation of seeds from the endangered insectivorous plant *Drosophyllum lusitanicum*. *Seed Science and Technology* 37: 485-490;
- Gupta S and Reed BM (2006) Cryopreservation of shoot tips of blackberry and raspberry by encapsulation-dehydration and vitrification. *CryoLetters* 27: 29-42;
- Haggman HM, Ryynanen LA, Aronen TS and Krainakova J (1998) Cryopreservation of embryogenic cultures of Scots pine. *Plant Cell, Tissue and Organ Culture* 54: 45-53;
- Harding K (2004) Genetic integrity of cryopreserved plant cells: a review. *CryoLetters* 25: 3-22;

- ICN (Instituto da Conservação da Natureza) (2006) Plano Sectorial da Rede Natura. Flora: *Thymus lotocephalus* G. López & R. Morales. [http://www.icn.pt/psrn2000/caracterizacao\\_valores\\_naturais/flora/Thymus%20lotocephalus.pdf](http://www.icn.pt/psrn2000/caracterizacao_valores_naturais/flora/Thymus%20lotocephalus.pdf). Accessed 29 April 2013;
- Kaity A, Drew RA and Ashmore SE (2013) Genetic and epigenetic integrity assessment of acclimatized papaya plants regenerated directly from shoot-tips following short- and long-term cryopreservation. *Plant Cell, Tissue and Organ Culture* 112: 75-89;
- Kartha KK, Leung NL and Mroginski LA (1982) *In vitro* growth responses and plant regeneration from cryopreserved meristems of Cassava (*Manihot esculenta* Crantz). *Zeitschrift für Pflanzenphysiologie* 107: 133-140;
- Marco-Medina A, Casas JL, Swennen R and Panis B (2010a) Cryopreservation of *Thymus moroderi* by droplet vitrification. *CryoLetters* 31: 14-13;
- Marco-Medina A, Casas JL and González-Benito ME (2010b) Comparison of vitrification and encapsulation-dehydration for cryopreservation of *Thymus moroderi* shoot tips. *CryoLetters* 31: 301-309;
- Marco-Medina A and Casas JL (2013) RAPD and phytochemical analysis of *Thymus moroderi* plantlets after cryopreservation. *CryoLetters* 34: 119-127;
- Martín C and González-Benito ME (2005) Survival and genetic stability of *Dendranthema grandiflora* Tzvelev shoot apices after cryopreservation by vitrification and encapsulation-dehydration. *Cryobiology* 51: 281-289;
- Martín C, Cervera MT and González-Benito ME (2011) Genetic stability analysis of chrysanthemum (*Chrysanthemum x morifolium* Ramat) after different stages of an encapsulation-dehydration cryopreservation protocol. *Journal of Plant Physiology* 168: 158-166;
- Martín C, Senula A, González I, Acosta A, Keller ERJ and González-Benito ME (2013) Genetic identity of three mint accessions stored by different conservation procedures: field collection, *in vitro* and cryopreservation. *Genetic Resources and Crop Evolution* 60: 243-249;
- Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497;
- Ozudogru EA and Kaya E (2012) Cryopreservation of *Thymus cariensis* and *T. vulgaris* shoot tips: comparison of three vitrification-based methods. *CryoLetters* 33: 363-375;
- Panis B, Piette B and Swennen R (2005) Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all *Musaceae*. *Plant Science* 168: 45-55;
- Pennycooke JC and Towill LE (2001) Medium alterations improve regrowth of sweet potato (*Ipomoea batatas* (L.) Lam.) shoot tips cryopreserved by vitrification and encapsulation-dehydration. *CryoLetters* 22: 381-389;
- Sakai A, Kobayashi S and Oiyama I (1990) Cryopreservation of nucellar cells and navel orange (*Citrus sinensis* Osb. Var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Reports* 9: 30-33;
- Sakai A and Engelmann F (2007) Vitrification, encapsulation-vitrification and droplet-vitrification: a review. *CryoLetters* 28: 151-172;

- Salaj T, Matušíková I, Fráterová L, Piršelová B and Salaj J (2011) Regrowth of embryogenic tissues of *Pinus nigra* following cryopreservation. *Plant Cell, Tissue and Organ Culture* 106: 55–61;
- Senula A, Keller ERJ, Sanduijav T and Yohannes T (2007) Cryopreservation of cold-acclimated mint (*Menta* spp.) shoot tips using a simple vitrification protocol. *CryoLetters* 28: 1-12;
- Stahl-Biskup E and Saéz F (2002) Thyme: The genus *Thymus*, Medicinal and Aromatic Plants – Industrial Profiles. Taylor & Francis, London;
- Tanaka A and Takahashi J (1995) Preservation of Cell Cultures of *Lavandula vera* (Lavender). In: Bajaj YPS (ed.) *Biotechnology in Agriculture and Forestry* 32: Cryopreservation of plant germplasm I (pp 500-508) Springer-Verlag, Berlin-Heidelberg;
- Trindade H, Costa M, Lima AS, Pedro LG, Figueiredo AC and Barroso JG (2008) Genetic diversity and chemical polymorphism of *Thymus caespititius* from Pico, São Jorge and Terceira islands (Azores). *Biochemical Systematics and Ecology* 36: 790-797;
- Uchendu EE and Reed BM (2008) A comparative study of three cryopreservation protocols for effective storage of *in vitro*-grown mint (*Mentha* spp.). *CryoLetters* 29: 181-188;
- Walters C, Wheeler L and Stanwood PC (2004) Longevity of cryogenically stored seeds. *Criobiology* 48: 229-244;
- Wang Q, Li P, Batuman O, Gafny R and Mawassi M (2003) Effect of benzyladenine on recovery of cryopreserved shoot tips of grapevine and citrus cultured *in vitro*. *CryoLetters* 24: 293-302;
- Yoon J-W, Kim H-H, Ko H-C, Hwang H-S, Hong E-S, Cho E-G and Engelmann F (2006) Cryopreservation of cultivated and wild potato varieties by droplet vitrification: effect of subculture of mother-plants and of preculture of shoot tips. *CryoLetters* 27: 211-222.



# CHAPTER 3



## *Plantago algarbiensis*

Coelho N, González-Benito ME and Romano A (2014) Approaches for the cryopreservation of *Plantago algarbiensis*, a rare endemic species of the Algarve. (Submitted)

Coelho N, Martín C, González-Benito ME and Romano A (2014) Estimation of genetic diversity in *Plantago algarbiensis* seedlings. (Submitted)



## 3.1

### **Approaches for the cryopreservation of *Plantago algarbiensis*, a rare endemic species of the Algarve**

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### **3.1.1. Abstract**

The main goal of this study was to investigate the viability of cryopreservation procedures in the conservation of seeds and nodal explants from *Plantago algarbiensis*, an endangered endemic species from the Algarve, Portugal. After 30 days in liquid nitrogen, the germination capacity of seeds was not affected. The two methods tested for the cryopreservation of nodal explants presented regrowth percentages of approximately 60%. With the droplet-vitrification method, a regrowth percentage of  $60.0 \pm 15.2\%$  was obtained after 120 min exposure to PVS2 (plant vitrification solution 2) and with encapsulation-dehydration method the highest percentage,  $63.3 \pm 9.6\%$ , was achieved after 3 h desiccation. For both methods, nodal segments were precultured on Murashige and Skoog (MS) medium and recovered on MS supplemented with  $0.2 \text{ mg l}^{-1}$  6-benzyladenine (BA), after freezing. Seed cryopreservation and cryopreservation of nodal segments by droplet-vitrification and encapsulation-dehydration are therefore effective approaches for the conservation of *P. algarbiensis*.

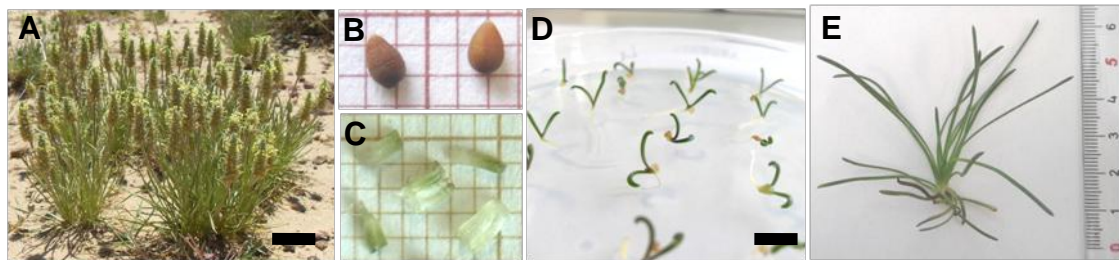
### 3.1.2. Introduction

Cryopreservation techniques are probably the most promising approach for preserving *ex situ* plant genetic resources and of extreme importance when applied to plant species in danger of extinction. The ultralow temperatures of the liquid nitrogen (LN, -196°C) allow the conservation of germplasm for a long period of time without deterioration, because at these temperatures all metabolic processes are believed to be drastically reduced (Engelmann 2004, Walters et al. 2004, Engelmann 2011).

Different plant materials, such as seeds, shoot tips, cell suspensions, dormant buds and others, can be cryopreserved. While orthodox seeds generally do not need any pretreatment before cryopreservation due to their natural dehydration processes, the majority of plant material used in cryopreservation possesses high quantity of cellular water, which can cause damages during the freezing process, due to the crystallization of water into ice (Engelmann 2011). In recent cryopreservation techniques, cells are dehydrated before cooling and plant material can be transferred directly to LN without the use of expensive apparatus to control the slow freezing process. In vitrification (*sensu stricto*) procedures cells are exposed to a highly concentrated cryoprotectant solution for short periods that, at ultralow temperatures, enables water to solidify into a meta-stable glass, without ice crystals formation. Droplet-vitrification is derived from the standard vitrification procedure in combination with the droplet-freezing technique, developed by Kartha et al. (1982). Plant material is placed in droplets of cryoprotective solution and cooled rapidly. In encapsulation-dehydration procedures cells are exposed to air-drying and water is removed by evaporation (Sakai and Engelman 2007; Engelmann 2011).

*Plantago algarbiensis* Samp. (Figure 3.1A) is an endemic species from the Algarve region (Portugal), considered endangered and legally protected by the European Habitats Directive 92/43/ CEE and Portuguese law (reference 140/99 of April 24). This species is limited to a few populations in a small area (approximately 50 ha) and with a low number of individuals. It can be found in clay-rich soils and areas located downstream from small springs or clearings of low acidophilic brushes (ICN 2007). For this species an *in vitro* propagation protocol has been developed as an *ex situ* conservation measure (Gonçalves et al. 2009b). The study of the germination requirements of *P. algarbiensis* seeds has also been reported (Martins et al. 2012). In addition, our group proved that *P. algarbiensis* accumulates considerable amounts of

aluminium (Martins et al. 2013), which can lead to the development and mass production of *in vitro* plants with enhanced performance in soils contaminated by aluminium. Cryopreservation would, therefore, broaden the *ex situ* conservation techniques used with this species and prolong the storage time of the propagules. To the best of our knowledge, cryopreservation of seeds has only been reported in another *Plantago* species, *P. cordata* (Pence and Clark 2005), and no information is available on the cryopreservation of vegetative explants within this genus. However, there are a number of studies reporting the use of explants from rare plants in cryopreservation, such as *Lithodora rosmarinifolia* (Barraco et al. 2013), *Hypericum richeri* ssp. *transsilvanicum* and *H. umbellatum* (Coste et al. 2012) and *Centaureum rigualli* (González-Benito et al. 1997), with positive results, proving that this technique plays an important role in conservation of genetic resources of rare species.



**Figure 3.1.** Cryopreservation of *Plantago algarbiensis*. Wild plant (bar: 7.5 cm approximately) (A). Seeds (B). Excised nodal segments 2-3 mm long (C). Seedlings from cryopreserved seeds (bar: 1 cm) (D). Plantlet from cryopreserved nodal segments by the droplet-vitrification method, eight weeks after culture (E).

The aims of this work were to evaluate seed cryopreservation tolerance and to develop cryopreservation protocols from nodal explants of *P. algarbiensis*, and therefore contribute to its conservation.

### 3.1.3. Materials and Methods

#### 3.1.3.1. Seed collection

Seeds (Figure 3.1B) were collected from three wild populations (Algoz, Tunes and Gambelas) in the Algarve region, Portugal, in July 2010. Collection was made from 10 to 30 individuals randomly selected from each population. After being extracted from the capsules, seeds from each population were kept in separate paper bags and stored

inside a hermetic sealed glass jar with silica gel and kept at  $25 \pm 2^\circ\text{C}$  in the dark for two months before the beginning of the trials.

### 3.1.3.2. Seed cryopreservation trials

Seeds were placed in 2-ml cryovials and directly immersed in LN for 30 days. After that period, cryovials were removed from the cryogenic storage and rewarming took place at room temperature for approximately 18 h. Seeds were sown in 11 cm glass Petri dishes on 0.6% (w/v) agar (Duchefa, The Netherlands) without nutrients and germinated at  $15^\circ\text{C}$  with a 16 h light/ 8 h dark photoperiod (cool white fluorescent lamps,  $69 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) (Martins et al. 2012). Control trials, without immersion in LN, were performed under the same conditions.

### 3.1.3.3. *In vitro* shoot cultures

Experiments were conducted with 2-3 mm long nodal segments (Figure 3.1C) dissected from *in vitro*-grown shoots of *P. algarbiensis*, clone P104. *In vitro* cultures, established from *P. algarbiensis* seeds from Algoz (Gonçalves et al. 2009b), were maintained on MS medium (Murashige and Skoog 1962) supplemented with  $0.2 \text{ mg l}^{-1}$  6-benzyladenine (BA), 2% (w/v) sucrose and solidified by adding 1% (w/v) agar, in a culture chamber at  $25^\circ\text{C}$  under a 16 h light/ 8 h dark photoperiod provided by cool-white fluorescent lamps at a photon flux density of  $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ , with a subculture period of 4 weeks.

### 3.1.3.4. Recovery medium

Prior to the cryopreservation experiments, *P. algarbiensis* segments were cultured on different containers and different media to assess their recovery. To test the different containers, 250 ml-Erlenmeyers and 6 cm Petri dishes, containing 60 ml and 10 ml, respectively, of semi-solid MS or MS supplemented with  $0.2 \text{ mg l}^{-1}$  BA, were used to culture the 2-3 mm segments. Also different media, dispensed only in the 250 ml-Erlenmeyers, were investigated: complete MS (1); complete MS supplemented with  $0.2 \text{ mg l}^{-1}$  BA (2); complete MS supplemented with 5% (w/v) glucose (instead of sucrose) (3); complete MS supplemented with  $0.004 \text{ mg l}^{-1}$  gibberellic acid ( $\text{GA}_3$ ) (4); complete MS supplemented with  $0.004 \text{ mg l}^{-1}$   $\text{GA}_3$  and  $0.1 \text{ mg l}^{-1}$  BA (5); MS with

reduced  $\text{NH}_4\text{NO}_3$  and  $\text{KH}_2\text{PO}_4$  (412.5 and 42.5  $\text{mg l}^{-1}$ , respectively) (6); and MS with reduced  $\text{NH}_4\text{NO}_3$  and  $\text{KH}_2\text{PO}_4$  supplemented with 0.004  $\text{mg l}^{-1}$   $\text{GA}_3$  and 0.1  $\text{mg l}^{-1}$  BA (7). After eight weeks in culture, the regrowth, multiplication and rooting percentage, the total number of new shoots produced per culture, the length of the longest shoot, the number of roots, the length of the longest root and the number of leaves were assessed.

### **3.1.3.5. Cryopreservation by droplet-vitrification**

Experiments were conducted following the droplet-vitrification protocol by Panis et al. (2005). Segments were excised and placed on semisolid MS medium or liquid MS medium containing 0.3 M sucrose for one day.

After the different pretreatments, segments were transferred onto two layers of sterile filter paper in 6 cm Petri dishes (ten segments per dish) containing 2 ml of loading solution (0.4 M sucrose and 2 M glycerol in MS medium, pH adjusted to 5.8 before autoclaving) and remained there for 20 min at room temperature. After loading, segments were transferred along with the filter paper to new 6 cm Petri dishes containing 2 ml of ice-cooled plant vitrification solution 2 (PVS2). The PVS2 solution consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO (dimethyl sulphoxide) dissolved in MS medium that contained 0.4 M sucrose, pH was adjusted to 5.8 and the solution was freshly prepared each time and filter sterilized (Sakai et al. 1990). Petri dishes were maintained on ice during the time segments were exposed to PVS2. The dehydration times tested were 0, 30, 60, 90 and 120 min. After PVS2 treatment, segments were placed on a sterile aluminium foil strip (approximately 25 x 5 x 0.03 mm, five segments per strip), each segment on a 2  $\mu\text{l}$  drop of PVS2. Strips were previously placed onto Petri dishes on top of a frozen cooling block, to keep the temperature of the strip close to 0°C. With the help of sterile forceps, each aluminium strip was placed inside a cryotube previously filled with LN, kept inside a polystyrene box containing LN. Strips remained in LN for at least 10 min. For rewarming, strips were rapidly transferred from the cryotube to a 6 cm Petri dish containing 4 ml of unloading solution (1.2 M sucrose in MS medium, pH adjusted to 5.8 before autoclaving), and kept there for 20 min at room temperature. Segments were cultured on semisolid MS medium in 6 cm Petri dishes for one night at 25°C in the dark. On the next day, segments were transferred to recovery medium (semisolid MS supplement with 0.2  $\text{mg l}^{-1}$  BA) in 250 ml-Erlenmeyers and were maintained one week in the dark

at 25°C before being transferred to light conditions (16 h light/ 8 h dark photoperiod provided by cool-white fluorescent lamps at a photon flux density of 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).

Control treatments refer to replicates carried out in the same conditions but without immersion in LN. For the treatment 0 min in PVS2, segments were transferred from the loading solution directly to LN (except for the control, which was directly transferred to the unloading solution).

### **3.1.3.6. Cryopreservation by encapsulation-dehydration**

Segments were excised and placed on semisolid or liquid MS medium containing 0.3 M sucrose for one day. Following the pretreatment, segments were immersed in a 3% (w/v) sodium alginate solution (prepared in MS medium containing 0.35 M sucrose, pH adjusted to 5.8 before autoclaving). With the help of a Pasteur pipette, drops, each containing one segment, were dispensed onto a 100 mM  $\text{CaCl}_2$  solution (prepared in MS medium containing 0.35 M sucrose, pH adjusted to 5.8 before autoclaving). The beads formed were kept in the  $\text{CaCl}_2$  solution for 30 min at room temperature and then transferred to liquid MS medium containing 0.75 M sucrose and kept there for 18-20 h at 25°C with continuous shaking, 120 rpm. Beads were removed from the previous solution, dried with sterile filter paper and placed in Petri dishes inside a laminar air flow cabinet for different periods of time (0, 1, 2, 3, 4, 5 and 6 h) at room temperature. Desiccated beads were placed in cryotubes (five beads per tube) and immersed in LN for at least 1 h. For rewarming, cryotubes were immersed in a water bath at 40°C for 2 min and beads were cultured on semisolid MS medium supplement with 0.2  $\text{mg l}^{-1}$  BA at 25°C in the dark. After one week, segments were transferred to light conditions (16 h light/ 8 h dark photoperiod provided by cool-white fluorescent lamps at a photon flux density of 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).

### **3.1.3.7. Statistical analysis**

In all seed cryopreservation trials, four replicates of 25 seeds each were tested. Germinating seeds (emerging radicle) were counted every two days over a total 30-day incubation period. At the end of the germination period, the final germination percentage (%) and the mean germination time (MGT, in days; Ellis and Roberts 1981) were calculated.

In the recovery medium trials, 45 segments were used per each medium tested. In the segments cryopreservation trials, 30 segments were used per each treatment of preculture medium and vitrification/desiccation time, for control and cryopreserved segments. Segment survival (segments remaining green or showing growth) and regrowth (segments developing into normal shoots) was recorded after eight weeks in culture, and expressed as the percentage over the number of segments cultured.

The values obtained were expressed as means  $\pm$  standard error. The data were subjected to analysis of variance (ANOVA) using the SPSS statistical package for Windows (release 18.0; SPSS Inc., Chicago, IL, USA). Significant differences between means were determined using Duncan's New Multiple Range Test ( $P = 0.05$ ). The original percentage data were arcsine transformed prior to statistical analysis.

### 3.1.4. Results

#### 3.1.4.1. Seed cryopreservation

In the present study, first it was investigated the feasibility of seed cryopreservation for the conservation of *P. algarbiensis* germplasm. Cryopreserved seeds (+LN) (Figure 3.1D) presented final germination percentages above 90% for all the populations studied (Table 3.1).

**Table 3.1.** – Final germination percentage and MGT of *Plantago algarbiensis* seeds from different populations after storage for 30 days in LN.

Population	Germination (%)		MGT (days)	
	-LN	+LN	-LN	+LN
Algoz	100.0 $\pm$ 0.0 a	100.0 $\pm$ 0.0 a	5.0 $\pm$ 0.0 b	5.1 $\pm$ 0.5 b
Tunes	99.0 $\pm$ 1.0 a	98.0 $\pm$ 2.0 a	6.5 $\pm$ 0.2 a	6.6 $\pm$ 0.2 a
Gambelas	97.0 $\pm$ 1.9 a	94.00 $\pm$ 1.2 a	5.2 $\pm$ 0.3 b	5.4 $\pm$ 0.7 b

Values represent means  $\pm$  SE of 4 replicates of 25 seeds each. For each parameter, in a column, values followed by the same letter are not significantly different at  $P < 0.05$  according to Duncan's Multiple Range Test. -LN: non-cryopreserved seeds (control); +LN: cryopreserved seeds.

There were no significant differences between the final germination percentage of cryopreserved seeds and the control (-LN) ( $P \geq 0.05$ ). As for MGT, values from both cryopreserved and control seeds were below 7 days and no significant differences were

observed between them ( $P \geq 0.05$ ). Among populations, also no significant differences were observed in the final germination percentage ( $P \geq 0.05$ ), but the MGT value was higher for Tunes population for cryopreserved and control seeds ( $P < 0.05$ ) (Table 3.1). Seed water content was  $8.84 \pm 0.75\%$  fresh weight basis.

#### **3.1.4.2. Recovery medium**

Recovery medium preliminary experiments suggested that the type of container could influence the regrowth of nodal explants. Based on that results (data not shown), the type of container for recovery of nodal explants was first tested: 250 ml-Erlenmeyers or 6 cm Petri dishes. After two weeks in culture, there were no significant differences ( $P \geq 0.05$ ) between the two types of containers, all presented regrowth percentages of 100%. However, after four weeks, shoots from segments cultured in the Petri dishes started to dry out and the respective regrowth percentages lowered. The results obtained after eight weeks of culture demonstrate that Erlenmeyers were a more suitable container for the regeneration of segments, with higher regrowth percentages for both media tested ( $P < 0.05$ ). The regrowth percentage using Erlenmeyers was  $100.0 \pm 0.0\%$  for both media and using Petri dishes was  $15.0 \pm 9.6$  and  $20.0 \pm 11.6\%$  for MS and MS supplemented with  $0.2 \text{ mg l}^{-1}$  BA, respectively.

Seven different media were then tested, using the Erlenmeyers, for the recovery of *P. algarbiensis* segments and results are presented in Table 3.2. These media were selected based on the results obtained during the development of *in vitro* propagation protocols for *P. algarbiensis* (Gonçalves et al. 2009b) and *P. major* (Mederos et al. 1997). All media presented regrowth percentages above 90%, without significant differences ( $P \geq 0.05$ ) between them. As for the other parameters measured, medium MS supplemented with  $0.2 \text{ mg l}^{-1}$  BA had the maximum multiplication percentage and the highest number of new shoots and shoot length ( $P < 0.05$ ). On the other hand, no root formation was observed in this medium. The highest rooting percentage and number of roots was obtained in medium MS supplemented with 5% (w/v) glucose ( $P < 0.05$ ). There were no significant differences in the number of leaves between the seven media tested ( $P \geq 0.05$ ). For the following cryopreservation experiments, the medium chosen for recovery was MS supplemented with  $0.2 \text{ mg l}^{-1}$  BA because it presented the best results when combining regeneration and multiplication.

**Table 3.2.** – Effect of different media on the regrowth, multiplication and rooting percentage, number of new shoots, shoot length, number of roots, root length and number of leaves of *Plantago algarbiensis* segments, eight weeks after culture.

Basal medium	Regrowth (%)	Multiplication (%)	No. new shoots	Shoot length (cm)	Rooting (%)	No. roots	Root length (cm)	No. leaves
1	100.0 ± 0.0 a	26.7 ± 9.9 c	1.3 ± 0.1 c	7.1 ± 0.3 b	23.3 ± 6.2 b	0.2 ± 0.1 b	9.4 ± 2.6 c	19.5 ± 1.1 a
2	100.0 ± 0.0 ab	100.0 ± 0.0 a	5.7 ± 0.4 a	8.4 ± 0.3 a	0.0 ± 0.0 c	-	-	21.7 ± 1.5 a
3	93.3 ± 3.3 ab	80.0 ± 6.3 b	3.3 ± 0.3 b	3.9 ± 0.3 d	64.0 ± 7.5 a	1.3 ± 0.3 a	9.1 ± 1.0 c	22.2 ± 1.6 a
4	97.8 ± 2.2 b	16.7 ± 6.2 c	1.2 ± 0.1 c	6.8 ± 0.4 b	16.7 ± 6.2 b	0.2 ± 0.1 b	11.4 ± 3.2 bc	19.5 ± 0.9 a
5	97.8 ± 2.2 a	83.3 ± 6.2 b	3.1 ± 0.3 b	7.4 ± 0.4 b	16.7 ± 8.0 bc	0.2 ± 0.1 b	19.6 ± 2.7 a	21.9 ± 1.2 a
6	97.8 ± 2.2 ab	23.3 ± 6.2 c	1.4 ± 0.2 c	5.1 ± 0.2 c	13.3 ± 4.2 bc	0.2 ± 0.1 b	18.4 ± 2.6 ab	18.8 ± 1.0 a
7	100.0 ± 0.0 a	83.3 ± 6.2 b	2.9 ± 0.2 b	4.4 ± 0.2 cd	16.7 ± 6.2 b	0.4 ± 0.2 b	13.1 ± 3.9 abc	21.2 ± 1.1 a

Values represent means ± SE of 9 replicates of 5 segments each. For each variable, values followed by the same letter are not significantly different at  $P < 0.05$  according to Duncan's Multiple Range Test. (1) complete MS; (2) complete MS supplemented with 0.2 mg l<sup>-1</sup> BA; (3) complete MS supplemented with 5% (w/v) glucose; (4) complete MS supplemented with 0.004 mg l<sup>-1</sup> GA<sub>3</sub>; (5) complete MS supplemented with 0.004 mg l<sup>-1</sup> GA<sub>3</sub> and 0.1 mg l<sup>-1</sup> BA; (6) MS with reduced NH<sub>4</sub>NO<sub>3</sub> and KH<sub>2</sub>PO<sub>4</sub>; (7) MS with reduced NH<sub>4</sub>NO<sub>3</sub> and KH<sub>2</sub>PO<sub>4</sub> supplemented with 0.004 mg l<sup>-1</sup> GA<sub>3</sub> and 0.1 mg l<sup>-1</sup> BA (7).

### 3.1.4.3. Droplet-vitrification

Effect of preculture and PVS2 exposure time without freezing – In a first droplet-vitrification experiment it was evaluated the effect of different exposure times to PVS2 on *P. algarbiensis* segments, precultured on semisolid MS medium or liquid MS medium containing 0.3 M sucrose for one day, without immersion in LN. Except for segments pretreated with MS medium containing 0.3 M sucrose followed by 120 min PVS2 exposure time, all treatments presented high survival and regrowth percentages, between 70.0 and 100.0% (Table 2.3). These results demonstrate a high level of regeneration after exposure to PVS2, meaning that the toxicity of the solution had no negative effect on the growth of *P. algarbiensis* segments.

**Table 2.3.** – Effect of preculture medium and PVS2 exposure time on the survival and regrowth percentages of *Plantago algarbiensis* segments by the droplet-vitrification method without freezing, eight weeks after culture.

Preculture medium	PVS2 exposure time (min)	Survival (%)	Regrowth (%)
MS	0	100.0 ± 0.0 a	100.0 ± 0.0 a
	30	93.3 ± 6.7 ab	93.3 ± 6.7 ab
	60	90.0 ± 6.8 ab	90.0 ± 6.8 ab
	90	76.7 ± 8.0 b	76.7 ± 8.0 b
	120	93.3 ± 4.2 ab	90.0 ± 4.5 ab
MS + 0.3 M sucrose	0	73.3 ± 8.4 b	70.0 ± 10.0 b
	30	83.3 ± 8.0 ab	83.3 ± 8.0 ab
	60	83.3 ± 6.2 ab	83.3 ± 6.2 ab
	90	80.0 ± 7.3 b	76.7 ± 8.0 b
	120	46.7 ± 8.4 c	43.3 ± 12.0 c

Values represent means ± SE of 6 replicates with 5 shoots tips each. In each column, values followed by the same letter are not significantly different at  $P < 0.05$  according to Duncan's Multiple Range Test.

Effect of PVS2 exposure time with freezing – *P. algarbiensis* segments precultured on semisolid MS medium were subsequently exposed to the same PVS2 dehydration times and immersed in LN. Eight weeks after rewarming, the results obtained for the control

(non-cryopreserved, -LN) again showed that the PVS2 has no negative effect on the regeneration of *P. algarbiensis* segments, with survival and regrowth percentages ranging between 80.0 and 100.0% (Table 3.4). However, for cryopreserved segments (+LN) (Figure 3.1E), the percentages decrease and were significantly influenced by exposure duration to PVS2 ( $P < 0.05$ ) (Table 3.4). The highest regrowth percentage,  $60.0 \pm 15.2\%$ , was obtained for segments exposed to PVS2 for 120 min, followed by 90 and 60 min exposure time (Table 3.4). These results indicate that segments withstand cryopreservation better when exposed to relatively higher PVS2 dehydration times.

**Table 3.4.** – Effect PVS2 exposure time on the survival and regrowth percentages of *Plantago algarbiensis* cryopreserved segments by the droplet-vitrification method, eight weeks after culture.

PVS2 exposure time (min)	-LN		+LN	
	Survival (%)	Regrowth (%)	Survival (%)	Regrowth (%)
0	100.0 ± 0.0 a	100.0 ± 0.0 a	10.0 ± 5.8 c	10.0 ± 5.8 c
30	95.0 ± 5.0 a	95.0 ± 5.0 a	25.0 ± 9.6 bc	25.0 ± 9.6 bc
60	90.0 ± 10.0 a	90.0 ± 10.0 a	40 ± 11.6 ab	40 ± 11.6 ab
90	80.0 ± 8.16 a	80.0 ± 8.2 a	50.0 ± 5.8 ab	50.0 ± 5.8 ab
120	95.0 ± 5.0 a	90.0 ± 5.8 a	60.0 ± 15.2 a	60.0 ± 15.2 a

Values represent means ± SE of 6 replicates with 5 shoots tips each. In each column, values followed by the same letter are not significantly different at  $P < 0.05$  according to Duncan's Multiple Range Test. -LN: non-cryopreserved segments (control); +LN: cryopreserved segments.

#### **3.1.4.4. Encapsulation-dehydration**

Effect of the different stages of the protocol – Initially it was evaluated the influence of the different stages (preculture, encapsulation, desiccation and immersion in LN) of the encapsulation-dehydration protocol on the survival and regrowth of *P. algarbiensis* segments, precultured on semisolid MS medium or liquid MS medium containing 0.3 M sucrose for one day. After eight weeks, segments precultured on MS medium presented higher survival and regrowth percentages in all steps of the protocol compared to segments precultured on MS medium containing 0.3 M sucrose (Table 3.5). The results also showed that the regrowth percentage decreased after the inclusion of each

additional step of the protocol, for both pretreatments (Table 3.5). Nevertheless, for segments precultured on MS medium, after immersion in LN, both survival and regrowth percentages were above 55% (Table 3.5).

**Table 3.5.** Effect of each encapsulation-dehydration stage on the regrowth and survival percentages of *Plantago algarbiensis* segments, eight weeks after culture.

<b>Preculture medium</b>	<b>Stage</b>	<b>Survival (%)</b>	<b>Regrowth (%)</b>
MS	PC	93.3 ± 6.7	93.3 ± 6.7
	E	66.7 ± 4.2	66.7 ± 4.2
	D	66.7 ± 6.7	60.0 ± 5.2
	LN+	56.67 ± 6.2	56.7 ± 6.2
MS + 0.3 M sucrose	PC	86.67 ± 6.7	86.7 ± 6.7
	E	60.00 ± 7.3	56.7 ± 6.2
	D	20.00 ± 10.3	20.0 ± 10.3
	LN+	13.33 ± 6.7	13.3 ± 6.7

Values represent means ± SE of 6 replications with 5 shoots tips. PC: preculture; E: encapsulation and culture in medium with 0.75 M sucrose; D: desiccation for 3 h; LN+: immersion in LN.

Effect of desiccation time –The response of cryopreserved segments exposed to different dehydration times was also evaluated (Table 3.6). In this experiment, segments were precultured on semisolid MS medium only. Non-cryopreserved segments (-LN) showed survival and regrowth percentages above 50%, except for beads desiccated for 6 h. The highest percentages were obtained in beads dehydrated for 1 h. As for cryopreserved segments (+LN), there was no regeneration for beads desiccated for 0 h and the highest survival and regrowth percentages were obtained after 3 h desiccation ( $P < 0.05$ ). The remaining desiccation times presented low percentages, varying between approximately 3 and 23%.

**Table 3.6.** – Effect desiccation time on the survival and regrowth percentages of *Plantago algarbiensis* cryopreserved segments by the encapsulation-dehydration method, eight weeks after culture.

Desiccation time (h)	-LN		+LN	
	Survival (%)	Regrowth (%)	Survival (%)	Regrowth (%)
0	73.3 ± 6.7 b	73.3 ± 6.7 ab	0.0 ± 0.0 d	0.0 ± 0.0 d
1	96.7 ± 3.3 a	93.3 ± 4.2 a	3.3 ± 3.3 cd	3.3 ± 3.3 cd
2	73.3 ± 9.9 b	70.0 ± 11.3 ab	23.3 ± 3.3 b	23.3 ± 3.3 b
3	70.0 ± 4.7 b	70.0 ± 4.5 b	63.3 ± 9.6 a	63.3 ± 9.6 a
4	70.0 ± 11.3 b	63.3 ± 12.0 b	16.7 ± 6.2 bc	16.7 ± 6.2 bc
5	50.0 ± 11.3 bc	50.0 ± 11.3 bc	13.3 ± 9.9 bcd	13.3 ± 9.9 bcd
6	36.7 ± 10.9 c	33.3 ± 11.2 c	6.7 ± 4.2 cd	6.7 ± 4.2 cd

Values represent means ± SE of 6 replicates with 5 shoots tips each. In each column, values followed by the same letter are not significantly different at  $P < 0.05$  according to Duncan's Multiple Range Test. -LN: non-cryopreserved segments (control); +LN: cryopreserved segments.

### 3.1.5. Discussion

Cryopreservation of seeds is one of the most important tools for *ex situ* preservation of endangered species. Besides allowing long-term conservation without deterioration of plant material, seed cryopreservation can be a simple, reliable and economical method. In many cases, the direct immersion of cryovials containing seeds into LN does not affect the germination behaviour and thus a gradual cooling rate is not required, which simplifies the cryopreservation process and considerably reduces the costs (Walters et al. 2004, Gonçalves and Romano 2009, Zaidi et al. 2010, Coelho et al. 2012). In the present study, from the obtained results, this fact was verified: the exposure of *P. algarbiensis* seeds to ultra low temperatures of LN had no negative effect on the seed germination and embryos grew into normal seedlings without any morphological abnormalities. Seeds from the rare *P. cordata* also survived exposure to LN, after being dried to 4-5% moisture content (Pence and Clark 2005). Cryopreservation of seeds proved to be a suitable procedure for the conservation of other endangered species from the Algarve region, such as *Thymus lotocephalus* (Coelho et al. 2012), *Tuberaria major* (Gonçalves et al. 2009a) and *Drosophyllum lusitanicum* (Gonçalves and Romano 2009).

As an alternative to seed cryopreservation, the cryopreservation of vegetative explants also offers an option for the long-term conservation of endangered plant species, especially when *in vitro* shoot cultures are already established. In the particular case of *P. algarbiensis*, 2-3 mm-long nodal segments from *in vitro* cultures were used in this study.

Preceding the cryopreservation experiments, different recovery media were assessed for the regeneration of *P. algarbiensis* segments. Several studies demonstrate the importance of the recovery medium in the regeneration of cryopreserved plant material (Pennycooke and Towill 2001, Wang et al. 2003, Marco-Medina et al. 2010a). The medium chosen for recovery of *P. algarbiensis* segments was the same as the medium used for multiplication of shoots, MS supplemented with 0.2 mg l<sup>-1</sup> BA (Gonçalves et al. 2009b).

Two cryopreservation methods, droplet-vitrification and encapsulation-dehydration, were tested in order to develop a cryopreservation protocol for segments excised from *in vitro*-grown shoots of *P. algarbiensis*. In a preliminary study, a third method, vitrification, was also tested, however, the regeneration was very low or inexistent for cryopreserved segments and results were not considered for this study (data not shown). In short, from the obtained results the highest regrowth percentages were around 60% for both methods. To develop a viable cryopreservation protocol, the time during which samples are dehydrated (either by exposure to the vitrification solution or to air-drying) needs to be optimized for each plant species. In the droplet-vitrification method, it was proved that the PVS2 solution was non-detrimental for non-cryopreserved segments, regardless the time of exposure. After immersion in LN, segments exposed to higher PVS2 dehydration times presented higher regeneration levels and the best result was obtained after 120 min in PVS2. Nodal segments of *L. rosmarinifolia*, a rare endemic species of the Mediterranean, were also cryopreserved using the droplet-vitrification method and the highest recovery was obtained after 60 min exposure to a vitrification solution (Barraco et al. 2013). Coste et al. (2012) successfully recovered 68 and 71% of cryopreserved axillary buds of *H. richeri* ssp. *transsilvanicum* and *H. umbellatum* (rare endemic species of Romania), respectively, after 10 and 15 min dehydration, using droplet-vitrification.

As for the encapsulation-dehydration method, the regeneration of non-cryopreserved *P. algarbiensis* segments was not dependent on the dehydration time, for the majority of

desiccation times tested. However, segments desiccated for 3 h survived cryopreservation better. This method was also applied to another endemic endangered plant species from Spain, *C. rigualli*, using nodal segments, and the highest survival was achieved after 4 h dehydration (González-Benito et al. 1997). Species belonging to the Plantaginaceae family, *Antirrhinum microphyllum* (González-Benito et al. 1998) and *Digitalis obscura* (Sales et al. 2001), were also effectively cryopreserved using the encapsulation-dehydration method using node segments or shoot tips as explants.

Before cryopreservation, several strategies can be employed to prepare plant material and improve survival after thawing. Preculture on medium with high levels of sugar reduces moisture content before freezing and therefore prevents the formation of ice-crystals (Sakai and Engelmann 2007, Benson 2008). Several studies report an enhancement in regeneration after freezing for explants exposed to medium with high sucrose concentrations (Sakai and Engelmann 2007). On the contrary, in the present study the use of higher sucrose concentrations in the preculture medium did not improve the regeneration of *P. algarbiensis* segments. This fact was also verified by Leunufna and Keller (2003), for *Dioscorea* spp, and Marco-Medina and co-workers (2010b), for *Thymus moroderi*.

In conclusion, we demonstrated for the first time that cryopreservation is a viable strategy for the long-term preservation of *P. algarbiensis*. Seeds were successfully cryopreserved without loss of their germination capacity and nodal segments can be cryopreserved using droplet-vitrification and encapsulation-dehydration methods. In this study, the best conditions were: preculture of excised nodal segments for one day on plain MS medium, followed by 120 min treatment in PVS2, for droplet-vitrification, or 3 h desiccation, for encapsulation-dehydration, and recovery medium MS supplemented with 0.2 mg l<sup>-1</sup> BA.

For an effective conservation of any plant species the use of different complementary conservation strategies is required, as no single conservation technique applied alone allows the preservation of the full range of genetic diversity. The *in vitro* propagation protocol previously developed (Gonçalves et al. 2009b) and the cryopreservation strategies studied in this report together represent an important contribution to the conservation of genetic resources of this endangered species.

### 3.1.6. References

- Barraco G, Sylvestre I, Iapichino G and Engelmann F (2013) Investigating the cryopreservation of nodal explants of *Lithodora rosmarinifolia* (Ten.) Johnst., a rare, endemic Mediterranean species. *Plant Biotechnology Reports* 7: 141-146;
- Benson EE (2008) Cryopreservation of Phytodiversity: A Critical Appraisal of Theory & Practice. *Critical Reviews in Plant Sciences* 27: 141-219;
- Coelho N, Gonçalves S, González-Benito ME and Romano A (2012) Germination and cryopreservation tolerance of seeds from the rare aromatic species *Thymus lotocephalus*. *Scientia Horticulturae* 145: 84-86;
- Coste A, Halmagyi A, Butiuc-Keul AL, Deliu C, Coldea G and Hurdu B (2012) *In vitro* propagation and cryopreservation of Romanian endemic and rare *Hypericum* species. *Plant Cell, Tissue and Organ Culture* 110: 213-226;
- Ellis RH and Roberts EH (1981) The quantification of ageing and survival in orthodox seeds. *Seed Science and Technology* 9: 373-409;
- Engelmann F (2004) Plant cryopreservation: progress and prospects. *In Vitro Cellular & Developmental Biology – Plant* 40: 427-433;
- Engelmann F (2011) Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cellular & Developmental Biology – Plant* 47: 5-16;
- Gonçalves S, Fernandes L, Pérez-García F, González-Benito ME and Romano A (2009a) Germination requirements and cryopreservation tolerance of seeds of the endangered species *Tuberaria major*. *Seed Science and Technology* 37: 480-484;
- Gonçalves S, Martins N and Romano A (2009b) Micropropagation and conservation of endangered species *Plantago algarbiensis* and *P. almogravensis*. *Biologia Plantarum* 53: 774-778;
- Gonçalves S and Romano A (2009) Cryopreservation of seeds from the endangered insectivorous plant *Drosophyllum lusitanicum*. *Seed Science and Technology* 37: 485-490;
- González-Benito ME, Pérez C and Viviani AB (1997) Cryopreservation of nodal explants of an endangered plant species (*Centaureum rigualii* Esteve) using the encapsulation-dehydration method. *Biodiversity and Conservation* 6: 583-590;
- González-Benito ME, Nunez-Moreno Y and Martin C (1998) A protocol to cryopreserve nodal explants of *Antirrhinum microphyllum* by encapsulation-dehydration. *CryoLetters* 19: 225-230;
- ICN (Instituto de Conservação da Natureza) (2007) Plano Nacional de Conservação da Flora em Perigo (1ª fase). Relatório final do Projecto Life – Natureza III P\8480. Volume IV. Instituto da Conservação da Natureza, Lisboa;
- Kartha KK, Leung NL and Mroginski LA (1982) *In vitro* growth responses and plant regeneration from cryopreserved meristems of Cassava (*Manihot esculenta* Crantz). *Zeitschrift für Pflanzenphysiologie* 107: 133-140;
- Leunufna S and Keller ERJ (2003) Investigating a new cryopreservation protocol for yams (*Dioscorea* spp.). *Plant Cell Reports* 21: 1159-1166;
- Marco-Medina A, Casas, JL, Swennen R and Panis B (2010a) Cryopreservation of *Thymus moroderi* by droplet vitrification. *CryoLetters* 31: 14-13;

- Marco-Medina A, Casas JL and González-Benito ME (2010b) Comparison of vitrification and encapsulation-dehydration for cryopreservation of *Thymus moroderi* shoot tips. *CryoLetters* 31: 301-309;
- Martins N, Gonçalves S, Palma T and Romano A (2012) Seed germination of two critically endangered plantain species, *Plantago algarbiensis* and *P. almogravensis* (Plantaginaceae). *Seed Science and Technology* 40: 144-149;
- Martins N, Gonçalves S and Romano A (2013) Metabolism and aluminum accumulation in *Plantago almogravensis* and *P. algarbiensis* in response to low pH and aluminum stress. *Biologia Plantarum* 57: 325-331;
- Mederos S, Martin C, Navarro E and Ayuso MJ (1997) Micropropagation of a medicinal plant, *Plantago major* L. *Biologia Plantarum* 40: 465-468;
- Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497;
- Panis B, Piette B and Swennen R (2005) Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all *Musaceae*. *Plant Science* 168: 45-55;
- Pence VC and Clark JR (2005) Desiccation, cryopreservation and germination of seeds of the rare wetland species, *Plantago cordata* Lam. *Seed Science and Technology* 33: 767-770;
- Pennycooke JC and Towill LE (2001) Medium alterations improve regrowth of sweet potato (*Ipomoea batatas* (L.) Lam.) shoot tips cryopreserved by vitrification and encapsulation-dehydration. *CryoLetters* 22: 381-389;
- Sakai A, Kobayashi S and Oiyama I (1990) Cryopreservation of nucellar cells and navel orange (*Citrus sinensis* Osb. Var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Reports* 9: 30-33;
- Sakai A and Engelmann F (2007) Vitrification, encapsulation-vitrification and droplet-vitrification: a review. *CryoLetters* 28: 151-172;
- Sales E, Nebauer SG, Arrillaga I and Segura J (2001) Cryopreservation of *Digitalis obscura* selected genotypes by encapsulation-dehydration. *Planta Medica* 67: 833-838;
- Walters C, Wheeler L and Stanwood PC (2004) Longevity of cryogenically stored seeds. *Criobiology* 48: 229-244;
- Wang Q, Li P, Batuman O, Gafny R and Mawassi M (2003) Effect of benzyladenine on recovery of cryopreserved shoot tips of grapevine and citrus cultured *in vitro*. *CryoLetters* 24: 293-302;
- Zaidi CA, González-Benito ME and Pérez-García F (2010) Morphology, germination and cryopreservation of seeds of the Mediterranean annual plant *Tuberaria macrosepala* (Cistaceae). *Plant Species Biology* 25: 149-157.



## 3.2

### Estimation of genetic diversity in *Plantago algarbiensis* seedlings

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### 3.2.1. Abstract

*Plantago algarbiensis* is an endangered endemic species from the Algarve, in the south of Portugal. In the present work the genetic diversity of three known natural populations was assessed by random amplified polymorphic DNA (RAPD) markers. Samples were amplified using ten primers that generated 145 markers, 80% of which were polymorphic. Tunes population presented the highest polymorphism percentage (73.68%) and Algoz population the lowest (67.67%). In the cluster analysis, two major groups were formed, one including individuals from Gambelas population and the other clustered together individuals from the other two populations. The highest level of genetic diversity, estimated by both Nei's gene diversity and Shannon's information measure, was found in Tunes and the lowest in Algoz. From the estimates of Shannon's index, the proportion of the diversity within populations was 86.12% and among populations was 13.88%. Similar results were obtained by analysis of molecular variance (AMOVA). A correlation was found between geographic and genetic distances among populations. The  $N_m$  values obtained suggest a high level of gene flow among populations, which was inversely proportional to the distance between populations. The use of RAPDs to determine the genetic diversity of *P. algarbiensis* populations has proved to be a useful tool. The data obtained can be used to develop effective conservation strategies to prevent the declining of populations.

### 3.2.2. Introduction

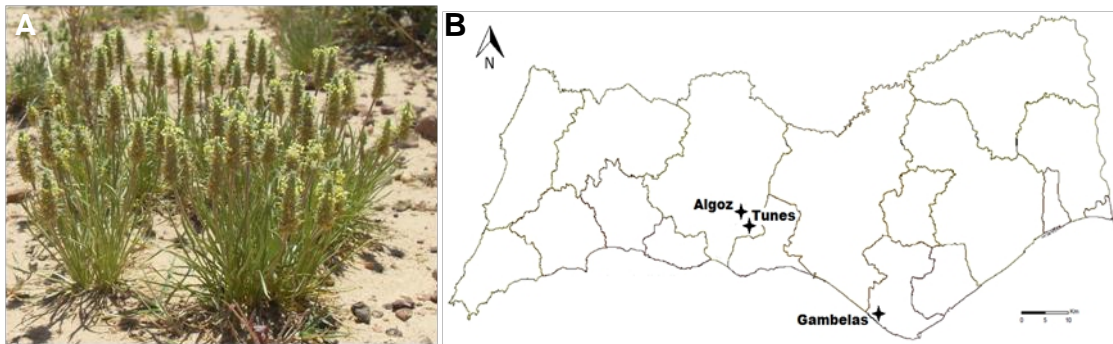
The genus *Plantago*, Plantaginaceae, is characterized by small perennial or annual herbs, with alternate leaves forming a basal rosette and inflorescence as a bracteate spike (Castroviejo 1986) and includes about 483 species distributed worldwide (Tutel et al. 2005, Ferreira et al. 2013). Most species are widely distributed and grow as weeds, but some others are restricted to a specific area, such as *Plantago algarbiensis* Samp. (Hoggard et al. 2003, ICN 2006). *Plantago* species have been used as medicinal herbs to treat various diseases related to the skin, digestive tract, reproductive system, blood circulation disorders and cancer (Chiang et al. 2003). In addition some species are tolerant to heavy metals in the soil (Remon et al. 2007, Malizia et al. 2012).

*P. algarbiensis* (Figure 3.2A) is an endangered endemic species to the Algarve, Portugal, limited to a few populations in a very small area. *P. algarbiensis* is a rosulate hemicryptophyte that grows up to 7 - 30 cm long and has woody stems, undivided or rarely bifid (Franco 1984). The species occurs on clay-rich soils that are temporarily flooded in winter and spring and it prefers areas that are located downstream from small springs or clearings containing acidophilic brushes (ICN 2006). The capacity of colonizing acid soils has been recently investigated by Martins et al. (2013a,b,c), indicating that *P. algarbiensis* accumulates considerable amounts of aluminium in its tissues. Of the, so far, three known populations, two are located in the interior of the region (Algoz and Tunes), widespread throughout an area of approximately 30 ha, and a third one (Gambelas) is located closer to the coast (Figure 3.2B). The major threats to this species are mining of clay soils for the production of construction materials, urbanization, trampling and grazing by livestock (ICN 2006). Due to its restricted distribution worldwide, *P. algarbiensis* is in risk of global extinction and is considered endangered and legally protected by the European Habitats Directive 92/43/ CEE and Portuguese law (reference 140/99 of April 24) (ICN 2006). Besides, an *in vitro* propagation protocol has already been developed, as an *ex situ* conservation measure, to support legal protection in the preservation of *P. algarbiensis* (Gonçalves et al. 2009).

However, for an effective implementation of any conservation programme, it is essential the understanding of genetic diversity and structure of natural populations. The information gathered can assist in the protection and management of populations and habitats, as well as in the strategy for germplasm collection, and genebank management. Molecular markers present a wide range of applications and are a useful tool in the

study of genetic diversity of plant populations.

Random amplified polymorphic DNA (RAPD) is one of the most commonly used methods in the genetic characterization and analysis of plant species, including endangered species (Martín et al. 2008, Silva et al. 2011). This method consists in the amplification of genomic DNA with single, arbitrary primers resulting in different amplification products (Williams et al. 1990, Harris 1999). RAPD does not need large amounts of DNA for amplification reactions; neither requires previous knowledge of DNA sequences, which can be very useful when working with wild plant species with unknown genome. Besides, it is a simple, fast and more economical method comparing to other molecular markers (Harris 1999, Martín et al. 2008, Poczai et al. 2013).



**Figure 3.2.** – *Plantago algarbiensis* wild plant from Algoz population (A) and map with the locations of the three populations studied in the present work (B).

There are several studies reporting the use of RAPD to assess the genetic diversity of *Plantago* species, such as *P. ovata* (Singh et al. 2009), *P. major* (Zubair et al. 2012) and others (Samantaray et al. 2010). Recently, Ferreira et al. (2013) reported the genetic diversity of *P. algarbiensis* and *P. almogravensis* (one population each) using RAPD and Inter-simple sequence repeat (ISSR) markers to provide information for their conservation but also to clarify their taxonomy, since in some literature both species are considered as one (Pedrol 2009). In this study we set out to evaluate the genetic diversity of the three known populations of *P. algarbiensis* and provide additional knowledge for the protection and management of this species.

### 3.2.3. Materials and Methods

#### 3.2.3.1. Plant material

Seeds were collected from three known wild populations in the Algarve region, Portugal (Algoz, Tunes and Gambelas) (Figure 3.2B). The number of individuals is estimated to be several thousand in Algoz population and less than 10,000 in Tunes population (ICN 2006). There are no official data concerning the number of individuals in Gambelas populations, but the area of occupancy is lower than any of the other populations. Collection was made from 10 to 30 individuals randomly selected from each population and the distance between the individuals sampled was at least 1 m. Seeds were germinated according to Martins et al. (2012). In short, for each population four replicates of 25 seeds were sown in glass Petri dishes on 0.6% (w/v) agar (Duchefa, The Netherlands) without nutrients and germinated at 15°C with a 16 h light/ 8 h dark photoperiod (cool white fluorescent lamps, 69  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Approximately eight weeks after sowing, the germination percentage was between 95 and 100% and 20 seedlings from each population were randomly selected and used for analysis.

#### 3.2.3.2. DNA extraction and RAPD amplification

DNA was extracted from the seedlings according to a slightly modified protocol described by Gawel and Jarret (1991). In short, seedlings were cut and placed on sterile eppendorf tubes and powdered with the help of a tissue lyser (Qiagen, Germany). Samples were incubated with CTAB (cetyl trimethylammonium bromide) buffer [2% CTAB, 1.4 M NaCl, 20 mM EDTA (ethylenediaminetetraacetic acid), 100 mM Tris HCl, 1% (p/v) PVP-40 (polyvinylpyrrolidone) and 0.2%  $\beta$ -mercaptoethanol] at 65°C for 1 h. A chloroform/isoamyl alcohol (24:1) extraction was performed and DNA was precipitated by adding cold isopropanol (-20°C). The precipitate was washed with 70% ethanol. The pellet was redissolved in 100  $\mu\text{l}$  of sterile distilled water and RNA was removed by digestion with DNase-free RNase A. The DNA was quantified in a 0.8% agarose gel. Twelve arbitrary primers from Operon Technology (Alameda/CA, USA) were tested for RAPD amplification. Ten were selected to evaluate the genetic variability of the samples: OPO-5 (5' CCCAGTCACT 3'), OPO-7 (5' CAGCACTGAC 3'), OPO-10 (5' TCAGAGCGCC 3'), OPO-20 (5' ACACACGCTG 3'), OPF-1 (5' ACGGATCCTG 3'), OPF-3 (5' CCTGATCACC 3'), OPF-4 (5' GGTGATCAGG 3'), OPF-10 (5' GGAAGCTTGG 3'), OPE-19 (5' ACGGCGTATG 3') and OPA-11 (5'

CAATCGCCGT 3'). DNA amplification reactions were performed in 25 µl reaction volume, containing approximately 10 ng template DNA, 0.4 or 0.8 µM primer, 5 µl 5x MyTaq reaction buffer and 1 U MyTaq DNA polymerase (Bioline, UK). Amplifications were performed in a thermal cycler (Eppendorf, Germany) with the following program: one cycle of 1 min at 95°C, 35 cycles of 15 s at 95°C, 15 s at 37°C and 10 s at 72°C, and a final cycle of 10 min at 72°C. Amplification products were separated in a 1.5% (w/v) agarose gel in 1x TBE (Tris-borate-EDTA) buffer, followed by staining with ethidium bromide. The gels were visualised and photographed under UV light. Molecular weights were estimated by reference to a 100 Base-Pair Ladder (GE Healthcare, UK). All the amplifications were repeated at least twice and only bands reproducible in both PCR (polymerase chain reaction) runs were considered for analysis.

### 3.2.3.3. Data analysis

Amplified fragments from the RAPD analysis were scored as present (1) or absent (0). Genetic similarities were calculated using the Jaccard similarity coefficient (Jaccard 1908). The resultant matrix was subjected to cluster analysis by the unweighted pair group method analysis (UPGMA) and a dendrogram was constructed from the clustering results. These analyses were performed using the program NTSYS-PC version 1.80 (Rohlf 1992).

The data matrix resulting from the presence/absence of RAPD fragments was analysed using GenAlEx version 6.5 (Peakall and Smouse 2006, 2012) to estimate Nei's gene diversity ( $h$ ) (Nei 1973) and to perform the analysis of molecular variance (AMOVA) (Excoffier et al. 1992).

From AMOVA analysis,  $\Phi_{PT}$  estimator (which is an analogue of  $F_{ST}$ ) was calculated.  $\Phi_{PT}$  estimates the proportion of the variance among populations. The use of  $\Phi_{PT}$  values allowed for the estimation of the effective number of migrants ( $Nm$ ) between populations [ $Nm = \frac{1}{4} (1 / \Phi_{PT} - 1)$ ] as an estimator of gene flow (Wright 1951, Freitas and Brehm 2001).

In addition, genetic diversity was estimated using Shannon's information measure,  $H' = - \sum p_i \log_2 p_i$  (Lewontin 1972), where  $p_i$  is the frequency of a given RAPD fragment. Shannon index was calculated for two levels: the average diversity within populations ( $H'_{pop}$ ), and the diversity within species ( $H'_{sp}$ ). The proportion of diversity

within populations was then estimated as  $H'_{pop}/H'_{sp}$ , and the proportion of diversity among populations as  $(H'_{sp} - H'_{pop})/H'_{sp}$ .

### 3.2.4. Results

#### 3.2.4.1. The RAPD profile

The ten primers used to amplify the 60 DNA samples from the three populations of *P. algarbiensis* (20 samples per population) produced 145 markers, ranging in size from approximately 290 to 1600 base pairs (bp). From these markers, 116 were polymorphic (80.00%) and 29 (20.00%) were present in all individuals.

At the species level, 124 markers (85.52%) were shared by the three populations. Tunes population presented the highest polymorphism percentage (73.68%) and Algoz population the lowest (67.67%) (Table 3.7). Four markers (2.76%) were specific to Algoz population, six (4.14%) were only found in Gambelas population and two (1.38%) were exclusive to Tunes population (Table 3.7). None of these markers was monomorphic within the corresponding population.

**Table 3.7.** – Total number of markers and number of polymorphic and population specific markers generated by RAPD primers for each *Plantago algarbiensis* population.

Population	No. markers	Polymorphic markers	Polymorphism (%)	Population specific markers
Algoz	133	90	67.67	4
Gambelas	137	93	67.88	6
Tunes	133	98	73.68	2

#### 3.2.4.2. Cluster analysis

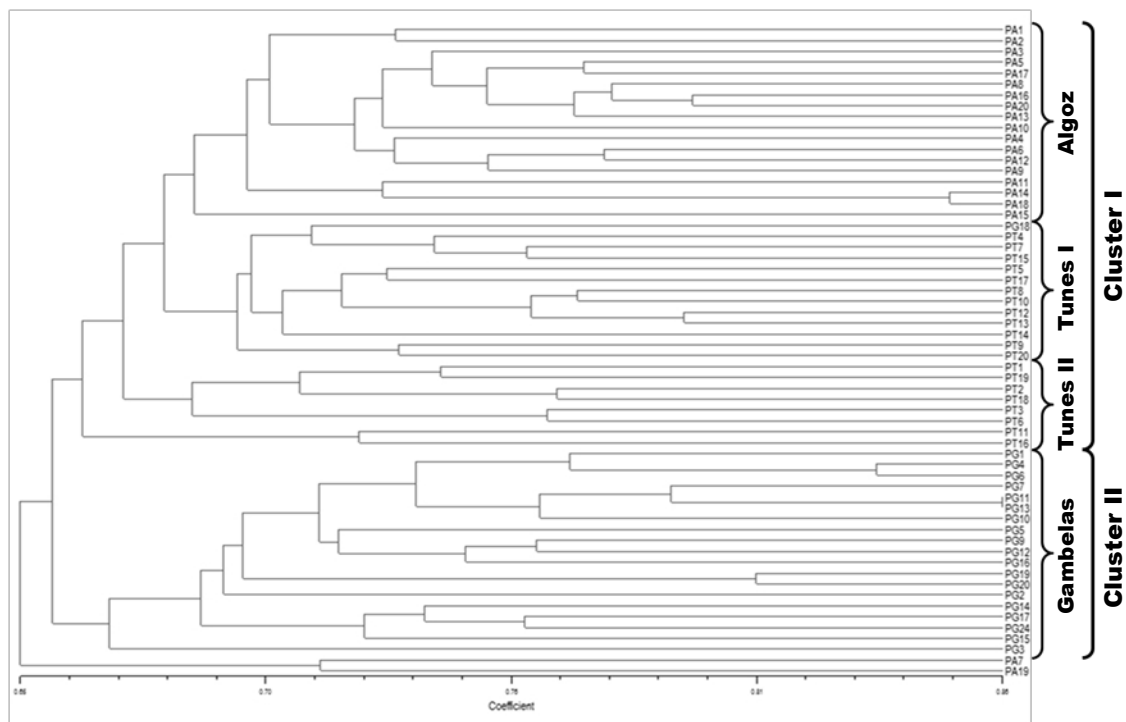
In the dendrogram obtained from the RAPD data (Figure 3.3), all individuals presented a unique RAPD phenotype.

The cluster analysis revealed two major groups, which clustered according to their geographical locations. The samples from Algoz and Tunes populations, which are geographically closer, were grouped together (cluster I) and separated from Gambelas

population (cluster II), which is located further to the east and closer to the coast in the Algarve region. According to the scale in the dendrogram, the two clusters were split at approximately 66% similarity.

Within cluster I, all individuals from Algoz population (except PA7 and PA19) clustered together in the same subgroup with a genetic similarity of 68.5%. The closest individuals in this group, PA14 and PA18, shared 85% similarity. Individuals from Tunes population, also included in cluster I, presented different levels of similarity between them. A subgroup of 12 individuals, Tunes I, was genetically closer to the Algoz subgroup, 68%, while the remaining 8 individuals, Tunes II, were 67% similar to Algoz and Tunes I. PT12 and PT13 were the closest individuals in Tunes population, sharing 79.5% similarity.

Cluster II included individuals from Gambelas population (except PG18) with a similarity of approximately 67%. The highest homogeneity was between PG11 and PG13 with 86% similarity.



**Figure 3.3.** – Dendrogram based on UPGMA showing the genetic similarity (Jaccard coefficient) among *Plantago algarbiensis* samples obtained by RAPD analysis. PA: Algoz; PT: Tunes; PG: Gambelas.

There were three individuals that were not grouped within their respective population: individual PG18, Gambelas population, was included in cluster I with Tunes population; and individuals PA7 and PA19, Algoz population, presented a similarity below 66% with all the other individuals and were not included in the two major clusters.

### 3.2.4.3. Genetic diversity

Genetic diversity in *P. algarbiensis* populations (Table 3.8) was estimated through Nei's gene diversity ( $h$ ; Nei 1973) and Shannon's information measure ( $H'$ ; Lewontin 1972). The Nei's gene diversity ( $h$ ) ranged from 0.205 in Algoz population to 0.219 in Tunes. Similar results were obtained with Shannon's index, the lowest level of within-population variability was found in Algoz ( $H' = 3.222$ ) and the highest diversity in Tunes ( $H' = 3.382$ ). The mean diversity within species ( $H'_{sp}$ ) and the mean diversity within populations ( $H'_{pop}$ ) that resulted from the estimates of Shannon's index of phenotypic diversity was 3.845 and 3.311, respectively. The proportion of the diversity within populations was 86.12%, whereas the diversity among populations resulted in 13.88% of the total diversity. Estimation of genetic variation by AMOVA revealed similar values: 86.00% of total variation was found within populations and 14.00% among populations.

**Table 3.8.** – Genetic variation of *Plantago algarbiensis* populations detected by RAPD markers

Population	$H'$	$h$
Algoz	3.222	0.205
Gambelas	3.329	0.215
Tunes	3.382	0.219

$H'$  - Shannon's information measure (Lewontin 1972);  $h$  - Nei's gene diversity (Nei 1973).

### 3.2.4.4. Relationships between populations

The distances among populations ( $\Phi_{PT}$ ) obtained from the AMOVA analysis are shown in Table 3.9.  $\Phi_{PT}$  values ranged from 0.094 between Algoz and Tunes, to 0.175 between

Algoz and Gambelas (Table 3.9). Each population was clearly differentiated, since all distances between pairs of populations were significantly different from zero. Geographic distance can be correlated to the genetic distance among populations: the geographically closest populations (Algoz and Tunes) had the lowest genetic distance and the farther populations (Algoz and Gambelas) the lowest. Therefore, the genetic differences between populations of *P. algarbiensis* can be explained by geographic movement.  $N_m$  values were higher than 1.0 (Table 3.9), suggesting a high level of gene flow between populations. Gene flow was inversely proportional to the distance between populations and can also be correlated to the geographic distances between pairs of populations.

**Table 3.9.** – Genetic distances/gene flow ( $N_m$ ) (below diagonal) and geographical distances (km) (above diagonal) between the three populations of *Plantago algarbiensis* studied.

Population	Algoz	Gambelas	Tunes
Algoz	-	32	5
Gambelas	0.175/2.353	-	29
Tunes	0.094/4.815	0.138/3.125	-

### 3.2.5. Discussion

The genetic diversity of three wild populations of *P. algarbiensis* was studied in the present work using RAPD markers. The high levels of polymorphism detected confirm that these markers are highly informative for the study of genetic parameters of *P. algarbiensis* populations. In addition, the analysis of these markers by different methods revealed very similar interpretations of the genetic structure of the populations studied. RAPD markers were also used to detect genetic differences between and within *P. major* populations (Zubair et al. 2012), the genetic variability of *P. ovata* accessions (Singh et al. 2009) and genetic relationships of several other *Plantago* species (Samantaray et al. 2010).

At the species level, the genetic diversity of *P. algarbiensis* populations, although geographically closed, can be considered high, as the results showed 80% of polymorphic bands. Ferreira et al. (2013) obtained percentages of polymorphic loci of

approximately 74 and 61% for *P. algarbiensis* and *P. almogravensis*, respectively, in a study comparing the genetic diversity of the two species, using RAPD and ISSR markers. These results are contrary to the general view that rare species are considered to maintain low levels of genetic diversity (Hamrick and Godt 1989, Gitzendanner and Soltis 2000). *Tuberaria major*, another endemic species of the Algarve region, also presented high levels of genetic diversity, 98% of polymorphic bands using ISSR markers (Trindade et al. 2012).

The *P. algarbiensis* population studied by Ferreira et al. (2013) was Algoz. They obtained, considering only RAPD data, higher percentage of polymorphic loci (83.7%) than in our study (67.7%), with the same number of primers and obtaining similar number of markers. These differences could be due to the number of individuals sampled.

Geographic distance can be correlated to the genetic distance among populations, the geographically closest populations (Algoz and Tunes) had the lowest genetic distance and the farther populations (Algoz and Gambelas) the higher.

The dendrogram obtained in the present study clearly distinguished *P. algarbiensis* populations. Individuals from Gambelas population, which is 32 and 29 km distant from Algoz and Tunes populations, respectively, formed a distinct group, sharing 66% similarity; while individuals from the other two populations, which are distant from each other 5 km, clustered together in the same group. Within this group, samples from Algoz population formed a well-defined sub-group and Tunes individuals were included in two different sub-groups. The high similarity found between individuals from Algoz and Tunes can be explained by their geographic closeness. However, geographical range and population differentiation not always are correlated; environmental differences across the range of a species may be the origin of population differentiation (Loveless and Hamrick 1984). In this case the short distance between both populations is in accordance with a homogeneous environment. The values that resulted from both the Nei's gene diversity ( $h$ ) (Nei 1973) and Shannon's information measure (Lewontin 1972) analysis were in agreement, revealing a higher genetic variability in Tunes population than in the other populations. As for the study of the structure of the populations, obtained from both Shannon's index and AMOVA, a high proportion of genetic variation in *P. algarbiensis* occurs within populations (86%) than among populations (14%). Several factors can influence genetic diversity: migration, natural

selection, genetic drift and reproductive system. Genetic differentiation among populations is promoted by natural selection and genetic drift, while allogamy and migration increase the levels of diversity within populations (Gómez-Gómez et al. 2012). The type of reproductive system in plants can therefore strongly affect the genetic diversity within and between populations. Mixed or outcrossing species present higher genetic variability within populations and less differentiation among populations than selfing species (Nybom and Bartish 2000, Nybom 2004). To the best of our knowledge, *P. algarbiensis* reproductive system is unknown, although according to our result would be presumable an outcrossing system of the species. Most *Plantago* species are described as allogamous (Pedrol 2009); however the genus *Plantago* also comprises species with a wide range of mating systems, from inbreeders to obligate outcrossers (Zubair et al. 2012). *P. major*, for instance, is an inbreeding species and a recent study demonstrated that the genetic diversity within populations of this species was lower than between populations (Zubair et al. 2012).

Geographic distribution can also influence the genetic diversity of populations. The overgeneralization is that species with a wide geographic distribution have higher genetic diversity. Hamrick and Godt (1989) reported that endemic and narrowly distributed species exhibit significantly lower diversity than widespread species. However, Gitzendanner and Soltis (2000) verified that there is no association: some rare species have reduced genetic variation and others keep equal or higher levels of diversity compared to widespread species. Nybom and Bartish (2000) also found no correlation between geographic range and within population diversity. In the particular case of *P. algarbiensis*, as previously stated, the three populations are spread over a relatively small area and therefore geographically close. The genetic distances between populations, obtained in the AMOVA analysis, although differentiated each population, revealed a low genetic differentiation among populations. This fact suggests a high gene flow among populations, which was corroborated by  $N_m$  values. The level of differentiation among populations appears to be associated with the geographic distance between populations of *P. algarbiensis* and can therefore be explained by geographical trends.

Genetic diversity results from the genetic differences between individuals of a variety or population of a species (Brown 1983, Ramanatha Rao and Hodgkin 2002). The maintenance of genetic diversity in natural populations is one of the main objectives of

nature conservation (Trindade et al. 2012). The determination of the extent and distribution of the different aspects of the genetic diversity in a species and structure of its natural populations is therefore an important and essential tool to create conservation strategies (Holsinger and Gottlieb 1991, Ramanatha Rao and Hodgkin 2002). In addition, for an effective conservation, the different methods applied should preserve as much diversity as possible (Trindade et al. 2012, Ferreira et al. 2013).

Overall, the diversity measured in this work can be considered high. However, due to the low populations' size and number, and their continuous declining, this level of diversity may be difficult to maintain. Genetic drift and inbreeding are some of the consequences associated with small and isolated populations that are responsible for a reduction of the genetic diversity (Ferreira et al. 2013). On the other hand, the survival of a species may not be directly related to its levels of diversity (Gitzendanner and Soltis 2000) and in this particular case, the main threat comes from human activities. Though *P. algarbiensis* is a protected species (ICN 2006), other conservation strategies are urgently required to avoid the decrease of individuals and populations and ultimately to preserve this species.

From the results presented here, and taking into account the management of the endangered populations, some actions should be regarded. Due to the low genetic differentiation found among populations and the small area of occupancy of this species, the three populations could be managed as a same unit. Nevertheless, *ex situ* conservation strategies, such as seed collection and storage, *in vitro* propagation or cryopreservation, should consider the different populations. In addition, it is also important to define a strategy that includes the restoration of natural populations, using *in vitro* propagated plants. This approach could be easily accomplished since a protocol for *in vitro* propagation of *P. algarbiensis* using seeds as explants has been already developed and the plants produced were successfully transferred to field conditions exhibiting normal development (Gonçalves et al. 2009). Also the seed germination requirements were studied (Martins et al. 2012). The use of seeds as starting material allows the preservation of a wider range of genetic information of the species and a way to increase the genetic diversity and the adaptation capacity of *P. algarbiensis* would be by transfer of germplasm between populations. As a rule, the reintroduction of plants propagated *ex situ* should be primarily made in the populations with less variability, which in the present work were Algoz and Gambelas. However, due to the small

number of individuals and the endangered status of the species, the reintroduction of *ex situ* propagated plants in Tunes population should also be prioritized.

In conclusion, RAPD markers provided valid and useful genetic information about the structure of *P. algarbiensis* populations. The implementation of conservation approaches should comprise both *in situ* and *ex situ* methods and genetic markers are a valuable tool to develop appropriate strategies. The measures presented here, along with the legal protection hold by this species, can contribute to increase the number of individuals in all populations and to preserve the species genetic resources and finally to prevent its extinction.

### 3.2.6. References

- Brown WL (1983) Genetic diversity and genetic vulnerability – an appraisal. *Economic Botany* 37: 4-12;
- Castroviejo S (coord. gen.) (1986-2012) *Flora iberica* 1-8, 10-15, 17-18, 21. Real Jardín Botánico, CSIC, Madrid;
- Chiang LC, Lean TNG, Chiang W, Chang MY and Lin CC (2003) Immunomodulatory activities of flavonoids, monoterpenoids, tripenoids, iridoid glycosides and phenolic compounds of *Plantago* species. *Planta Medica* 69: 600-604;
- Excoffier NC, Smouse PE and Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA restriction data. *Genetics* 131: 479-491;
- Franco JA (1984) *Nova Flora de Portugal (Continente e Acores)*. Vol. II, Sociedade Astoria, Lisboa;
- Ferreira V, Matos M, Correia S, Martins N, Gonçalves S, Romano A and Pinto-Carnide O (2013) Genetic diversity of two endemic and endangered *Plantago* species. *Biochemical Systematics and Ecology* 51: 37-44;
- Freitas H and Brehm A (2001) Genetic diversity of the Macaronesian leafy liverwort *Porella canariensis* inferred from RAPD markers. *Journal of Heredity* 92: 339-345;
- Gawel NJ and Jarret RL (1991) A modified CTAB DNA extraction procedure for *Musa* and *Ipomoea*. *Plant Molecular Biology Reporter* 9: 262-266;
- Gitzendanner MA and Soltis PS (2000) Patterns of genetic variation in rare and widespread plant congeners. *American Journal of Botany* 87: 783-792;
- Gómez-Gómez L, Ahrazem O, Herranz JM and Ferrandis P (2012) Genetic characterization and variation within and among populations of *Anthyllis rupestris* Coss., and endangered endemism of southern Spain. *Biochemical Systematics and Ecology* 45: 138-147;
- Gonçalves S, Martins N and Romano A (2009) Micropropagation and conservation of endangered species *Plantago algarbiensis* and *P. almogravensis*. *Biologia Plantarum* 53: 774-778;
- Hamrick JL and Godt MJW (1989) Allozyme diversity in plant species. In: Brown AHD, Clegg MT, Kahler AL and Weir BS (eds) *Plant population genetics, breeding, and genetic resources* (pp 43-63) Sinauer, Sunderland, Massachusetts;
- Harris SA (1999) *Molecular Approaches to Assessing Plant Diversity*. In: Benson EE (ed) *Plant Conservation Biotechnology* (pp 11-24) Taylor & Francis, London;
- Hoggard RK, Kores PJ, Molvray M, Hoggard GD and Broughton DA (2003) Molecular systematics and biogeography of the amphibious genus *Littorella* (Plantaginaceae). *American Journal of Botany* 90:429-435;
- Holsinger KE and Gottlieb LD (1991) Conservation of rare and endangered plants: principles and prospects. In: Falk DA and Holsinger KE (eds.) *Genetics and conservation of rare plants* (pp 195-223) Oxford University Press, New York;
- ICN (Instituto da Conservação da Natureza) (2006) Plano Sectorial da Rede Natura. Flora: *Plantago algarbiensis* Samp. <<http://www.icnf.pt/portal/naturaclas/rn2000/resource/rn-plan-set/flora/plant-alg>> Accessed 3 May 2014;

- Jaccard P (1908) Nouvelles recherches sur la distribution florale. Bulletin de la Societe Vaudoise des Sciences Naturelles 44: 223-70;
- Lewontin RC (1972) The apportionment of human diversity. Evolutionary Biology 6: 381-394;
- Loveless MD and Hamrick JL (1984) Ecological determinants of genetic structure in plant populations. Annual Review of Ecology and Systematics 15: 65-95;
- Malizia D, Giuliano A, Ortaggu G and Masotti A (2012) Common plants as alternative analytical tool to monitor heavy metals in soil. Chemistry Central Journal 6:S6;
- Martín C, Parra T, Clemente-Muñoz M and Hernandez-Bermejo JE (2008) Genetic diversity and structure of the endangered *Betula pendula* subsp. *fontqueri* populations in the south of Spain. Silva Fennica 42: 487-498;
- Martins N, Gonçalves S, Palma T and Romano A (2012) Seed germination of two critically endangered plantain species, *Plantago algarbiensis* and *P. almogravensis* (Plantaginaceae). Seed Science & Technology 40: 144-149;
- Martins N, Gonçalves S and Romano A (2013a) Metabolism and aluminum accumulation in *Plantago almogravensis* and *P. algarbiensis* in response to low pH and aluminum stress. Biologia Plantarum 57: 325-331;
- Martins N, Osório ML, Gonçalves S, Osório J, Palma T and Romano A (2013b) Physiological responses of *Plantago algarbiensis* and *P. almogravensis* shoots and plantlets to low pH and aluminum stress. Acta Physiologiae Plantarum 35: 615–625;
- Martins N, Osório ML, Gonçalves S, Osório J and Romano A (2013c) Impact of low pH and aluminum on the oxidative stress, energy partitioning and antioxidant responses in roots and leaves of *Plantago algarbiensis* and *P. almogravensis*. Biometals 26:427–437;
- Nei M (1973) Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences of the USA 70: 3321-3323;
- Nybom H and Bartish IV (2000) Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. Perspectives in Plant Ecology, Evolution and Systematics 3: 93-114;
- Nybom H (2004) Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Molecular Ecology 13: 1143-1155;
- Pedrol J (2009) *Plantago* L. In: Benedí González C and Rico E (Eds) Flora Ibérica – Plantas vasculares de la Peninsula Iberica e Islas Baleares (pp 4-38) Real Jardín Botánico, CSIC, Madrid;
- Peakall R and Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6: 288-295;
- Peakall R and Smouse PE (2012) GenAIEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics 28: 2537-2539.
- Poczai P, Varga I, Laos M, Cseh A, Bell N, Valkonen JPT and Hyvönen J (2013) Advances in plant gene-targeted and functional markers: a review. Plant Methods 9: 6;

- Ramanatha Rao V and Hodgkin T (2002) Genetic diversity and conservation and utilization of plant genetic resources. *Plant Cell, Tissue and Organ Culture* 68: 1-19;
- Remon E, Bouchardon J-L and Faure O (2007) Multi-tolerance to heavy metals in *Plantago arenaria* Waldst. & Kit.: adaptative versus constitutive characters. *Chemosphere* 69: 41-47;
- Rohlf FJ (1992) NTSYS-PC: numerical taxonomy and multivariate analysis system. Exeter Software, New York;
- Samantaray S, Dhagat UM and Maiti S (2010) Evaluation of genetic relationships in *Plantago* species using Random Amplified Polymorphic DNA (RAPD) markers. *Plant Biotechnology* 27: 297-303;
- Silva L, Elias RB, Moura M, Meimberg H and Dias E (2011) Genetic variability and differentiation among populations of the Azorean endemic gymnosperm *Juniperus brevifolia*: baseline information for a conservation and restoration perspective. *Biochemical Genetics* 49: 715-734;
- Singh N, Lal RK and Shasany AK (2009) Phenotypic and RAPD diversity among 80 germplasm accessions of the medicinal plant isabgol (*Plantago ovata*, Plantaginaceae). *Genetics and Molecular Research* 8: 1273-1284;
- Trindade H, Sena I, Gonçalves S and Romano A (2012) Genetic diversity of wild populations of *Tuberaria major* (Cistaceae), an endangered species endemic to the Algarve region (Portugal), using ISSR markers. *Biochemical Systematics and Ecology* 45: 49-56;
- Tutel B, Kandemir I, Kuş S and Kence A (2005) Classification of Turkish *Plantago* L. species using numerical taxonomy. *Turkish Journal of Botany* 29: 51-61;
- Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV (1990) DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* 18: 6531-6535;
- Wright S (1951) The genetical structure of populations. *Annals of Eugenetics* 15: 323-354;
- Zubair M, Nybom H, Ahnlund M and Rumpunen K (2012) Detection of genetic and phytochemical differences between and within populations of *Plantago major* L. (plantain). *Scientia Horticulturae* 136: 9-16.

# CHAPTER 4



## *Tuberaria major*

Coelho N, González-Benito ME and Romano A (2014) Cryopreservation of shoot tips from the rare endemic species *Tuberaria major*. (Submitted)



## 4.1

### **Cryopreservation of seeds and shoot tips from the rare endemic species *Tuberaria major***

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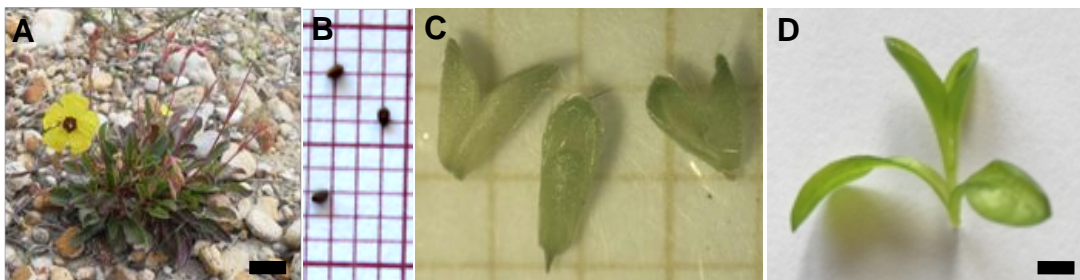


#### 4.1.1. Abstract

*Tuberaria major* is an endangered endemic species from the Algarve, in the south of Portugal. We investigated a new pretreatment to improve the germination response and subsequently the cryopreservation of seeds. Scarification presented higher germination percentages (approximately 95%) compared to a heat shock pretreatment previously used (approximately 65%). In this study we also investigated different techniques for the cryopreservation of *T. major* shoot tips. Vitrification and encapsulation-dehydration presented the highest regrowth percentages, approximately 60 and 65%, respectively. The best times were 60 min exposure to PVS2 (plant vitrification solution 2) for vitrification and 3 h desiccation for encapsulation-dehydration. For the droplet-vitrification method the highest regrowth percentage achieved was below 40%. The use of the cryopreservation techniques tested here proved to be an important asset in the conservation of this endangered species and will complement the conservation strategies previously developed.

#### 4.1.2. Introduction

*Tuberaria major* (Willk.) P. Silva & Rozeira (Cistaceae) (Figure 4.1A) is a small perennial plant endemic to the Algarve, in the south of Portugal. The few populations left are degraded and fragmented and the main threats to this species are the constant human pressure and increasing urbanization in the region. *T. major* is considered an endangered and priority species by European legislation and is legally protected by the European Habitats Directive 92/43/CEE and Portuguese law (reference 140/99 from April 24) (ICN 2006).



**Figure 4.1.** – Cryopreservation of *Tuberaria major*. Wild plant (bar: 6 cm approximately) (A). Seeds (B). Excised shoot tips approximately 2 mm long (C). Plantlet from cryopreserved shoot tips by the encapsulation-dehydration method, eight weeks after culture (bar: 0.5 cm) (D).

The legal protection involves the preservation of ecosystems and biodiversity in their natural habitats, i.e. *in situ* conservation, but it may not always be sufficient to preserve the genetic diversity of a given species. Thus it is important to integrate different conservation strategies that will complement and support each other. The cryopreservation of *T. major* seeds was already accomplished (Gonçalves et al. 2009) and an efficient *in vitro* propagation protocol was also developed (Gonçalves et al. 2010), representing important contributions for the *ex situ* preservation of this species. *T. major* plantlets produced *in vitro* can now be used to assess the feasibility of cryopreservation in the long-term conservation of plant material different to seeds.

Cryopreservation is one of the most promising techniques for the long-term *ex situ* conservation of plant genetic resources and it can be applied to different plant materials. In new cryopreservation techniques, which can be used for the cryopreservation of shoot tips, plant material is dehydrated by exposure to highly concentrated cryoprotective solutions (vitrification procedures) and/or air desiccation (encapsulation-

dehydration procedures), followed by rapid immersion in liquid nitrogen (LN) (Sakai and Engelman 2007, Engelmann 2011). The droplet-vitrification technique is derived from the droplet-freezing technique (Kartha et al. 1982) and the standard vitrification procedure based on the use of plant vitrification solutions. In this technique shoot tips are placed in small droplets of cryoprotective solution and cooled rapidly (Sakai and Engelman 2007). To the best of our knowledge, there are no reports describing the cryopreservation of shoot tips from species of the Cistaceae family.

Both micropropagation and cryopreservation are powerful tools in the *ex situ* conservation of plant genetic resources and together can provide means to replenish small populations and secure plant germplasm in cryobanks (Benson 2008). It was recently proved by Osório et al. (2013) that *T. major* micropropagated plants can be successfully acclimatised to field conditions and therefore used to restore natural populations.

The aims of this study were to improve the cryopreservation of *T. major* seeds by improving the germination percentage and to evaluate the response of cryopreserved shoot tips using different cryopreservation techniques.

#### **4.1.3. Materials and Methods**

##### **4.1.3.1. Seed collection**

Seeds (Figure 4.1B) were collected from a wild population, located in Gambelas, in the Algarve, Portugal, in July 2010. Collection was made from 10 to 30 individuals randomly selected. After being extracted from the capsules, seeds were kept in paper bags and stored inside a hermetic sealed glass jar with silica gel and kept at  $25 \pm 2^\circ\text{C}$  in the dark for two months before the beginning of the trials.

##### **4.1.3.2. Seed germination and cryopreservation trials**

Seeds were placed in an oven at  $100^\circ\text{C}$  for 60 min (dry heat) (Gonçalves et al. 2009) or scarified by abrasion between two sheets of fine-grained sandpaper. After the pretreatments, seeds were aseptically sown in Petri dishes (11-cm inner diameter) on 0.6% (w/v) agar (Duchefa, The Netherlands) without nutrients and incubated under a 16 h light/ 8 h dark photoperiod provided by cool-white fluorescent lamps at a photon

flux density of  $69 \mu\text{mol m}^{-2} \text{s}^{-1}$  at  $25^{\circ}\text{C}$  for 30 days. Control seeds were sown in the same incubation conditions without pretreatment.

For the cryopreservation trials, seeds were placed in 2-ml cryovials and directly immersed in LN for 30 days. After that period, cryovials were removed from the cryogenic storage and rewarming took place at room temperature for approximately 18 h. Seeds were scarified before sowing and incubation conditions were the same as described above. A control treatment, without immersion in LN, was performed under the same conditions.

#### **4.1.3.3. *In vitro* shoot cultures**

*T. major in vitro* shoot cultures (clone T16) were used to obtain apical and nodal segments. Cultures were established from two-months old seedlings and maintained on MS medium (Murashige and Skoog 1962) supplemented with  $0.2 \text{ mg l}^{-1}$  zeatin (ZEA), 2% (w/v) sucrose and solidified by adding 1% (w/v) agar, and incubated at  $25^{\circ}\text{C}$  under a 16 h light/ 8 h dark photoperiod provided by cool-white fluorescent lamps at a photon flux density of  $40 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Gonçalves et al. 2010), with a subculture period of 4-6 weeks. The nodal segments were cultured on the same medium, freshly prepared, for one week, under low irradiance (approximately  $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), before extraction of 2 mm long shoot tips (Figure 4.1C). Before the cryopreservation experiments, shoot tips were precultured for one day on liquid MS medium containing 0.3 M sucrose.

#### **4.1.3.4. Cryopreservation by vitrification**

Following the pretreatment, shoot tips were immersed in 1 ml of loading solution (0.4 M sucrose and 2 M glycerol in MS medium, pH adjusted to 5.8 before autoclaving), for 20 min at room temperature in 2 ml cryotubes (five shoot tips per tube). The loading solution was removed with a hypodermic needle and replaced by ice-cooled Plant Vitrification Solution 2 (PVS2; Sakai et al. 1990). The PVS2 solution consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO (dimethyl sulphoxide) dissolved in MS medium that contained 0.4 M sucrose, the pH was adjusted to 5.8 and the solution was freshly prepared each time and filter sterilized (Sakai et al. 1990). Cryotubes were maintained on ice during the time shoot tips were exposed to PVS2. The dehydration times tested were 0, 30, 60, 90 and 120 min. After PVS2 treatment, cryotubes were directly plunged in LN and remained there for at least

1 h. For rewarming, cryotubes were immersed in a water bath at 40°C for 2 min. PVS2 was removed and 1 ml of unloading solution (1.2 M sucrose in MS medium, adjusted to pH 5.8 before autoclaving) was added to each cryotube to wash the shoot tips, for 20 min at room temperature. Shoot tips were cultured on semisolid MS medium for one night at 25°C in the dark. On the next day, apices were transferred to recovery medium (semisolid MS supplement with 0.2 mg l<sup>-1</sup> ZEA) and were maintained in the dark at 25°C for one week before being transferred to light conditions (16 h light/ 8 h dark photoperiod provided by cool-white fluorescent lamps at a photon flux density of 40 µmol m<sup>-2</sup> s<sup>-1</sup>). Control treatments refer to replicates carried out in the same conditions but without immersion in LN. For the treatment 0 min in PVS2, segments were transferred from the loading solution directly to LN (except for the control, which was directly transferred to the unloading solution).

#### **4.1.3.5. Cryopreservation by droplet-vitrification**

Experiments were conducted following the droplet-vitrification protocol by Panis et al. (2005). After the pretreatment, shoot tips were transferred onto two layers of sterile filter paper in 6 cm Petri dishes (ten shoot tips per dish) containing 2 ml of loading solution, for 20 min at room temperature. After loading, shoot tips were transferred along with one layer of filter paper to a Petri dish containing 2 ml of ice-cooled PVS2. Petri dishes were maintained on ice during the time shoot tips were exposed to PVS2. The dehydration times tested were 0, 30, 60, 90 and 120 min. After PVS2 treatment, shoot tips were placed on a sterile aluminium foil strip (approximately 25 × 5 × 0.03 mm, five segments per strip), with each shoot tip in a 2 µl drop of PVS2. Strips were previously placed in Petri dishes on top of a frozen cooling block, to keep the temperature close to 0°C. With the help of sterile forceps, each aluminium strip was placed inside a cryotube previously filled with LN, kept inside a polystyrene box containing LN. Strips remained in LN for at least 10 min. For rewarming, strips were rapidly transferred from the cryotube to a Petri dish containing 4 ml of unloading solution, for 20 min at room temperature. Shoot tips were cultured under the same conditions as described above.

#### 4.1.3.6. Cryopreservation by encapsulation-dehydration

Following the pretreatment, shoot tips were immersed in a 3% (w/v) sodium alginate solution (prepared in MS medium containing 0.35 M sucrose and the pH adjusted to 5.8 before autoclaving). With the help of a Pasteur pipette, drops, each containing one shoot tip, were dispensed on a 100 mM CaCl<sub>2</sub> solution (prepared in MS medium containing 0.35 M sucrose, with the pH adjusted to 5.8 before autoclaving). The beads formed were kept in the CaCl<sub>2</sub> solution for 30 min at room temperature and then transferred to liquid MS medium containing 0.75 M sucrose for 18-20 h at 25°C with continuous shaking, 120 rpm. Beads were removed from the previous solution, dried with sterile filter paper and placed in Petri dishes inside a laminar air flow cabinet for different periods of time (0, 1, 2, 3, 4, 5 and 6 h) at room temperature. Desiccated beads were placed in cryotubes (five beads per tube) and immersed in LN for at least 1 h. For rewarming, cryotubes were immersed in a water bath at 40°C for 2 min and beads were cultured on semisolid MS medium supplement with 0.2 mg l<sup>-1</sup> ZEA at 25°C in the dark. After one week, beads containing shoot tips were transferred to light conditions (16 h light/ 8 h dark photoperiod provided by cool-white fluorescent lamps at a photon flux density of 40 μmol m<sup>-2</sup> s<sup>-1</sup>).

#### 4.1.3.7. Statistical analysis

In all seed germination and cryopreservation trials, four replicates of 25 seeds each were tested. Germinating seeds (emerging radicle) were counted every two days over a total 30-day incubation period. At the end of the germination period, the final germination percentage (%) and the mean germination time (MGT, in days; Ellis and Roberts 1981) were calculated.

In the cryopreservation trials, 30 shoot tips were used per treatment. Shoot tip regrowth (shoot tips developing into normal shoots) was recorded after eight weeks in culture and expressed as the percentage over the number of shoot tips cultured.

The values obtained were expressed as means ± standard error. The data were subjected to analysis of variance (ANOVA) using the SPSS statistical package for Windows (release 20.0; SPSS Inc., Chicago, USA). Significant differences between means were determined using Duncan's New Multiple Range Test ( $P = 0.05$ ). The original percentage data were arcsine transformed prior to statistical analysis.

#### 4.1.4. Results

##### 4.1.4.1. Seed germination and cryopreservation

To improve the germination percentage of *T. major* seeds, scarification was tested as pretreatment before sowing. Compared to the control and to dry heat, the previous pretreatment tested that presented the best results (Gonçalves et al. 2009), scarification significantly improved the germination percentage with a value close to 100% ( $P < 0.05$ ) (Table 4.1). As for the MGT values, there were no significant differences between pretreated seeds ( $P \geq 0.05$ ) and control seeds took longer to germinate ( $P < 0.05$ ) (Table 4.1). Scarification was then used to pretreat cryopreserved seeds after storage in LN. The results demonstrate that there were no significant differences between the final germination percentages of cryopreserved,  $95.0 \pm 1.0\%$ , and non-cryopreserved seeds,  $97.0 \pm 1.0\%$  ( $P \geq 0.05$ ), when they were submitted to scarification.

**Table 4.1.** – Final germination percentage and MGT of *Tuberaria major* seeds after different pretreatments.

Pretreatment	Germination (%)	MGT (days)
Scarification	$95.0 \pm 1.9$ a	$12.4 \pm 1.1$ a
Dry heat	$65.0 \pm 3.4$ b	$13.4 \pm 1.1$ a
Control	$2.1 \pm 0.9$ c	$17.3 \pm 1.8$ b

Values represent means  $\pm$  SE of 4 replicates of 25 seeds each. For each parameter, in a column, values followed by the same letter are not significantly different at  $P < 0.05$  according to Duncan's Multiple Range Test.

##### 4.1.4.2. Vitrification

The response of *T. major* shoot tips exposed to different PVS2 dehydration times was evaluated, using the vitrification method. The optimization of the exposure time to the vitrification solution is of major importance for successful shoot tip regeneration after cryopreservation. Non-cryopreserved shoot tips (-LN) presented high regrowth percentages, indicating that the PVS2 treatment, regardless the time of exposure, did not diminish the regenerating capacity of the shoot tips (Table 4.2). After immersion in LN (+LN), there was a reduction of the regrowth percentages and the duration of exposure to PVS2 significantly affected the regrowth ( $P < 0.05$ ) (Table 4.2). Nevertheless, 60%

recovery was obtained after 60 min exposure to PVS2. The other exposure times tested resulted in regeneration lower than 30%.

**Table 4.2.** – Effect of PVS2 exposure time on the regrowth percentages of *Tuberaria major* cryopreserved shoot tips by the vitrification and droplet-vitrification methods, eight weeks after culture.

PVS2 exposure time (min)	Regrowth (%)		Droplet-vitrification	
	Vitrification -LN	+LN	-LN	+LN
0	96.7 ± 3.3 a	3.3 ± 3.3 c	100.0 ± 0.0 a	13.3 ± 9.9 ab
30	90.0 ± 4.5 a	20.0 ± 8.9 bc	83.3 ± 3.3 b	23.3 ± 9.6 ab
60	93.3 ± 6.7 a	60.0 ± 5.2 a	96.7 ± 3.3 a	26.7 ± 8.4 a
90	93.3 ± 4.2 a	30.0 ± 6.8 b	93.3 ± 6.7 a	-
120	86.7 ± 6.7 a	26.7 ± 11.3 b	90.0 ± 4.5 ab	33.3 ± 11.3 a

Values represent means ± SE of 6 replicates with 5 shoots tips each. In each column, values followed by the same letter are not significantly different at  $P < 0.05$  according to Duncan's Multiple Range Test. -LN: non-cryopreserved shoot tips (control); +LN: cryopreserved shoot tips; -: explants lost by contamination.

#### 4.1.4.3. Droplet-vitrification

The droplet-vitrification method, derived from the vitrification technique, was also tested for *T. major* shoot tips after exposure to different PVS2 dehydration times. As previously proved with the vitrification method, the PVS2 was non-detrimental for the regeneration capacity of non-cryopreserved shoot tips (-LN), since high regrowth percentages, above 80%, were obtained for all the exposure times tested (Table 4.2). As for the cryopreserved shoot tips (+LN), with the vitrification method, a decrease in the regrowth percentages was expected compared to shoot tips not immersed in LN. However, the results were much lower using droplet-vitrification and the highest regrowth percentages were below 40% (Table 4.2). Shoot tips exposed to PVS2 for 60 and 120 min presented the highest percentages after cryopreservation by droplet-vitrification ( $P < 0.05$ ) (Table 4.2).

#### 4.1.4.4. Encapsulation-dehydration

Encapsulation-dehydration was the third method tested for the cryopreservation of *T. major* shoot tips. This method does not use vitrification solutions to dehydrate cells before freezing, but air drying. Nonetheless, the desiccation time needs to be optimized like in the vitrification-based procedures. The results obtained are presented in Table 4.3. For non-cryopreserved shoot tips (-LN), the highest regrowth percentage, close to 100%, was achieved after 1 h desiccation ( $P < 0.05$ ). For the following desiccation times, as the time increased, the regrowth percentage decreased. Cryopreserved shoot tips (+LN) (Figure 4.1D) desiccated for 0 and 1 h, presented no regeneration after eight weeks in culture. The highest regrowth percentage, above 65%, was observed after 3 h desiccation ( $P < 0.05$ ). For longer desiccation times, the percentages were gradually lower.

**Table 4.3.** – Effect of desiccation time on the regrowth percentages of *Tuberaria major* cryopreserved shoot tips by the encapsulation-dehydration method, eight weeks after culture.

Desiccation time (h)	Regrowth (%)	
	-LN	+LN
0	76.7 ± 6.2 b	0.0 ± 0.0 d
1	96.7 ± 3.3 a	0.0 ± 0.0 d
2	80.0 ± 7.3 ab	6.7 ± 4.2 cd
3	80.0 ± 5.2 b	66.7 ± 11.2 a
4	63.3 ± 8.0 b	30.0 ± 8.6 b
5	23.3 ± 9.6 c	16.7 ± 6.2 bc
6	16.7 ± 8.0 c	3.3 ± 3.3 cd

Values represent means ± SE of 6 replicates with 5 shoots tips each. In each column, values followed by the same letter are not significantly different at  $P < 0.05$  according to Duncan's Multiple Range Test. -LN: non-cryopreserved shoot tips (control); +LN: cryopreserved shoot tips.

#### 4.1.5. Discussion

Cryopreservation of seeds is an important tool for *ex situ* preservation of endangered species, allowing long-term conservation without deterioration of plant material

(Walters et al. 2004, Gonçalves and Romano 2009, Coelho et al. 2012). Before any study of seed cryopreservation tolerance, it is important to assess seed germination requirements (Kadis and Georghiou 2010, Zaidi et al. 2010, Coelho et al. 2012). Usually Cistaceae species possess dormant seeds with a hard seed coat that needs to be broken so that germination can carry on (Thanos et al. 1992, Zaidi et al. 2010). Gonçalves et al. (2009) proved that a heat shock significantly improved the germination of *T. major* seeds compared to non-pretreated seeds. In addition, the germination capacity of cryopreserved seeds that were submitted to the same heat pretreatment was not affected (Gonçalves et al. 2009). However, the highest germination percentages obtained by Gonçalves et al. (2009) with the heat shock pretreatment (dry heat) were approximately 65%. In the present study, a different pretreatment was tested to improve the germination of *T. major* seeds and therefore, their cryopreservation. The use of scarification before sowing significantly improved the germination percentage up to 30% compared to the use of dry heat as a pretreatment. And, as in the previous report (Gonçalves et al. 2009), cryopreserved seeds germinated without any loss of their capacity, after scarification, with a germination percentage around 95%. The germination percentage of *T. macrosepala* was also significantly improved by scarification pretreatment and the storage of seeds in LN did not diminish their germinating performance (Zaidi et al. 2010). Studies with other Cistaceae species confirm the use of scarification to improve germination (Pérez-García and González-Benito 2005, 2006) and the suitability of cryopreservation for the long-term conservation of seeds (Pérez-García and González-Benito 2008).

The cryopreservation of shoot tips can also provide an option for the long-term conservation of germplasm from endangered plant species and complement other conservation strategies, in particular when the number of available seeds is limited. In the present work, vitrification, droplet-vitrification and encapsulation-dehydration were the methods tested to develop a cryopreservation protocol for apices from *in vitro*-grown shoots of *T. major*. There are several reports describing protocols for the cryopreservation of shoot tips from endangered plant species using the same methods tested here (Mandal and Dixit-Sharma 2007, Mallon et al. 2008, Sen-Rong and Ming-Hua 2009, Ozudogru and Kaya 2012, Rabba'a et al. 2012). The key for an efficient cryopreservation protocol using these techniques is to dehydrate cells enough so that solutions are able to vitrify upon rapid cooling in LN, without damages to cells (Sakai

and Engelmann 2007). The results obtained demonstrated that vitrification and encapsulation-dehydration were the best methods, presenting regrowth percentages of approximately 60 and 65%, respectively. Shoot tips exposed to PVS2 for 60 min, in the vitrification method, and beads dehydrated for 3 h, in the encapsulation-dehydration method, were the most appropriate conditions tested. With the droplet-vitrification method, the regrowth percentages were lower than 40%, for all PVS2 exposure times tested.

The regrowth percentage of cryopreserved *T. major* shoot tips was slightly higher with the encapsulation-dehydration method than with the vitrification method. Encapsulation-dehydration is frequently a good choice when plant material is sensitive to the toxicity of the cryoprotective solutions used in vitrification-based procedures (Benson 2008). In the present study that seems not to be the case, since the cryoprotective solution used, PVS2, was non-detrimental for non-cryopreserved shoot tips. On the other hand, the use of alginate beads to encapsulate shoot tips can be highly advantageous, because it allows a drastic desiccation, normally lethal to non-encapsulated ones, thus preventing the formation of ice crystals and devitrification, without causing any injuries to the cells (González-Arno and Engelmann 2006). Encapsulation-dehydration proved to be a better method compared to vitrification in the cryopreservation of *Gentiana* (Tanaka et al. 2004) and *Rubus* (Gupta and Reed 2006) shoot tips.

The droplet-vitrification and vitrification methods presented quite different results, although the first derives from the second. Usually with the droplet-vitrification method better results are achieved when compared to standard vitrification, because the use of a very small volume of cryoprotective solution allows ultra rapid cooling and warming rates (Leunufna and Keller 2003, Panis et al. 2005, Sakai and Engelmann 2007, Ozudogru and Kaya 2012). However in this study the opposite occurred, the regrowth percentage of cryopreserved shoot tips was considerably higher using the vitrification method.

To summarize, cryopreservation is a viable option for the long-term conservation of *T. major* germplasm. We confirmed that cryopreservation did not affect the germination response of seeds and demonstrated that vitrification and encapsulation-dehydration are suitable methods for the cryopreservation of shoot tips. Further studies should be performed in order to improve the regeneration capacity of cryopreserved shoot tips and

to test the protocol with several genotypes. Several preconditioning approaches and preculture conditions can be optimized to improve the dehydration tolerance of samples before freezing. The most common are cold acclimation and precultured on medium with high levels of sugar for different time periods (Sakai and Engelmann 2007, Benson 2008). Nevertheless with the present report a major contribution was given for the preservation of this endangered species.

#### 4.1.6. References

- Benson EE (2008) Cryopreservation of Phytodiversity: A Critical Appraisal of Theory & Practice. *Critical Reviews in Plant Sciences* 27: 141-219;
- Coelho N, Gonçalves S, González-Benito ME and Romano A (2012) Germination and cryopreservation tolerance of seeds from the rare aromatic species *Thymus lotocephalus*. *Scientia Horticulturae* 145: 84-86;
- Ellis RH and Roberts EH (1981) The quantification of ageing and survival in orthodox seeds. *Seed Science and Technology* 9: 373-409;
- Engelmann F (2011) Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cellular & Developmental Biology – Plant* 47: 5-16;
- Gonçalves S, Fernandes L, Pérez-García F, González-Benito ME and Romano A (2009) Germination requirements and cryopreservation tolerance of seeds of the endangered species *Tuberaria major*. *Seed Science and Technology* 37: 480-484;
- Gonçalves S and Romano A (2009) Cryopreservation of seeds from the endangered insectivorous plant *Drosophyllum lusitanicum*. *Seed Science and Technology* 37: 485-490;
- Gonçalves S, Fernandes L and Romano A (2010) High-frequency *in vitro* propagation of the endangered species *Tuberaria major*. *Plant Cell, Tissue & Organ Culture* 101: 359-363;
- González-Arnao MT and Engelmann F (2006) Cryopreservation of plant germplasm using the encapsulation–dehydration technique: review and case study on sugarcane. *CryoLetters* 27: 155-168;
- Gupta S and Reed BM (2006) Cryopreservation of shoot tips of blackberry and raspberry by encapsulation-dehydration and vitrification. *CryoLetters* 27: 29-42;
- ICN (Instituto da Conservação da Natureza) (2006) Plano Sectorial da Rede Natura. Flora: *Tuberaria major* (Willk.) P. Silva & Rozeira <<http://www.icnf.pt/portal/naturaclas/rn2000/resource/rn-plan-set/flora/tub-maj>> Accessed 20 May 2014;
- Kadis C and Georghiou K (2010) Seed dispersal and germination behavior of three threatened endemic labiates of Cyprus. *Plant Species Biology* 25: 77-84;
- Kartha KK, Leung NL and Mroginski LA (1982) *In vitro* growth responses and plant regeneration from cryopreserved meristems of Cassava (*Manihot esculenta* Crantz). *Zeitschrift für Pflanzenphysiologie* 107: 133-140
- Leunufna S and Keller ERJ (2003) Investigating a new cryopreservation protocol for yams (*Dioscorea* spp.). *Plant Cell Reports* 21: 1159-1166;
- Mallon R, Bunn E, Turner SR and Gonzalez ML (2008) Cryopreservation of *Centaurea ulreiae* (Compositae) a critically endangered species from Galicia (Spain). *CryoLetters* 29: 363-370;
- Mandal BB and Dixit-Sharma S (2007) Cryopreservation of *in vitro* shoot tips of *Dioscorea deltoidea* Wall., an endangered medicinal plant: Effect of cryogenic procedure and storage duration. *Cryoletters* 28: 461-470;
- Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497;

- Osório ML, Gonçalves S, Coelho N, Osório J and A Romano (2013) Morphological, physiological and oxidative stress markers during acclimatization and field transfer of micropropagated *Tuberaria major* plants. *Plant Cell, Tissue & Organ Culture* 115: 85-97;
- Ozudogru EA and Kaya E (2012) Cryopreservation of *Thymus cariensis* and *T. vulgaris* shoot tips: comparison of three vitrification-based methods. *CryoLetters* 33:363-375;
- Panis B, Piette B and Swennen R (2005) Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all *Musaceae*. *Plant Science* 168: 45-55;
- Pérez-García F and González-Benito ME (2005) Effects of temperature and different pre-treatments on seed germination of four *Halimium* species. *Seed Science and Technology* 33: 505-509
- Pérez-García F and González-Benito ME (2006) Seed germination of five *Helianthemum* species: Effect of temperature and pre-sowing treatments. *Journal of Arid Environments* 65: 688-693;
- Pérez-García F and González-Benito ME (2008) Seed cryopreservation of *Halimium* and *Helianthemum* species. *CryoLetters* 29: 271-276;
- Rabba'a MM, Shibli RA and Shatnawi MA (2012) Cryopreservation of *Teucrium polium* L. shoot-tips by vitrification and encapsulation-dehydration. *Plant Cell, Tissue & Organ Culture* 110: 371-382;
- Sakai A, Kobayashi S and Oiyama I (1990) Cryopreservation of nucellar cells and navel orange (*Citrus sinensis* Osb. Var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Reports* 9:30-33
- Sakai A and Engelmann F (2007) Vitrification, encapsulation-vitrification and droplet-vitrification: a review. *CryoLetters* 28: 151-172;
- Sen-Rong H and Ming-Hua Y (2009) High-efficiency vitrification protocols for cryopreservation of *in vitro* grown shoot tips of rare and endangered plant *Emmenopterys henryi* Oliv. *Plant Cell, Tissue & Organ Culture* 99: 217-226;
- Tanaka D, Niino T, Isuzugawa K, Hikage T and Uemura M (2004) Cryopreservation of shoot apices of *in-vitro* grown gentian plants: Comparison of vitrification and encapsulation-vitrification protocols. *CryoLetters* 25: 167-176;
- Thanos CA, Georghiou K, Kadis C and Pantazi C (1992) Cistaceae: A plant family with hard seeds. *Israel Journal of Botany* 41: 251-263;
- Walters C, Wheeler L and Stanwood PC (2004) Longevity of cryogenically stored seeds. *Criobiology* 48: 229-244;
- Zaidi CA, González-Benito ME and Pérez-García F (2010) Morphology, germination and cryopreservation of seeds of the Mediterranean annual plant *Tuberaria macrosepala* (Cistaceae). *Plant Species Biology* 25: 149-157.

# CHAPTER 5



## General discussion and conclusions



### 5.1. General discussion

All over the globe, plant biodiversity is at risk and the number of threatened species increases year after year. Human activities are the primary cause for the loss of natural populations or entire species and therefore the loss of genetic diversity. Though in recent years many efforts have been done to minimize the declining of biodiversity, it is still not sufficient and additional conservation measures are immediately needed (Sarasan et al. 2006, Reed et al. 2011).

*In situ* conservation, which includes the management of wild populations and natural habitats, is the most effective conservation approach in the preservation of species, including threatened species. However, *in situ* conservation alone may not be sufficient and *ex situ* techniques can and should be used to complement this strategy, especially in the case of endangered and rare species. For some of these species *ex situ* conservation may also be the only available option (Sarasan et al. 2006, Engelmann 2011, Reed et al. 2011).

The majority of actions dealing with *ex situ* conservation of plant biodiversity have focussed on crop species, due to their economical and food value (Engelmann 2011). The publication in international journals of matters dealing with threatened plant conservation is limited and not encouraging (Sarasan et al. 2006). Nevertheless, the conservation of the genetic diversity of wild and rare plant species is highly important and is becoming a major concern (Engelmann 2011, Reed et al. 2011).

Plant conservation has greatly benefited from the application of conservation strategies based on biotechnology (Benson 1999). In particular, *in vitro* techniques provide tools that can be used in a variety of ways and their use in germplasm conservation is increasing. The development of successful storage methods enables the establishment of extensive collections, with representative genetic diversity, which is of particularly interest in the conservation of rare species. *In vitro* techniques have been successfully developed for the propagation and cryopreservation of many species, including several threatened species (Sarasan et al. 2006, Reed et al. 2011). In addition, the biotechnological tools based on molecular biology can assist in the collection of plant material, management of ecosystems and in the development of more effective propagation and storage protocols (Reed et al. 2011).

All these measures can greatly aid in the conservation of the genetic diversity of rare and threatened plant species from around the world. In the present work, three endemic species, *Thymus lotocephalus*, *Plantago algarbiensis* and *Tuberaria major*, of the southern region of Portugal, Algarve, were chosen for the development of *ex situ* conservation strategies due to their vulnerability and risk of extinction. These plant species are geographically and ecologically rare because their distribution is restricted to a very small area in the world wide context and are consistent with a particular habitat at a regional comparison scale level. The main threat common to the three species is the destruction of their natural habitat due to human interference. All three species are legally protected under national and European legislation. However, as previously highlighted, additional conservation measures are needed to ensure the survival of these species.

#### **5.1.1. *In vitro* propagation**

*In vitro* propagation refers to the vegetative propagation of plants in an aseptic controlled environment. This technique offers several advantages for plant conservation, in particular the possibility of *in vitro* storage of germplasm and the use of *in vitro*-propagated plants for reintroduction or restoration of rare species in the wild. *In vitro* cultures can also provide large quantities of genetically identical plant material from a single explant in a relatively short time and space, throughout the whole year. Plants are usually free from contaminations, thus ensuring the production of disease free stocks and simplifying the procedures for exchange of germplasm (Debnath et al. 2006, Engelmann 2011, Reed et al. 2011). The use of a reduced storing space also contributes for a reduction in labour costs (Engelmann 2011).

From the three species studied in this work, *T. lotocephalus* was the only one that had no *in vitro* propagation protocol already developed. So the first step was to initiate the *in vitro* propagation of this species, not just as complementary conservation measure to the *in situ* protection, but also to obtain enough plant material for further conservation approaches and other relevant studies.

In Chapter 2.1 *in vitro* cultures of *T. lotocephalus* were initiated from seedlings and one genotype was chosen to initiate shoot proliferation. After testing different media, this species revealed a high shoot proliferation capacity since all shoots multiplied despite the medium used. Murashige and Skoog (MS, 1962) medium without growth regulators

proved to be the most suitable medium for the proliferation of *T. lotocephalus*, considering the combination of the different parameters tested: number of new shoots, shoot length and hyperhydric shoots. The MS media supplemented with 6-benzyladenine (BA) presented the highest number of new shoots; however, it also produced a high number of hyperhydric shoots, which did not occur in any of the other media tested. The longest shoots were obtained on MS medium supplemented with a combination of cytokinin and auxin [zeatin (ZEA) and indole-3-acetic acid (IAA), respectively].

During the proliferation stage there was no simultaneous root formation and therefore a subsequent stage was necessary to induce rooting. The rooting frequency was significantly improved by gradually lower concentrations of MS medium macronutrients and  $\frac{1}{4}$ MS proved to be the best concentration for the formation of roots. The addition of IAA to  $\frac{1}{4}$ MS improved the number of roots and root length. A different approach to induce root formation was also tested: dipping the basal end of the shoots in a concentrated auxin solution followed by culture in auxin-free medium. Media with lower macronutrient concentration showed the best results, however there was no significant improvement compared to the experiments without dipping.

After the rooting stage, plantlets with well-developed roots were selected for acclimatisation and were progressively exposed to reduced relative humidity. Plantlets acclimatised in a plant growth chamber obtained high survival rates and no morphological variations were detected.

This protocol was later successfully applied to different genotypes and shoots produced *in vitro* were used to excise shoot tips for the following cryopreservation studies. Besides, other biochemical studies were performed (Costa et al. 2012a,b) and others are undergoing using this plant material produced *in vitro*, avoiding collection from the wild.

The *in vitro* propagation of *P. algarbiensis* and *T. major* were also initiated using seedlings as explants following previously published protocols (respectively, Gonçalves et al. 2009b, Gonçalves et al. 2010). The effect of cytokinin type and concentration on the proliferation capacity of both species were evaluated. For *T. major*, the type of explant, nodal or apical, was also studied. In terms of multiplication response, the best results were obtained on MS medium supplemented with 0.2 or 0.5 mg l<sup>-1</sup> BA and MS medium supplemented with 0.2 mg l<sup>-1</sup> ZEA, for *P. algarbiensis* and *T. major*,

respectively. Nodal segments showed a higher proliferation capacity than apical segments, in the case of *T. major*.

Contrary to *T. lotocephalus*, spontaneous rooting was observed during the proliferation phase of both species and plantlets were prepared to be directly acclimatised without passing through a rooting phase. However, additional experiments, using different MS macronutrient concentrations and auxins, were performed to improve the rooting frequency. For *P. algarbiensis*, the rooting frequency was not affected by MS macronutrient concentration nor improved by the use of auxins. Nevertheless, rooting frequencies were above 90%. As for *T. major*, the rooting frequency was also not affected by the addition of auxins to the medium, but ½MS improved the results comparing to full MS medium.

For both species, plants obtained *in vitro* using these protocols were easily acclimatised to *ex vitro* conditions and successfully transferred to field conditions, exhibiting a normal development.

The shoots produced *in vitro* following these protocols were used to excise nodal segments and apices, for *P. algarbiensis* and *T. major*, respectively, for the following cryopreservation studies.

Recently, a more thorough study was performed to evaluate the morphological and physiological traits of *T. major in vitro*-propagated plants during acclimatisation and field transfer (Osório et al. 2013). The study included the analysis of several parameters throughout the different stages of acclimatisation (*in vitro* rooting, growth chamber, greenhouse and field). The results demonstrated that the adaptation of *in vitro*-propagated *T. major* plants to field conditions results from changes in their morphological and physiological characteristics. In the natural habitat, these plants were capable of surviving without loss of photosynthetic and growth capacity compared to wild plants. This study proved that the *in vitro* propagation protocol for *T. major* developed by Gonçalves et al. (2010) combined with the acclimatisation procedure described can be used for the restoration of depleted populations of this species.

*P. algarbiensis in vitro*-propagated plants were also used to evaluate the capacity of this species to accumulate aluminium (Martins et al. 2011, 2013a,c,d,e). These experiments were performed based on the fact that another endemic plantain species from the southwest coast of Portugal, *P. almogravensis*, is considered an aluminium

hyperaccumulator (Branquinho et al. 2007) and that both species colonize acidic soils and are morphologically similar. The main conclusion withdraw from these studies is that *P. algarbiensis* accumulates considerable amounts of aluminium and that is moderately tolerant to low pH and aluminium.

The development of individual protocols for each species is the major drawback of *in vitro* conservation (Maxted et al. 1997, González-Benito et al. 2004, Reed et al. 2011). In the present work, an effective *in vitro* propagation protocol for *T. lotocephalus* was developed (Chapter 2.1), thus surpassing this limitation. For the other two species, *P. algarbiensis* and *T. major*, the protocols had already been previously established (Gonçalves et al. 2009b, Gonçalves et al. 2010, respectively). *In vitro* propagation will allow the short and medium-term storage of plant germplasm in culture and simultaneously, can provide plant material for cryopreservation. With the appropriate facilities and manpower, culture collections can be maintained for different genotypes originating from the different populations in germplasm banks. In addition, plants produced *in vitro* can support investigations following different paths, avoiding their collection from the wild. In the case of *T. lotocephalus*, its aromatic and medicinal properties are under study using *in vitro*-cultured plants. As for *P. algarbiensis*, due to its capacity to accumulate aluminium, the development and mass production of *in vitro* plants to clean soils contaminated by aluminium is something to consider.

### **5.1.2. Seed cryopreservation**

As previously mentioned, seed storage is the simplest and most efficient method for *ex situ* conservation of plant germplasm. Cryopreservation is an important tool to store seeds for long periods and has been successfully applied in the preservation of several wild and endangered species (Pence and Clark 2005, Gonçalves and Romano 2009). However, prior to the study of seed cryopreservation tolerance, it is important to assess seed germination behaviour and seed morphology (Fenner and Thompson 2005, Kadis and Georghiou 2010). Moreover, because variability in the germination response among seeds of the same species is relatively frequent in wild plants, studies should include different populations (Albert et al. 2002, Pérez-García et al. 2003).

The germination requirements and the cryopreservation tolerance of *T. lotocephalus* seeds were investigated in Chapter 2.2. Several studies were performed using seeds collected from different populations (Algoz, Tunes, Gambelas and Olhão). *T.*

*lotocephalus* produces very small rounded seeds and the seed morphologic study revealed that seed length ranged from  $1009.58 \pm 7.56 \mu\text{m}$  to  $1156.93 \pm 9.48 \mu\text{m}$ , while seed width ranged from  $924.11 \pm 7.36 \mu\text{m}$  to  $1099.70 \pm 8.29 \mu\text{m}$ . The weight of 100 seeds was approximately between 50-55 mg for all populations.

The germination percentage of *T. lotocephalus* seeds (Chapter 2.1) was 44% at 25°C without any pretreatment. A preliminary study was performed to evaluate the effect of different pretreatments in the germination behaviour of seeds from Algoz population (Coelho et al. 2010). The pretreatments applied to improve seed germination were: soaking in water, soaking in gibberellic acid solution (GA<sub>3</sub>), cold stratification, dry heat, hand scarification, mechanical scarification and acid scarification. The best final germination percentage, approximately 86%, was obtained after immersion of seeds in a GA<sub>3</sub> solution. From this preliminary study it can be concluded that *T. lotocephalus* seeds could have a physiological dormancy, easily breakable by immersing the seeds in a GA<sub>3</sub> solution.

Subsequently, using seeds from the four populations, different incubation conditions were tested, without pretreatment (Chapter 2.2). The final germination percentage and mean germination time (MGT) were assessed for two germination temperatures, 15 and 25°C, under photoperiod or dark incubation. Both variables were affected by germination temperature. At 15°C, more seeds germinated (final germination percentages approximately between 80 and 100%) and faster (MGT values below 10 days for all populations) than at 25°C, despite the light conditions. Between populations, no major differences were observed in the different parameters studied and the best results depended on the germination conditions used. According to these results, where final germination percentages above 80% were obtained at 15°C without any pretreatment, it was concluded that *T. lotocephalus* seeds can be considered non-dormant. When non-optimum incubation conditions are used (25°C, Chapter 2.1.) germination percentages could be increased by GA<sub>3</sub> pretreatment.

The exposure of *T. lotocephalus* seeds to liquid nitrogen (LN) proved to be non-detrimental to seed germination. Germination percentages were above 80% and MGT values below 10 days for the three populations tested (Algoz, Tunes and Olhão), with minor differences between cryopreserved and control seeds.

As for *P. algarbiensis* seeds, the morphological features and the effects of temperature and light on their germination were previously investigated by Martins et al. (2012).

Using seeds from one wild population (Algoz) collected in two consecutive years (2009 and 2010), constant (15 and 25°C) and alternating temperature regime (25/15°C), combined with light/dark photoperiod or constant darkness conditions were studied. The results showed that *P. algarbiensis* has non-dormant seeds. The optimal germination temperature was 15°C under light or darkness, along with the lowest MGT values. Although, no differences were observed in the germination capacity of *P. algarbiensis* seeds collected in two different years, differences were detected in the morphological features.

*P. algarbiensis* seeds were also used to study the effect of aluminium on germination and seedling development (Martins et al. 2013b). Results indicate that seeds are able to tolerate the presence of aluminium and germinate in acidic soils, where the species occurs. However, high amounts of aluminium inhibit root growth during early seedling development, which may compromise plants establishment in their natural habitats.

In Chapter 3.1, the feasibility of cryopreservation to preserve *P. algarbiensis* seeds was studied. Seeds from three wild populations (Algoz, Tunes and Gambelas) were directly immersed in LN for 30 days and set to germinate according to the best conditions determined by Martins et al. (2012). Germination percentages of cryopreserved seeds were above 90% and MGT values below 7 days, with no differences between cryopreserved and control seeds and among all populations, except Tunes.

Chapter 4.1 firstly describes the improvement of the germination capacity of *T. major* seeds and consequent improvement in seed cryopreservation. The study of the germination requirements and the cryopreservation tolerance of *T. major* seeds were initially reported by Gonçalves et al. (2009a). In that study, the germination percentage was improved by a heat shock pretreatment when compared to non-pretreated seeds. Different incubation temperatures were also studied (constant 15°C, 20°C or 25°C, or alternating 25°C/15°C) and the results demonstrate that there was no effect on the final germination percentage. After cryopreservation, the germination capacity was not affected but the heat shock pretreatment (dry heat, 100°C for 60 min) was also essential to obtain a germination percentage of 65%.

In the present study (Chapter 4.1), based on results from other *Cistaceae* species (Pérez-García and González-Benito 2005, 2006, Zaidi et al. 2010), scarification was tested as pretreatment to improve the germination percentage of *T. major* seeds. Comparing to dry heat (Gonçalves et al. 2009a), scarification improved the germination percentage

with a result of 95%. Once more it was proved that cryopreservation was non-detrimental for seeds as the germination percentage, after immersion in LN followed by scarification pretreatment, was not affected.

Germination is an important event in the life cycle of any plant. Its timing largely predetermines the chances of survival of a seedling up to maturity. Therefore, it is very important to study the germination behaviour of a plant species before setting up any conservation measure. Plants regulate their germination time by having specific requirements for various environmental factors such as moisture, temperature and light. These factors not only can regulate the dormancy breaking process, but also influence subsequent germination, allowing seedling establishment during suitable conditions to maximize survival (Bewley et al. 2013).

*T. lotocephalus* (Chapter 2.2) and *P. algarbiensis* (Martins et al. 2012) seeds suffered no influence of the different light conditions tested in their germination process. However, the germination temperature was decisive to improve the final germination percentages. For both species the best temperature was 15°C. On the contrary, *T. major* seeds were not affected by the germination temperature (Gonçalves et al. 2009a). The crucial step to improve final germination percentage was the scarification pretreatment before sowing (Chapter 4.1).

As for seed cryopreservation, the germination capacity was not affected after exposure to LN for the three species (Chapters 2.2, 3.1 and 4.1). In addition there was no need to use slow cooling rates and therefore expensive or complicated devices are not necessary for lowering the temperature. The long-term storage of *T. lotocephalus*, *P. algarbiensis* and *T. major* germplasm, and ultimately their conservation, can therefore be assured by the cryopreservation of seeds. Because these are rare species, the collection of seeds is limited. On the other hand, since cryostorage increases the longevity of stored seeds (Walters et al. 2004), every year a small number of seeds can be collected from the wild and maintained in seed or cryobanks for hypothetically an unlimited time period. Compared to other conservation approaches, seed storage is the most economical and simple method.

### 5.1.3. Cryopreservation of vegetative explants

Cryopreservation is the most promising technique for the long-term *in vitro* conservation of plant germplasm. This technique greatly benefited from the development of biotechnology and it is being applied to an increasing number of plant species in the last years, in particular to species for which is not possible the traditional conservation in seed banks (Benson 2008, Reed et al. 2011). Several types of plant material can be cryopreserved without loss of their viability, since it is believed that the storing conditions in LN (-196°C) suspend most chemical reactions, avoiding cell division and therefore their degeneration. The techniques applied are relatively simple with a low maintenance cost associated and small space requirements. In addition, it can be applied to different genotypes and the need for regular subcultures is eliminated, keeping the genetic stability of plant material (Harding 2004, Walters et al. 2004, Engelmann 2011).

By combining cryopreservation with *in vitro* propagation, a powerful strategy is created: the numbers of individuals from a declining, small population can be increased; their germplasm can be secured in cryobanks; and in storage at-risk germplasm can be hold until a time is suitable for re-introduction into a safe environment (Benson 2008).

Shoot tips were used in the development of cryopreservation protocols of vegetative explants from *T. lotocephalus* and *T. major*. Apices were excised from *in vitro*-grown shoots cultured according to the protocols developed in Chapter 2.1 and by Gonçalves et al. (2010), for *T. lotocephalus* and *T. major*, respectively. In the case of *P. algarbiensis*, nodal segments of 2-3 mm-long excised from *in vitro* cultures (Gonçalves et al. 2009b) were used to perform the experiments. Initially shoot tips were tested, however due to several difficulties, nodal segments proved to be a more suitable type of explant for this species. Shoot tips were both very small and almost transparent, which made them very difficult to excise, or too big, depending on the subculture period of *in vitro*-donor plants. Besides, the few experiments conducted presented rather low survival and regrowth percentages (data not shown).

The development of the protocols for *T. lotocephalus* and *P. algarbiensis* were preceded by the evaluation of the best recovery medium. The composition of the medium for recovery after cryopreservation is very important for the regeneration of plantlets. Plant growth regulators play an important role and an appropriate medium allows a high survival and fast regrowth of cryopreserved plant material, without callus formation,

which is not desirable, because it can cause somaclonal variations (Wang et al. 2003). For *T. lotocephalus* (Chapter 2.3), two media were tested to evaluate the regeneration of shoot tips. MS medium supplemented with 0.2 mg l<sup>-1</sup> ZEA presented regrowth percentages of 100% and was chosen as recovery medium for the subsequent experiments. As for *P. algarbiensis* (Chapter 3.1), first it was necessary to evaluate the most appropriate container for regeneration. The results obtained showed that Erlenmeyers were better than Petri dishes, as used with *T. lotocephalus*, for the regeneration of segments. All media presented regrowth percentages above 90% and multiplication capacity. Considering the different multiplication and regeneration parameters assessed, the medium MS supplemented with 0.2 mg l<sup>-1</sup> BA was selected as recovery medium for *P. algarbiensis* segments, as it presented the best results.

Vitrification, droplet-vitrification and encapsulation-dehydration were the methods tested to develop cryopreservation protocols for the three species. Overall, droplet-vitrification was the method that had the best results for *T. lotocephalus* shoot tips and encapsulation-dehydration was more effective for the cryopreservation of *P. algarbiensis* nodal segments and *T. major* shoot tips.

Several parameters were optimized using the droplet-vitrification method to achieve the best combination for the recovery of *T. lotocephalus* cryopreserved shoot tips (Chapter 2.3). The conditions improved included shoot tip size, exposure time to plant vitrification solution 2 (PVS2), preculture and cold-hardening and subculture period of *in vitro*-donor plants. All parameters, except cold-hardening, had a significant effect on the regeneration of cryopreserved shoot tips. The highest regrowth percentage, 67%, was accomplished using 1 mm shoot tips excised from shoots grown for 4 weeks since the last subculture at 25°C, and precultured for one day on liquid MS medium containing 0.3 M sucrose, followed by dehydration for 60 min in PVS2. With the encapsulation-dehydration method a regrowth percentage of 44% was obtained after desiccating the beads containing the shoot tips for 4 h before freezing. As for the vitrification method, no regrowth of cryopreserved shoot tips was observed.

For *P. algarbiensis* (Chapter 3.1), results using the vitrification method were later excluded because the survival of cryopreserved segments was very low and there was no regrowth. With the droplet-vitrification method, firstly it was studied the effect of preculture and PVS2 exposure time without freezing. The results demonstrated that PVS2 solution was non-detrimental for non-cryopreserved segments, for all exposure

times tested. The regeneration of segments was not improved by preculture on medium with high levels of sugar. After cryopreservation, the highest regrowth percentage, 60%, was obtained for segments exposed to PVS2 for 120 min. Using the encapsulation-dehydration method, the effect of the different stages of the protocol was first evaluated. The regrowth percentage of *P. algarbiensis* segments gradually decreased along the steps of the protocol. The study of the effect of the desiccation time demonstrated that the highest survival and regrowth percentages, 63%, were obtained after 3 h desiccation for cryopreserved segments.

As for *T. major* (Chapter 4.1), the shoot tips used in the experiments were 2 mm long and were excised from apical and nodal segments cultured for one week under low irradiance. The exposure of shoot tips to PVS2, using both vitrification-based procedures (vitrification and droplet-vitrification), was non-detrimental for the regeneration of non-cryopreserved shoot tips. However, after cryopreservation, results were quite different in both methods. With the vitrification method a regrowth percentage of 60% was obtained for 60 min exposure time to PVS2, while with the droplet-vitrification method, the highest percentage achieved was much lower, 33%, and was obtained after 120 min exposure time. These results are not in agreement with many other studies, including the results obtained for *T. lotocephalus* and *P. algarbiensis* in the present work, where droplet-vitrification frequently presents better results than the standard vitrification method (Leunufna and Keller 2003, Panis et al. 2005, Sakai and Engelmann 2007, Ozudogru and Kaya 2012). The encapsulation-dehydration method presented the highest regrowth percentage of the three methods tested for the cryopreservation of *T. major* shoot tips. A percentage of 67% was obtained for beads dehydrated for 3 h in a laminar air flow cabinet before immersed in LN.

The results obtained in the development of a protocol for the cryopreservation of *T. major* shoot tips, though preliminary, were very positive. Without any optimization of the conditions tested, except exposure times to PVS2 and air-drying, regrowth percentages of 60% and above were obtained for cryopreserved shoot tips with the vitrification and encapsulation-dehydration methods, respectively. However, an improvement of the results is possible, by optimizing certain conditions, such as preconditioning of *in vitro*-donor plants, preculture medium and time and other steps of

the protocols (Sakai and Engelmann 2007, Benson 2008). These two methods are therefore a viable approach for the cryopreservation of *T. major* germplasm.

The cryopreservation of plant germplasm is usually associated with tissue and cell culture, except for orthodox seeds and dormant buds (Benson 2008). Several factors influence survival after thawing (Benson 2008) and the optimization of cryoprotection is very important in the development of all cryopreservation protocols (Fuller 2004). Therefore, pretreatments are applied before cryoprotection to improve the efficacy of cryoprotectants and survival of cryopreserved material (Benson 2008, Reed and Uchendu 2008). Vitrification-based cryoprotection can inflict extreme biophysical and chemical stress that can be damaging to germplasm because vitrification solutions are applied at high concentrations (Benson 2008). In the present work, the exposure of shoot tips and nodal segments to PVS2 was non-detrimental in the regeneration of non-cryopreserved shoot tips for the three species, since regrowth percentages close to 100% were achieved after culture. In the case of *T. lotocephalus* (Chapter 2.3), pretreatment with medium with high levels of sucrose enhanced the regeneration of cryopreserved shoot tips. On the contrary, with *P. algarbiensis* (Chapter 3.1), the exposure of segments to a sugar rich medium before cryoprotection did not improve the post-freezing survival. Also for *T. lotocephalus* cold hardening of *in vitro*-donor plants had no influence on the final regrowth percentages.

The success of any storage protocol is finally achieved when the genetic stability of recovered plants can be guaranteed. During *in vitro* culture and cryopreservation plant tissues are exposed to several stresses that may affect the genome (Harding 2004). In the case of rare and endangered species, that may have a small number of populations with few individuals, any further loss of material due to genetic degradation can be a major concern. In Chapter 2.3, the genetic stability of *T. lotocephalus* shoot tips was assessed using random amplified polymorphic DNA (RAPD) markers and samples from regenerated non-cryopreserved and cryopreserved shoot tips. The RAPD analysis produced 97 scorable bands and the total number of RAPD fragments was 4656. A few variations were identified between cryopreserved and non-cryopreserved material, with a variation frequency of 0.06%. However, no morphological abnormalities were detected and the plants recovered from *T. lotocephalus* cryopreserved shoot tips developed normally.

As with *in vitro* propagation, protocol development and optimization is the most labour-intensive phase of cryopreservation. After creating cryobank accessions and once samples are stored in LN, little maintenance is required. This is highly advantageous, when comparing to other *in vitro* conservation procedures based on slow-growth, because it saves on personnel time and resources and reduces the risks of contamination occurring during routine subculture. The cost-effectiveness of the process is increased by reducing production and waste disposal costs, the making of culture media, the purchasing of consumables and washing, sterilizing and recycling culture vessels (Benson 2008).

Alternatively to seed cryopreservation, which is limited due to the risk of extinction of these species, the cryopreservation of vegetative explants obtained from *in vitro*-grown plants will also provide the long-term storage and conservation of *T. lotocephalus*, *P. algarbiensis* and *T. major* germplasm.

#### **5.1.4. Genetic diversity of wild populations**

In order to define strategies for the conservation of endangered species it is also important to know the levels and distribution of genetic diversity (Holsinger and Gottlieb 1991, Ramanatha Rao and Hodgkin 2002). To reduce the risk of extinction, plants produced *in vitro* can be used to enhance and restore natural populations. The information on gene distribution and population structures will support appropriate strategies for sample collection and reintroduction of micropropagated plants.

Studies concerning the genetic diversity of *T. lotocephalus* populations were recently initiated. Seedlings obtained from seeds collected from five wild populations (Algoz, Tunes, Belharucas, Gambelas and Olhão) are being used to collect samples for RAPD analysis on the genetic diversity (undergoing work).

In Chapter 3.2, the genetic diversity of three populations (Algoz, Tunes and Gambelas) of *P. algarbiensis* was assessed by RAPD markers. High levels of polymorphism were detected and the genetic diversity of *P. algarbiensis* populations was considered high. Tunes population presented the highest genetic diversity of the three populations studied. In the cluster analysis, the populations of Tunes and Algoz grouped together and Gambelas was separated, which was in agreement with their geographical locations. The proportion of the diversity within populations (86%) was higher than among

populations (14%) and a correlation was found between geographic and genetic distances among populations. The level of gene flow among populations was high and was inversely proportional to the distance between populations. The results obtained in this study will be useful in the implementation and management of different conservation strategies and therefore in the preservation of *P. algarbiensis* genetic resources.

The genetic variation of one population of each *P. algarbiensis* and *P. almogravensis* was assessed using RAPD and inter-simple sequence repeat (ISSR) markers (Ferreira et al. 2013). The two species are morphologically very similar and some authors considered them as a single species (Pedrol 2009). Ferreira et al. (2013) set out to elucidate their taxonomy and contributed with extra information at the molecular level for their conservation, since both species are considered endangered. *P. algarbiensis* revealed higher genetic variability than *P. almogravensis* and both showed a high level of genetic diversity. The cluster analysis revealed two main, clearly separate clusters, which directly corresponded to the plants isolated from each species.

The study on the genetic diversity of *T. major* wild populations was also recently performed (Trindade et al. 2012). Samples were collected from three populations (Tunes, Gambelas and Olhão) and genetic variation within and among populations were evaluated using ISSR markers. The results demonstrated that the population with the highest genetic variability was Olhão and the lowest was Gambelas. In the cluster analysis, Tunes and Olhão populations clustered together, while Gambelas was separated. The three populations revealed a high level of genetic diversity and also a high level of gene flow was observed among populations. The authors concluded that Gambelas population should be a priority for conservation, because it constitutes a distinctive population.

Both *P. algarbiensis* and *T. major* presented high genetic diversity, despite the fact that these are rare species that are generally considered to have low levels of genetic variability (Hamrick and Godt 1989, Gitzendanner and Soltis 2000). Trindade et al. (2012) for *T. major* and Ferreira et al. (2013) for *P. algarbiensis* and *P. almogravensis* proposed that before the destruction of their habitats, these species were more widely distributed and that during their evolutionary process considerable genetic variability has been accumulated. Nowadays, even with major losses of populations and

individuals, a large amount of variability has been well preserved in these small and isolated populations, exhibiting high intraspecific genetic diversity.

The use of molecular markers has proved to be a valuable tool to determine the genetic structure of wild populations from endangered species (for instance, Martín et al. 2008, Trindade et al. 2012, Ferreira et al. 2013). The identification of a species and the knowledge about the extent and distribution of its genetic variation is of prime importance in conservation management. Molecular markers can provide that information and are considered to be the most suitable way for estimating genetic diversity because of their abundant polymorphism and the fact that they are independent of the environment (Samantaray et al. 2010). In the particular case of RAPDs, they are also able to detect polymorphism without any prior knowledge of the genome, only requiring little genomic DNA as template (Harris 1999, Poczai 2013).

## 5.2. General conclusions

In the present work, different conservation strategies based on biotechnological tools were developed to complement and support *in situ* protection and previously developed *ex situ* strategies for three rare and endemic species of the Algarve, *T. lotocephalus*, *P. algarbiensis* and *T. major*. Overall, the main objective of this thesis was accomplished.

Previous to this study, *T. lotocephalus* preservation was dependent only on legal protection. In the course of this work, different *ex situ* conservation approaches were experimented and successfully implemented. An *in vitro* propagation protocol, using seedlings as initial explant, was developed and applied to different genotypes. For the multiplication stage, MS medium presented the best results in the set of the different parameters tested. A rooting stage was necessary to induce root formation and the best medium was ¼MS. Plants were easily acclimatised in a plant growth chamber without visible variations. From the shoots produced *in vitro*, apices were excised and used to test three cryopreservation methods. The outcome was a protocol for the cryopreservation of shoot tips based on the droplet-vitrification technique. Shoot tips 1 mm long were precultured in a sucrose rich medium for one day and dehydrated with PVS2 for 60 min before being plunged into LN. In addition, the genetic stability of the cryopreserved plant material was confirmed using molecular markers. A study on the germination requirements of *T. lotocephalus* seeds was also conducted. The best germinating conditions were set at a germination temperature of 15°C, without any pretreatment. These conditions were then used to germinate cryopreserved seeds that had been directly immersed in LN. Cryopreservation proved to be a suitable method for the conservation of seeds from *T. lotocephalus*.

In the present work, the objectives for *P. algarbiensis* were focused on the cryopreservation of plant germplasm (seeds and nodal segments) since an *in vitro* propagation protocol and the germination requirements were previously established. After testing different methods for the development of a cryopreservation protocol, encapsulation-dehydration and droplet-vitrification presented the highest regrowth percentages. These two methods are therefore the most appropriate for the cryopreservation of nodal segments of this species. The cryopreservation of seeds was also successfully accomplished.

The study for *P. algarbiensis* was completed with the assessment of the genetic diversity of populations. This revealed high levels of genetic diversity within populations and though the number of individuals is scarce, this can be a major advantage in conservation programs.

The goals for *T. major* in this work were directed to the improvement of the germination conditions and cryopreservation of seeds and to the investigation of shoot-tip cryopreservation methods. An *in vitro* propagation protocol was already established and the study of the germination and cryopreservation of seeds was also reported, but with low results. The germination was significantly improved by the use of scarification as pretreatment, which was positively reflected in the germination of the cryopreserved seeds, which was not affected by the low temperatures of the LN. The application of three different methods in the cryopreservation of shoot tips revealed relatively good results, even without major optimizations of the several conditions in the protocols. The method that showed the highest regrowth percentages was encapsulation-dehydration.

Several *ex situ* conservation approaches had now been developed during the course of this thesis that will aid and contribute to the preservation of *T. lotocephalus*, *P. algarbiensis* and *T. major*.

### 5.3. Final considerations

*In situ* protection is often the first step and possibly the most important in the protection of endangered species. The species studied in this work besides being legally protected by Portuguese and European laws, can also be found in National protected areas in the Algarve region. However, the constant pressure of tourism and construction puts at risk the ecological and scientific value of these protected areas, because of economic interests. For instance, at the moment construction is undergoing in some of the places where there are known populations of the species studied. As a result, the official protection given to different habitats and endangered species, including the ones in this work, is frequently nothing more than just a written law. There is a lack of practical and visible measures that will effectively contribute for their conservation. For these reasons, *ex situ* conservation measures are extremely important to aid *in situ* strategies.

In the present work, and previous studies, different *ex situ* conservation strategies were developed. *In vitro* propagation and vegetative explants cryopreservation protocols were developed for the three species; however, improvements can still be performed to optimize the shoot tips cryopreservation protocol of *T. major*. The genetic stability of *T. lotocephalus* cryopreserved shoot tips was assessed and confirmed. In the cases of *P. algarbiensis* and *T. major*, the study on the genetic integrity of cryopreserved material still needs to be performed. The viability of cryopreserved seeds was also established for the three species. Finally, the genetic diversity of wild populations was investigated for *P. algarbiensis* and *T. major*, as for *T. lotocephalus*, it is still a work in progress. For a better understanding, the different approaches developed for the preservation of the three species investigated are summarized in Table 5.1.

The work developed can considerably contribute for the preservation of these three species. However, to fully benefit from it, the measures need to be set into practice. Plant material, in particular seeds, should be collected taking into consideration the results obtained from the evaluation of the genetic diversity of the wild populations. The majority of seeds can then be stored, cryogenically and/or conventionally, while a small fraction should be used to initiate and establish new lines of *in vitro* collections, based on the protocols developed. Ideally, seeds should be collected every year for these purposes. In addition, a constant supervising of the populations should be undertaken.

**Table 5.1.** – Summary of the different conservation approaches based on biotechnological tools developed in the present work, or by other authors, for the preservation of *Thymus lotocephalus*, *Plantago algarbiensis* and *Tuberaria major*.

Species	Conservation approach	Previous work	Chapter
<i>T. lotocephalus</i>	<i>In vitro</i> propagation	---	Chapter 2.1
	Germination requirements	---	Chapter 2.2
	Seed cryopreservation	---	Chapter 2.2
	Shoot tips cryopreservation	---	Chapter 2.3
	Genetic stability	---	Chapter 2.3
	Genetic diversity	Undergoing work	
<i>P. algarbiensis</i>	<i>In vitro</i> propagation	Gonçalves et al. 2009b	---
	Germination requirements	Martins et al. 2013b	---
	Seed cryopreservation	---	Chapter 3.1
	Segments cryopreservation	---	Chapter 3.1
	Genetic stability	Future work	
	Genetic diversity	Ferreira et al. 2013	Chapter 3.2
<i>T. major</i>	<i>In vitro</i> propagation	Gonçalves et al. 2010, Osório et al 2013	---
	Germination requirements	Gonçalves et al. 2009a	Chapter 4.1
	Seed cryopreservation	Gonçalves et al. 2009a	Chapter 4.1
	Shoot tips cryopreservation	---	Chapter 4.1
	Genetic stability	Future work	
	Genetic diversity	Trindade et al. 2013	

A valid conservation strategy should comprise all these measures in agreement with the *in situ* protection. But, the reality is that probably it will be very difficult to maintain this entire course of action due to the lack of funds, manpower and facilities to keep a high number of seeds and *in vitro* collections. It is now up to the official authorities to direct this information and manage the different strategies according to the available resources in order to postpone and, preferably, avoid the extinction of *T. lotocephalus*, *P. algarbiensis* and *T. major*.

#### 5.4. References

- Albert MJ, Iriondo JM and Pérez-García F (2002) Effects of temperature and pretreatments on seed germination of nine semiarid species from NE Spain. *Israel Journal of Plant Sciences* 50: 103-112;
- Benson EE (1999) An Introduction to Plant Conservation Biotechnology. In: Benson EE (ed) *Plant Conservation Biotechnology* (pp 3-10) Taylor & Francis, London;
- Benson EE (2008) Cryopreservation of Phytodiversity: A Critical Appraisal of Theory & Practice. *Critical Reviews in Plant Sciences* 27: 141-219;
- Bewley JD, Bradford KJ, Hilhorst HW and Nonogaki H (2013) Dormancy and the Control of Germination. In: Bewley JD, Bradford KJ, Hilhorst HW and Nonogaki H (eds) *Seeds: Physiology of Development, Germination and Dormancy*, 3<sup>rd</sup> Edition (pp 247-297) Springer, New York;
- Branquinho C, Serrano HC, Pinto MJ, Martins-Loução MA (2007) Revisiting the plant hyperaccumulation criteria to rare plants and earth abundant elements. *Environmental Pollution* 146: 437-443;
- Coelho N, Gonçalves S, González-Benito ME and Romano A (2010) Seed germination of the endangered species *Thymus lotocephalus* G. López & R. Morales (Lamiaceae). XVII Congress of the Federation of European Societies of Plant Biology (FESPB), 4-9 July, Valencia, Spain, pp. 101;
- Costa P, Gonçalves S, Grosso C, Andrade PB, Valentão P, Bernardo-Gil MG and Romano A (2012a) Chemical profiling and biological screening of *Thymus lotocephalus* extracts obtained by supercritical fluid extraction and hydrodistillation. *Industrial Crops and Products* 36: 246-256;
- Costa P, Gonçalves S, Grosso C, Valentão P, Andrade PB, Coelho N and Romano A (2012b) *Thymus lotocephalus* wild plants and *in vitro* cultures produce different profiles of phenolic compounds with antioxidant activity. *Food Chemistry* 135: 1253-1260;
- Debnath M, Malik CP and Bisen PS (2006) Micropropagation: A Tool for the Production of High Quality Plant-based Medicines. *Current Pharmaceutical Biotechnology* 7: 33-49;
- Engelmann F (2011) Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cellular & Developmental Biology – Plant* 47: 5-16;
- Fuller BJ (2004) Cryoprotectants: the essential antifreezes to protect life in the frozen state. *CryoLetters* 25: 375-388;
- Fenner M and Thompson K (2005) *The ecology of seeds*. Cambridge University Press, Cambridge;
- Ferreira V, Matos M, Correia S, Martins N, Gonçalves S, Romano A and Pinto-Carnide O (2013) Genetic diversity of two endemic and endangered *Plantago* species. *Biochemical Systematics and Ecology* 51: 37-44;
- Gitzendanner MA and Soltis PS (2000) Patterns of genetic variation in rare and widespread plant congeners. *American Journal of Botany* 87: 783-792;
- Gonçalves S, Fernandes L, Pérez-García F, González-Benito ME and Romano A (2009a) Germination requirements and cryopreservation tolerance of seeds of the endangered species *Tuberaria major*. *Seed Science & Technology* 37: 480-484;

- Gonçalves S, Martins N and Romano A (2009b) Micropropagation and conservation of endangered species *Plantago algarbiensis* and *P. almogravensis*. *Biologia Plantarum* 53: 774-778;
- Gonçalves S and Romano A (2009) Cryopreservation of seeds from the endangered insectivorous plant *Drosophyllum lusitanicum*. *Seed Science & Technology* 37: 485-490;
- Gonçalves S, Fernandes L and Romano A (2010) High-frequency *in vitro* propagation of the endangered species *Tuberaria major*. *Plant Cell, Tissue and Organ Culture* 101: 359-363;
- González-Benito ME, Clavero-Ramírez I and López-Aranda JM (2004) Review. The use of cryopreservation for germplasm conservation of vegetatively propagated crops. *Spanish Journal of Agricultural Research* 2: 341-351;
- Hamrick JL and Godt MJW (1989) Allozyme diversity in plant species. In: Brown AHD, Clegg MT, Kahler AL and Weir BS (eds) *Plant population genetics, breeding, and genetic resources* (pp 43-63) Sinauer, Sunderland, Massachusetts;
- Harding K (2004) Genetic integrity of cryopreserved plant cells: a review. *CryoLetters* 25: 3-22;
- Harris SA (1999) Molecular Approaches to Assessing Plant Diversity. In: Benson EE (ed) *Plant Conservation Biotechnology* (pp 11-24) Taylor & Francis, London;
- Holsinger KE and Gottlieb LD (1991) Conservation of rare and endangered plants: principles and prospects. In: Falk DA and Holsinger KE (eds) *Genetics and conservation of rare plants* (pp 195-223) Oxford University Press, New York;
- Kadis C and Georghiou K (2010) Seed dispersal and germination behavior of three threatened endemic labiates of Cyprus. *Plant Species Biology* 25: 77-84;
- Leunufna S and Keller ERJ (2003) Investigating a new cryopreservation protocol for yams (*Dioscorea* spp.). *Plant Cell Reports* 21: 1159-1166;
- Martín C, Parra T, Clemente-Muñoz M and Hernandez-Bermejo JE (2008) Genetic diversity and structure of the endangered *Betula pendula* subsp. *fontqueri* populations in the south of Spain. *Silva Fennica* 42: 487-498;
- Martins N, Gonçalves S, Palma T and Romano A (2011) The influence of low pH on *in vitro* growth and biochemical parameters of *Plantago almogravensis* and *P. algarbiensis*. *Plant Cell, Tissue and Organ Culture* 107: 113-121;
- Martins N, Gonçalves S, Palma T and Romano A (2012) Seed germination of two critically endangered plantain species, *Plantago algarbiensis* and *P. almogravensis* (Plantaginaceae). *Seed Science & Technology* 40: 144-149;
- Martins N, Gonçalves S, Andrade PB, Valentão P and Romano A (2013a) Changes on organic acid secretion and accumulation in *Plantago almogravensis* Franco and *Plantago algarbiensis* Samp. under aluminum stress. *Plant Science* 198: 1-6;
- Martins N, Gonçalves S and Romano A (2013b) Aluminum inhibits root growth and induces hydrogen peroxide accumulation in *Plantago algarbiensis* and *P. almogravensis* seedlings. *Protoplasma* 250: 1295-1302;
- Martins N, Gonçalves S and Romano A (2013c) Metabolism and aluminum accumulation in *Plantago almogravensis* and *P. algarbiensis* in response to low pH and aluminum stress. *Biologia Plantarum* 57: 325-331;

- Martins N, Osório ML, Gonçalves S, Osório J, Palma T and Romano A (2013d) Physiological responses of *Plantago algarbiensis* and *P. almogravensis* shoots and plantlets to low pH and aluminum stress. *Acta Physiologiae Plantarum* 35: 615-625;
- Martins N, Osório ML, Gonçalves S, Osório J and Romano A (2013e) Differences in Al tolerance between *Plantago algarbiensis* and *P. almogravensis* reflect their ability to respond to oxidative stress. *Biometals* 26: 427-437;
- Maxted N, Ford-Lloyd BV and Hawkes JG (1997) Complementary conservation strategies. In: Maxted N, Ford-Lloyd BV and Hawkes JG (eds) *Plant Genetic Conservation: The In Situ Approach* (pp 15-40) Chapman and Hall, London;
- Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497;
- Osório ML, Gonçalves S, Coelho N, Osório J and A Romano (2013) Morphological, physiological and oxidative stress markers during acclimatization and field transfer of micropropagated *Tuberaria major* plants. *Plant Cell, Tissue and Organ Culture* 115: 85-97;
- Ozudogru EA and Kaya E (2012) Cryopreservation of *Thymus cariensis* and *T. vulgaris* shoot tips: comparison of three vitrification-based methods. *CryoLetters* 33:363-375;
- Panis B, Piette B and Swennen R (2005) Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all *Musaceae*. *Plant Science* 168: 45-55;
- Pedrol J (2009) *Plantago* L. In: Benedí González C and Rico E (eds) *Flora Ibérica – Plantas vasculares de la Península Iberica e Islas Baleares* (pp. 4–38) Real Jardín Botánico, CSIC, Madrid;
- Pence VC and Clark JR (2005) Desiccation, cryopreservation and germination of seeds of the rare wetland species, *Plantago cordata* Lam. *Seed Science and Technology* 33: 767-770;
- Pérez-García F, Hornero J and González-Benito ME (2003) Interpopulation variation in seed germination of five Mediterranean Labiatae shrubby species. *Israel Journal of Plant Sciences* 51: 117-124;
- Pérez-García F and González-Benito ME (2005) Effects of temperature and different pre-treatments on seed germination of four *Halimium* species. *Seed Science Technology* 33: 505-509;
- Pérez-García F and González-Benito ME (2006) Seed germination of five *Helianthemum* species: Effect of temperature and pre-sowing treatments. *Journal Arid Environments* 65: 688-693;
- Poczai P, Varga I, Laos M, Cseh A, Bell N, Valkonen JPT and Hyvönen J (2013) Advances in plant gene-targeted and functional markers: a review. *Plant Methods* 9: 6;
- Ramanatha Rao V and Hodgkin T (2002) Genetic diversity and conservation and utilization of plant genetic resources. *Plant Cell, Tissue and Organ Culture* 68: 1-19;
- Reed BM and Uchendu E (2008) Controlled Rate Cooling. In: Reed BM (ed) *Plant Cryopreservation: A Practical Guide* (pp 77-92) Springer, New York;
- Reed BM, Sarasan V, Kane M, Bunn E and Pence VC (2011) Biodiversity conservation and conservation biotechnology tools. In *Vitro Cellular & Developmental Biology – Plant* 47: 1-4;

- Sakai A and Engelmann F (2007) Vitrification, encapsulation-vitrification and droplet-vitrification: a review. *CryoLetters* 28: 151-172;
- Samantaray S, Dhagat UM and Maiti S (2010) Evaluation of genetic relationships in *Plantago* species using Random Amplified Polymorphic DNA (RAPD) markers. *Plant Biotechnology* 27: 297-303;
- Sarasan V, Cripps R, Ramsay MM, Atherton C, McMichen M, Prendergast G and Rowntree JK (2006) Conservation *in vitro* of threatened plants - progress in the past decade. *In Vitro Cellular and Developmental Biology - Plant* 42: 206-214;
- Trindade H, Sena I, Gonçalves S and Romano A (2012) Genetic diversity of wild populations of *Tuberaria major* (Cistaceae), an endangered species endemic to the Algarve region (Portugal), using ISSR markers. *Biochemical Systematics and Ecology* 45: 49-56;
- Walters C, Wheeler L and Stanwood PC (2004) Longevity of cryogenically stored seeds. *Criobiology* 48: 229-244;
- Wang Q, Li P, Batuman O, Gafny R and Mawassi M (2003) Effect of benzyladenine on recovery of cryopreserved shoot tips of grapevine and citrus cultured *in vitro*. *CryoLetters* 24: 293-302;
- Zaidi CA, González-Benito ME and Pérez-García F (2010) Morphology, germination and cryopreservation of seeds of the Mediterranean annual plant *Tuberaria macrosepala* (Cistaceae). *Plant Species Biology* 25: 149-157.

