Universidade do Algarve
Faculdade de Engenharia dos Recurso Naturais

In vitro propagation of insectivorous plants for phytochemical purposes

Natacha Coelho

Mestrado Integrado em Engenharia Biológica

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2009

This is a master thesis project work and the student is responsible for the results and discussions in the report.

Declaração

Na presente dissertação apresentam-se resultados que foram, ou serão, alvo de publicação em colaboração com A Romano, S Gonçalves, T Grevenstuk.

A autora declara que interveio na concepção e execução do trabalho experimental, na interpretação dos resultados e na redacção dos manuscritos enviados para publicação.

Faro, Fevereiro 2009

Natacha Rodrigues Coelho

Abstract

The aims of this research work were to establish *in vitro* propagation protocols for the insectivorous plants *D. intermedia, D. rotundifolia* and *P. vulgaris*, using seeds as explants, in order to produce plants for biocompound extraction and to replenish natural populations. The antioxidant capacity of the extracts prepared from *in vitro* cultures of these species was evaluated.

The establishment of a simple protocol for *in vitro* propagation of *D. intermedia* is reported. Protocols for micropropagation of *P. vulgaris* and *D. rotundifolia* were initiated but still need to be improved. Cultures of all three species were initiated from shoots of *in vitro* produced seedlings. The effect of three MS medium concentrations, ¼MS, ½MS and MS, without PGR were tested in *D. intermedia* and *P. vulgaris*. The same media supplemented with 0.1 mg l^{-1} KIN were investigated in *D. intermedia* and ¼MS supplemented with 0.5 mg l^{-1} KIN or ZEA were investigated in *P. vulgaris*. In all media *D. intermedia* shoots rooted during the proliferation phase, presenting a multiplication and rooting percentage above 90%. The highest mean number of shoots was attained in ¼MS and ¼MS supplemented with 0.1 mg l^{-1} KIN. All the *D. intermedia* plants were successfully acclimatized to *ex vitro* conditions. The formation of roots also occurred during *P. vulgaris* proliferation phase, though with poorer results for both proliferation and rooting. Nevertheless, the best results were obtained in the media supplemented with cytokinins. In *D. rotundifolia* the media tested were ¼MS and MS. Multiplication percentage and mean number of shoots was higher in ¼MS medium and no rooting was observed.

Antioxidant activity of *D. intermedia*, *D. rotundifolia* and *P. lusitanica* extracts was measured using three different methods: ORAC, F-C phenolics and TEAC. Methanol and standard maceration improved the efficiency of the antioxidant extraction procedure. For all tested methods *P. lusitanica* presented the highest antioxidant activity, representing a possible good source of antioxidants.

Keywords: Insectivorous plants, *Drosera*, *Pinguicula*, micropropagation, antioxidants.

Resumo

As plantas insectívoras possuem a capacidade única de capturar e digerir artrópodes. Em Portugal, existem algumas espécies destas plantas. Porém, encontram-se ameaçadas e em perigo de extinção como resultado da destruição dos seus habitats naturais e da colheita excessiva devido às suas propriedades medicinais. Neste projecto, as plantas estudadas pertencem aos géneros *Drosera* (*Drosera intermedia* e *D. rotundifolia*) e *Pinguicula* (*Pinguicula vulgaris* e *P. lusitanica*). De forma a evitar a colheita de plantas ameaçadas do seu ambiente natural, pode-se recorrer à propagação *in vitro*, na qual a partir de pequenas quantidades de tecido é possível obter grandes quantidades de material vegetal de uma forma rápida. Consequentemente, o material vegetal obtido por micropropagação poderá ser usado para realizar estudos biológicos, extrair compostos e posteriormente para renovar as populações naturais de tais espécies. Estabelecer protocolos para a micropropagação de *D. intermedia*, *D. rotundifolia* e *P. vulgaris* era um dos objectivos deste estudo. A propagação *in vitro* de *P. lusitanica* já havia sido anteriormente estabelecida.

Neste relatório, descreve-se um protocolo simples de propagação *in vitro* de *D. intermedia*. Os protocolos para *D. rotundifolia* e *P. vulgaris* necessitam de alguns ajustes para que o processo fique completo. As culturas das três espécies foram iniciadas a partir de rebentos provenientes de germinantes produzidos *in vitro*. Obtiveram-se elevadas percentagens de germinação das sementes de *D. intermedia*, sendo o ensaio controlo melhor que o tratamento no frio por uma semana. Já as percentagens de germinação de sementes de *P. vulgaris* foram muito baixas para ambos os tratamentos.

O efeito da concentração do meio MS sem reguladores de crescimento foi testado em todas as espécies. Em *D. intermedia* foram testados meios com as concentrações ½ e ¼ de MS para além de MS completo e os mesmos meios suplementados com 0.1 mg l^{-1} KIN. A proliferação foi elevada em todos os meios, obtendo-se percentagens de multiplicação acima dos 90%. O número médio de rebentos foi superior em ¼MS e ¼MS suplementado com 0.1 mg l^{-1} KIN. Durante a fase de proliferação observou-se a formação de raízes com percentagens de enraizamento na ordem dos 100% em todos os meios. O número de raízes foi maior

nos meios ¼MS e ¼ e ½MS suplementados com KIN, e as raízes mais longas foram observadas em ½MS. Curiosamente, ocorreu enraizamento dos novos rebentos produzidos com percentagens de enraizamento elevadas, entre 77 e 93%. Tendo em conta os elevados resultados obtidos para o enraizamento durante a fase de proliferação, concluiu-se que uma fase posterior de enraizamento seria desnecessária. Durante a fase de aclimatização todas as plântulas foram aclimatizadas com sucesso às condições *ex vitro*.

Em *D. rotundifolia* foram testados os meios MS e ¼MS. A percentagem de multiplicação e número médio de rebentos foi superior em meio ¼MS e não se observou a formação de raízes. Os rebentos obtidos eram muito pequenos, devendo ser considerada uma fase de elongação e uma fase de indução de raízes para esta espécie de forma a obter rebentos mais longos e enraizados.

Em *P. vulgaris* foram testados os meios com as mesmas concentrações de MS que em *D. intermedia* (¼ e ½ de MS e MS completo), para além do meio ¼MS suplementado com 0.5 mg l^{-1} KIN ou 0.5 mg l^{-1} ZEA. A proliferação de rebentos não foi muito elevada em qualquer dos meios testados, porém, tal como em *D. intermedia*, também ocorreu enraizamento durante esta fase em todos os meios. Devido à limitação de material vegetal, inicialmente, testaram-se apenas os meios sem citocininas. Os valores mais elevados tanto para a percentagem de multiplicação e número médio de rebentos como para a percentagem de enraizamento, número de raízes e comprimento foram obtidos em meio ¼MS. Por esta razão, este meio foi suplementado com citocininas com as concentrações anteriormente referidas. Os resultados, embora superiores aos anteriores, não foram significativamente diferentes. O meio em que se obteve melhores resultados de percentagem de multiplicação e número médio de rebentos foi o ¼MS suplementado com 0.5 mg l^{-1} ZEA. Os resultados de enraizamento foram superiores em meio ¼MS suplementado com 0.5 mg l^{-1} KIN. Para se obter um protocolo eficiente para a micropropagação de *P. vulgaris* e plantas aclimatizadas às condições *ex vitro* terão que ser melhoradas várias condições.

Neste projecto também foi avaliada a capacidade antioxidante de extractos preparados a partir de culturas *in vitro* de *D. intermedia*, *D. rotundifolia* e *P. lusitanica*. Os antioxidantes são substâncias orgânicas, algumas das quais

enzimas, capazes de neutralizar o efeito nocivo de radicais livres e espécies de oxigénio reactivas. Crê-se que a ingestão de alimentos ricos em antioxidantes, como frutas e vegetais, diminui o risco de doenças relacionadas com o stress oxidativo, tais como cancro, doenças cardiovasculares, entre outras. Muitos métodos têm sido desenvolvidos para avaliar a actividade antioxidante, porém nenhum deles reflecte efectivamente a actividade antioxidante total de uma amostra. É também importante que seja desenvolvido um método uniforme, que até à data não existe, para se poderem comparar resultados entre produtos botânicos, alimentares ou outros. A actividade antioxidante destas espécies foi avaliada usando três métodos: ORAC, F-C phenolics e TEAC. Os resultados obtidos foram consistentes em todos os métodos, embora com uma amplitude de valores diferente, provavelmente devido aos diferentes mecanismos de reacção que cada método segue. Para todos os métodos testados o extracto de *P. lusitanica* apresentou a actividade antioxidante mais elevada das três espécies, representando uma possível fonte de antioxidantes. Diferentes solventes de extracção foram testados (metanol, água e hexano). O extracto em que se obteve uma capacidade antioxidante mais elevada foi o de metanol e a mais baixa foi o de hexano. Também foi testado o tipo de maceração, maceração "standard" e maceração com azoto líquido. O primeiro tipo melhorou a eficiência do procedimento de extracção de antioxidantes. De futuro, é importante identificar os compostos responsáveis pela actividade antioxidante nestas plantas.

Um importante objectivo futuro será o reforço das populações naturais através da reintrodução das plantas micropropagadas após aclimatização no seu habitat natural reduzindo o risco de extinção de espécies ou populações ameaçadas.

Palavras-chave: Plantas insectívoras, *Drosera*, *Pinguicula*, micropropagação, antioxidantes.

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Abbreviations

AAPH – 2,2'-azobis(2- amidinopropane) dihydrochloride;

AOC – Antioxidant capacity;

AUC – Area under the curve;

ET – Electron transfer;

Ext – Extract;

F-C – Folin-Ciocalteu;

GAE – Gallic acid equivalents;

HAT – Hydrogen atom transfer;

ICN – Instituto da Conservação da Natureza;

KIN – Kinetin;

MS – Murashige and Skoog's (1962) medium;

ORAC – Oxygen radical absorbance capacity;

PGR – Plant growth regulator;

ROS – Reactive oxygen species;

TE – Trolox equivalents;

TEAC – Trolox equivalent antioxidant capacity;

ZEA – Zeatin;

½MS – MS medium with ½ concentration of MS macronutrients;

¼MS – MS medium with ¼ concentration of MS macronutrients.

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1. Introduction

1.1 Insectivorous plants and their importance

Insectivorous plants have developed a highly specialized way of taking up nutrients. Such plants are able to catch or trap preys, mostly arthropods, absorb metabolites from them and utilize these metabolites in their growth and development (Aldenius et al. 1983). Insectivorous plants usually grow in moist habitats, poor in nutrients such as nitrogen and phosphorous (Aldenius et al. 1983; Juniper et al. 1989; Okabea et al. 2005). The limitations of low soil nutrient resource resulted in the adaptation of these plants to capture insects providing an alternative nutrient source (Stewart & Nilsen 1993; Millett et al. 2003).

All insectivorous plants share common characteristics; they have smaller root systems, lower photosynthetic capacity, lower photosynthetic nitrogen use efficiency and weaker competitive ability than non-insectivorous plants (Aldenius et al. 1983; Thorén et al. 2003).

To attract preys the plants secret nectar or exhale a sweet odor or exhibit bright colors (Adlassnig et al. 2005). Then the highly specialized leaves of these plants will work as traps; once the insects lay on the plant leaves, they are captured and unable to escape (Millett et al. 2003; Adlassnig et al. 2005; Wolf et al. 2006). Different insectivorous plants retain their prey in different ways and five types of trapping mechanisms can be distinguished: adhesive (or “fly-paper”) traps use a sticky mucilage; snap traps utilize rapid leaf movements; pitcher (or pitfall) traps trap the prey in a rolled leaf that contains a pool of digestive enzymes or bacteria; eel (or “lobster pot”) traps force prey to move towards a digestive organ with inward pointing hairs; and suction traps suck in prey with a bladder that generates an internal vacuum (Adlassnig et al. 2005; Wolf et al. 2006). The plants studied in this report have all a “fly-paper” type of trapping mechanism.

During the process of prey digestion, a digestive fluid is secreted by these plants. Only in the “flypaper” plants is the fluid also involved in insect capture in addition to its digestive role (Gaume & Forterre 2007). The nutrients are absorbed by tentacles, glands or tiny hairs on the surface of leaves (Quintanilha 1926).

According to Juniper et al. (1989) the insectivorous plant group consists of nine families, seventeen genera and about 550-600 species. A considerable number of these

plants are included in the pharmacopeias. The compounds with pharmacological activity mainly found in insectivorous plants are the flavonoids quercetin, hyperoside and isoquercetin. The naphtoquinones plumbagin and 7-methyljuglone can be found in some *Drosera* species (Budzianowsky 1996; Hook 2001; Melzig et al. 2001; Marczak et al. 2005; Paper et al. 2005; Grevenstuk et al. 2008a). The latest compounds are of extreme importance due to their broad-range of biological activities: phytotoxic, insecticidal, antibacterial and fungicidal (Babula et al. 2006). Moreover, plants are typically good sources of antioxidants and insectivorous plants are possibly not an exception, though the study of their antioxidant content has not hitherto been reported.

However the increasing interest in the medicinal potential of insectivorous plants along with collectors' curiosity and destruction of their habitats resulted in overharvesting from their natural sites. Some species are considered threatened or endangered (ICN 2000). As an alternative, *in vitro* propagation may be used to obtain plant material rapidly from a small amount of tissue, avoiding collection of plants from their natural environment (Anthony 1992; Hook 2001). A number of insectivorous plants have already been successfully propagated *in vitro*, such as *Dionea muscipula* (Jang et al. 2003), *Drosera peltata* (Kim & Jang 2004), *Drosophyllum lusitanicum* (Gonçalves & Romano 2005), *Drosera indica* (Jayaram & Prasad 2006), *Pinguicula lusitanica* (Gonçalves et al. 2008), among others.

1.1.1 *Drosera*

The genus *Drosera*, family Droseraceae, is a large group of insectivorous plants comprising about 90 species, spread world wide, although the biggest group can be found in Australia (Heslop-Harrison 1976; Marckzak et al. 2005). In Europe, natural populations of *Drosera* species are becoming increasingly scarce, due to uncontrolled collections and to its medicinal properties. Plants belonging to this genus have been commonly used in the treatment of convulsive or whooping cough for many years and are the source for the drug Droserae Herba. (Melzig et al. 2001; Kawiak et al. 2003; Paper et al. 2005). Leaves are covered with stalked mucilagenous glands (pedunculated glands) which secret a fluid in the form of drops of sticky mucilage rich in carbohydrates, where insects are lured and adhere, most of the time irremediably (Gaume & Forterre 2007). Subsequently sessile glands, also on leaves, produce enzymes which will digest the prey. Such enzymes enable the plant to decompose the

insect body and to assimilate nitrogen, phosphorus and sulphur (Matusíková et al. 2005).

In Portugal there are two known *Drosera* species: *Drosera intermedia* Hayne and *Drosera rotundifolia* Linneaus. *D. intermedia* (Figure 1.1), commonly known as Spoonleaf Sundew, lives in shallow waters and bogs and it is also found in eastern Canada, United States, West Indies and South America. Spoonleaf Sundew is a small perennial herb which forms a semi-erect stemless rosette of spatulate leaves. White flowers bloom on one side of a leafless stalk curved at the base, arising from beneath the leaves. During winter, in temperate regions, these plants undergo dormancy forming a winter bud, or hibernaculum, which is a mass of small leaves that protect the apex from desiccation. The fruit is a dry capsule containing several seeds up to 1 mm long.

Figure 1.1 – *D. intermedia* plant.

D. rotundifolia (Figure 1.2), Common Sundew or round-leaved sundew, is one of the most widespread *Drosera* species and it grows in Europe, North America and Asia. *D. rotundifolia* grows mostly on acidic soils or substrates that are often deficient in nitrogen and phosphorus (Matusíková et al. 2005). It is a small perennial herb with round leaves formed in a basal rosette and lying flat on the ground. Flowers, white or pink, grow on one side of a leafless stalk that comes up from the centre of the leaf rosette. As *D. intermedia*, Common Sundew also forms a hibernaculum during the winter to be protected from the cold. Seeds of about 1 mm long are enclosed in a capsule (fruit). This plant can be found in open bogs.

In vitro propagation of *D. rotundifolia* from leaf explants has been previously accomplished (Anthony 1992; Bobák et al. 1995). Budzianowsky (1996) produced

D. intermedia plants by *in vitro* propagation to obtain isolated naphtoquinones, however the micropropagation protocol was not described by the author.

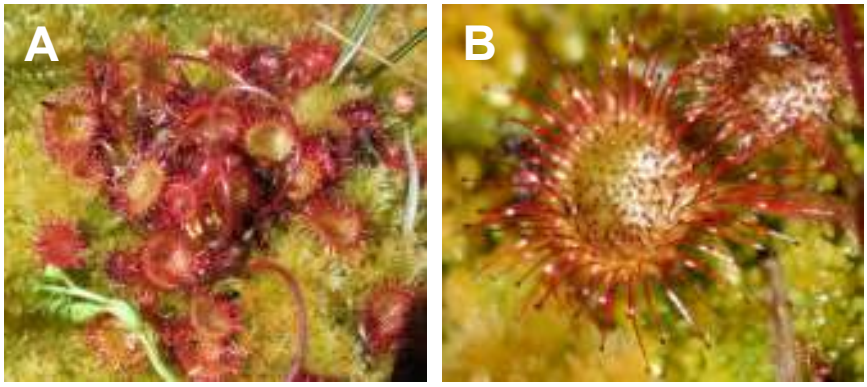


Figure 1.2 – *D. rotundifolia* plants (A) and leaf detail (B).

1.1.2 *Pinguicula*

The genus *Pinguicula*, commonly known as Butterworts, family Lentibulariaceae, consists of about 50 species (Casper 1966) which are present on all continents except Australia. In Europe there are 12 species and nine of those occur on the Iberian Peninsula, of which five are endemics (Heslop-Harrison 2004). *Pinguicula* are herbaceous plants, relatively short-lived perennials which form a rosette during their active growth and usually live in wet and relatively open sites. Their roots are fibrous, tufted and often ephemeral. The leaves lie close to the ground and, as the plants from the *Drosera* genus, are adapted to insect capture (Heslop-Harrison 2004). Two types of glands can be found on the upper surfaces of *Pinguicula*'s leaves, stalked and sessile. The stalked glands carry permanent mucilaginous droplets, causing the shiny appearance of the leaves that led to the common name ‘‘butterwort”. Insects are caught and digested by the gland secretions and then absorb by the same glands (Heslop-Harrison 2004; Cieslak et al. 2005). Many of the species reproduce vegetatively by means of bulbils and/or epiphyllous buds which later take roots (Heslop-Harrison 2004). Extracts from several species of *Pinguicula* are currently used in pharmaceutical formulations as cough suppressant and mucolytic.

Pinguicula vulgaris and *P. lusitanica* are two *Pinguicula* species present in Portugal. *P. vulgaris* Linneaus (Figure 1.3), Common Butterwort, is a small perennial herb with weakly developed roots and a basal rosette of 4-7 light green to yellow green leaves close to the ground, during the summer, and resting as a hibernaculum during the

winter (Aldenius et al. 1983; Heslop-Harrison 2004) . The funnel shaped flowers are normally lavender-purple and occasionally white. This plant grows in bogs and swamps, in low or subalpine elevations and it can be found in Europe, Northwest Asia and North America.

Figure 1.3 – *P. vulgaris* plants (A) and flower detail (B).

P. lusitanica Linneaus (Figure 1.4), common name Pale Butterwort, grows wild in areas along coastal Western Europe from Scotland to Iberia and Morocco in north-western Africa. It is a perennial plant consisting of a rosette of 5-12 oval leaves, lying close to the ground and contrary to *P. vulgaris*, overwinters as a rosette. Its pale mauve flowers with a yellow throat are held on a leafless stem. Normally it grows in bogs and wet heaths.

Figure 1.4 - *P. lusitanica* plant (A) and flower detail (B).

In vitro propagation of *P. lusitanica* from seeds has been recently reported by Gonçalves et al. (2008). To date, only one other member of the *Pinguicula* genus has been cultured *in vitro*, namely *P. moranensis* starting from leaf explants (Adams et al. 1979).

1.2 Micropropagation

The development of vegetative propagation techniques emerged from the necessity to produce plants genetically equal to mother plant in order to retain its more favourable characteristics, avoiding the gametes genetic recombination and therefore the phenotypic diversity of the plant. These techniques are based on the totipotency theory, which states the capacity of a single cell to regenerate the phenotype and genotype of the original organism and originate a new plant.

Along the centuries several vegetative propagation techniques have been created. However, not every plant species responds positively to the conventional methods and an *in vitro* technique was developed. Micropropagation can be defined as the *in vitro* propagation of plants from cells or organs in a controlled environment, through the employ of aseptic techniques and the use of proper containers, and in a defined medium.

There are three different micropropagation techniques, meristem culture, organogenesis and somatic embryogenesis, though the first one is the technique that expresses best the cells regeneration capacity, given that the meristems have non-differentiated cells which are more reactive.

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

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

Micropropagation begins with the selection and preparation of plant material to be propagated – stage 0. Mother plant or its parts are raised under more hygienic conditions, by the use of sterilization agents and growing it in greenhouse, to fight possible contaminations before initiation of culture. During this phase it is also

important to manipulate certain parameters to make an explant more suitable or more reliable as starting material. Temperature, light, photoperiod and growth regulators are controlled to obtain explants more reactive to *in vitro* growth (Debergh & Zimmerman 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 & Zimmerman 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. 1990). The most often used growth regulators in this phase are cytokinins, as they promote the formation and development of axillary shoots. Metabolism of phenolic compounds might be stimulated at this time in the explants due to stress of being isolated from mother plant and to adaptation to the new conditions. As a result these products provoke blackening of the explants and medium, leading to a growth inhibition of the explants and consequently their death (Debergh & Zimmerman 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. 1990).

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. 1990). 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 (Hartmann et al. 1990; Debergh & Zimmerman 1991). Multiplication may be repeated several times to increase the supply of material to a predetermined level for subsequent rooting and transplanting (Hartmann et al. 1990). 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 & Zimmerman 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. 1990). There is an increasing interest for rooting *ex vitro* due to its many advantages, essentially its simplicity and lower costs (Debergh & Zimmerman 1991). This procedure consists in the direct rooting of the plantlet in the acclimatization substrate, after dipping the shoot's base into a rich auxin solution.

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. 1990).

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 (Hartmann et al. 1990). 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 (Hartmann et al. 1990). 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. 1990). The use of specific treatments, like stratification, temperature or chemical treatment, will break this state and consequently germination takes place.

Micropropagation is a fast process; from a single explant it is possible to obtain thousands of plants in a short period of time. In the present study this feature is extremely important, avoiding the collection of endangered plants from their natural environment for biological activities tests and allowing to replenishing natural populations. Plants free from contaminations, the possibility to propagate species impossible to clone *in vivo* and the use of a reduced storing space are other of the advantages of micropropagation.

1.3 Antioxidant capacity (AOC)

Antioxidants are organic substances, some of them enzymes, which are capable of counteracting the damaging effects of free radicals and reactive oxygen species (ROS). These forms are highly reactive and unstable, resulting from metabolism of living organisms. Normally, their concentration in the organism is low and controllable, though almost all adverse environmental conditions, such as UV radiation, pollution, drought, heat stress, may increase their production (Huang et al. 2005; Gillespie et al. 2007). High concentrations can result in unrestricted oxidation of DNA, proteins and membrane lipids, which will lead to oxidative destruction of the cell (Gillespie et al. 2007).

In food industry antioxidants are very important since they include components that prevent fats in food from becoming rancid (Huang et al. 2005). The formation of toxic products in foods not only decreases their quality as well lipid oxidation is associated with the occurrence of oxidative stress related diseases, including inflammation, cardiovascular disease, cancer, and aging-related disorders. The ingestion of fruits and vegetables showed an inverse correlation with these diseases, possibly due to their high content of antioxidants (Fukumoto & Mazza 2000; Huang et al. 2005). Hence it is of great importance the study of the antioxidant capacity (AOC) not only in the foods we consume, as well in other biological samples in order to isolate and use the compounds related to this activity for the prevention and treatment of such diseases (Middleton et al. 2000; Huang et al. 2005).

Various methods to measure antioxidant activity have been developed among the years. However, none of the methods accurately reflects the "total antioxidant activity" of a sample, because there are diverse antioxidants and free radical and oxidant sources with different chemical and physical characteristics involved in a complex system. It is of extreme importance to obtain consistent results for comparisons within botanical, food or other commercial products, but due to these difficulties a standardized AOC method as not yet been developed. The "total antioxidant capacity" needs to reflect both lipophilic and hydrophilic capacity, and at least for physiological activity it needs to reflect and differentiate both hydrogen atom transfer (radical quenching) and electron transfer (radical reduction) (Prior et al. 2005). Taking into consideration these issues, Prior et al. (2005) proposed three methods that should be standardized for use in the routine quality control and measurement of AOC of dietary supplements and other

botanicals: oxygen radical absorbance capacity (ORAC), Folin-Ciocalteu (F-C) phenolics and trolox equivalent antioxidant capacity (TEAC) (Prior et al. 2005). These will also be the methods used in the present study to assess the AOC of the plant extracts.

Depending upon the chemical reactions involved, major antioxidant capacity assays can be classified into two types: assays based on hydrogen atom transfer (HAT) reactions and assays based on single electron transfer (ET) reactions (Huang et al. 2005). HAT-based assays measure the classical ability of an antioxidant to quench free radicals by hydrogen donation. They are generally composed of a synthetic free radical generator, an oxidizable molecular probe, and an antioxidant. ET-based assays detect the ability of potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls and radicals. The compounds when reduced will change colour and the degree of colour change is correlated with the sample's antioxidant concentrations. Both are intended to measure the radical (or oxidant) scavenging capacity, instead of the preventive antioxidant capacity of a sample. ORAC assay follows the HAT mechanism while the other two follow the ET mechanism (Huang et al. 2005, Prior et al. 2005).

1.3.1 ORAC

In the ORAC assay the free radical damages a fluorescent probe by changing its fluorescence intensity. Therefore, the change of fluorescence intensity indicates the level of destruction by the free radical. When an antioxidant is present, the action of the free radical over the fluorescent probe is inhibited, which corresponds to the AOC of the antioxidant against the free radical (Figure 1.5) (Huang et al. 2002). This reaction is driven to completion and the effect of the antioxidant is measured by assessing the area under the fluorescence decay curve (AUC) of the sample as compared to that of the blank in which no antioxidant is present (AUCsample - AUCblank) (Ou et al. 2001; Huang et al. 2002). A standard curve is then obtained by plotting the AUC and the concentration of trolox (standard antioxidant) and used to calculate the trolox equivalents of a sample (Huang et al. 2005). Due to the AUC technique, the ORAC assay is, until now, the only method that combines both inhibition time and inhibition degree of the free radical damage by the antioxidant into a single quantity (Huang et al. 2002).

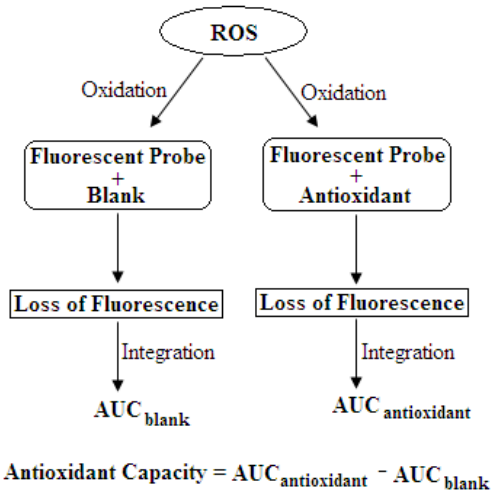


Figure 1.5 – Schematic illustration of the principle of the ORAC assay, adapted from Huang et al. (2002).

In practise, ORAC measures the antioxidant activity against peroxyl radical induced by AAPH [2,2'-azobis(2- amidinopropane) dihydrochloride] at 37ºC, using fluorescein as fluorescent probe (Ou et al. 2001) (Figure 1.6).

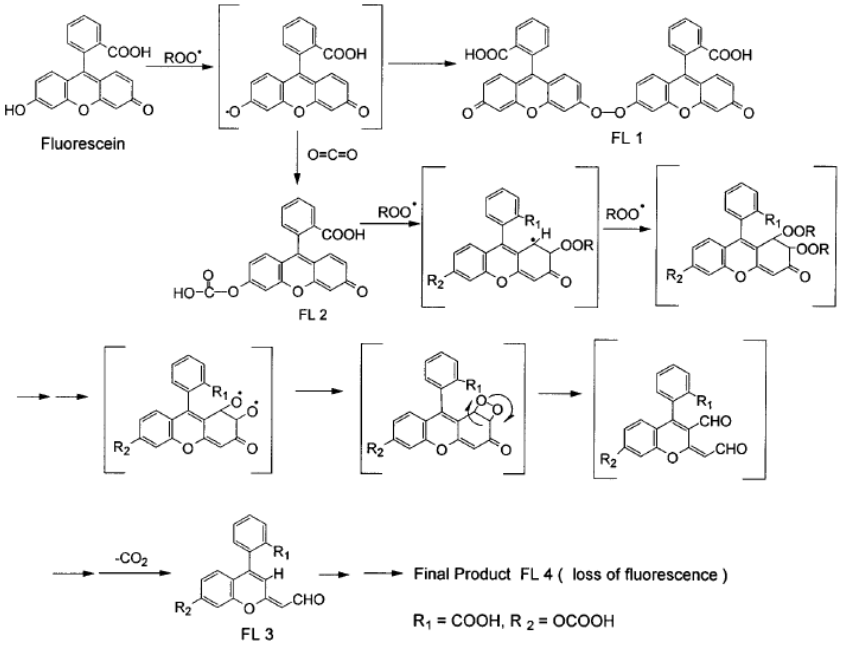


Figure 1.6 – Proposed fluorescein oxidation pathway in the presence of AAPH (Ou et al. 2001).

The ORAC assay has been widely used in the nutritional field, but only recently a protocol was developed for the measurement of AOC in plants (Gillespie et al. 2007).

1.3.2 F-C phenolics assay

The F-C phenolics assay, improved by Singleton & Rossi (1965), is based on the reaction of phenolic compounds with a colorimetric reagent, which allows measurement in the visible portion of the spectrum (Ainsworth & Gillespie 2007). It has been proposed as a standardized method for use in the routine quality control and measurement of antioxidant capacity of food products and dietary supplements (Prior et al. 2005). The F-C reagent (Folin & Ciocalteu 1927) consists of a mixture of phosphomolybdic and phosphotungstic acids (Ikawa et al. 2003). In highly basic medium, phenolic compounds are oxidized resulting in formation of O_2^-, and electrons are transferred to the phosphomolybdic and phosphotungstic acid complexes to form blue complexes that are determined spectroscopically at approximately 760 nm (Ainsworth & Gillespie 2007; Roginsky et al. 2005). Total phenolics is then frequently calculated as gallic acid equivalents using the regression equation between gallic acid standards and the absorbance measured (Roginsky & Lissi 2005; Ainsworth & Gillespie 2007).

Because the oxidation/reduction reaction mechanism of the F-C phenolics assay is non-specific, other oxidation substrates besides phenols may interfere (Prior et al. 2005; Ainsworth & Gillespie 2007). Therefore, F-C phenolics assay not only measures total phenols in a certain sample but also other oxidation substrates. Despite this concern, the F-C assay is a convenient, simple and reproducible method that has been commonly used for many years to measure the total phenolics content (Huang et al. 2005; Ainsworth & Gillespie 2007). Recently a specific protocol for plant tissues has been developed by Ainsworth & Gillespie (2007).

1.3.3 TEAC

The TEAC assay is based in the generation of the ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation ($ABTS^{\bullet+}$) and its decay caused by the addition of a antioxidant-containing sample (Figure 1.7). $ABTS^{\bullet+}$ has a strong absorption in the range of 600–750 nm and can be easily determined spectrophotometrically (Re et al. 1999; Roginsky & Lissi 2005).

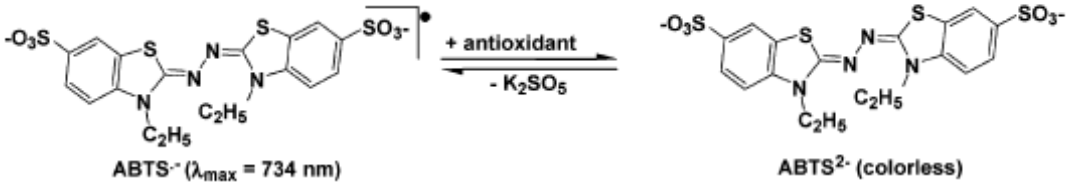


Figure 1.7 – Reaction of ABTS in the presence of an antioxidant.

This method was initially described by Miller et al. (1993) and afterwards improved by Re et al. (1998). In the original TEAC assay, there is an activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation, in the presence or absence of antioxidants (Miller et al. 1993). Since an intermediate is formed, the faster reacting antioxidants may also contribute to the reduction of the metmyoglobin, overestimating the results. The improved method, based on a decolorization technique, generates directly the radical in a stable form before the reaction with antioxidants avoiding the involvement of an intermediary radical. Thus the blue/green $ABTS^{\bullet+}$ is generated by oxidation of ABTS with potassium persulfate and is then reduced in the presence of hydrogen-donating antioxidants. The level of decolorization corresponds to the percentage inhibition of $ABTS^{\bullet+}$ and is determined as a function of concentration and calculated relative to the reactivity of trolox as a standard, under the same conditions (Re et al. 1999).

This method is relative simple allowing its application for routine determinations in any laboratory and is applicable to the study of both lipophilic and hydrophilic antioxidants, pure compounds and food extracts (Re et al. 1999; Roginsky & Lissi 2005).

1.4 Aim

In this research work the species studied are threatened or endangered and to minimize collection from their natural populations *in vitro* propagation is indicated because many plants may be rapidly produced from a small amount of tissue. As a result several biological studies concerning these plants can be performed using the *in vitro* produced material that in other way would not be possible to carry out. A number of insectivorous plants have pharmacological and medical interest. Some of their

compounds have been identified and the biological activity of several of these plant extracts has also been evaluated. However there are no studies regarding the antioxidant activity of insectivorous plants.

Considering all these subjects, the aims of this research work were:

- to establish an *in vitro* propagation protocol for the insectivorous plants *D. intermedia, D. rotundifolia* and *P. vulgaris*, using seeds as explants;
- to evaluate the antioxidant capacity of extracts prepared from *in vitro* cultures of *D. intermedia, D. rotundifolia* and *P. lusitanica*;
- to contribute to the conservation of these species.

2. Materials and methods

2.1 *In vitro* propagation

2.1.1 *Drosera intermedia*

D. intermedia seeds were collected from plants growing in a natural population near the Sado estuary (Portugal) in July 2007 by T. Grevenstuk and S. Gonçalves. Seeds were surface sterilized with 20% (v/v) commercial bleach (5% sodium hypochlorite) and a few drops of Tween-20 for 20 min and rinsed 3 times in sterile water. Afterwards, part of the seeds was aseptically inoculated in test tubes containing 10 ml of plant growth regulator (PGR) free ¼MS medium (Murashige and Skoog 1962), 2% (w/v) sucrose and 1% (w/v) agar. Medium pH was adjusted to 5.75 prior to autoclaving at 121ºC for 20 min. The remaining seeds were immersed in sterile water and stored in a refrigerator at 5ºC for one week period before inoculation under the same conditions. All cultures were incubated under a 16 h photoperiod provided by cool-white fluorescent lamps at a photon flux density of 53.1 μmol m^{-2} s^{-1} at a temperature of 25 ± 2ºC and monitored every week for germination for a period of 7 weeks. For each treatment 3 replicates of 10 seeds were tested.

Seedlings had their roots removed and the entire shoots were then sub-cultured onto full strength MS medium to obtain enough shoots for the subsequent assays. As the available plant material began to increase, shoot proliferation assays were initiated. Three concentrations of MS macronutrients (total MS, ½MS and ¼MS) without cytokinins or supplemented with 0.1 mg l^{-1} of kinetin (KIN) were tested. For each medium 4 repetitions with 10 explants each were performed. The conditions of the growth room were the same as described above. After 8 weeks of culture the proliferation capacity was evaluated, based on the multiplication percentage and total number of shoots produced per culture. Since rooting occurred in all tested media, shoots producing roots, number of roots and root length were also analysed.

Plantlets with well-developed roots and a developed aerial part originating from ¼MS medium were selected for acclimatization and transplanted to 100 ml plastic pots containing a mixture of peat and vermiculite (3:1, v/v). The pots were then placed into transparent polyethylene boxes and kept under the same growth conditions as referred before for 6 weeks. After this period, the boxes were gradually opened in order to expose the plants to a progressively reduced relative humidity, up to 10 weeks in total.

The number of surviving plants was recorded. Four assays with 10 plantlets each were carried out.

2.1.2 *Drosera rotundifolia*

A natural population of *D. rotundifolia* was found at a peat bog near the highest point of Serra da Estrela (Portugal) in August 2007 and seeds were collected by T. Grevenstuk, S. Gonçalves and A. Romano. *In vitro* cultures were initiated from seeds previously surface sterilized with commercial bleach 20% (v/v) (5% of sodium hypochlorite) and a few drops of Tween-20 for 20 min and washed 3 times in sterile water before being aseptically transferred into test tubes containing 10 ml ¼MS medium without PGR, 2% (w/v) sucrose and 1% (w/v) agar. The medium and growth conditions were the same as described above.

New shoots originated from seeds were subcultured onto full strength MS medium till enough plant material was obtained. Cultures proliferation was then analysed on two different media: PGR free MS and ¼MS. The growth conditions were the same as before. The multiplication percentage and the total number of shoots per culture were recorded after 8 weeks in culture. In total, 40 explants divided into 4 repetitions were studied for each medium.

2.1.3 *Pinguicula vulgaris*

P. vulgaris seeds were gently obtained from Dr. Duarte Silva of the Instituto da Conservação da Natureza (ICN). Seeds were collected by Mr. António Rebelo from a population near Cagarouço in Parque Natural da Peneda Gerês (Portugal), in late September 2007. Seeds were surface sterilized with 20% (v/v) commercial bleach (5% sodium hypochlorite) and a few drops of Tween-20 for 20 min and rinsed 3 times in sterile water. Afterwards part of the seeds was aseptically inoculated in test tubes containing 10 ml PGR free ¼MS medium, 2% (w/v) sucrose and 1% (w/v) agar. The remaining seeds were immersed in sterile water and stored in a refrigerator at 5ºC for one or two week period before inoculation. The medium and growth conditions were equal to the ones described above. All seeds were monitored every week for germination for a period of 7 weeks. For each treatment 3 replicates of 10 seeds were tested.

Following the 7 weeks period, seedlings were transferred to regulator free ¼MS medium for shoot proliferation. For assay purposes, shoots were subcultured on

different MS medium concentrations (total MS, ½MS and ¼MS) without PGR and analysed. Subsequently, the effect of ¼MS medium supplemented with 0.5 mg l^{-1} zeatin (ZEA) or 0.5 mg l^{-1} KIN was also studied. Assays of 10 explants each were carried out with a total of 4 repetitions per medium. The growth conditions were the same described above. Data were collected after 8 weeks in culture. Besides multiplication rate and total number of shoots, rooting rate, number of roots and root length were also recorded.

Cultures are maintained in ¼MS medium for further research. Due to the reduced plant material produced during the proliferation stage, other media could not be tested and the evaluation of *P. vulgaris* antioxidant activity was not accomplished.

2.1.4 *Pinguicula lusitanica*

In vitro cultures of *P. lusitanica* have been previously established by Gonçalves et al. (2008) and could be readily used. These cultures were initiated from seeds collected by Mr. Jorge Jesus in 2007 from a population located near Algoz in the Algarve region. These were surface sterilized and transferred to tests tubes containing ¼MS medium. The cultures were maintained since then in ¼MS medium without growth regulators with a subculture period of 2 months.

2.1.5 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 15.0; 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 mean ± standard error.

2.2 Evaluation of antioxidant activity

2.2.1 Extracts preparation

All plant material of *D. intermedia*, *D. rotundifolia* and *P. lusitanica* growing *in vitro* for 10 weeks in medium ¼MS (*D. intermedia* and *P. lusitanica*) and MS (*D. rotundifolia*) was ground in a mortar with liquid nitrogen for extraction. The grinded

material, approximately 20 g, was then extracted twice with 200 ml of solvent for 24 h with agitation. Methanol was used as solvent for all species and hexane and water were also used as solvent for *D. intermedia* (Table 2.1). A second methanol extract was prepared for *D. intermedia* where the plant material was macerated without adding liquid nitrogen and left to rest for 30 min before being extracted (Extract II).

Table 2.1 – Description of the prepared extracts.

Extract	Description
I	*D. intermedia* extracted with methanol
II	*D. intermedia* extracted with methanol standard maceration
III	*D. intermedia* extracted with water
IV	*D. intermedia* extracted with hexane
V	*D. rotundifolia* extracted with methanol
VI	*P. lusitanica* extracted with methanol

The extracts were filtered and methanol and hexane extracts were concentrated in a rotary-evaporator under reduced pressure at 50°C until dry and afterwards dissolved in water. The final extracts were lyophilized until dry and dissolved in phosphate buffer (75 mM, pH 7.0) at different concentrations (0.001; 0.01; 0.05; 0.1; 0.5; 1 and 10 mg ml^{-1}).

2.2.2 ORAC assay

The ORAC assay was carried out according to the protocol proposed by Gillespie et al. (2007) and using the extracts prepared previously. Phosphate buffer (75 mM, pH 7.0), fluorescein (Panreac, Spain) (0.08 mM), AAPH (Acros, USA) (150 µM) and trolox (Calbiochem, USA & Canada) (50, 25, 12.5 and 6.25 µM) solutions were prepared in advance. To each well of a black microplate were added 150 µl of fluorescein solution and 25 µl of phosphate buffer (blank) or trolox standard or sample (extracts I and II, with the concentrations 0.1, 0.05 and 0.01 mg ml^{-1}; extracts III and IV, with the concentrations 1, 0.1 and 0.01 mg ml^{-1}; extracts V and VI, with the concentrations 0.1, 0.01 and 0.001 mg ml^{-1}) (Figure 2.1). The plate was preheated at 37ºC for 10 min. After addition of 25 µl of the AAPH solution to each well, the kinetic read was instantly started. Fluorescence was measured every minute for 90 min with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. All mixtures were analysed in triplicate in each microplate and assays were repeated three times.

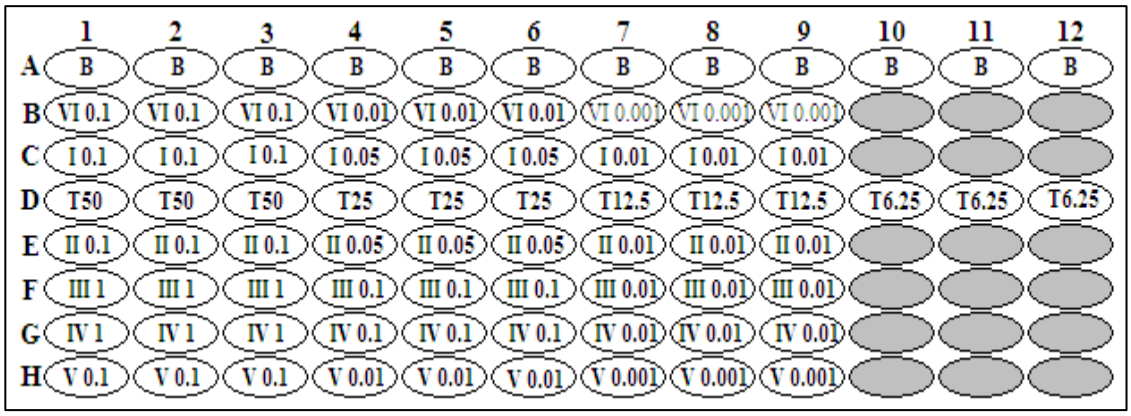


Figure 2.1 - Layout of the 96-well microplate for the ORAC assay with the different extracts and concentrations. T: Trolox standards, concentrations 50 (T50), 25 (T25), 12.5 (T12.5), 6.25 (T6.25) µM; B: blank; I: *D. intermedia* extracted with methanol; II: *D. intermedia* extracted with methanol standard maceration; III: *D. intermedia* extracted with water; IV: *D. intermedia* extracted with hexane; V: *D. rotundifolia* extracted with methanol; VI: *P. lusitanica* extracted with methanol.

The average AUC of the blanks was subtracted from the AUC of each sample and standard (trolox) to obtain the net AUC. A standard curve was obtained by plotting the trolox standards against the average AUC (Figure 2.2) and final ORAC values were calculated using the regression equation between trolox equivalents (TE) and the net AUC.

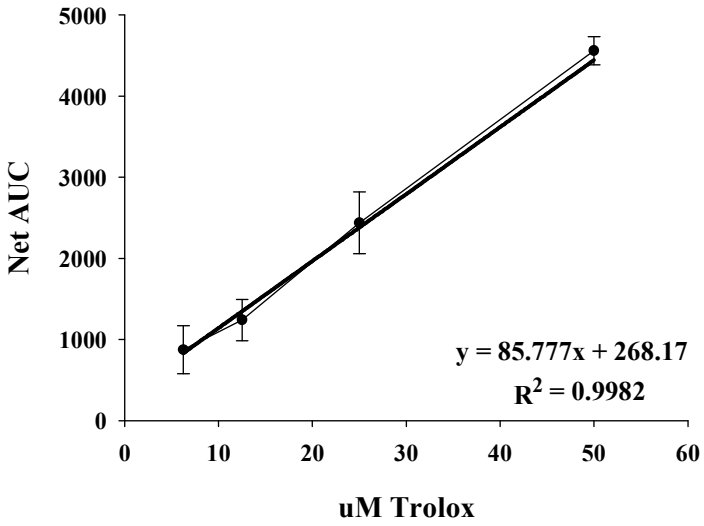
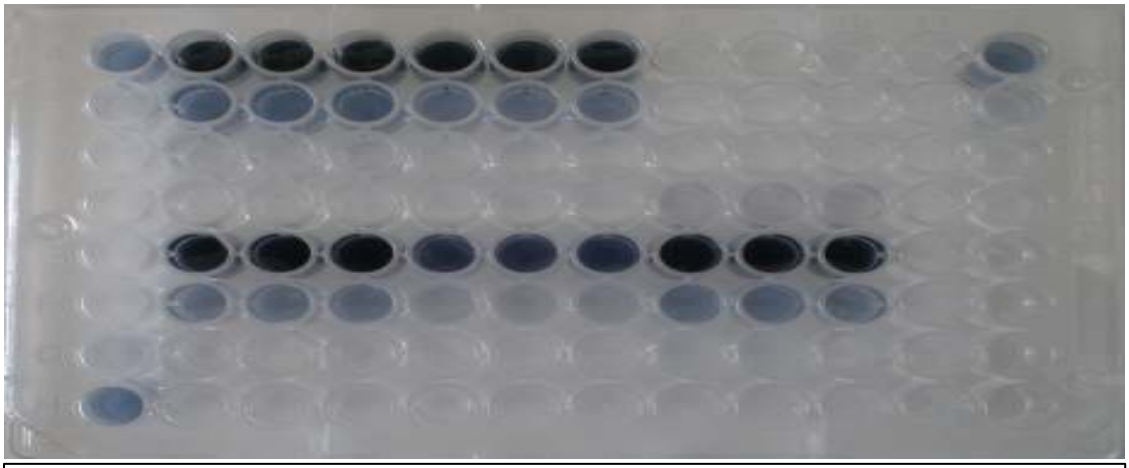


Figure 2.2 – Trolox standard curve for the ORAC assay.

2.2.3 F-C phenolics assay

The Folin-Ciocalteu (F-C) phenolics assay was carried out according to the protocol proposed by Ainsworth and Gillespie (2007). The sodium carbonate (700 mM Na_2CO_3), gallic acid (Fluka, Spain) (0.5 mM - 4 µM) and phosphate buffer (75 mM, pH 7.0) solutions were prepared in advance. To 2 ml microtubes were added 100 µl of sample (concentrations 10, 1, 0.1 and 0.01 mg ml^{-1} for all extracts) or gallic acid standard or phosphate buffer (blank) and 200 µl 10% (v:v) F-C reagent (Fluka, Switzerland). The tubes were vortexed and 800 µl of 700 mM Na_2CO_3 were then added into each tube. The different mixtures were incubated at room temperature for 2 h. From all tubes 200 µl were transferred to a 96-well clear transparent microplate (Figure 2.3). The absorbance of each well was read at 765 nm. All mixtures were analysed in triplicate and assays were performed three times.



	1	2	3	4	5	6	7	8	9	10	11	12
A	G0.5	VI 10	VI 10	VI 10	II 10	II 10	II 10	IV 10	IV 10	IV 10	G0.5	B
B	G0.1	VI 1	VI 1	VI 1	II 1	II 1	II 1	IV 1	IV 1	IV 1	G0.1	B
C	G0.02	VI 0.1	VI 0.1	VI 0.1	II 0.1	II 0.1	II 0.1	IV 0.1	IV 0.1	IV 0.1	G0.02	B
D	G0.004	VI 0.01	VI 0.01	VI 0.01	II 0.01	II 0.01	II 0.01	IV 0.01	IV 0.01	IV 0.01	G0.004	B
E	G0.004	I 10	I 10	I 10	III 10	III 10	III 10	V 10	V 10	V 10		B
F	G0.02	I 1	I 1	I 1	III 1	III 1	III 1	V 1	V 1	V 1		B
G	G0.1	I 0.1	I 0.1	I 0.1	III 0.1	III 0.1	III 0.1	V 0.1	V 0.1	V 0.1		B
H	G0.5	I 0.01	I 0.01	I 0.01	III 0.01	III 0.01	III 0.01	V 0.01	V 0.01	V 0.01		B

Figure 2.3 - Layout of the 96-well microplate for the F-C assay with the different extracts and concentrations. G: Gallic acid standards, concentrations 0.5 (G0.5), 0.1 (G0.1), 0.02 (G0.02), 0.004 (G0.004) mM; B: blank; I: *D. intermedia* extracted with methanol; II: *D. intermedia* extracted with methanol standard maceration; III: *D. intermedia* extracted with water; IV: *D. intermedia* extracted with hexane; V: *D. rotundifolia* extracted with methanol; VI: *P. lusitanica* extracted with methanol.

A standard curve of the gallic acid standards and the blank-corrected absorbance measured at 765 nm was calculated (Figure 2.4) and the regression equation was obtained. Total phenolics were calculated as gallic acid equivalents (GAE) using the previous regression equation.

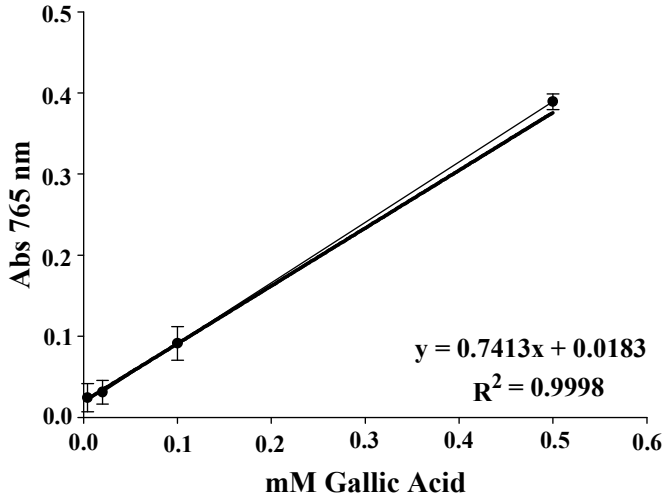
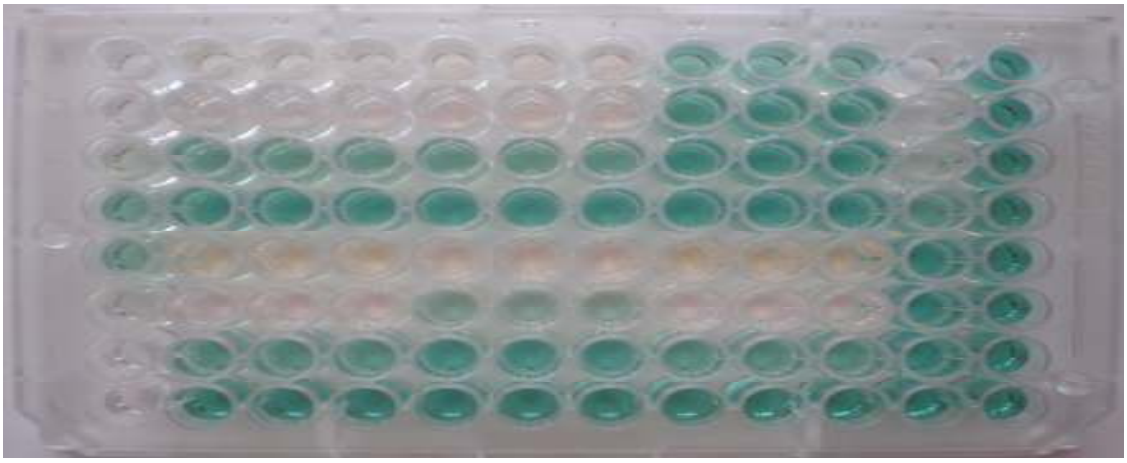


Figure 2.4 – Gallic acid standard curve.

2.2.4 TEAC assay

The TEAC assay was carried out according to the protocol proposed by Re et al. (1999) and adapted by Silva et al. (2007). The ABTS radical cation was produced by reacting an ABTS tablet (Sigma, USA) with a 2.45 mM potassium persulfate solution to give a final concentration of 7 mM. The mixture was left in the dark at room temperature for 12-16 h before being used. The $ABTS^{+\bullet}$ working solution was produced by diluting the previous solution in H_2O to an absorption of 0.70 ± 0.02 at 734 nm. The trolox solutions (0.4 – 0.1 mM) and phosphate buffer (75 mM, pH 7.0) were also prepared in advance. To a 96-well clear microplate were added 10 µl of phosphate buffer (blank) or trolox standards or sample (extracts I, II, III, V and VI, with the concentrations 1, 0.5 and 0.1 mg ml^{-1}; extract IV, with the concentration 10 mg ml^{-1}) (Figure 2.5). The reaction began after the addition of 190 µl diluted $ABTS^{+\bullet}$ working solution. The absorbance was read after 1 min at 734 nm, at 30ºC. All mixtures were analysed in triplicate and assays were performed three times.



	1	2	3	4	5	6	7	8	9	10	11	12
A	T0.4	VI 1	VI 1	VI 1	II 1	II 1	II 1	IV 10	IV 10	IV 10	T0.4	B
B	T0.3	VI 0.5	VI 0.5	VI 0.5	II 0.5	II 0.5	II 0.5				T0.3	B
C	T0.2	VI 0.1	VI 0.1	VI 0.1	II 0.1	II 0.1	II 0.1				T0.2	B
D	T0.1										T0.1	B
E	T0.1	I 1	I 1	I 1	III 1	III 1	III 1	V 1	V 1	V 1		B
F	T0.2	I 0.5	I 0.5	I 0.5	III 0.5	III 0.5	III 0.5	V 0.5	V 0.5	V 0.5		B
G	T0.3	I 0.1	I 0.1	I 0.1	III 0.1	III 0.1	III 0.1	V 0.1	V 0.1	V 0.1		B
H	T0.4											

Figure 2.5 - Layout of the 96-well microplate for the TEAC assay with the different extracts and concentrations. T: Trolox standards, concentrations 0.4 (T0.4), 0.3 (T0.3), 0.2 (T0.2), 0.1 (T0.1) mM; B: blank; I: *D. intermedia* extracted with methanol; II: *D. intermedia* extracted with methanol standard maceration; III: *D. intermedia* extracted with water; IV: *D. intermedia* extracted with hexane; V: *D. rotundifolia* extracted with methanol; VI: *P. lusitanica* extracted with methanol.

The percentage of activity inhibition, calculated by the equation 2.1

$$\frac{Abs_{Blank} - Abs_{Sample}}{Abs_{Blank}} \times 100 \qquad \textbf{Equation 2.1}$$

in relation to the control at 734 nm was plotted as a function of concentration of trolox for the standard curve (Figure 2.6). The antioxidant activity of the extracts was calculated from this curve as TE.

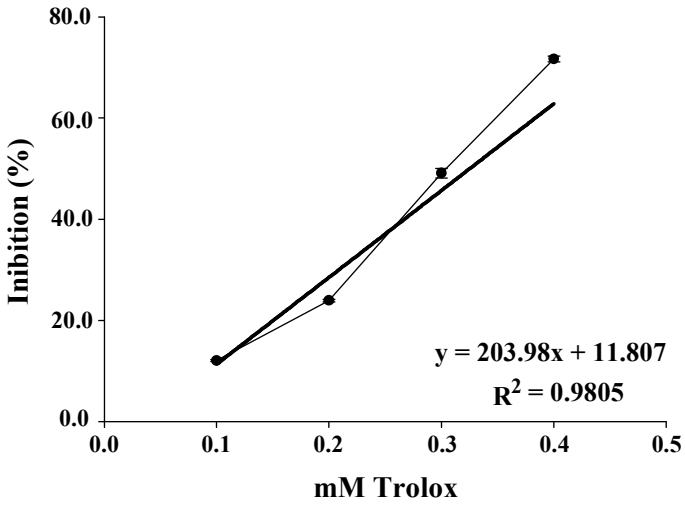


Figure 2.6 – Trolox standard curve for the TEAC assay.

3. Results and discussion

3.1 *In vitro* propagation

3.1.1 *Drosera intermedia*

D. intermedia seeds were all free from contaminations after the applied sterilization procedure (Figure 3.4A). The greatest part of the seeds, for both treatments, germinated during the second week (Figure 3.1).

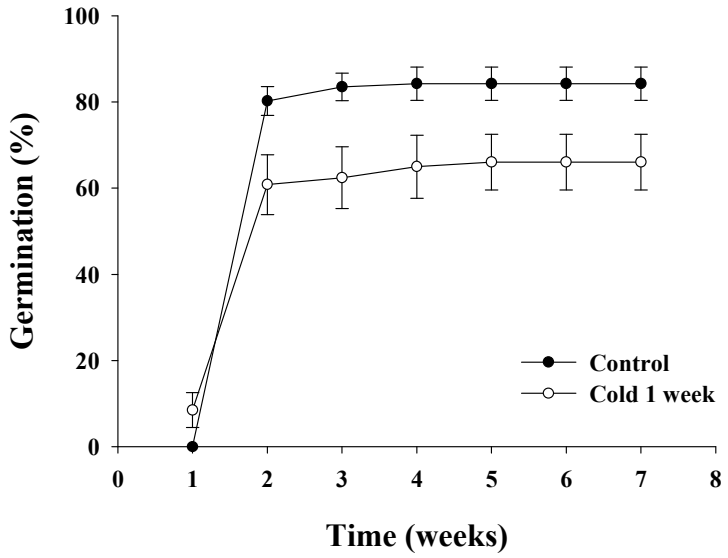


Figure 3.1 – Percentage of seeds of *D. intermedia* that germinated during a 7 week period after two different treatments. Values represent means ± SE of 3 replications.

Many of the *Drosera* species seeds need a cold treatment (4°C) to break dormancy and germinate (Jayaram & Prasad 2006). Kim & Jang (2004) obtained significantly higher germination rates of *D. peltata* seeds treated with cold. However, in this work the control treatment showed higher germination rate than the seeds submitted to cold treatment for one week, 84.22 ± 3.85 and 65.70 ± 6.71, respectively, although the germination rates were not significantly different. For other *Drosera* species, the results are diverse: Kawiak et al. (2003) successfully germinated *in vitro* seeds of *D. anglica* and *D. cuneifolia*, after ethanol treatment; while Jayaram & Prasad (2006) were not able to germinate *D. indica* and *D. burmanii* seeds even after cold stratification.

After a 7 week period, shoot cultures were established from the developed seedlings, originating shoots with a high proliferation capacity. Cultures for shoot proliferation were initiated on PGR free media and subsequently in the same basal

media supplemented with KIN, in a total of six different media. A high proliferation capacity was observed in all the media tested (Figure 3.4E). With respect to the multiplication percentage (percentage of shoots showing multiplication capacity), although results showed no significant differences among media, the highest values were observed in the media with lower concentration of MS macronutrients, ¼MS and ¼MS with KIN, which reached the 100% (Figure 3.2A, $P < 0.05$). The mean number of shoots was influenced by MS medium strength. Higher mean number of shoots was obtained for the lowest concentrations of MS macronutrients, hence the more effective were ¼MS and ¼MS with KIN (18.16 ± 1.91 and 18.18 ± 1.23, respectively) (Figure 3.2B, $P < 0.05$). These data are in agreement with the fact that carnivorous plants grow normally in nutrient-poor areas. Other *in vitro* propagation protocols from seeds or leaf explants developed for *Drosera* species reported similar results, even though ½MS was usually the suitable MS concentration for inducing shoot proliferation (Anthony 1992; Bobák et al. 1995; Kawiak et al. 2003; Kim & Jang 2004).

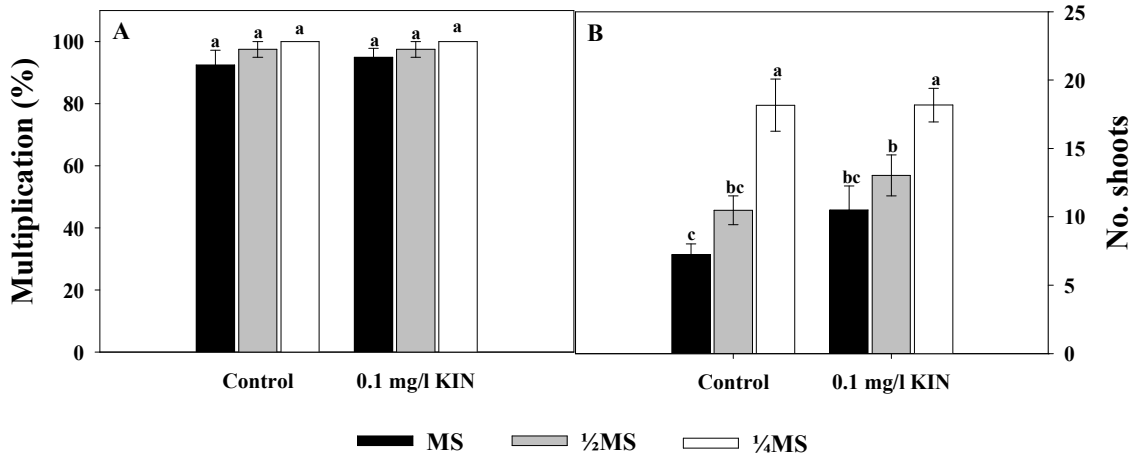


Figure 3.2 – Effect of MS medium concentration and KIN on multiplication percentage (A) and mean number of shoots (B) of *D. intermedia* shoots. Control: media without PGR. Values represent means ± SE of 4 replications with 10 shoots. In each graph, bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

On all tested media shoots produced extensive root systems during the proliferation phase (Figure 3.4D). The formation of roots in this phase has previously been observed in *D. rotundifolia, D. capensis* and *D. binata* (Anthony 1992) and in *P. lusitanica* (Gonçalves et al. 2008). There were no significant differences between cultures on the rooting rate (Figure 3.3A, $P < 0.05$), and on four of the tested media

(¼MS, ¼MS with KIN, ½MS and ½MS with KIN) a percentage of 100% was observed. The mean number of roots was significantly influenced by MS strength (Figure 3.3C, $P < 0.05$), and the best results were obtained in ¼MS, ¼MS and ½MS supplemented with KIN (8.40 ± 0.44, 8.85 ± 0.48 and 8.90 ± 0.51, respectively). As for root length, ½MS showed the longest roots (3.60 ± 0.13 cm) (Figure 3.3D, $P < 0.05$).

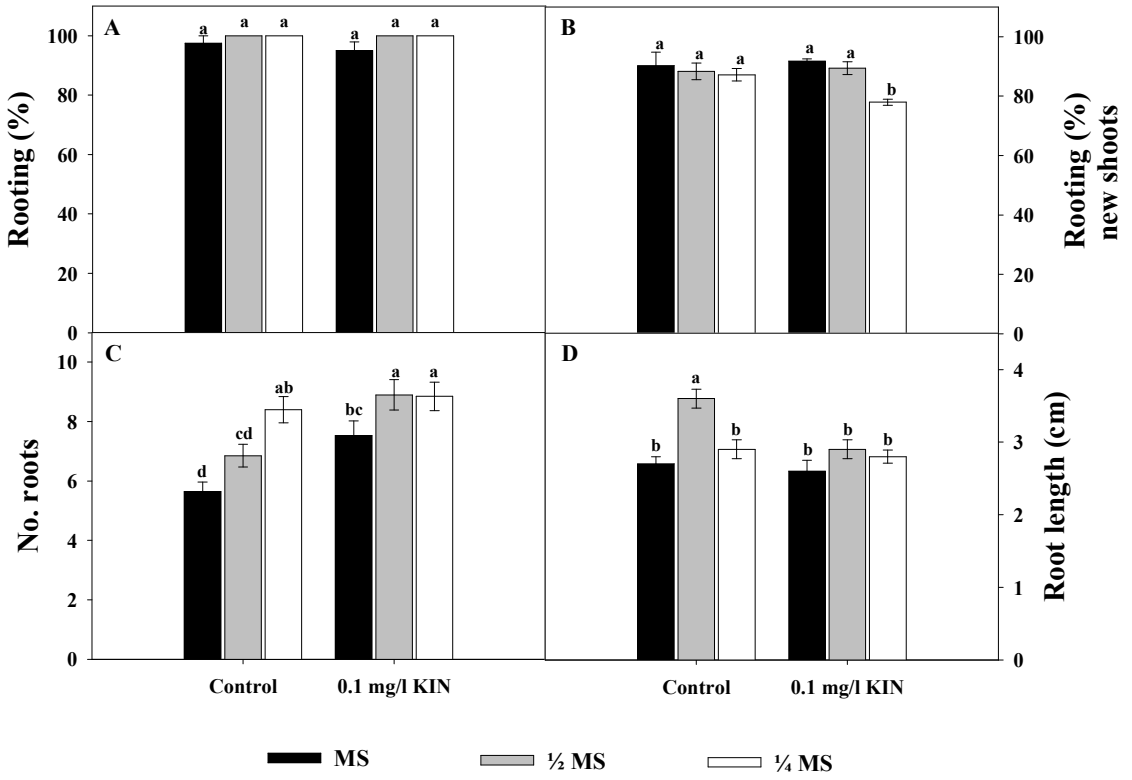


Figure 3.3 – Effect of MS medium concentration and KIN on rooting rate (A), rooting rate of the new shoots (B), mean number of roots (C) and root length (D) of *D. intermedia* micropropagated shoots. Control: media without PGR. Values represent means ± SE of 4 replications with 10 shoots. In each graph, bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Curiously, higher percentage of the new shoots already developed during the period of culture rooted on all the media tested. Contrary to the rooting of the initial shoots, the highest rooting percentages of new shoots occurred on the media with highest concentrations of MS macronutrients, MS and MS with KIN (90.33 ± 4.48 and 91.84 ± 0.77, respectively), even though there were no significant differences between the tested media (Figure 3.3B, $P < 0.05$), except for ¼MS that showed the lowest rooting percentage (77.98 ± 0.98). These results indicate that large numbers of

D. intermedia plantlets can be produced in one single step. On average, one shoot cultured on PGR free ¼MS medium produces 15.8 plantlets after 8 week period.

Taking into account that rooting percentages close to 100% were obtained in all tested media, an additional rooting phase with auxins-supplemented media was unnecessary.

The plantlets produced *in vitro* were then gradually exposed to natural environment. All plantlets were successfully acclimatized to *ex vitro* conditions and their leaves were functional and able to catch insects (Figure 3.4 F).

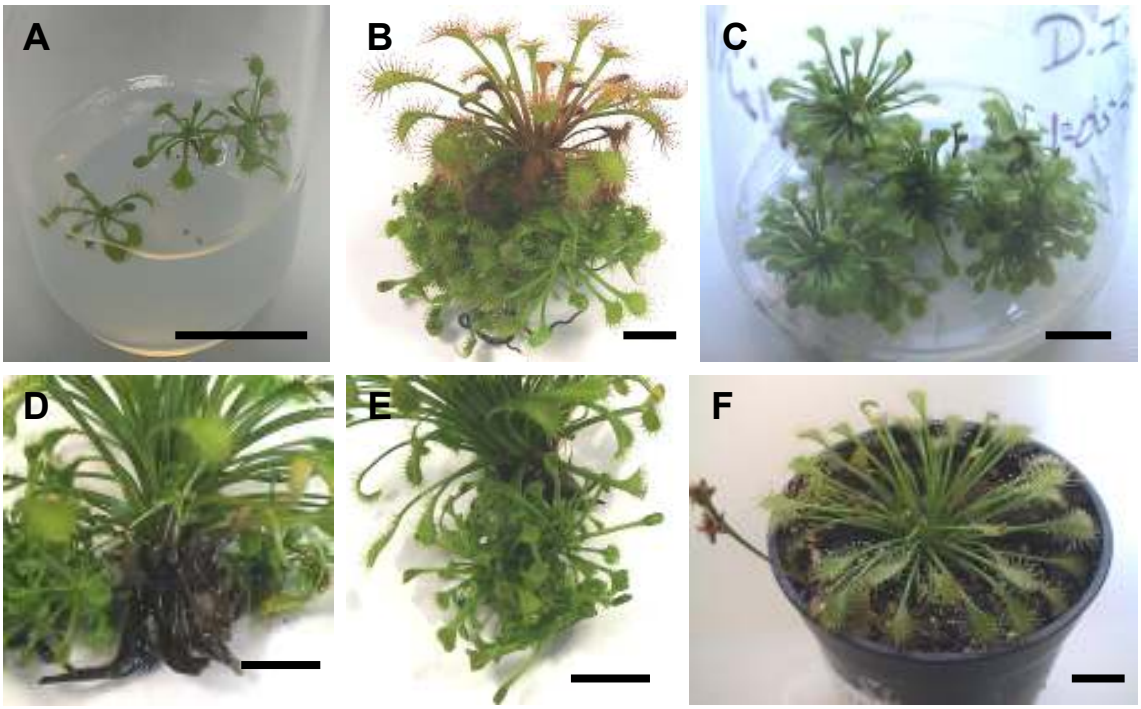


Figure 3.4 - Micropropagation of *D. intermedia*. Germinated seeds after 5weeks (A). Shoots at the end of proliferation phase in ¼MS + KIN (B) and MS + KIN (C) media. Roots (D), and new formed shoots detail (E) after 8 weeks in culture. Acclimatized plant (F). (Bars = 1 cm)

3.1.2 *Drosera rotundifolia*

Seeds from *D. rotundifolia* germinated successfully (data not showed) and from the new produced seedlings cultures were established. According to Anthony (1992) and Bobák et al. (1995), media without PGR enhanced shoot proliferation from leaf explants of *D. rotundifolia*. Therefore, shoot proliferation assays were carried out on two different media MS and ¼MS without PGR. The shoots and the new formed shoots after 8 weeks of culture were very small in size (Figure 3.5).

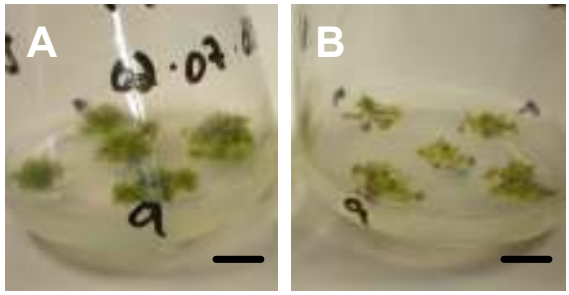


Figure 3.5 - Micropropagated shoots of *D. rotundifolia* at the end of proliferation phase in ¼MS (A) and MS (B) medium. (Bars = 1 cm)

For both multiplication percentage and number of shoots, the results were higher in the medium with lower MS concentration (90.00% ± 4.08 and 10.68 ± 1.14 shoots, respectively) (Figure 3.6, $P < 0.05$). However, the multiplication percentage was not significantly different in the two tested media (Figure 3.6A, $P < 0.05$).

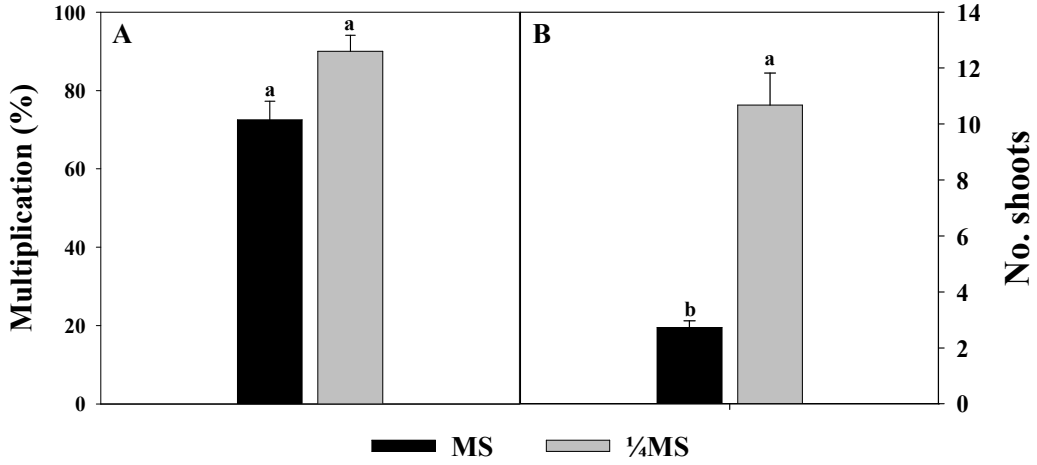


Figure 3.6 - Effect of MS medium concentration on multiplication percentage (A) and mean number of shoots (B) of *D. rotundifolia* shoots. Values represent means ± SE of 4 replications with 10 shoots. In each graph bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Under the tested conditions no root formation was observed, contrary to previous works referred above (Anthony 1992; Bobák et al. 1995) and the present study (*D. intermedia* and *P. vulgaris*). As the formed shoots were very short an elongation and root induction or development stage should be considered in order to obtain longer and rooted shoots.

3.1.3 *Pinguicula vulgaris*

Seeds of *P. vulgaris* were all free from contaminations after the applied sterilization procedure. To break dormancy *P. vulgaris* seeds need to pass through a cold period (Heslop-Harrison 2004), therefore, a cold treatment at 5ºC was tested for one and two weeks. The germination rates attained were very low and curiously the highest rate was obtained in the control (seeds inoculated without cold treatment), 24.24% (Figure 3.7A). Such results may be due to the fact that the seeds were collected several months before the initiation of this project and their viability can be uncertain.

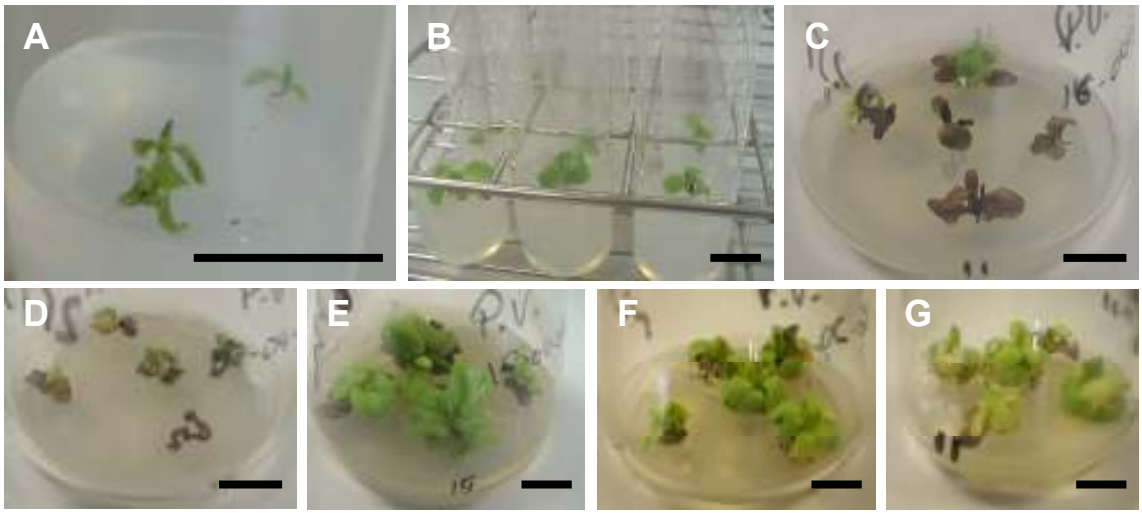


Figure 3.7 – Micropropagation of *P. vulgaris*. Germinated seeds (A). Seedling explants used in the assays (B). Shoots at the end of proliferation phase on mediums MS (C), ½ MS (D), ¼ MS (E), ¼ MS + KIN (F), and ¼ MS + ZEA (G). (Bars = 1 cm)

Despite the low germination rates, the plant material obtained during this phase was enough to establish shoot cultures from the new produced seedlings (Figure 3.7B) and to start the proliferation assays. The study of shoot proliferation was initiated by testing different MS medium concentrations (total MS, ½MS and ¼MS) without PGR. According to the statistical analysis, the multiplication percentage and the mean number of shoots was not significantly different between the tested media (Table 3.1, $P < 0.05$), most likely due to the high standard errors obtained. Nevertheless, the highest values for both variables were obtained in ¼MS medium (Table 3.1). Moreover, the shoots cultured on the other two media dried out easily (Figures 3.7C, 3.7D and 3.7E). As was the case for *D. intermedia* and other carnivorous plants (Adams et al. 1979; Kim & Jang 2004; Gonçalves et al. 2008), lowering the macronutrients concentrations in the medium increased the shoot proliferation of *P. vulgaris*.

Table 3.1 - Effect of MS medium concentration on multiplication percentage, mean number of shoots, rooting rate, mean number of roots and root length of *P. vulgaris* micropropagated shoots.

Basal medium	Multiplication (%)	No. new shoots	Rooting (%)	No. roots	Root length (cm)
MS	32.50 ± 9.46 a	2.11 ± 0.22 a	45.00 ± 13.23 a	2.39 ± 0.24 a	0.70 ± 0.08 a
½MS	52.50 ± 14.36 a	2.14 ± 0.15 a	62.50 ± 12.50 a	2.72 ± 0.33 a	0.70 ± 0.08 a
¼MS	57.50 ± 12.50 a	2.53 ± 0.31 a	75.00 ± 8.66 a	2.45 ± 0.30 a	0.80 ± 0.07 a

Values represent means ± SE of 4 replications with 10 shoots. 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 formation of small roots also occurred during the shoot proliferation stage. Rooting rate and root length were higher in ¼MS medium, while the mean number of roots was higher in ½MS (Table 3.1). But like the previous results, there were no significant differences between the media on the parameters analysed (Table 3.1, $P < 0.05$).

Since the best proliferation results were obtained in ¼MS medium, this medium was then supplemented with cytokinins. Due to small amounts of plant material, only two media were tested, ¼MS supplemented with 0.5 mg l^{-1} KIN or 0.5 mg l^{-1} ZEA. Similar to results obtained by Gonçalves et al. (2008) with *P. lusitanica*, better results were attained with the addition of cytokinins to the medium in the multiplication percentage and mean number of shoots, though in this case those differences were not significantly different from the control, ¼MS (Table 3.2, $P < 0.05$). The shoots showed no morphological differences on the tested media (Figures 3.7E, 3.7F and 3.7G).

To enhance the shoot proliferation of *P. vulgaris*, further media with different cytokinins and concentrations, and combinations of cytokinins and auxins should be tested. Furthermore, since MS concentration was the most significant factor for both proliferation and rooting of shoots, other MS concentrations could also be studied as well as other basal media.

As for the formation of roots, the mean number of roots increased significantly in the presence of KIN and ZEA in the culture medium and the longest roots were obtained in medium with KIN (Table 3.2, $P < 0.05$). The latter medium also showed the highest rooting rate, which was not significantly different from the other media (Table 3.2, $P < 0.05$). According to these results it is uncertain whether an additional rooting phase is necessary or not. To increase the formation and development of roots, medium supplemented with auxins should be investigated (Hartmann et al. 1990).

Table 3.2 - Effect of cytokinin type on multiplication percentage, mean number of shoots, rooting rate, mean number of roots and root length of *P. vulgaris* micropropagated shoots.

Basal medium	Multiplication (%)	No. new shoots	Rooting (%)	No. roots	Root length (cm)
Control	57.50 ± 12.50 a	2.53 ± 0.31 a	75.00 ± 8.66 a	2.45 ± 0.30 b	0.80 ± 0.07 b
KIN	62.50 ± 10.31 a	3.05 ± 0.35 a	82.50 ± 8.54 a	4.36 ± 0.47 a	1.20 ± 0.10 a
ZEA	72.50 ± 7.50 a	3.87 ± 0.48 a	62.50 ± 10.31 a	3.92 ± 0.42 a	1.00 ± 0.08 ab

Control: ¼MS media without growth regulators; KIN and ZEA: ¼MS media supplemented with 0.5 mg l^{-1} of each cytokinin. Values represent means ± SE of 4 replications with 10 shoots. For each variable, values followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Several conditions should be improved in order to develop an efficient protocol for the micropropagation of *P. vulgaris* and to obtain plantlets acclimatized to *ex vitro* conditions. As an alternative, induction of shoots from leaf explants should be considered for this species, since it has been a successful procedure among insectivorous plants (Anthony 1992; Bobák et al. 1995; Kawiak et al. 2003).

3.2 Evaluation of antioxidant activity

Antioxidants are scavengers of reactive oxygen radicals (ROS) that attack polyunsaturated fatty acids in cell membranes and inhibit or delay the oxidation of biological molecules by inhibiting the initiation or propagation of oxidizing chain reactions. Therefore antioxidants play an important role in protecting tissues from ROS (Huang et al. 2005; Gillespie et al. 2007). The study of antioxidant activity in foods, beverage and medicinal or edible plants has been widely reported (Prior et al. 2005; Magalhães et al. 2006). However, different methods were used in different studies which make it difficult to compare the results. To our knowledge, after an extensive search on the data bases, there are no reports on the evaluation of the antioxidant activity of insectivorous plants, though several of these plants are known to have pharmacological and medical properties.

In this study extracts from *P. lusitanica*, *D. intermedia* and *D. rotundifolia* were evaluated for their AOC. For this purpose the following methods were used: ORAC, TEAC and F-C assays. These methods are based on different radicals and reaction mechanisms. The ORAC method is based on a hydrogen atom transfer (HAT) reaction

mechanism, while TEAC and F-C assay on an electron transfer (ET) based method. Thus, the values obtained for the AOC should not be compared between methods as they have different underlying reaction mechanisms.

The range of values obtained for the different methods were: ORAC from 4.11 to 335.80 $\mu mol_{TE}/g_{ext}$; F-C assay from 10.23 to 677.68 $\mu mol_{GAE}/g_{ext}$; and TEAC from 12.93 to 341.41 $\mu mol_{TE}/g_{ext}$ (Figure 3.8). Analysing these results it is inconclusive whether the antioxidants of the species tested in this study react better under a HAT or an ET mechanism. The values for ORAC and TEAC were closer than the values for F-C and TEAC, both ET based methods. Although the F–C assay has commonly been used to measure "total phenolic content", it has been proved that the assay also measures other readily oxidized substances (Huang et al. 2005; Ainsworth & Gillespie 2007). As for TEAC and ORAC, the results obtained were not in agreement with those attained by Silva et al. (2007) for several Amazonian plants species, in which ORAC values were considerably higher than TEAC.

Even with a range of values different in all methods, the results were consistent when considering the relative AOC between extracts (Figure 3.8). Comparing the methanol extracts macerated with liquid nitrogen (extracts I, V and VI, *D. intermedia*, *D. rotundifolia* and *P. lusitanica*, respectively), the highest value was presented by extract VI and therefore *P. lusitanica* has the highest AOC of the three species studied, followed by *D. rotundifolia* (V) and *D. intermedia* (I), the results were, respectively, 335.80, 91.11 and 64.67 $\mu mol_{TE}/g_{ext}$ in the ORAC method; 677.68, 333.04 and 293.11 $\mu mol_{GAE}/g_{ext}$ in the F-C method; and 341.41, 271.88 and 293.92 $\mu mol_{TE}/g_{ext}$ in the TEAC method (Figure 3.8).

Extracts I and II, both *D. intermedia* extracts, differ in the type of maceration of plant material. Interestingly, the extract prepared without liquid nitrogen maceration (extract II) showed higher antioxidant capacity in all methods comparing to the extract prepared with liquid nitrogen maceration (extract I), respectively, 74.17 and 64.67 $\mu mol_{TE}/g_{ext}$ in ORAC; 378.57 and 293.11 $\mu mol_{GAE}/g_{ext}$ in F-C; and 332.18 and 293.92 $\mu mol_{TE}/g_{ext}$ in TEAC (Figure 3.8). Many secondary metabolites, including antioxidants, are bound to sugar units in the plant. Possibly, the mechanical damage of the plant tissue releases enzymes localized in specific cell compartments, activating the respective aglicones by removal of the sugar group (Konno et al. 1999). In the extract I, plant material is directly extracted with methanol, ceasing enzymatic activity.

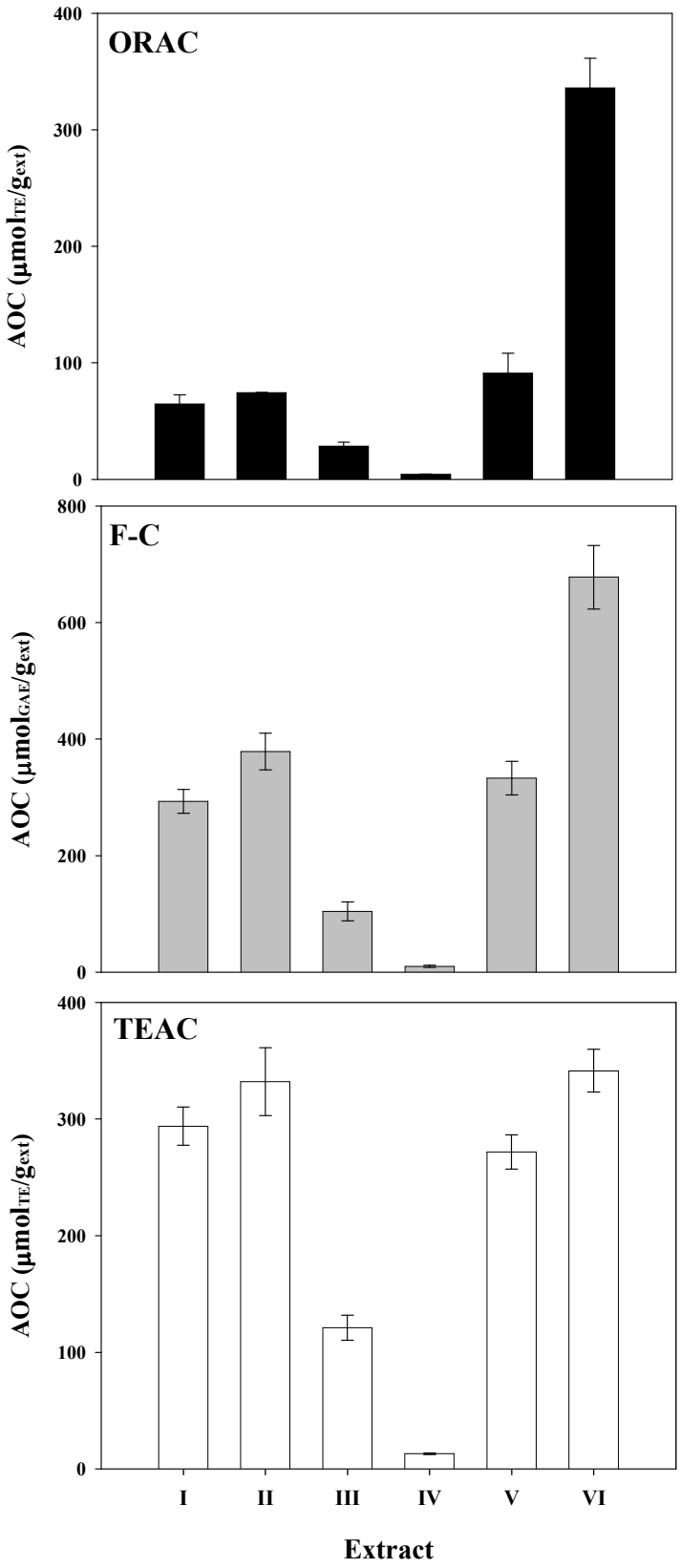


Figure 3.8 – Antioxidant activity of extracts from *D. intermedia*, *D. rotundifolia* and *P. vulgaris* by ORAC, F-C and TEAC methods. I - *D. intermedia* extracted with methanol; II - *D. intermedia* extracted with methanol standard maceration; III - *D. intermedia* extracted with water; IV - *D. intermedia* extracted with hexane; V - *D. rotundifolia* extracted with methanol; VI - *P. lusitanica* extracted with methanol.

As for the different solvents used (extracts I, III and IV), extraction with hexane (extract IV) was the less efficient (4.11 $\mu mol_{TE}/g_{ext}$ in ORAC, 10.23 $\mu mol_{GAE}/g_{ext}$ in F-C and 12.93 $\mu mol_{TE}/g_{ext}$ in TEAC, Figure 3.8) and the highest AOC values were obtained with the methanol extract (extract I) (64.6684 $\mu mol_{TE}/g_{ext}$ in ORAC, 293.11 $\mu mol_{GAE}/g_{ext}$ in F-C and 293.92 $\mu mol_{TE}/g_{ext}$ in TEAC, Figure 3.8).

Methanol usually extracts a larger number of compounds than other solvents (Cowan 1999), though it is not unexpected that the same occurs with antioxidants. In extracts prepared from *Drosophyllum lusitanicum*, hexane extracted mainly naphtoquinones (Grevenstuk et al. 2008a), which are also known to be present in *D. intermedia*. This group of natural compounds does not have antioxidant activity, which can explain the low AOC of extract IV.

Of all the tested extracts, methanol extract of *P. lusitanica* (extract VI) presented the highest values in the three tested methods. This plant may be considered as a natural source of antioxidants. The methanolic extract of *P. lusitanica* has been previously analysed by Grevenstuk et al. (2008b). The authors identified several compounds in the groups of iridoid glycosides (mussaenosidic acid, scutellarioside II and globularin) and caffeoyl phenylethanoid glycosides (R/S campneoside I, R/S campneoside II, and acteoside) (Grevenstuk et al. 2008b). Both groups of natural compounds identified in *P. lusitanica* exhibit antioxidant activity, among with other biological activities, such as antiviral, antitumor, antibacterial and diuretic for iridoid glycosides compounds, and immunosuppressant, cytotoxic, antibacterial, analgesic for caffeoyl phenylethanoid glycosides compounds (Wong et al. 2001; Harput et al. 2002; Dinda et al. 2007). The identification of the compounds effectively responsible for the AOC in *P. lusitanica* and in the other insectivorous plants studied in this research project is essential in the future. Subsequently these compounds can be isolated and used for diverse purposes.

4. Conclusions

The development of a protocol for the *in vitro* propagation of *D. intermedia*, *D. rotundifolia* and *P. vulgaris* from seeds was one of the objectives of this study. The first species was successfully micropropagated showing a high proliferation of shoots and a high rooting capacity. All tested media induced multiplication of the shoots and rooting percentages above 90%. The highest mean number of new shoots was observed in ¼MS and in the same medium supplemented with 0.1 mg l^{-1} KIN, which together with ½MS containing 0.1 mg l^{-1} KIN were the media with higher number of roots. Root length was higher in ½MS. New shoots also rooted during the proliferation phase with a rooting frequency between 77 and 93%. Due to the high rooting capacity of the shoots during the proliferation phase, an independent rooting phase turned out to be unnecessary. At the end of acclimatization phase, new developed plants of *D. intermedia* were able to catch insects.

Concerning the micropropagation protocols for *P. vulgaris* and *D. rotundifolia*, both need some improvements. However, the best results for the multiplication rate and mean number of new shoots of *P. vulgaris* were attained in ¼MS supplemented with 0.5 mg l^{-1} ZEA. As for the rooting rate, number of roots and root length, the highest results were observed in ¼MS supplemented with 0.5 mg l^{-1} KIN.

The best results for proliferation rate and mean number of new shoots of *D. rotundifolia* were obtained in ¼MS. No root formation was observed and a subsequent root induction phase should be carried out.

Results of antioxidant activity of *D. intermedia*, *D. rotundifolia* and *P. lusitanica* extracts were obtained using three different methods, ORAC, F-C phenolics assay and TEAC. *P. lusitanica* showed high antioxidant activity as compared to the other two species on all tested methods. The use of methanol instead of water or hexane as solvents increases the efficiency of the extraction procedure. Moreover, standard maceration as an alternative to maceration with liquid nitrogen also raised the antioxidant activity of the extract. The compounds present in the extracts that are responsible for their antioxidant activity should be further identified. *P. lusitanica* is a possible good source of antioxidants.

The *in vitro* propagation of *D. intermedia*, *D. rotundifolia* and *P. vulgaris* is an important alternative to collect them from the wild due to their potential medicinal

applications. In addition, *in vitro* propagated plants can be used for biological studies and for extraction of important biocompounds. In the future, accomplishing the last objective of this study, conservation of the species by "returning" new plants to their natural habitat is imperative.

5. Future work

The *in vitro* propagation protocols of *P. vulgaris* and *D. rotundifolia* starting from seeds need to be improved. A high proliferation capacity was attained for *D. rotundifolia* shoots on ¼MS medium. However, the new produced shoots were very small in size and an additional elongation phase should be performed. Since no root formation occurred, it is also essential to include a rooting phase on this plant micropropagation protocol.

The low proliferation capacity that *P. vulgaris* shoots presented during this study, suggests that different cytokinins and combinations of cytokinins and auxins, different MS concentrations and other basal media could be tested to increase the proliferation of shoots. Despite the high rooting percentages obtained, an independent rooting phase is unsure to be necessary, because on the other parameters measured, number of roots and root length, the results were low. The answer to this concern may be overwhelmed by the adjustments proposed initially.

Once the protocols of these two species are completed and the plants produced *in vitro* are successfully adapted to *ex vitro* conditions, they, along with the new formed *D. intermedia* plants accomplish in the present study, will be used to replenish wild populations.

Concerning the AOC studies, the antioxidant activity of *P. vulgaris* extracts still needs to be measured as soon as enough plant material is available. It is also important to identify and study the different compounds related to AOC of all the extracts in a near the future.

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Annexe

Annexe 1

Basal composition of MS medium (Murashige & Skoog, 1962)

Components	mg l^{-1}
Macronutrients	
KNO_3	1900
$MgSO_4.7H2O$	370
$CaCl_2.2H_2O$	440
NH_4NO_3	1650
KH_2PO_4	170
Micronutrients	
$MnSO_4.4H_2O$	16.90
H_3BO_3	6.20
$ZnSO_4.7H_2O$	8.60
$Na_2MoO_4.2H_2O$	0.25
KI	0.83
$CoCl_2.6H2O$	0.025
$CuSO_4.5H_2O$	0.025
Iron source	
$Na_2.EDTA.2H_2O$	37.25
$FeSO_4.7H_2O$	27.85
Aditivs	
Mio-inositol	100
Nicotinic acid	0.5
Piridoxin.HCl	0.5
Thiamine.HCl	0.1
Glycine	2
Agar	7000
Sucrose	20000