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MICROPROPAGATION

In vitro propagation of the wild carrot *Daucus carota* L. subsp. *halophilus* (Brot.) A. Pujadas for conservation purposes

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Abstract *Daucus carota* subsp. *halophilus*, is a wild crop relative of domestic carrot. It is an aromatic plant widely used in folk medicine due to recognized therapeutic properties of its essential oils. Experiments were carried out to evaluate the potential of *in vitro* propagation techniques to the conservation of this endemic and endangered taxon. The results showed that shoot tips of *in vitro* germinated seeds were able to proliferate in the presence of benzyladenine, with the best results being achieved using 4.4 μM, both in the first and second cultures. Shoots rooted after being transferred to 1/2-Murashige and Skoog basal medium. The results indicated that the concentration of benzyladenine used during the multiplication phase did not interfere with the rate of root formation. The obtained plantlets were morphologically and anatomically identical to those obtained by seeds. Some of the *in vitro* produced shoots developed flowers that produced viable pollen. Plant regeneration was also achieved by somatic embryogenesis induction in cotyledons and root segments cultured in the presence of 4.5 μM 2,4-dichlorophenoxyacetic acid. Somatic embryos converted into plantlets in a medium without growth regulators. Plants obtained either by shoot proliferation or somatic embryogenesis were acclimatized and are now growing at the Coimbra Botanical Garden. The first attempts to reintroduce these plants in the original habitat were successful. It can be concluded that the protocols developed are a useful approach to the conservation of this endemic species.

Keywords Acclimatization · *In vitro* flowering · Plant conservation · Rooting · Shoot proliferation · Somatic embryogenesis

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Introduction

The Iberian Peninsula possesses a great diversity of environments supporting a diversified flora where many endemisms have been reported (Parker 1981; Giménez et al. 2004). Among these endemisms, the family Apiaceae (Umbelliferae) is particularly well-represented since at least 65 apiaceous taxa are endemic of this region (Pujadas Salvá 2003). One of these endemic taxa, *Daucus carota* subsp. *halophilus*, is exclusively found along Portugal's coast, mainly in the provinces of Algarve, Alentejo, and Estremadura (Pujadas Salvá 2002, 2003). This subspecies, a crop wild relative (CWR) of the domestic carrot, grows spontaneously in coastal dunes, escarpments, and plateaus in saline rich soils (as suggested by its subspecific designation), usually 10–15 m above sea level (Pujadas Salvá 2002). Flowering occurs from March to June, and each plant produces a large number of seeds that germinate well *in vitro*. Like other members of the same species, individuals of *D. carota* subsp. *halophilus* perennates through a small tap root. Considering the economic importance of several *Daucus* species in terms of essential oil production (Ekiert 2000), the oils of *D. carota* subsp. *halophilus* and their antifungal activity have been evaluated (Tavares et al. 2008), trying to find an economic

support for its conservation as suggested by some conservationists (Kareiva and Marvier 2007). These studies showed that the oils of *D. carota* subsp. *halophilus* have a strong antifungal activity against dermatophyte strains opening the possibility for practical applications and its commercial use (Tavares et al. 2008). From an ecological point of view, this taxon is also interesting, since it can be used for the rehabilitation of impoverished saline soils and to sustain dunes, thus contributing to the fixation of sands near the coast. The importance of CWR species is now well-recognized as a source of genes that can be used to improve the characteristics of food or industrial crops (Maxted et al. 2006). Due to anthropogenic pressures, the natural habitats of this subspecies are rapidly decreasing with the consequent reduction in the number of plants and wild populations growing in their natural ecosystems.

In the last two decades, public concerns about the loss of many habitats and species have increased pressure for the development of more effective ways to reduce environment damage and species extinction (Vitousek et al. 1997; Dobson 1998; Meyers et al. 2000). *In situ* conservation strategies have been applied with relative success to the management of plant genetic resources (Dobson 1998; Jarvis et al. 2000; Graudal et al. 2001). In addition, biotechnological methods are becoming increasingly important for plant conservation of both cultivated crops and endangered species (Pence 1999). Three specific biotechnological tools have been successfully used in several programs of plant conservation, namely, (1) tissue culture techniques for *in vitro* propagation (Almeida et al. 2005; Gonçalves and Romano 2005; Sarasan et al. 2006), (2) the use of molecular markers to assess the degree of variability among populations (Gaudeul et al. 2002; Sergio and Gianni 2005), and (3) techniques of long-term conservation such as encapsulation and cryopreservation (Engelmann 2004). Plant tissue culture techniques are particularly relevant since they can be used for large-scale propagation of individuals that are threatened, and the plants thus obtained can be further reintroduced into their natural environments or in new ones. Other advantages of these techniques are the reduced space requirements necessary to maintain a large number of individuals and the possibility of keeping collections of the endangered species *in vitro* at the same time that the reintroduction is being done or until the conditions to do that are established. Based on these methods, the Kew and Missouri Botantical Gardens and other institutions are now using these approaches to propagate and maintain rare plants. However, it must be emphasized that *in vitro* methods are not exempted from problems such as the possibility of genetic changes (somaclonal variation) in the propagated material (Wilkinson et al. 2003) and the static approach subjacent to these methods (Kjær et al. 2004).

Recently, we have started a project for plant conservation of several endemic Iberian Apiaceae through tissue culture techniques. One of these taxa is *D. carota* subsp. *halophilus*. To our knowledge, the only reports concerning the *in vitro* culture of *D. carota* subsp. *halophilus* are those of Imani et al. (2001) and Thi and Pleschka (2005). As it is well-known, somatic embryogenesis was first achieved in carrot (Reinert 1958; Steward et al. 1958), and since then, many reports about micropropagation and somatic embryogenesis have been published for carrot and other Apiaceae species (Ekiert 2000; Thorpe and Stasolla 2001; Makunga et al. 2005).

The objective of this study was the large-scale propagation of *D. carota* subsp. *halophilus* to obtain a large number of plants that can be used for reintroduction purposes.

Materials and Methods

Seed germination. Seeds of *D. carota* subsp. *halophilus* were collected in their natural habitat (Fig. 1*a*) at St. Vicent's Cape, Algarve (37°01′22.54 N; 8°59′47.17 W) during the month of June when the fruits are ripened and kept at room temperature until the initiation of the experiments. Voucher specimens collected on the indicated location were deposited at the Herbarium of the Department of Botany of the University of Coimbra (COI).

The collected seeds were soaked in an ethanol solution (90% *v*/*v*) for 1 min and then surface-sterilized in a 7% (*w*/*v*) hypochlorite solution containing two to three drops of Tween-20 for 20 min. Following three washes in sterile double-distilled water, the seeds were left in sterilized water overnight and surface-sterilized again. Seeds were then inoculated in test tubes containing 1/2-strength MS (Murashige and Skoog 1962) medium and 0.029 M sucrose for seed germination. Cultures were kept in a growth room under aseptic conditions and submitted to a daily illumination regime (16 h light) of 15–20 µmol $m^{-2}s^{-1}$ photosynthetically active radiation provided by cool-white fluorescent lamps.

Shoot proliferation and embryogenesis. After 3–4 wk of germination, seedling shoot tips (5 mm) were cultured on MS medium supplemented with 0.44, 0.88, 1.76, 3.52, or 4.4 µM benzyladenine (BA) and 0.087 M sucrose. A medium without BA was used as a control. Root segments and cotyledons (0.5–1.0 cm length) obtained from the same seedlings were cultured in the same basal medium containing 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D). All media were gelled by adding 0.62% agar (Merck), and the

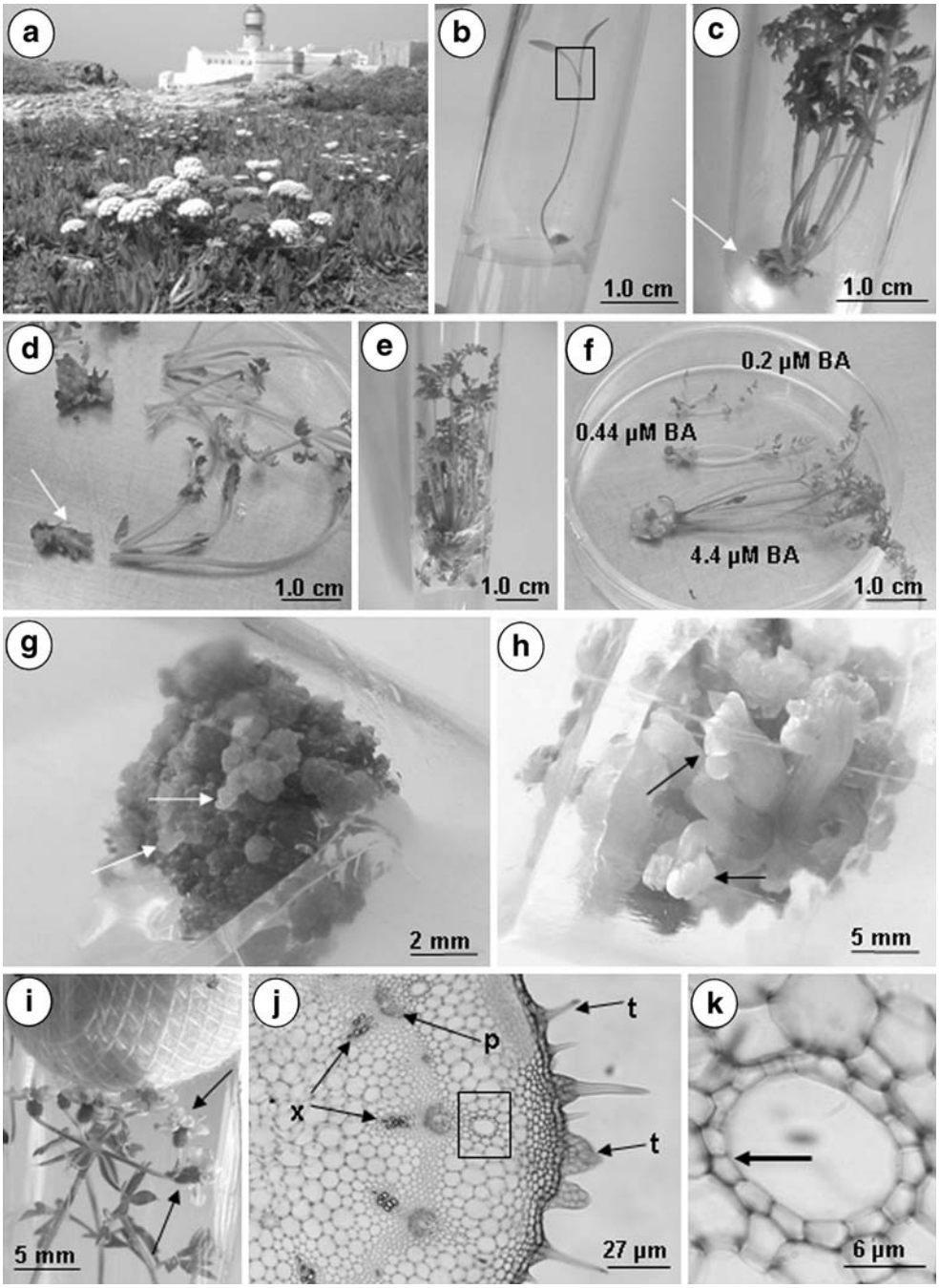

Figure 1. Morphological aspects of shoot proliferation. (*a*) Location where seeds of *Daucus carota* subsp. *halophilus* were collected. (*b*) Four-week germinated seedling. The *boxed zone* indicates the explant cultured in the first experiment. (*c*) Shoots obtained in a medium containing 4.4 µM BA after 4 wk of shoot tip culture. (*d*) General aspect of several shoots obtained after the first cycle of shoot proliferation. The *arrow* indicates the explant used in the second cycle of multiplication. (*e*) Shoots obtained in the second cycle of proliferation. (*f*) Comparison between shoots obtained in media containing different BA concentrations. (*g*) Organogenic callus produced at the base of shoots cultured on a medium containing 4.4 µM BA. The *arrows* point out to adventitious shoots. (*h*) Rudimentary flowers (*arrows*) developed from shoots produced *in vitro*. (*i*) Well-formed flowers (*arrows*) produced in developing shoots. (*j*) Cross-section of a floral peduncle from an *in vitro* produced shoot. The *boxed zone* indicates a secretory cavity. *p* phloem, *t* trichomes, *x* xylem. (*k*) Detail of the secretory cavity *boxed* in (*j*).

pH was adjusted to 5.8 with KOH (0.1–1.0 N) before autoclaving at 121°C (120 kPa) for 20 min. Cultures were kept in a growth room under the same conditions used for seed germination. The number of shoots formed from shoot tips was recorded after 4 wk of culture, and the initial explant (shoot stump) was inoculated for another 4 wk under the same culture conditions after which a new evaluation of shoot formation was taken.

After 2.5 mo of culture on 2,4-D containing medium, callus formation and somatic embryogenesis were evaluated. The calluses thus obtained were subcultured under the same conditions, whereas somatic embryos were used in the assays of conversion.

Rooting and plant regeneration from somatic embryos. Isolated shoots (2.0–3.0 cm) formed in the referred conditions were induced to root on MS medium containing 4.9 µM indole-3-butyric acid (IBA) or in the same basal medium (full or 1/2-strength) without growth regulators. Rooting was evaluated after 1 mo of culture. Rooted plantlets (root length of at least 2 cm) were transferred to pots containing a mixture of one garden

soil/one sand (v/v) and hardened in an acclimatization chamber for 3 mo before being transferred to field conditions at the Botanical Garden. After 2–5 mo of growth at the Botanical Garden, some plants were then transferred to the location where seeds were collected.

For embryo conversion, isolated somatic embryos or groups of somatic embryos with small portions of the calluses attached were transferred to full or 1/2-strength MS basal medium. The obtained emblings were potted and acclimatized under the same conditions of shoot proliferation. To assess the viability of the pollen grains produced by *in vitro* developed flowers, the pollen was cultured on a simple medium containing 1% agar (*w*/*v*) and 0.2 M sucrose.

Histological studies. Peduncle segments from *in vitro* propagated plantlets and from field growing plants were fixed in a formalin aceto-alcohol solution (5:5:90, *v*/*v*), embedded in paraffin wax, and the sections (10–12 µm) were stained by the safranine-light method described by Cruz et al. (1990).

Statistical analysis. All experiments were conducted at least three times. For each treatment, a minimum of 14 and a maximum of 25 explants were used. For statistical analysis, all quantitative data expressed as percentages were first submitted to arcsine transformation and the means corrected for bias before a new conversion of the means and standard error back into percentages (Zar 1996). Statistical analysis was performed by ANOVA (Statistica 7), and significantly different means were identified using Tukey's test (P=0.05).

Results

Seed germination and shoot proliferation. After 3–4 wk of culture in the germination medium, 50% of the seeds germinated, and their shoot tips were used for shoot proliferation (Fig. 1*b*).

The results indicated that the percentage of shoot tips showing shoot proliferation (Fig. 1*c*) reached 100% in all tested media, the only exception being the medium containing 1.76 µM BA, which resulted in 90% induction (Table 1). The number of shoots/explant increased with increasing BA concentration reaching a maximum of 4.4 shoots for the medium containing 4.4 µM BA (Table 1). The medium without BA was also able to support shoot proliferation but with the least number of shoots (2.7 shoots/explant).

In a second set of experiments and after removal of all proliferating shoots, the shoot stump (Fig. 1*d*) was inoculated again in the same culture medium for a new cycle of shoot multiplication. Since the number of shoots formed in MS basal medium was quite low, this medium was not tested in the second phase. Data from this experiment showed that induction ranged from 89% to 100% (Table 2) with no statistical differences among media ($P \leq 0.05$). The number of shoots was also the greatest (7.9 shoots/explant) using 4.4 µM BA (Table 2, Fig. 1*e*) but was the least (3.8 shoots/explant) using the lowest concentration of BA. It was observed that, for all BA concentrations, the number of shoots was higher in the second cycle of culture than in the first culture (Tables 1 and 2).

Table 1. Effect of BA concentration on shoot proliferation of shoot tips obtained from 3–4 wk-old seedlings of *D. carota* subsp. *halophilus*

BA (µM)	Number of explants cultured	Induction (%)	Number of shoots/explant
Control	51	100[z] a	2.7±0.03 d
0.44	51	100 a	3.3±0.14 c
0.88	51	100 a	3.6±0.04 bc
1.76	70	90.1±8.7 a	3.5±0.12 c
3.52	75	100 a	3.9±0.08 b
4.40	74	100 a	4.4±0.14 a

[z] Each value is the mean with standard error of three replicates. Values within *columns* followed by the same *letter* are not significantly different (Tukey test, P<0.05)

Although the length of shoots was not recorded, it was observed that shoot length was, in general, higher on media containing higher BA concentrations than on media with reduced levels of the BA (Fig. 1*f*). In some of the stumps, in particular in the presence of 3.52 or 4.4 µM BA, an organogenic callus (Fig. 1*g*) developed at the cut edge of the explant, giving origin to adventitious shoots that increased the total number of shoots formed. Further inoculation of the explants showed that the organogenic potential of the explant was maintained for at least 8 mo (data not presented) without loss of the shoot proliferation capacity.

Flowering was observed in some of the shoots. Two types of flowering could be seen: one in which shoots, at early stages of development, differentiated rudimentary flowers that did not develop further (Fig. 1*h*) and a second type in which well-developed shoots formed inflorescences at the shoot tip that developed in a considerable number of flowers (Fig. 1*i*). Preliminary assays of pollen viability carried out with flowers of the latter type indicated that some of the pollen grains were able to germinate *in vitro* (data not presented). Histological studies carried out in peduncles of the *in vitro* produced flowers showed an anatomic structure (Fig. 1*j*) similar to those formed in the field including the presence of secretory cavities (Fig. 1*j*, *k*) characteristics of the Apiaceae family.

Table 2. Shoot proliferation in *D. carota* subsp. *halophilus* in a second cycle of culture

[z] Each value is the mean with standard error of three replicates. Values within *columns* followed by the same *letter* are not significantly different (Tukey test, $P<0.05$).

BA (μM)	Number of explants cultured	Induction (%)	Number of shoots/explant
0.44	51	100[z] a	3.8±0.15 c
0.88	51	96.1±4.0 a	4.6±0.36 c
1.76	70	100 a	6.1±0.39 b
3.52	75	90.7±5.3 a	6.0±0.16 b
4.40	74	89.2±7.0 a	7.9±0.41 a

Rooting and acclimatization When shoots (2–3 cm) were isolated (Fig. 2*a*) and transferred to MS medium without growth regulators, root formation was observed (Fig. 2*b*). Roots started to appear 2–3 wk following transfer to MS medium, and in general, two to three roots were produced in each shoot (Fig. 2*b*). Rooting, which ranged from 46% to 60%, was not affected by BA concentrations in the shoot multiplication media (Table 3). Furthermore, the rate of root

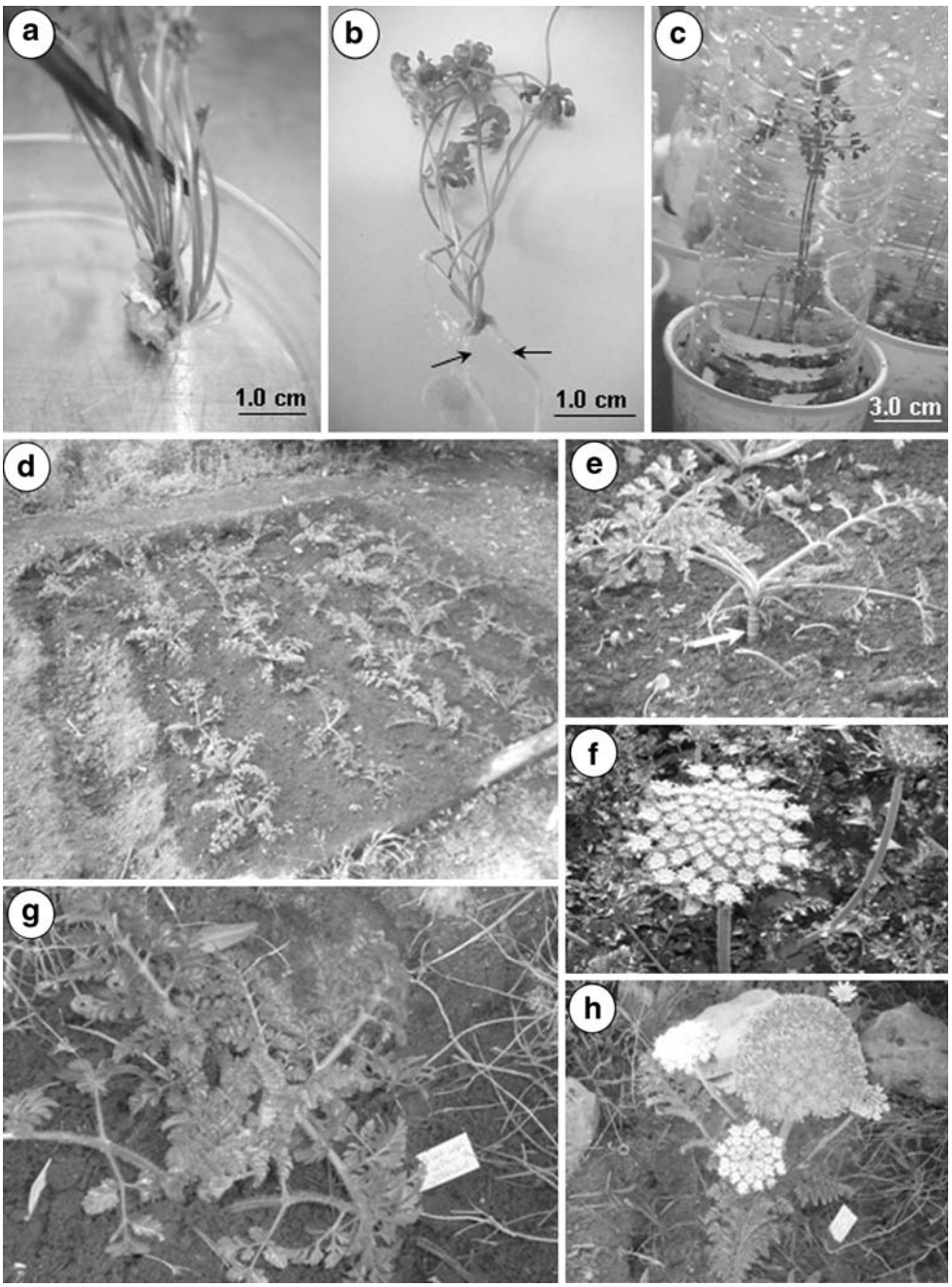

Figure 2. Rooting and acclimatization of *D. carota* subsp. *halophilus* plantlets obtained *in vitro*. (*a*) Shoots just before transference to the rooting medium. (*b*) Rooted shoot showing two well-formed roots (*arrows*). (*c*) Plantlets during the process of acclimatization. (*d*) Plants growing in the field, at the Coimbra Botanical Garden, 8 mo after culture initiation. (*e*) Detail of a field-growing plant showing the tap root (*arrow*). (*f*) Flowering plant. (*g*, *h*) *In vitro* propagated plants growing in their natural habitat (St. Vincent Cape) at the time of plantation (*g*) and 3 mo later.

Table 3. Rooting of *D. carota* subsp. *halophilus* microshoots formed on a medium containing different concentrations of BA

BA (μM) in the induction medium	Inoculated explants	Number rooted explants	Rooted shoots (%)
0.44	285	132	45.7±5.6[z] a
0.88	303	193	59.7±5.8 a
1.76	752	447	59.3±2.5 a
3.52	615	311	54.1±7.8 a
4.40	456	223	55.6±4.4 a

Rooting was induced on a 1/2-strength MS basal medium

[z] Each value is the mean with standard error of three replicates. Values within *columns* followed by the same *letter* are not significantly different (Tukey test, $P<0.05$)

formation did not increase in the presence of IBA in the medium.

Rooted shoots were transferred to pots covered with plastic covertures to reduce water loss and increase shoot hardening (Fig. 2*c*). About 95% of the plantlets survived and were successfully planted in the field (Fig. 2*d*), where they produced tap roots (Fig. 2*e*) and flowers (Fig. 2*f*) without morphologically detected variations. Following a period of 3–6 mo at the Coimbra Botanical Garden, the plants were transferred to the original habitat where they grew normally (Fig. 2*g*, *h*).

Somatic embryogenesis and plant regeneration. Cotyledons and root segments (see Fig. 1*b*) of the germinated plantlets cultured on MS medium containing 2,4-D produced well-developed calluses (Fig. 3*a*) from which somatic embryos later formed (Fig. 3*b*). Cotyledons were more effective for embryogenic callus formation than roots. In fact, in the latter type of explant, callus formation occurred only sporadically. Callus started to form after 30 d of culture, and after another 30–60 d, somatic embryos could be seen. Some of the embryos germinated precociously (Fig. 3*c*), but most of them developed through the phases of embryo development (globular, heart-shaped, torpedo, and cotyledonar). Somatic embryo formation was not synchronized, and all the phases of embryo development could be found in the same explant (Fig. 3*b*). The number of somatic embryos per explant was also quite variable with some explants producing only a few embryos when others showed more than 100 embryos (Fig. 3*b*). Somatic embryos attained the cotyledonary stage of development in the 2,4-D-containing medium (Fig. 3*d*) and were then transferred to an auxin-free medium where they germinated and developed into plantlets (Fig. 3*e*, *f*). Plantlets were then removed from the test tubes (Fig. 3*g*) and transferred to pots (Fig. 3*h*) under conditions similar to those described on the previous section. The rate of plant survival was near 100%, but the plantlets were, in general, thinner and weaker than those obtained by shoot proliferation.

Discussion

The results presented in this paper showed that *D. carota* subsp. *halophilus* can be successfully propagated *in vitro* both by shoot proliferation and somatic embryogenesis. Since the pioneering works of Reinert (1958) and Steward et al. (1958) on carrot somatic embryogenesis, these techniques have been applied to the propagation of a large number of Apiaceae species with particular incidence on somatic embryo formation and conversion into plantlets (Brown et al. 1995; Ekiert 2000; Thorpe and Stasolla 2001). Although methods for *in vitro* propagation have been mainly used for crop species (Engelmann 1999; Chawla 2002), there has been an increasing interest on their applicability to propagate rare or threatened species for purposes of plant conservation (Jaramillo and Baena 2002; Engels 2003; Sarasan et al. 2006). Propagation of endangered species is important not only for conservation purposes but also because wild taxa are a source of genetic diversity that can be used to improve or develop crops with new genetic characteristics through hybridization (sexual or somatic) or plant genetic transformation (Bajaj 1994; Pellegrineschi 2005; Maxted et al. 2006).

As part of a strategy for the conservation of endemic and threatened species of the Portuguese flora, we decided to apply *in vitro* culture techniques to the propagation of *D. carota* subsp. *halophilus*, an endemic Apiaceae located in one (Mediterranean basin) of the 25 world biodiversity hotspots (Meyers et al. 2000; Ricketts 2001). This Portuguese endemism is also a salt-tolerant taxon that, as above stated, might be used to transfer this characteristic to domestic carrot cultivars. Moreover, it is an essential-oil-producing plant that is now being evaluated for potential medicinal purposes (Tavares et al. 2008).

As far as we know, previous reports concerning *D. carota* subsp. *halophilus in vitro* culture were made by Imani et al. (2001) and Thi and Pleschka (2005). In both cases, somatic embryogenesis was the technique used, but no data were presented about the rates of plant regeneration and acclimatization. Imani et al. (2001) evaluated the

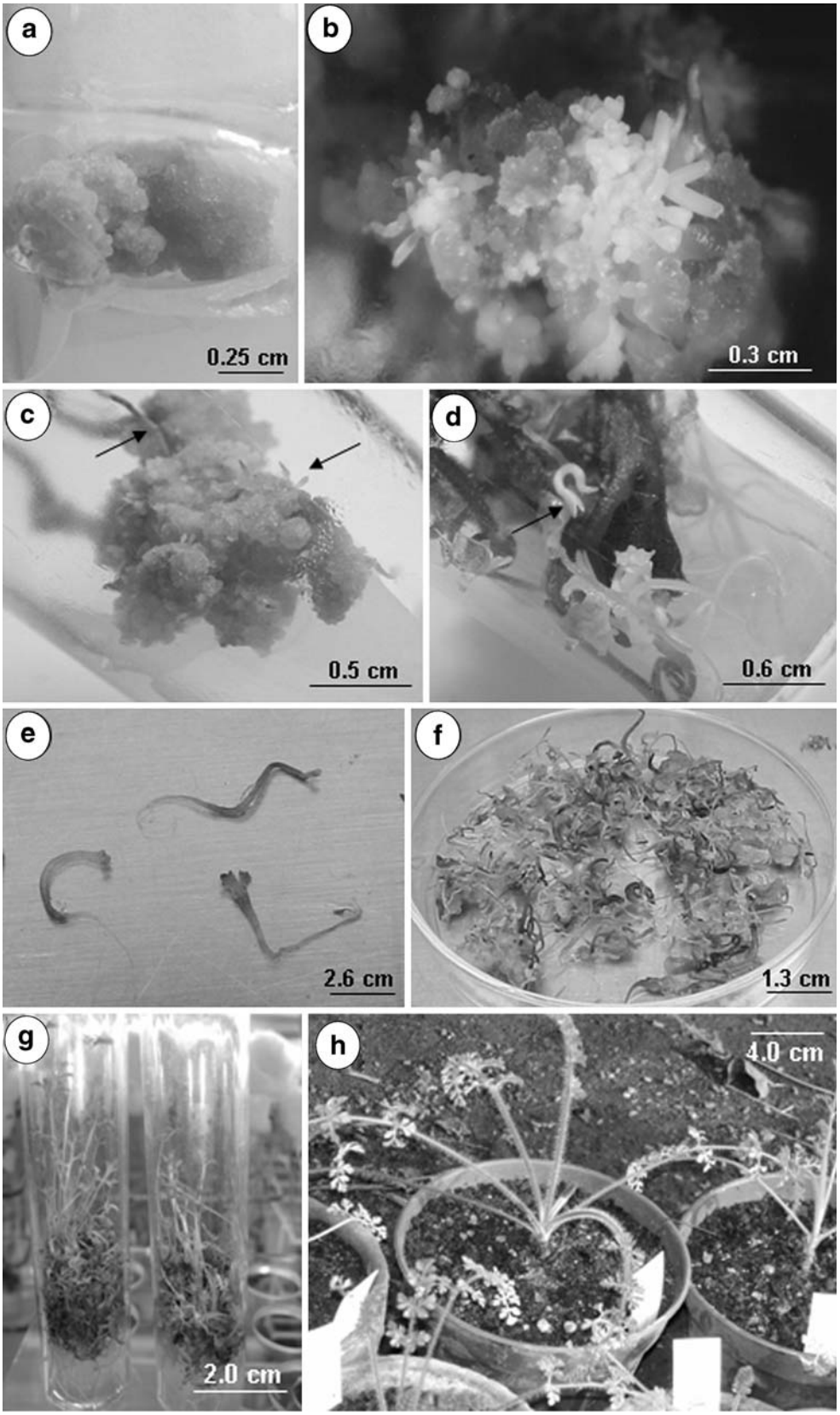

Figure 3. Somatic embryogenesis induction and plant conversion. (*a*) Callus of cotyledonary origin after 1 mo of culture. (*b*) Somatic embryos in different developmental stages formed on a cotyledonary derived callus. (*c*) Embryogenic callus showing various precociously germinated somatic embryos (*arrows*). (*d*) Cotyledonar somatic embryo (*arrow*) after 2 mo of culture in the induction medium. (*e*) Three somatic embryo derived plantlets after 3 wk of conversion. (*f*) Large number of somatic embryo-derived plantlets obtained from a single explant. (*g*) Plantlets just before the acclimatization phase. (*h*) Plant of *Daucus carota* subsp. *halophilus* obtained by somatic embryogenesis and growing at the Coimbra Botanical Garden.

potential of six *Daucus* species and six carrot subspecies for somatic embryogenesis induction, concluding that *D. carota* subsp. *halophilus* was one of the subspecies showing the capacity to form somatic embryos. These were obtained from petiolar explants through a two-step process in which embryogenic calluses were first induced in the presence of indol-3-acetic acid (1.13×10^{-5}M), whereas further embryo development occurred in an auxin-free medium. More recently, Thi and Pleschka (2005) showed that the ability of several *D. carota* species and subspecies to undergo somatic embryogenesis was strongly correlated with the endogenous levels of ABA before culture of the petioles. Explants showing the lowest endogenous ABA concentration were the most effective for somatic embryogenesis induction. Our work confirmed the potential of *D. carota* subsp. *halophilus* to be propagated by somatic embryogenesis and showed that other explants, such as germinated cotyledons and root segments, could also be used for somatic embryogenesis induction. The conditions used in our experiments were also different since somatic embryos were induced and attained the cotyledonar stage of development in the original medium containing 2,4-D. This one-step process for somatic embryogenesis induction reduces the time for somatic embryo formation and plant regeneration. Attempts to optimize this protocol are now being carried out to reduce the number of precociously germinated somatic embryos observed in some cultures and to synchronize somatic embryo development, which is quite variable even in the same explant. Both goals are essential to improve the rates of plant regeneration and the effectiveness of the process. Previous work at our lab showed that the manipulation of the culture media can increase both the rate of somatic embryogenesis induction as well as the quality of the embryos (Canhoto and Cruz 1994). Besides somatic embryogenesis induction and conversion, *D. carota* subsp. *halophilus* was also propagated by axillary shoot proliferation from shoot tips. This technique has been applied to *in vitro* propagation of a large number of crops and endangered species (Engelmann 1999; Chawla 2002). Through this method, high rates of multiplication can be obtained, and the regenerated plants are genetically uniform (George and Debergh 2008). In our experiments, *D. carota* subsp. *halophilus* was, for the first time, propagated by this method. The explants used were shoot tips from germinated seedlings, which means that the obtained plantlets were seed clones and not clones of particular adult plants, thus assuring the genetic diversity of the propagated plantlets. Genetic variability is particularly important for plant conservation since the main objective is not to clone a particular genotype but to assure that genetic diversity is maintained or increased (Graudal et al. 2001). The results of our experiments showed that the concentration of BA slightly affects the number of shoots produced, with the highest concentration used giving the highest number of shoots. The role of cytokinins on shoot proliferation is well-known (van Staden et al. 2008), and the type and concentrations most effective greatly vary among the different species. The data also indicated an increase in shoot proliferation in the second assay that can be explained by variations in the endogenous levels of cytokinins or other hormones. A habituation to the cytokinins (Geneve et al. 2007) or an increase in the number of adventitious shoots formed from small organogenic calluses originated at the base of the explants might also have contributed to this increase.

Rooting of the *in vitro* obtained shoots is an essential step for the success of plant regeneration (Németh 1986; Bennett et al. 1994). In the current study, shoots were able to root without auxin treatment. Moreover, the presence of IBA did not increase the rate of root formation. Due to the high number of shoots formed, the rates of root formation observed are enough to assure that a considerable number of rooted plantlets could be obtained. However, attempts are being made to increase the rate of rooted shoots by treatments with other concentrations of IBA and other auxins and by the application of auxinic shocks, a procedure routinely used in many species. Our data also showed that root formation was not conditioned by the levels of BA used for shoot proliferation. However, other authors have pointed out that cytokinins seem to negatively interfere with further root formation (van Staden et al. 2008). It is possible that the concentrations used in the present study are not high enough to induce this inhibitory effect.

Shoots of *D. carota* subsp. *halophilus* were able to flower *in vitro* a kind of morphogenesis that has been observed in different species (Handro and Floh 2001; Lin et al. 2005). Flower formation was randomly observed, and the physical (light intensity and photoperiod) and chemical conditions (*e.g.*, plant growth regulators and sugars) that can control flowering were not evaluated. Preliminary observations showed that pollen grains of these flowers seem to be viable, since they germinated *in vitro* in a jellified medium. It would be interesting to optimize the conditions for *in vitro* flower production and determine if the life cycle of this species could be completed *in vitro* as occurs in other plants such as *Arabidopsis*.

Plants of *D. carota* subsp. *halophilus* obtained either by axillary shoot proliferation or somatic embryogenesis were successfully acclimatized and are now growing at the Coimbra Botanical Garden and in their original habitat, thus showing the potential of this methodology for its conservation. This strategy should reduce pressure on wild populations. Further studies on this taxon will be focused on the evaluation of their genetic diversity through molecular markers and in the comparison between the essential oils produced *in vitro* and in the field.

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References

Almeida R.; Gonçalves S.; Romano A. *In vitro* micropropagation of endangered *Rhododendron ponticum* L. subsp. *baeticum* (Boisson & Reuter) Handel-Mazzeti. *Biodivers. Conserv.* 14: 1059–1069; 2005.

Bajaj Y. P. S. Somatic hybridization—a rich source of genetic variability. In: Bajaj Y. P. S. (ed) Biotechnology in agriculture and forestry, vol 27. Somatic hybridization in crop improvement I. Springer, Berlin, pp 3–32; 1994.

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

Brown D. C. W.; Finstad K. I.; Watson E. M. Somatic embryogenesis in herbaceous dicots. In: Thorpe T. A. (ed) *In vitro* embryogenesis in plants, vol. 1. Kluwer, Dordrecht, pp 345–415; 1995.

Canhoto J. M.; Cruz G. S. Improvement of somatic embryogenesis in *Feijoa sellowiana* Berg. (Myrtaceae) by manipulation of culture media composition. *In Vitro Cell. Dev. Biol. Plant* 30: 21–25; 1994.

Chawla H. S. Introduction to plant biotechnology. 2nd ed. Science, Enfield; 2002.

Cruz G. S.; Canhoto J. M.; Abreu M. A. Somatic embryogenesis and plant regeneration from zygotic embryos of *Feijoa sellowiana* Berg. *Plant Sci.* 66: 263–270; 1990.

Dobson A. P. Conservation and biodiversity. Scientific American Library, New York; 1998.

Ekiert H. Medicinal plant biotechnology: Apiaceae family as example of rapid development. *Pharmazie* 55: 561–567; 2000.

Engelmann F. *In vitro* conservation of horticultural genetic resources: review of the state of the art. *Acta Hortic.* 495: 245–250; 1999.

Engelmann F. Plant cryopreservation: progress and prospects. *In Vitro Cell. Dev. Biol. Plant* 40: 427–433; 2004.

Engels J. M. M. Plant genetic resources management and conservation strategies: problems and progress. *Acta Hortic.* 623: 179–191; 2003.

Gaudeul M.; Naciri-Graven Y.; Gauthier P.; Pompanon F. Isolation and characterization of microsatellites in a perennial Apiaceae, *Erygium alpinum* L. *Mol. Ecol. Notes* 2: 107; 2002.

Geneve R. L.; Kester S. T.; Pomper K. W. Cytokinin habituation for autonomous shoot initiation in pawpaw. *Acta Hortic.* 738: 371–374; 2007.

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

Giménez E.; Melendo M.; Valle F.; Gómez-Mercado F.; Cano E. Endemic flora biodversity in the south of the Iberian Peninsula: altitudinal distribution, life forms and dispersal modes. *Biodivers. Conserv.* 13: 2641–2660; 2004.

Gonçalves S.; Romano A. Micropropagation of *Drosophyllum lusitanicum* (Dewy pine), an endangered West Meditarranean endemic insectivorous plant. *Biodivers. Conserv.* 14: 1071–1081; 2005.

Graudal L.; Thomson L.; Kjaer E. Selection and management of *in situ* gene conservation areas for target species. In: FAO, DFS, IPGRI (ed) Forest genetic resources conservation and management, vol 2. In managed natural forests and protected areas (*in situ*). International Plant Genetic Resources Institute, Rome, pp 5–12; 2001.

Handro W.; Floh E. I. S. Neo-formation of flower buds and other morphogenetic responses in tissue cultures of *Melia azedarach*. *Plant Cell Tissue Organ Cult.* 64: 73–76; 2001.

Imani J.; Tran Thi L.; Langen G.; Arnholdt-Schmitt B.; Roy S.; Lein C.; Kumar A.; Neumann K.-H. Somatic embryogenesis and DNA organization of genomes from selected *Daucus* species. *Plant Cell Rep.* 20: 537–541; 2001.

Jaramillo S.; Baena M. Ex situ conservation of plant genetic resources: training module. International Plant Genetic Resources Institute, Rome; 2002.

Jarvis D. I.; Myer L.; Klemick H.; Guarino L.; Smale M.; Brown A. D. H.; Sadiki M.; Sthapit B.; Hodgkin T. A training guide for *in situ* conservation on-farm. International Plant Genetic Resources Institute, Rome; 2000.

Kareiva P.; Marvier M. Conservation for the people. *Sci. Am.* 297: 50–57; 2007.

Kjær E.; Amaral W.; Yanchuk A.; Graudal L. Strategies for conservation of forest genetic resources. In: FAO, DFS, IPGRI (ed) Forest genetic resources conservation and management, vol 1. Overview, concepts and some systematic approaches. International Plant Genetic Resources Institute, Rome, pp 5–24; 2004.

Lin C.-S.; Chen C.-T.; Hsiao W.-W.; Chang W.-C. Effects of growth regulators on direct flowering of isolated ginseng buds *in vitro*. *Plant Cell Tissue Organ Cult.* 83: 241–244; 2005.

Makunga N. P.; Jäger A. K.; Van Staden J. An improved system for the *in vitro* regeneration of *Thapsia garganica* via direct organogenesis—influence of auxins and cytokinins. *Plant Cell Tissue Organ Cult.* 82: 271–280; 2005.

Maxted N.; Ford-Lloyd B. V.; Jury S. L.; Kell S. P.; Scholten M. A. Towards a definition of a crop wild relative. *Biodivers. Conserv.* 15: 2673–2685; 2006.

Meyers N.; Mittermeier A.; Mittermeier C. G.; da Fonseca G. A. B.; Kent J. Biodiversity hotspots for conservation priorities. *Nature* 403: 853–858; 2000.

Murashige T.; Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473–497; 1962.

Németh G. Induction of rooting. In: Bajaj Y. P. S. (ed) Biotechnology in agriculture and forestry, vol. 1. Springer, Berlin, pp 49–64; 1986.

Parker P. F. The endemic plants of metropolitan Portugal, a survey. *Bol. Soc. Brot. Sér.* 2(53): 943–994; 1981.

Pellegrineschi A. The use of biotech for the introgression of new genetic variability in wheat varieties for developing countries. In: Tuberosa R.; Phillips R. L.; Gale M. (eds) Proceedings of the international congress. "In the wake of the double helix: from the green revolution to the gene revolution", Bologna, May 2003. University of Bologna, Italy; 2005.

Pence V. C. The application of biotechnology for the conservation of endangered species. In: Benson E. E. (ed) Plant conservation biotechnology. Taylor and Francis, London, pp 227–241; 1999.

Pujadas Salvá A. J. El complejo de *Daucus carota* L (Apiaceae) en la flora Ibérica. *An. Jard. Bot. Madr.* 59: 368–375; 2002.

Pujadas Salvá A. J. *Daucus* L. In: Nieto Feliner G.; Jury S. L.; Herrero A. (eds) Flora iberica, vol X. Real Jadín Botánico, CSIC, Madrid; 2003.

Reinert J. Über die kontrolle der morphogenese und die induktion von adventivembryonene an gewebekulturen aus karotten. *Planta* 53: 318–333; 1958.

Ricketts T. H. Conservative biology and biodiversity. In: Encyclopedia of life sciences. Wiley, New York. http://www.els.net. Cited 25 Apr 2001.

Sarasan V. A.; Cripps R.; Ramsay M. M.; Atherton C.; McMichen P. G.; Rowntree J. K. Conservation *in vitro* of threatened plants—progress in the past decade. *Plant Cell Tissue Organ Cult.* 42: 206–214; 2006.

Sergio L.; Gianni B. Molecular markers based analysis for crop germplasm preservation. Paper presented at the FAO Meeting on the role of biotechnology for the characterisation and conservation of crops, forestry, animal and fishery genetic resources, Turin, Italy, 5–7 March 2005.

Steward F. C.; Mapes M. O.; Mears K. Growth and organized development of cultured cells. II. Organization in culture grown freely suspended cells. *Am. J. Bot.* 45: 705–708; 1958.

Tavares A. C.; Gonçalves M. J.; Cavaleiro C.; Cruz M. T.; Lopes M. C.; Canhoto J.; Salgueiro L. Essential oil of *Daucus carota subsp*. *halophilus*: composition, antifungal activity and citotoxicity. *J. Ethnopharmacol.* 119: 129–134; 2008.

Thi L. T.; Pleschka E. Somatic embryogenesis of some *Daucus* species influenced by ABA. *J. Appl. Bot. Food Qual.* 79: 1–4; 2005.

Thorpe T. A.; Stasolla C. Somatic embryogenesis. In: Bhojwani S. S.; Soh W. Y. (eds) Current trends in the embryology of angiosperms. Kluwer, Dordrecht, pp 279–336; 2001.

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

Vitousek P. M.; Mooney H. A.; Luhchenco J.; Melillo J. M. Human domination of Earth's ecosystems. *Science* 297: 494–499; 1997.

Wilkinson T.; Wetten A.; Prychid C.; Fay M. F. Suitability of cryopreservation for the long-term storage of rare and endangered species: a case history for *Cosmos atrosanguineus*. *Ann. Bot.* 91: 65–74; 2003.

Zar J. H. Biostatistical analysis. 3rd ed. Prentice Hall, Upper Saddle River; 1996.