

# ***In vitro* conservation of *Angelica pachycarpa*, an Iberian endemic *Apiaceae* of the Portuguese Berlenga Islands**

Ana Cristina Pessoa Tavares\*, Lígia Salgueiro\*\* & Jorge Canhoto\*

\*Jardim Botânico, Universidade de Coimbra. Portugal.

\*\*Laboratório de Farmacognosia/CEF, Universidade de Coimbra. Portugal.

## **INTRODUCTION**

Continuing our research on the *in vitro* and *ex situ* conservation of the Iberian endemic *Apiaceae* in Portugal (Tavares & *al.* 2007), we present the first results on the micropropagation of *Angelica pachycarpa* Lange. This species requires particular conservation measures since its geographical distribution in Portugal is restricted to very small areas of the west coast in the north of Lisbon and in the Berlenga Islands (Parker, 1981; Castroviejo & *al.*, 2003; Gimenez & *al.* 2004).

For a long time, plants from the *Apiaceae* family have been used as spices or drugs and herbal medicinal products from this botanic family are actually described in some Pharmacopoeias, as having antiseptic, expectorant, diuretic, carminative, vasodilator, or spasmolytic actions (Ekiert 2000; Nalawade & *al.* 2003).

As other members of the *Apiaceae* family, *Angelica* L. is an essential oil producing plant and is largely cultivated in Europe due to its flavouring qualities for food and liquor production (Eeva & *al.* 2003) and also for the anti-oxidant properties of the essential oils (Wei & Shibamoto 2007).

Most of the pharmaceutical industry is highly dependent on wild population for the supply of raw materials for extraction of medicinally important compounds. Due to a lack of proper cultivation practices, destruction of plant habitats, and illegal and indiscriminate collection of plants from these habitats, many medicinal plants are severely threatened. Advanced biotechnological methods of culturing plant, cells and tissues should provide new means of conservation and propagation of valuable, rare and endangered medicinal plants (Nalawade & *al.* 2003).

Somatic embryogenesis and other techniques of micropropagation provide a means for large scale plant production and seems to be an ideal method to achieve the multiplication of species for purposes of plant conservation. Since the first report of the induction of somatic embryogenesis in *Daucus carota* (Steward & *al.* 1958), this technique of micropropagation has been

successfully applied to the propagation of a great number of angiosperms and gymnosperms, including some genera of the Iberian *Apiaceae* endemic in Portugal (Castroviejo, S. & *al.* 2003), such as *Bunium persicum* (Grewal, 1996), *Thapsia garganica* (Jager & *al.* 1993), *Eryngium foetidum* (Ignacimuthu & *al.* 1999) and *Angelica sinensis* (Tsay & Huang 1998).

The objective of the present study was to establish reliable protocols for the propagation of *Angelica pachycarpa* Lange that can be further used for essential oil production and conservation.

## **MATERIAL AND METHODS**

Mature seeds of *Angelica pachycarpa* were collected in the field (Berlenga Islands) and kept at room temperature until the initiation of the experiments.

Seeds were soaked in ethanol solution (90% v/v) for 1 min., surface sterilized in 7% (v/v) hypochlorite solution for 20 min. and left in sterilized water overnight. Following three washes in sterile double-distilled water the seeds were germinated in test tubes containing MS (Murashige & Skoog 1962) medium reduced at half strength and 0.087M sucrose. Cultures were kept in a greenhouse and submitted to a daily illumination regime of 15-20  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation provided by cool-white fluorescent lamps.

For shoot proliferation shoot tips (5 mm) from 4-6 week-old seedlings were cultured on MS medium supplemented with 0.44, 1.76 and 4.4  $\mu\text{M}$  BA (benzyladenine) and 0.087 M sucrose. All experiments were carried out three times. For each treatment a minimum of 13 and a maximum of 40 explants were used. The number of shoots formed from shoot tips was evaluated after 4 weeks of culture and the initial explant inoculated for another 4 weeks in the same culture conditions.

Roots obtained from the same seedlings were cut into segments of 0.5-1.0 cm long and cultured in the same basal medium containing 4.5  $\mu\text{M}$  2,4-D (2,4-dichlorophenoxyacetic acid) to induce somatic embryo formation. Root segments from *in vitro* rooted plantlets were also used for the same purpose. All media were

gelled by adding 0.62% agar (Merck) and the pH was adjusted to 5.8 with KOH (0.1-1.0N) before autoclaving at 121°C for 20 min. Cultures were kept in a greenhouse in the same conditions used for seed germination.

For rooting, isolated shoots (1.5-2.0 cm) were placed on a medium containing 5.3  $\mu$ M IBA (indole-3-butyric acid) and then transferred to the same medium without auxin.

A voucher specimen was deposited at the COI Herbarium, Department of Botany, University of Coimbra, Portugal.

## RESULTS

Seed germination tests performed *in vitro* showed that about 50% of the seeds developed into plantlets. It was also observed that seed germination was rapidly lost since 6 month collected seeds were unable to germinate.

When shoot tips were inoculated in the media tested the results indicate that shoot proliferation (Figure 1A) was achieved in all media, even when BA was not present (Table I). The frequencies of induction were similar and reached values near 100%. When the number of shoot per explants was evaluated the data showed that the medium containing 4.4  $\mu$ M BA gave the best results with 2.5 shoots per explant, after the first inoculation (Table I). Results of the second inoculation showed again that the medium containing 4.4  $\mu$ M BA was the most effective for shoot formation (Table I).



**Fig. 1.** *In vitro* shoot formation of *Angelica pachycarpa*. A- culture of shoot tips; B- rooting of shoots; C- *in vitro* obtained plantlet (Photo: A. C. Pessoa Tavares).

**TABLE I**

*In vitro* shoot formation after two inoculations of the shoot tips of *Angelica pachycarpa*.

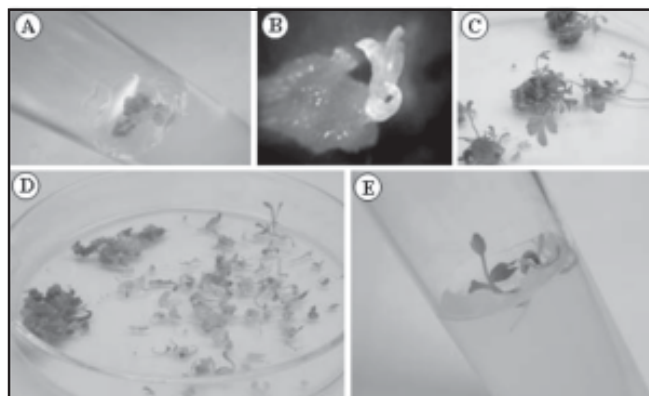
| BA ( $\mu$ M) | Number of shoots per inoculated explant* |                             | Total number of shoots |
|---------------|--|-----------------------------|------------------------|
|               | 1 <sup>st</sup> inoculation              | 2 <sup>nd</sup> inoculation |                        |
| 0.00          | 2.1                                      | 1.7                         | 3.8                    |
| 0.44          | 2.0                                      | 1.8                         | 3.8                    |
| 1.76          | 1.9                                      | 1.2                         | 3.1                    |
| 4.40          | 2.5                                      | 1.5                         | 4.0                    |

\* Each experiment was repeated at least three times and 14 to 40 explants were used per treatment.

Root formation in the obtained shoots was achieved (Figure 1B and 1C) after treatment with IBA and the percentage of shoots producing roots was 31.21% (958 shoots produced 299 roots).

Cultured root segments produced masses of callus (Figure 2A) after 2.5 months of culture on a medium containing 4.5  $\mu$ M 2,4-D from which somatic embryos start to appear (Figure 2B). These embryogenic calli could be maintained in the same culture conditions for a year without loss of their potential to produce somatic embryos. Somatic embryos were able to attain the cotyledonary stage of development in the 2,4-D supplemented medium and some of them germinated precociously (Figure 2C). Somatic embryo development was quite asynchronous with the different stages (globular, heart-shaped, torpedo and cotyledonar) being observed in the same explant (Figure 2D).

Somatic embryo conversion into plantlets was achieved when the embryos were cultured on a medium without 2,4-D or with reduced amounts of 2,4-D (0.45  $\mu$ M) with frequencies of germination ranging from 73 to 100% (Figure 2E).



**Fig. 2.** Somatic embryogenesis induction and plant regeneration in *Angelica pachycarpa*. A- callus from a root segment; B - cotyledonary somatic embryos; C- somatic embryos precociously germinated; D- different stages of somatic embryo development in the same explant; E- Somatic embryo conversion into plantlets (Photo: A. C. Pessoa Tavares).

The results also indicated that *Angelica pachycarpa* somatic embryos have the potential to develop secondary somatic embryos thus increasing the final number of embryos produced.

Attempts to transfer the somatic embryo-derived plantlets to soil and to field conditions are now being carried out.

## DISCUSSION

The results so far obtained in the experiments with *Angelica pachycarpa* have shown that this species, like other *Apiaceae* plants, has a strong potential for *in vitro* propagation, a favorable condition for studies of plant conservation. Moreover, it was found that *Angelica pachycarpa*

can be propagated *in vitro* by different methods of micropropagation such as shoot proliferation and somatic embryogenesis induction making possible to adopt different strategies of multiplication according with the type of explants available and with the objectives of the conservation. In fact, whereas shoot proliferation is more indicated to obtain true-to-type plants, somatic embryogenesis from long-term calli is often responsible for the regeneration of plants displaying some kind of variability that can be used to increase the genetic diversity of a population.

According with some conservationists, conservation is accomplished easily if human populations are advised of the economic value of the species that must be protected (Kareiva & Marvier 2007). Thus, together with the studies of plant conservation of some endemic *Apiaceae* plants of the Portuguese flora, we are also trying to find characteristics of these plants that can be explored and that may have some economic interest. Since members of the *Apiaceae* family produce essential oils our goal is to characterize and to evaluate the biological activities of the essential oils produced by some of these species. Also, concerning this aspect, *in vitro* culture micropropagation techniques are a powerful tool to help in plant conservation since essential oils or other compounds of interest can be isolated from these plants instead of using field-growing plants thus avoiding the overexploration of natural populations.

Further studies on the *in vitro* propagation of *Angelica pachycarpa* will be focused on the optimization of the established protocols mainly in the conditions necessary to increase the yield and quality of the embryogenic process. This can be achieved either by reducing the precocious germination of the embryos and with attempts to improve the rates of somatic embryo germination and conversion into plantlets.

A crucial step in processes of micropropagation is the acclimatization of the *in vitro* obtained propagules. In the case of *Angelica pachycarpa* these assays are now being carried out and we do not have yet consistent data about this phase. However, similar experiments made by our group in *Daucus carota* subsp. *halophilus* showed that the *in vitro* regenerated plantlets acclimatized a higher rates and the plants were phenotypically normal (Tavares & al. 2007).

## REFERENCES

Castroviejo, S., Laínz, M., López González, G., Montserrat, P., Muñoz Garmendia, F., Paiva, J. & Villar, L. – (eds.). 2003. Flora Ibérica, Plantas vasculares de la Península Ibérica, e Islas Baleares, vol. X, ARALIACEAE-UMBELLIFERAE. Real Jardín Botánico, C.S.I.C. Madrid.

Eeva, M., Ojala, T., Tammela, P., Galambosi, B., Vuorela, H., Hiltunen, R., Fagerstedt, K. & Vuorela, P. 2003 - Propagation of *Angelica archangelica* Plants in an Air-Sparged Bioreactor from a Novel Embryogenic Cell Line, and their Production of Coumarins. *Biologia Plantarum* 46(3):343-347.

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

Gimenez, E., Melendo, M., Valle, F., Gómez-Mercado & Cano, E., 2004 - Endemic flora biodiversity in the south of the Iberian Peninsula: altitudinal distribution, life forms and dispersal modes. *Biodiversity and Conservation* 13: 2641–2660.

Grewal, S., 1996. Microtubers from somatic embryos of *Bunium persicum*. *Indian J. Exp. Biol.*, 34: 813-815.

Lignacimuthu, S., Arockiasamy S., Antonysamy M. & Ravichandran P., 1999 - Plant regeneration through somatic embryogenesis from mature leaf explants of *Eryngium foetidum*, a condiment. *Plant Cell, Tissue and Organ Culture* 56: 131–137.

Jager, A. K., Sehottlinder, B., Smitt U. W. & f. Nyman U., 1993 - Somatic embryogenesis in cell cultures of *Thapsia garganica*. Correlation between the state of differentiation and the content of thapsigargin. *Plant Cell Reports* 12:517-520.

Kareiva, P. & Marvier, M., 2007. Conservation for the people. *Scientific American*, Oct:27-33.

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

Nalawade, S. M., Sagare A. P., Lee C. Y., Kao C. L. & Tsay H. S., 2003 – Studies on tissue culture of Chinese medicinal plant resources in Taiwan and their sustainable utilization. *Bot. Bull. Acad. Sin.* 44:79–98

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

Steward, F. C., Mapes, M. O. & Mears, K., 1958. Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. *Amer. J. Bot.*, 45:705-709.

Tavares, A. C., Salgueiro, L. R. & Canhoto, J. M., 2007. An efficient *in vitro* protocol for conservation of *Daucus carota* subsp. *halophilus*, an endemic Portuguese species. IX Simpósio da Associação Ibero-Macaronésica de Jardins Botânicos, Departamento de Botânica/Jardim Botânico da FCTUC, 4-6 de Junho. pg. 54.

Tsay, H. S. & Uang, H. L., 1998. Somatic embryo formation from immature embryo-derived suspension-cultured cells of *Angelica sinensis* (Oliv.) Diels. *Plant Cell Rep.*, 17:670-674.

Wei, A. & Shibamoto, T., 2007. Antioxidant activities and volatile constituents of various essential oils. *J. Agric. Food Chem.*, 55:1737-1742.

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**Direcc. de los autores:** \* Jardim Botânico/Departamento de Botânica, Universidade de Coimbra 3001-455 Coimbra – Portugal; actavar@bot.uc.pt; \*\*Laboratório de Farmacognosia/CEF, Faculdade de Farmácia, Universidade de Coimbra. Rua do Norte 3000-295 Coimbra – Portugal