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BRIEF COMMUNICATION

In vitro minimum growth for conservation of *Drosophyllum lusitanicum*

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Abstract

The present paper reports a protocol for minimum growth conservation of *Drosophyllum lusitanicum* (L.) Link. *in vitro*. Double-node cuttings were maintained for 4, 8 and 12 months at 5 or 25 ºC in the dark. The effects of sucrose either alone at 5, 20, 30, 40 and 60 g dm^{-3} or at 20, 40 and 60 g dm^{-3} in combination with 20 g dm^{-3} mannitol, on survival and post-storage shoot multiplication efficiency were investigated. The cultures could effectively be conserved under minimum growth at 5 ºC for 8 months on Murashige and Skoog's medium supplemented with 60 g dm^{-3} sucrose, 20 g dm^{-3} mannitol and 0.91 μM zeatin. Following extended conservation, the cultures could be successfully regenerated into new shoots, and they were morphologically similar to those of non-stored controls.

Additional key words: endangered species, germplasm conservation, osmotic stress.

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It has long been recognized that no single conservation technique applied alone for conservation of plant genetic resources adequately conserves the full range of genetic diversity of a target species or gene pool (Withers and Engelmann 1997). Each method has its own advantages and disadvantages and complementary strategies are required for effective sustainable conservation of a maximum range of genetic diversity in a given taxon. In recent years, the traditional approaches of germplasm conservation are complemented by *in vitro* conservation methods that can be used in combination with conventional practices, offering added security for field gene banks (Martin and Pradeep 2003).

Elite germplasm of various rare and endangered plant species have been conserved by *in vitro* methods (Martin and Pradeep 2003, Tyagi *et al*. 2004, Islam *et al*. 2005, González *et al*. 2006). Minimum growth is the most direct way of restricting the growth and development of plantlets *in vitro*, which can be attained by employing controlled osmotic stress using sugar alcohols and sucrose in combination with other treatments such as reduced temperature, low irradiance and hermetic vessel closures (Benson 1999, Sarkar *et al*. 2005). However, the successful application of minimum growth methodologies requires the establishment of crop-specific protocols (Watt *et al*. 2000).

The Portuguese sundew [*Drosophyllum lusitanicum* (L.) Link.], an insectivorous plant of the family *Drosophyllaceae*, is native to the southern part of the Iberian Peninsula and northern Morocco (Garrido *et al*. 2003). *D. lusitanicum* is heavily endangered (Garrido *et al*. 2003), although the reasons for this remain puzzling (Adlassnig *et al*. 2006). *In vitro* propagation of *D. lusitanicum* has been previously accomplished (Gonçalves and Romano 2005). The objective of this study was to develop a simple and reliable method for *in vitro* medium-term conservation of *D. lusitanicum* under minimum growth.

The cultures of *D. lusitanicum* were initiated *in vitro* as described earlier (Gonçalves and Romano 2005), and were multiplied and maintained on MS medium (Murashige and Skoog 1962) supplemented with 20 g dm^{-3} sucrose and 0.91 μM zeatin at an interval of 6 weeks. The medium was solidified with 7 g dm^{-3} agar, and the pH was adjusted to 5.8 before autoclaving at 121 ºC for 20 min. The cultures were grown in a growth

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Abbreviations: MS - Murashige and Skoog (1962) medium.
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chamber under a 16-h photoperiod from cool-white fluorescent tubes [irradiance of 60 μmol(photon) m^{-2} s^{-1}] at 25 ± 2 ºC.

For minimum growth experiments, sucrose was tested either alone at 5, 20, 30, 40 and 60 g dm^{-3} or at 20, 40 and 60 g dm^{-3} in combination with 20 g dm^{-3} mannitol supplemented in the same basal medium (MS + 0.91 μM zeatin). Five double-node cuttings (2.0 cm in length) (Fig. 1*A*) were cultured per glass-capped culture bottle (250 cm^3 capacity) containing 50 cm^3 of medium. After respective conservation period (4, 8 and 12 months) at 5 or 25 ºC in the dark, all the cultures were subcultured in multiplication medium under standard conditions as described above, and the percentage of viable cultures (cultures that can regenerate) was evaluated 6 weeks after subculturing. The shoot multiplication rate was assessed by scoring the total number of shoots produced per culture over the first three multiplication cycles, each of 6 weeks, following conservation.

The experiment was conducted with 4 replications, each representing 2 culture bottles (*i.e*. 10 double-node cuttings). The data were analysed by one-way analyses of variance (*ANOVA*) for each conservation period followed by mean separation by Duncan's New Multiple Range Test (P = 0.05) using the *SPSS* statistical package for *Windows* (release *11.0*, *SPSS Inc*., CA, USA). To analyze the data on rooting percentages, arcsin square root transformation was used.

The cultures of *D. lusitanicum* under conditions for minimum growth had much longer internodes than those not conserved. They continued to produce the lateral buds during conservation (Fig. 1*B*), but most of them degenerated over extended storage period. No regeneration occurred in the cultures conserved for 12 months independent of the culture medium or conservation temperature. In the present study, a reduced storage temperature was found to be critical for successful re-growth of cultures. No regeneration occurred when the cultures were conserved at 25 ºC after both 4 and 8 months. In fact, a reduced temperature has been successfully employed for minimum growth conservation of several plant species (Bekheet 2000, Bekheet *et al*. 2002).

None of the cultures maintained at 5 ºC in medium supplemented with a concentration of sucrose lower than 40 g dm^{-3} survived after 4 or 8 months of conservation. The increase of sucrose concentration from 40 to 60 g dm^{-3} resulted in a significant increase in culture

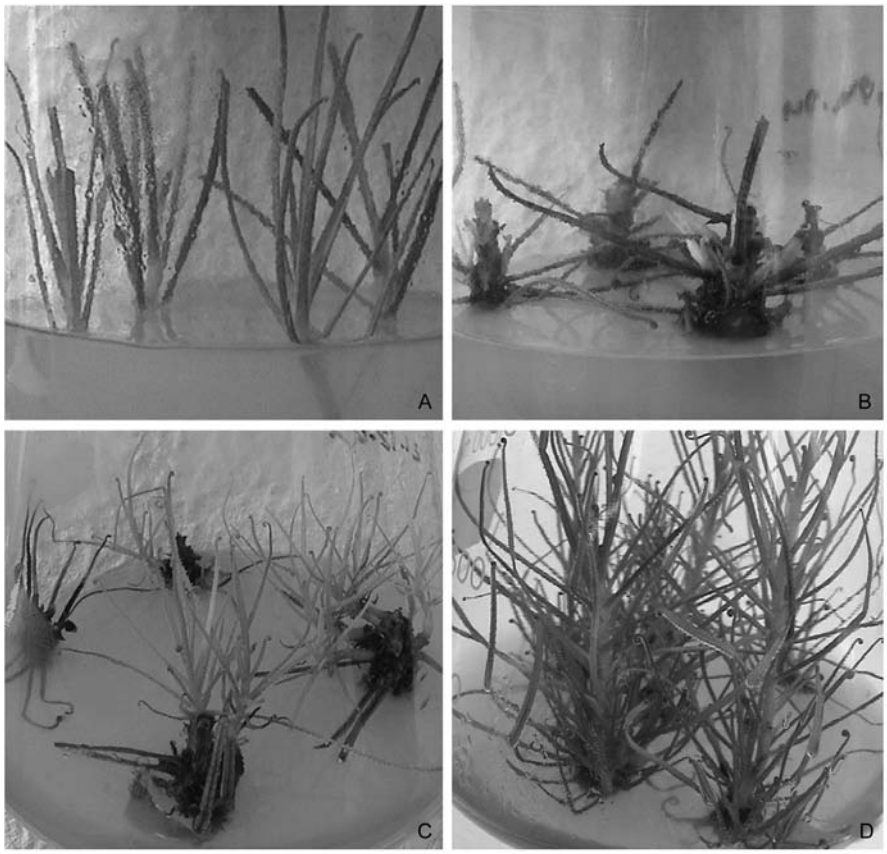

Fig. 1. *Drosophyllum lusitanicum* shoot cultures: *A* - before conservation; *B* - after 8 months at 5 ºC in the dark, in medium containing 40 g dm^{-3} sucrose and 20 g dm^{-3} mannitol; *C* - after 6 weeks on normal growth conditions, and *D* - during the second subculture on normal growth conditions.

Table 1. Percentage of viable cultures after 6 weeks on standard conditions and multiplication rate of *D. lusitanicum* cultures after conservation in different culture media at 5 ºC and in the dark. For multiplication rate, each value represents a mean ± SE of the first three multiplication cycles, each of 6 weeks, of 4 replications with 10 shoots. For each variable and conservation period, means with common letters are not significantly different at $P \leq 0.05$ (one-way *ANOVA*, Duncan's New Multiple Range Test).

Medium	Viability [%]		Multiplication rate	
	4 months	8 months	4 months	8 months
Control	-	-	3.8 ± 0.3 ab	3.8 ± 0.3 ab
40 g dm^{-3} sucrose	33 c	18 e	2.9 ± 0.4 bc	2.9 ± 0.3 cd
60 g dm^{-3} sucrose	70 d	38 d	3.6 ± 0.2 ab	4.3 ± 0.4 a
20 g dm^{-3} sucrose + 20 g dm^{-3} mannitol	85 a	68 c	2.6 ± 0.3 c	2.4 ± 0.2 d
40 g dm^{-3} sucrose + 20 g dm^{-3} mannitol	85 a	95 b	4.4 ± 0.4 a	3.4 ± 0.3 bc
60 g dm^{-3} sucrose + 20 g dm^{-3} mannitol	83 a	100 a	4.1 ± 0.3 a	3.7 ± 0.2 abc

survival (33 to 70 % and 18 to 38 %, after 4 and 8 months of conservation, respectively; Fig. 1*C*, Table 1). According to Gollagunta *et al*. (2005), growth, storage potential and post-storage performance of cultures are closely related to the sugar content. Several authors have observed increased survival of *in vitro* conserved cultures in culture medium containing high sugar concentration (Fletcher 1994, Sarkar *et al*. 1998, Gollagunta *et al*. 2005).

To restrict the growth of *D. lusitanicum* cultures, the modification of conservation medium by the incorporation of 20 g dm^{-3} mannitol was also investigated, because this non-metabolisable sugar alcohol has been reported to inhibit the growth of various species *in vitro* (Negash *et al*. 2001, Borges *et al*. 2004). The addition of mannitol significantly improved the survival of *D. lusitanicum* cultures ($P \leq 0.05$) (Table 1). After 8 months, maximum survival (100 %) was observed when the cultures were conserved on medium containing 60 g dm^{-3} sucrose plus 20 g dm^{-3} mannitol. The slightly higher values for survival observed in some culture media after 8 months of conservation, compared to 4 months could indicate adaptation to low-temperature (Negash *et al*. 2001).

The multiplication rate of cultures conserved for 4 months in medium with 60 g dm^{-3} sucrose, 40 g dm^{-3} sucrose + 20 g dm^{-3} mannitol or 60 g dm^{-3} sucrose + 20 g dm^{-3} mannitol was not significantly different from the control ($P > 0.05$; Table 1). When the conservation period was extended to 8 months the results were similar, excepting for cultures conserved in medium containing 40 g dm^{-3} sucrose + 20 g dm^{-3} mannitol. Healthy shoots with vigorous growth and without vitrification symptoms or other apparent abnormalities were observed after the third subculture (Fig. 1*D*).

This study describes an efficient and simple protocol for conservation of *D. lusitanicum* cultures under minimum growth conditions. The results presented show that cultures can be conserved *in vitro* for 8 months in medium containing high sucrose concentration and mannitol at 5 ºC and in the dark without any subculturing. The recovered shoots multiplied normally, without losing regeneration capacity and without apparent morphological changes.

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