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BRIEF COMMUNICATION

In vitro propagation of *Drosera intermedia* in a single step

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Abstract

A simple and efficient protocol for the micropropagation of *Drosera intermedia,* using cultures initiated from *in vitro* produced seedlings, is described. Shoot proliferation was significantly influenced by Murashige and Skoog (MS) macronutrient concentration, showing higher multiplication rates for ¼ MS (the lowest concentration), but was not affected by the addition of 0.1 mg dm^{-3} kinetin. In all cases a multiplication percentage above 90 % was recorded. High rooting percentages (up to 100 %) were obtained in multiplication phase on ¼ MS medium without growth regulators. In average 15.8 plantlets per initial shoot was produced after 8 weeks of culture. All plantlets were successfully acclimatized to *ex vitro* conditions, exhibiting normal development.

Additional key words: carnivorous plant, *Droseraceae*, kinetin.

Drosera is a widely distributed genus, comprising 152 carnivorous species occurring globally, with its centre of distribution in Oceania, where over 40 species are found in southwest Australia alone (Juniper *et al.* 1989, Bekesiová *et al.* 1999). Several compounds with biological activity, mainly naphthoquinones and flavonoids have been identified in *Drosera* species (Marckzak *et al.* 2005). The naphthoquinone plumbagin, a compound with a broad spectrum of biological and pharmacological activities (Didry *et al*. 1998, Sugie *et al*. 1998, Min *et al*. 2002), has been identified in *D. intermedia* (Budzianowski 1996). *D. intermedia* (Fig. 1*A*) is an obligate wetland species that is typically found in nutrient-poor peat lands including rain-fed bogs and poor fens (Juniper *et al.* 1989). However, dewatering, drainage in order to create productive land and cattle grazing are serious threats to these habitats and the conservation of *D. intermedia*. Taking into account the declining natural populations, the development of efficient micropropagation protocols is critical as they can be used to replenish wild stocks and provide an alternative source of plant material for bioactivity assays (Chen *et al*. 2008, Rout *et al*. 2008). In fact, micropropagation protocols for several carnivorous species have been described lately (Jang *et al.* 2003, Gonçalves and Romano 2005, Gonçalves *et al.* 2008). Most micropropagation protocols of *Drosera* species use leaf segments or shoot tips as starting material (Bobák *et al*. 1995, Kawiak *et al.* 2003) while in this work cultures were initiated from seeds. *In vitro* cultures of *D. intermedia* have been used to study its content in naphthoquinones, however, a micropropagation procedure for *D. intermedia* has not been described hitherto. The aim of the present work was to develop a simple and efficient method to produce large amounts of *D. intermedia* plants to replenish natural populations and for plumbagin extraction.

Seeds of *Drosera intermedia* Hayne were collected from plants growing in a natural population near the Sado estuary, Portugal. The plant material was authenticated by Dr. A.I. Correia from the Botanical Garden of the University of Lisbon and a specimen voucher has been preserved under the number LISU231581. Seeds were surface sterilized with commercial bleach 15 % (v/v) (5 % of sodium hypochlorite) for 15 min, and washed 3 times in sterile water. Seeds were inoculated directly (control) or immersed in sterile water and stored at 5 ºC for one week before inoculation (cold stratification treatment). For each treatment 3 replicates of 10 seeds were tested. Seeds were germinated in test tubes

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Abbreviations: KIN - kinetin; MS - Murashige and Skoog; PGR - plant growth regulator.

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containing 10 cm^3 of ¼ Murashige and Skoog (1962; MS) medium, 2 % (m/v) sucrose and 1 % (m/v) agar. Media pH was adjusted to 5.75 before autoclaving at 121 ºC for 20 min. All cultures were incubated under a 16-h photoperiod provided by cool white fluorescent lamps at a photon flux density of 40 µmol m^{-2} s^{-1}, at a temperature of 25 ± 2 ºC and monitored every week for germination for a period of 7 weeks.

Seedlings (Fig. 1*B*) 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. Media with three concentrations of MS macronutrients (total MS, ½ MS and ¼ MS) supplemented with 0.1 mg dm^{-3} of KIN or without plant growth regulators (PGR) were tested. For each medium 4 repetitions with 10 explants each were performed. 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 on all tested media, rooting frequency, number of roots and root length were also evaluated. Plantlets with well-developed roots and shoots originating from ¼ MS medium were selected for acclimatization. They were transplanted to 100 cm^3 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, after which they were completely opened. The success of acclimatization was determined as the survival rate. Four replicates with 10 plantlets each were carried out. 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, USA). Significant differences between means were determined using Duncan's new multiple range test ($P = 0.05$).

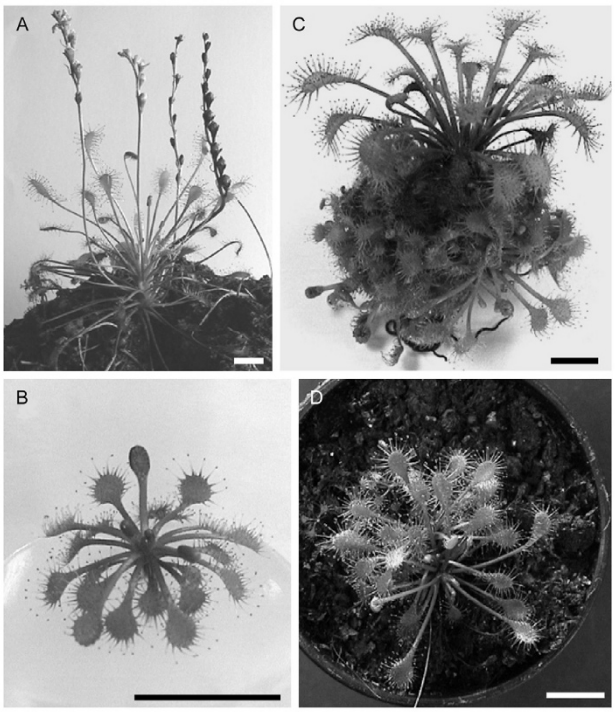

Fig. 1. Micropropagation of *D. intermedia*: *A* - field specimen, *B* - seedling explant, *C* - shoot after 8 weeks of culture, *D* - acclimatized plant after 10 weeks in *ex vitro* conditions (*bars* = 1 cm).

All seeds were successfully sterilized and germination occurred during the second week after inoculation. Higher germination rates were obtained for the control (84.2 ± 3.9 %) than for the cold stratification treatment (65.7 ± 6.7 %), although differences were not significant ($P \geq 0.05$). These results are interesting as many *Drosera* species need a cold treatment for breaking dormancy and germinate (Jang and Park 1999, Jang *et al*. 2003, Jayaram and Prasad 2006). Seedlings had a high proliferation capacity, producing enough explants for the subsequent experiments.

The cultures had a high proliferation capacity in PGR-free media and in media supplemented with KIN (Figs. 1*C*, 2*A*). As concerns the percentage of shoots with proliferation capacity, no significant differences were observed either among the MS macronutrient concentrations or between the control or KIN supplemented media. In all cases multiplication percentages above 90 % were recorded. The highest number of shoots was obtained on PGR-free ¼ MS medium (18.2 ± 1.9) and ¼ MS medium with KIN (18.2 ± 1.2). No significant differences were observed between the PGR-free and the KIN supplemented media for the mean number of shoots (Fig. 2*B*). From these results, we can conclude that decreasing the MS macronutrient concentration significantly enhances the multiplication of *D. intermedia* shoots and that the use of KIN does not affect proliferation. The promotion of shoot proliferation by media with low concentration of macronutrients has similarly been reported for other carnivorous plants (Jang *et al*. 2003, Kim and Jang 2004, Gonçalves *et al*. 2008) and seems to be a characteristic for this group.

Rooting frequency of initial explants was not affected by MS macronutrient concentration or by the presence of KIN (Fig. 2*C*). High rooting percentages were obtained in PGR-free media and also in media with KIN, reaching 100 % in some cases (Fig. 2*C*). Taking into account that rooting percentages close to 100 % were obtained in all tested media, an additional rooting phase on medium with auxins was unnecessary. Noteworthy, the new developed shoots also rooted in very high percentages (Fig. 2*D*). Over 80 % of the produced shoots rooted in all tested media, except for shoots cultured in ¼ MS medium supplemented with KIN which was statistically the less effective medium ($P < 0.05$). These results show that large amounts of *D. intermedia* plantlets can be produced in one single step. On average, one shoot cultured on PGR-free ¼ MS medium produces 15.8 plantlets in an 8 weeks period.

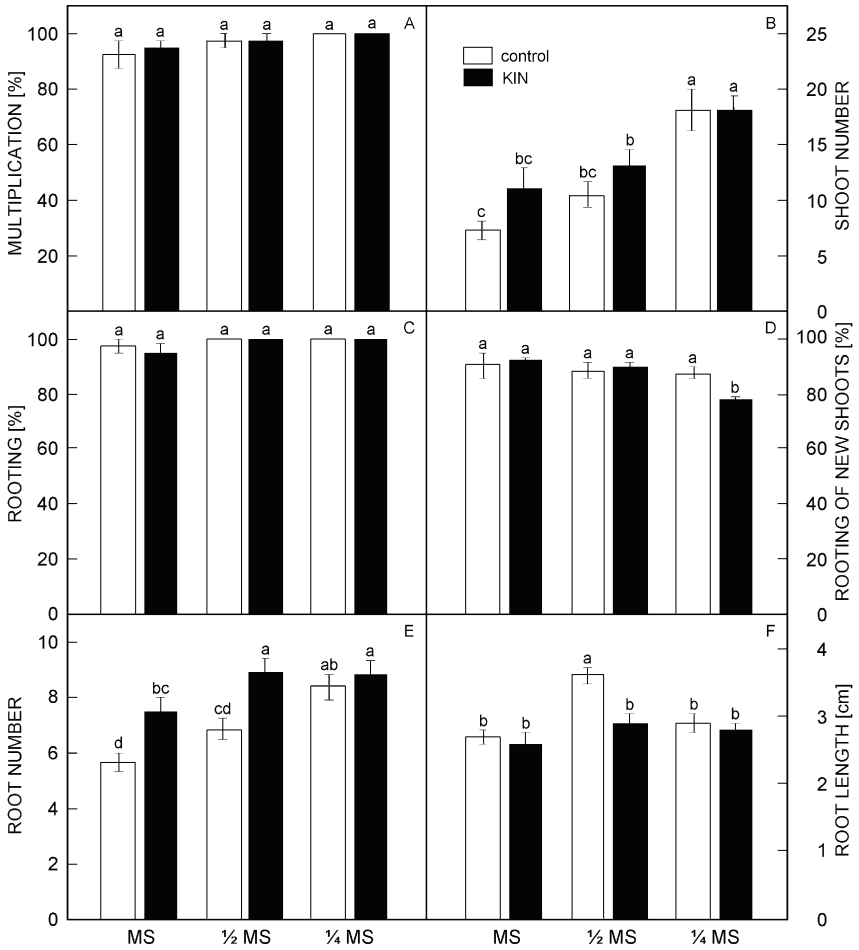

Fig. 2. Effect of MS medium concentration and addition of KIN (0.1 mg dm^{-3}) on proliferation and rooting of *D. intermedia* shoots: *A* - multiplication percentage, *B* - mean number of developed shoots, *C* - rooting percentage of initial explant, *D* - rooting percentage of the new produced shoots, *E* - mean number of developed roots per initial shoot, *F* - longest root length in initial shoot. Control: PGR-free media. 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.

Culture media supplemented with KIN significantly enhanced the number of roots produced ($P < 0.05$), with ¼ MS (8.9 ± 0.5) and ½ MS (8.9 ± 0.5) being the most effective (Fig. 2*E*). On the contrary, the longest roots were obtained in ½ MS medium without PGR, with an average length of 3.6 ± 0.1 cm (Fig. 2*F*). *In vitro* produced plantlets did not show any apparent morphological variation and 100 % of regenerated plantlets were successfully acclimatized to *ex vitro* conditions (Fig. 1*D*). Their leaves were functional and able to catch preys, and their flowers produced capsules and seeds normally.

This study reports a simple and efficient protocol producing a high number of *D. intermedia* plants. Shoots, obtained from seedlings, showed high proliferation and rooting capacity and overall, the medium found to be best was PGR-free ¼ MS medium. An average of 15.8 plantlets per initial explant can be obtained in one single step. The regenerated plantlets could be used to replenish declining populations in the nature, making a valuable contribution to the conservation of the species.

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