In Vitro Growth and Regeneration of *Drosera spatulata* Labill. on Various Media

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Abstract. Conditions for in vitro multiplication and flowering of *Drosera spatulata* plants were established. Shoot tips of greenhouse-grown plants were sterilized with 1% or 0.5% sodium hypochlorite. The influence of different media concentrations, hormone supplementation, and pH was investigated. Full MS medium without growth regulators was the best for regeneration and multiplication of plants. Regenerated shoots rooted spontaneously on medium without growth regulators and without transfer to additional medium. In 3 months, 100 to 200 plants were generated per explant. Flowering was induced on media supplemented with plant growth regulators. Plants were acclimatized on sterile peat.

Plumbagin, a phytochemical produced by the carnivorous genus *Drosera* (Bonnet et al., 1984b; Crouch et al., 1990; Gupta et al., 1993), is extracted for pharmaceutical preparations because of its antispasmodic and antibiotic activity (Didry et al., 1986; Wurm et al., 1984). If carnivorous plants are to be used for investigations or extractions of useful chemicals, many plants must be available. Since their natural populations are scarce, in vitro propagation would be useful. Until now, the following species of genus *Drosera* have been successfully cultured in vitro: *D. binata* La Billardiere (Anthony, 1992; Caniato et al., 1989), *D. capensis* L. (Anthony, 1992; Caniato et al., 1989; Crouch et al., 1990), *D. natalensis* Diels (Crouch and Van Staden, 1988; Crouch et al., 1990; Finnie and Van Staden, 1993), *Drosera-regia* Stephens (Janssens, 1986), *D. rotundifolia* L. (Anthony, 1992; Bonnet et al., 1984a; Kukulczanka and Czastka, 1987; Simola, 1978), and *D. spatulata* Labill. (Blehova et al., 1990, 1992). However, a completely defined medium for *D. spatulata* (the sundew) has not been developed and seeds have been the only material successfully sterilized for primary culture establishment. Consequently, investigations were conducted to determine the most suitable, fully defined medium for rapid propagation and flowering.

Materials and Methods

Shoottips, 4 to 6 mm long, of well-developed, greenhouse-grown *D. spatulata* (>2.5 cm in diameter) were used as initial explants. Roots and lower leaves were cut off and shoot apices were sterilized with either 70% ethanol (5 sec), 3% sodium hypochlorite (SH) from Izosan G (Pliva, Zagreb) (5 min); 70% ethanol (5 sec), 2% SH (5 min); 1.5% SH (5 min); 1% SH (5 min); 0.5% SH (7 min); 6% H₂O₂ (15 min); 3% H₂O₂ (20 min). All of the methods used were followed by three 3-min washes in sterile distilled water.

Explants were inoculated separately on full-strength MS medium (Murashige and Skoog, 1962) supplemented with sucrose (30 g·L⁻¹) and agar (8 g·L⁻¹) (Biolife, Milano), in glass culture tubes (30 × 120 mm) containing 10 mL of medium. Medium was adjusted to pH 5.7 with NaOH before autoclaving. After 4 months in culture, plants were subcultured on full-strength MS medium and 3/4, 1/2, 1/4, 1/8 macro salts dilutions of the medium supplemented as noted, or on B medium (Boxus, 1974) and its 1/2, 1/4, and 1/8 macro salts dilutions supplemented with glucose (40 g·L⁻¹) and agar (8 g·L⁻¹). In this experiment, in vitro-derived single leaves, 12 to 14 mm long, also were inoculated. In all other experiments, whole plants (≈8 to 15 mm in diameter) were used for subculturing. Each experiment had 24 replicate cultures per treatment and every experiment was conducted twice. All cultures were maintained at 24 ± 4 °C under a 16-h photoperiod of cool-white light (40 μmol·m⁻²·s⁻¹).

Effects of growth regulators also were investigated. 1/4 MS medium was supplemented with (in μM): indole-3-butyric acid (IBA) (1.97, 4.9, 9.8); indole-3-acetic acid (IAA) (5.7) plus kinetin (K) (9.3); IAA (22.8) plus K (9.3); α-naphthaleneacetic acid (NAA) (0.54 or 5.4)

plus K (9.3); IAA (0.143) plus 6-benzylaminopurine (BA) (0.44); IBA (0.12) plus BA (0.44); NAA (0.13) plus BA (0.44); IAA (0.143) plus K (0.46); IBA (0.12) plus K (0.46); NAA (0.13) plus K (0.46). B medium was supplemented with (in μM): IBA (4.9), BA (4.44) plus IBA (4.9).

To estimate the influence of pH on plant growth, whole plants were transferred from 1/4 MS (pH 5.7) to 1/4 MS medium, adjusted to pH values of 4.0, 5.0, 6.0, and 7.0, before autoclaving. Average size of plants was estimated 8 weeks after inoculation.

Twenty plants grown on full-strength and 20 grown on each diluted MS medium were acclimatized on sterile peat in plastic containers (6.5 cm in diameter) enclosed in vented plastic bags. After 18 ± 2 days of growth in a temperature-controlled room at 24 ± 4 °C and a 16-h photoperiod of cool-white light (40 μmol·m⁻²·s⁻¹), plastic bags were gradually removed. Plants were transferred to a greenhouse and surviving plants were counted 2 months after transfer to ex vitro conditions.

Mean values and standard deviations were used for analysis and interpretation of the results.

Results and Discussion

The main problem with establishment of carnivorous plants in vitro is effective sterilization of explants (Anthony, 1992). In our experiment, shoot tips disinfected with 1% or 0.5% SH yielded 20% and 10% sterile explants, respectively. Ethanol used in combination with 2% or 3% SH or 1.5% SH alone caused all explants to turn brown and decay. Sterilization with H₂O₂ was unsuccessful.

Presumably, sterile explants were inoculated separately on full-strength, hormone-free MS medium, and after 4 months in culture each tube contained 200 to 300 plantlets. Plants and single leaves derived from that primary culture were subcultured on full-strength and diluted MS and B media. Regeneration of plants was high on both tested media (Table 1). When single leaves were subcultured, regeneration occurred 2 weeks after that on whole plants, but was high (83% to 100%) nevertheless.

MS medium proved to be more suitable for *D. spatulata* growth than B medium. Multiplication of plants decreased with diluting the medium (Table 1). This result was unexpected, since carnivorous plants normally grow in nutrient-poor areas (Juniper et al., 1989), and experimental work on the genus *Drosera* shows that these plants readily and even more successfully grow on diluted culture media (Anthony, 1992; Crouch and Van Staden, 1988; Janssens, 1986; Kukulczanka and Czastka, 1988; Simola, 1969).

Addition of growth regulators in the combinations and concentrations used by us retarded plant growth and multiplication rate. When NAA was used alone or in combination with other growth regulators, shoots formed multiple and extremely thick roots and only 50% of the plants had regenerated shoots 6 weeks after subculturing. In contrast, plants

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Table 1. Effect of medium (hormone-free) and strength on regeneration and multiplication of *Drosera spatulata* plants 6 weeks after subculturing of whole plants.

Medium ^a and strength	Regeneration (%)	Plants/tube (mean values $\pm$ SD)
MS		
Full	100	43.0 $\pm$ 2.1
3/4	92	40.8 $\pm$ 3.2
1/2	100	39.0 $\pm$ 2.4
1/4	100	37.7 $\pm$ 2.6
1/8	83	35.2 $\pm$ 3.3
B		
Full	92	17.2 $\pm$ 1.5
1/2	100	14.2 $\pm$ 2.7
1/4	100	12.2 $\pm$ 2.4
1/8	83	10.8 $\pm$ 2.1

^aMS = Murashige and Skoog medium; B = Boxus medium.

subcultured on MS medium without growth regulators usually produced adventitious shoots before root development.

In vitro-produced plants flowered in the following media: 100% on 1/4 MS with 0.12 μ M IBA plus 0.44 μ M BA; 67% on 1/4 MS with 0.143 μ M IAA plus 0.44 μ M BA; 50% on 1/4 MS with 0.143 μ M IAA plus 0.46 μ M K; and 50% on B medium supplemented with 4.9 μ M IBA plus 4.44 μ M BA. Although *Drosera* species spontaneously flower under in vitro conditions, the results of our experiments show that it is possible to control time of flowering with growth regulators, which permits having flowering plants in various periods of the year, thus allowing investigation of related biochemical, physiological, and other properties.

Plant growth was strongly affected by medium pH. Plants inoculated on 1/4 MS medium adjusted to pH 4 had the biggest average diameter (3 cm). Average diameter of plants grown on the medium adjusted to pH 5, pH 6, and pH 7 was 2.3, 2.2, and 2.1 cm, respectively. However, since sundews grow on acidic soils (Juniper et al., 1989), we expected that growth would be better under low pH.

Plants grown on full-strength and diluted MS media were acclimatized, and after 2

months of growth ex vitro, 80% of plants survived. Media dilution had no effect on plant survival.

Growth and multiplication of *D. spatulata* was possible on all media tested. In our experiments, we found that MS medium without growth regulators was the best for regeneration and multiplication of plants. It was not necessary to use the usual three-stage system for shoot induction, multiplication, and rooting, respectively. Shoots rooted spontaneously on medium without growth regulators and without transfer to additional medium.

The main problem with *Drosera* tissue culture is sterilization of plant material. Even in subcultures, contamination was not completely eliminated, probably due to the symbiotic microorganisms in plants (Anthony, 1992). We managed to apparently sterilize shoot tips of *D. spatulata* and establish optimal conditions for rapid multiplication and flowering of this species. Although most of the plant material is lost during sterilization, this is negligible in comparison to the number of plantlets that could be generated in culture in the short time. In our experiments, we obtained 100 to 200 regenerated plantlets from a single explant (plant or leaf) in 3 months.

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