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In vitro propagation of *Drosera intermedia* as influenced by cytokinins, pH, sucrose, and nutrient concentration

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Abstract

This study was aimed to optimize *in vitro* propagation of *Drosera intermedia* for commercial and conservation purposes. The effect of concentration of MS nutrients (⅛ MS, ¼ MS, ½ MS and, MS), various pH (3.7-7.7), sucrose concentration (10-40 g l^{-1}) and cytokinins (0.1-3 mg l^{-1}), namely BA (N6-benzyladenine), kinetin and zeatin were evaluated. After 60 days of shoot cultivation, growth and developmental characteristics (plant height, number of shoots per explant, diameter of rosette, number of roots per explant, length of roots) were recorded. No significant differences were found for various levels of pH and sucrose. On the contrary, plant height was negatively influenced by an increase of nutrients in the medium. The plants on 1/8 MS medium were significantly taller, and displayed higher proliferation capacity compared with those cultivated on full-strength MS medium. Shoot multiplication and growth was suppressed by supplementation of BA and kinetin, regardless of concentration used. Zeatin at the lowest concentration (0.1 mg l^{-1}) provided the best results for shoot proliferation of all 26 treatments and can be recommended for micropropagation of *D. intermedia*.

Key words: Droseraceae, Micropropagation, Nutrient concentration, Oblong-leaved sundew, and Zeatin

Introduction

The *Drosera* genus (Droseraceae) consists of carnivorous plants with active flypaper traps and includes nearly 150 species distributed in Australia, Africa, and South America, with some Northern Hemisphere species (Rivadavia et al., 2003). Some species, mainly *Drosera rotundifolia* L., *D. intermedia* Hayne and *D. anglica* Huds., are commonly used in traditional medicine as the therapy of respiratory tract (Paper et al., 2005; Fukushima et al., 2009). Recently, several bioactive compounds from sundew leaves and roots including flavonoids and quinones have been found (Hook, 2001; Marczak et al., 2005; Putalun et al., 2010). These compounds can be considered as very important metabolites (Milella et al., 2011; Padula et al., 2013). In addition, to the use for pharmaceutical applications (Biteau et al., 2012), *Drosera* species have captured the interest of botanists and horticulturists because of their unique biology and carnivorous habit (Kawiak et al., 2003; Wolf et al., 2006), and today they are considered economically important ornamental plants (Barthlott et al., 2004).

D. intermedia is an attractive species of potential horticultural value. It is sized between *D. anglica* and *D. rotundifolia*, bearing wedge-shaped leaves, 7-12 mm long, and 4-10 mm wide, in a rosette. All the tentacles on the leaf surface are of symmetric length. Inflorescences are cymose or racemose, more robust than those of *D. rotundifolia* composed of white flowers (Wynne, 1944; Crowder et al., 1990). *D. intermedia* had been used for artificial hybridization with *D. anglica* and *D. capillaris* (Kusakebe, 1979). Subesquently, a spontaneous hybrid *D. intermedia* x *D. capillaris*, interspread between colonies of the two species, was discovered (Sheridan, 1987).

In nature, *D. intermedia* grows in the acidic parts of valley mires that are flooded in winter, and subject to drying out in summer, but it is also widespread in persistent pools. In most natural sites of Europe and North America, it is becoming less frequent because of land drainage and uncontrolled collections for medicinal and ornamental purposes (Crowder et al., 1990; Kawiak et al., 2003; Kawiak and Lojkowska, 2011). Therefore, *in vitro* propagation would be a useful tool for conservation

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of germplasm and as a source of plants for commercialization of the species (Jimenez et al., 2011; Swart et al., 2012).

In *Drosera* genus, micropropagation has been successfully optimized for *D. indica* (Jayaram and Prasat, 2007), *D. aliciae* (Kawiak and Lojkowska, 2011), *D. peltata* (Kwang-Soo and Go-Won, 2004), *D. rotundifolia* (Bobak et al., 1995), *D. capensis* (Jiménez et al., 2011), *D. spatulata* (Bobak et al., 1993, Perica and Berljak, 1996), and *D. anglica*, *D. binata* and *D. cuneifolia* (Kawiak et al., 2003).

Recently, there was just one brief report on *in vitro* micropropagation of *D. intermedia* (Grevenstuk et al., 2010). Thus, the aim of this study was to establish an efficient micropropagation protocol for this vulnerable and attractive species, extensively testing responses to different abiotic factors, especially on the concentration of mineral salts, plant growth regulators and sucrose in cultivation media, as well as various pH of the media.

Materials and Methods

Plant material and establishment of *in vitro* culture

As the initial plant material for the experiment, the seeds of *Drosera intermedia* (Droseraceae) were used. They were obtained via *Index Seminum* from Universitatea Babeş-Bolyai, Gradina botanica-Al Borza, Cluj-Napoca, Romania, in the year 2009. The seeds were disinfected in 70% ethanol for 1 min, followed by 20-25 minutes in 2% NaClO containing Tween 20 (1ml l^{-1}). After sterilization, the plant material was rinsed three times for one minute in sterile, distilled water, and placed on half-strength MS medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 8 g l^{-1} agar, 100 mg l^{-1} *myo*-inositol and pH adjusted to 5.7. Cultures were maintained at 25/23°C under a 16/8 h light/dark regime with 36 µmol m^{-2} s^{-1} of cool white fluorescent light. The seedlings (ca 5 mm in height), obtained from the seeds after 30 days of cultivation were used for multiplication experiment.

In vitro multiplication of plants

For multiplication, in total of 25 treatments were tested. All media were derived from basal medium, consisting of half-strength MS medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 8 g l^{-1} agar, 100 mg l^{-1} *myo*-inositol and pH adjusted to 5.7. This medium was also used as control.

In order to investigate optimal concentration of sucrose for multiplication, media containing 10 g l^{-1}, 20 g l^{-1}, 30 g l^{-1} and 40 g l^{-1} sucrose were tested. The effect of nutrient concentration was investigated using full-strength MS, ½ MS, ¼ MS and ⅛ MS media. To detect the influence of various pH, the media pH was adjusted to 3.7, 4.7, 5.7, 6.7 and 7.7 using KOH (1 M). The cytokinin effect was tested on media enriched by cytokinins, namely kinetin, N6-benzyladenine (BA) and zeatin at four concentrations (0.1, 0.5, 1, 3 mg l^{-1}). The media were prepared in Erlenmeyer flasks (100 ml) and sterilized by autoclaving (20 min, 121°C, 100 kPa).

Cultures were maintained under the cultivation conditions described above. After 60 days of cultivation, plant height, the number of shoots per plant, diameter of rosettes, and the number and length of roots were recorded.

Experimental design and statistical analysis

All experiments were repeated twice with 15 replications per treatment. The experiment was arranged in a completely randomized block design. The data were subjected to analysis of variance (ANOVA) and the least significant ($p < 0.05$) differences among mean values were estimated using Fisher's LSD test [StatSoft STATISTICA 12.0].

Ex vitro transfer and acclimatization

Well rooted plants (having at least 4 roots longer than 10 mm) were rinsed with tap water to remove the adhering medium and then planted separately in pots (50 x 50 mm) filled with a mixture of peat moss and perlite 1:1 (v/v). The plants were grown under high air humidity (85-90%) for 7 days. These conditions were maintained by covering the potted plants with pored polythene bags. From the 15th day, the plastic bags were gradually removed to reduce the relative humidity (ca 70%). The 30th day, the plastic bags were completely removed. The success of acclimatization was determined after 8 weeks based on survival percentage.

Results

Multiplication

The results revealed that plants cultivated on medium with lower concentrations of sucrose (10 or 20 g l^{-1}) show higher proliferation capacity and had a higher diameter of rosettes, although the differences among these characteristics were not statistically significant when compared to other treatments. Moreover, these plants were rather tiny and displayed the tendency to produce flowers under *in vitro* conditions. On the contrary, a treatment containing 40 g l^{-1} sucrose produced thick lower plants, with smaller diameter of rosettes, in some individuals of abnormal morphology. In all treatments, plant height, number of roots per plant and their length, were comparable (Table 1).

Table 1. Effect of nutrient concentration, pH, sucrose level and cytokinins on growth and developmental characteristics in *Drosera intermedia.* Data were recorded after 60 days of culture.

Tested treatments	Plant height (mm)	No of shoots/explant	Diameter of rosette (mm)	No of roots/explant	Length of roots (mm)
Nutrient concentration					
⅛ MS	24.34±3.60ef	4.27±1.32ab	20.16±0.04cd	6.67±1.03abc	37.50±2.74efg
¼ MS	21.35±3.32def	2.18±1.07ab	27.52±2.74d	6.63±1.39abc	39.13±4.05fg
½ MS	20.10±4.46def	2.60±2.08ab	26.03±8.28d	8.67±2.41abc	37.51±2.72efg
MS	12.30±2.24bcd	3.17±1.83ab	17.72±4.74bcd	11.57±2.71c	25.73±4.66bcd
pH					
½ MS pH 3.7	15.22±3.85cde	2.17±1.47ab	22.89±5.27cd	5.65±3.47abc	39.23±4.03fg
½ MS pH 4.7	17.47±4.17def	2.51±2.36ab	26.67±7.63d	6.77±5.82abc	46.57±5.17g
½ MS pH 5.7	20.03±5.21def	2.48±2.17ab	25.13±7.88d	8.97±2.28abc	36.48±2.54efg
½ MS pH 6.7	14.82±4.73cde	2.44±1.75ab	21.47±2.54cd	4.08±1.89ab	34.07±4.90def
½ MS pH 7.7	15.20±3.70cde	3.13±2.16ab	23.13±4.28cd	9.51±5.68abc	28.29±8.16cde
Sucrose concentration					
10 g l^{-1} sucrose	25.27±9.43f	4.13±2.16ab	26.56±4.58d	11.50±4.10c	21.33±9.88bc
20 g l^{-1}sucrose	18.33±3.98def	4.32±2.75ab	25.93±7.72d	9.46± 4.93abc	28.33±4.06cde
30 g l^{-1} sucrose	19.02±4.44def	3.16±2.04ab	25.41±8.35d	9.67±3.34abc	37.63±2.54efg
40 g l^{-1} sucrose	17.91±6.43def	3.33±2.42ab	20.85±8.07cd	10.51±4.48bc	25.04±4.45bcd
Cytokinins					
0	19.51±4.37def	2.53±2.10ab	26.03±7.37d	8.58±2.32abc	36.99±2.64efg
Kinetin (mg l^{-1})					
0.1	4.79±0.43ab	1.00±0.00a	5.32±1.19ab	0.00±0.00	0.00±0.00
0.5	4.50±2.27ab	1.00±0.00a	4.87±0.42ab	0.00±0.00	0.00±0.00
1	4.18±0.96ab	1.00±0.00a	5.03±1.27ab	0.00±0.00	0.00±0.00
3	4.02±1.24ab	1.00±0.00a	4.66±1.03ab	0.00±0.00	0.00±0.00
BA (mg l^{-1})					
0.1	6.19±2.08abc	1.00±0.00a	4.42±1.51ab	0.00±0.00	0.00±0.00
0.5	6.28±2.12abc	1.00±0.00a	3.67±1.52ab	0.00±0.00	0.00±0.00
1	4.99±0.89ab	1.00±0.00a	3.33±1.05ab	0.00±0.00	0.00±0.00
3	1.96±1.45a	2.32±1.51ab	1.75±1.30a	2.57±2.06a	2.33±2.06a
Zeatin (mg l^{-1})					
0.1	25.92±3.66f	10.51±2.50c	41.34±4.12e	8.64±3.68abc	18.31±2.41b
0.5	17.27±8.68def	5.17±1.17b	27.17±11.52d	5.84±1.57abc	6.17±2.86a
1	13.19±5.76bcd	4.54±2.27ab	19.67±9.27cd	2.67±1.96a	6.00±2.90a
3	5.85±2.14abc	2.65±0.53ab	10.82±2.06abc	0.00±0.00	0.00±0.00

[] Mean values in column followed by the different letters are significantly different according to the Fisher's LSD test ($p < 0.05$).

The effect of nutrient concentration on measured characteristics after 60 day cultivation is shown in Table 1. It is evident that plant height was negatively influenced by an increase of nutrients in the medium. The plants on ⅛ MS medium were significantly higher compared to those cultivated on full strength MS medium. In addition, they displayed high proliferation capacity, though the statistical differences were not confirmed for this characteristic. Reducing the concentration of nutrients did not have any effect on diameter of resettes and number of roots. On the contrary, the roots of plants cultivated on media with decreased concentrations of nutrients were significantly longer and thinner in comparison to those of plants grown on full-strength medium (data for root thickness are not shown).

When the effect of different pH values on growth and development characteristics was investigated, almost no significant differences among treatments were observed (Table 1). Overall, media with lower pH level (3.7, 4.7) produced tiny plants, while individuals cultivated on media with higher values of pH (6.7, 7.7) were rather thick and robust. These differences, however, did not have any impact on success of *ex vitro* transfer and acclimatization of plants.

Plant growth and development was strongly affected by cytokinins used. Homogeneous behavior was observed for the cultures on media, supplemented with BA and kinetin at all concentrations (Table 1). These treatments produced extremely dwarf plants, morphologically abnormal (Figure 1A). Red pigmentation and necrosis in these cultures abundantly occurred. Of these treatments, only 3 mg l^{-1} BA provided some multiplication and root formation (Table 1). However, the plants were even smaller compared to the other treatments, and the necrosis was more frequent in these cultures.

In contrast, zeatin proved to be optimal for *D. intermedia* propagation. This treatment provided the highest shoot proliferation of all 26 media tested (Figure 1B). Also the diameter of rosettes and length of roots were significantly higher when compared to other cytokinin treatments. The increasing concentration of zeatin suppressed the growth and development of cultures.

Ex vitro transfer and acclimatization

Shoot clusters developed on media were divided into separate shoots and transferred *ex vitro*. Only well rooted plants were used for *ex vitro* transfer (in total 500 plants), survival rate after 8 weeks was 98.6%. Plants displayed vital and vigorous growth. Any abnormalities, observed *in vitro* gradually disappeared during further 4 weeks.

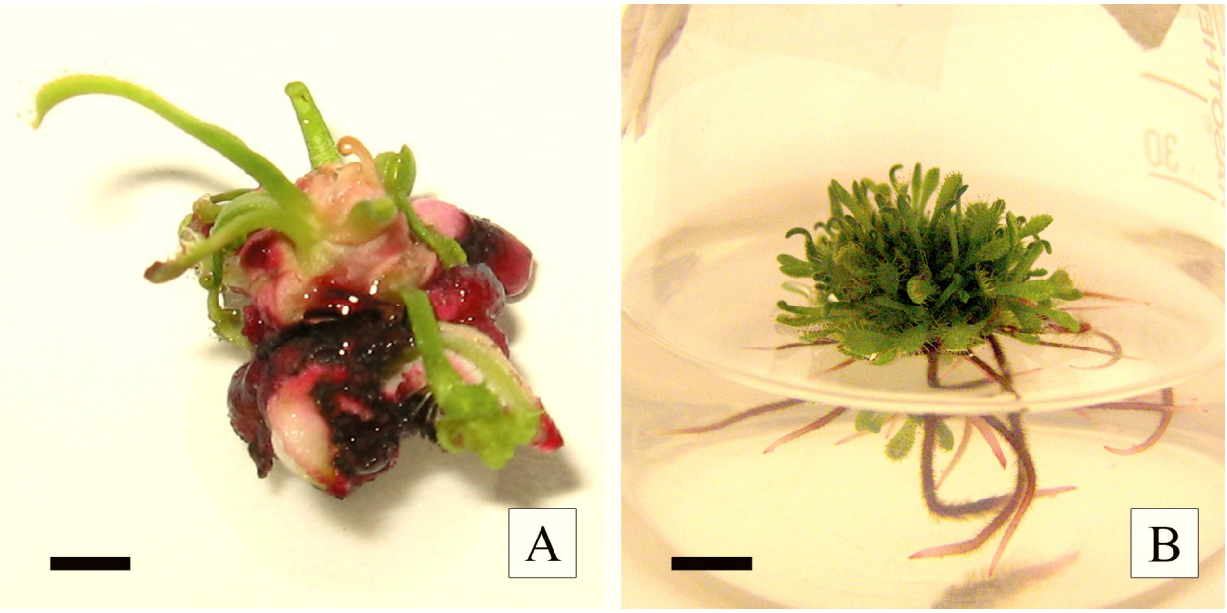

Figure 1. Micropropagation of *D. intermedia*. (A) Plant after 60 days of cultivation on ½ MS medium with 1 mg l^{-1} kinetin; bar = 1 mm. (B) Shoot multiplication on ½ MS with 0.1 mg l^{-1} zeatin after 60 days of cultivation; bar = 10 mm.

Discussion

In the present work, protocol for effective *in vitro* propagation of *Drosera intermedia* was established. This is the first complex study on multiplication of this species, extending the previous research of Grevenstuk et al. (2010).

Since *Drosera* plants grow normally in nutrient poor habitats (Jayaram and Prasad, 2007), different strengths of MS medium had been tested. The superiority of ⅛ MS medium over other concentration of MS medium was shown, although ¼ MS and ½ MS media also provided satisfactory results. Similarly, Grevenstuk et al. (2010) recommended for multiplication of the same species ¼ MS medium, the least concentrated medium, which had been tested in the study. Unlike our results, in *D. spatulata*, shoot proliferation was significantly decreased on diluted MS medium (Perica and Berljak, 1996), and in *D. indica*, the plant multiplication was not markedly influenced by different nutrient concentrations (Jayaram and Prasad, 2007).

Surprisingly in this study, no significant differences were detected between different pH (3.7-7.7). Since sundews grow in natural sites on acid soils, where pH ranges between 3.5 and 4.7 (Juniper et al., 1989; Crowder et al., 1990), markedly better results for multiplication had been expected under lower pH. These data do not correspond with those of Kwang-Soo and Go-Won (2004) for *D. peltata*, where significantly higher shoot proliferation was achieved at pH 5.7, while pH 3.7 substantially decreased this characteristic.

For *in vitro* cultures, the main source of carbohydrates providing energy for growth and biosynthetic processes is sucrose (Ferreira et al., 2011). Thus, the effect of its concentration on growth and developmental characteristics was also examined. No statistical significant differences between concentrations, however, were detected. These findings confirm those of Jayaram and Prasad (2007) for *D. indica*, where three sucrose concentrations provided very similar results. Jimenez et al. (2011) in *D. capensis* suggested that the highest increase in fresh weight can be achieved by low concentration of sucrose, but the optimal level of sucrose may depend upon other components of the culture medium, such as mineral salts and organic substances.

The inductive effect of cytokinins on morphological characteristics had been tested in our study. Synthetic cytokinins, i.e. BA and kinetin were not suitable as they did not allow good shoot proliferation when compared to the effect of zeatin. In addition, BA and kinetin supplementations caused extreme suppression of growth and hyperhydricity of *in vitro* tissues. Grevenstuk et al. (2010), did not report in the study on *D. intermedia,* a negative effect of kinetin on shoot multiplication when compared to control medium without plant grow regulators. Likewise, BA alone or in combination with NAA provided high micropropagation coefficient in *D. aliciae*, *D. anglica* and *D. cuneifolia* (Kawiak et al., 2003; Kawiak and Lojkowska, 2011).

Zeatin proved to be the most suitable cytokinin for multiplication of *D. intermedia*. The lowest tested concentration used (0.1 mg l^{-1} zeatin) provided the highest number of shoots per plant from all 26 tested media. Superior effect of zeatin over kinetin and BA had been reported also for micropropagation of other species (Peixe at al., 2007; Hendrawati et al., 2012). Although zeatin is very expensive chemical compound, it is the only growth regulator capable of inducing satisfactory growth and multiplication, and thus it is not possible to replace it by another cytokinin.

Acclimatization to greenhouse conditions was successfully achieved in most plants (98.6%), which may be attributed to the fact that for *ex vitro* transfer were used only well developed and rooted plants. Furthermore, a mixture of peat moss and perlite represents the optimal substrate for acclimatization and continuous growth of plants, as it allows a certain degree of water retention, and permits good drainage and aeration of roots (Jimenez et al., 2011).

Conclusion

To summarize, various levels of abiotic factors may greatly affect growth and development of *in vitro* cultivated plants of *D. intermedia*. A decreased concentration of nutrients in cultivation medium and supplementation of zeatin at low concentrations seems to be adequate for micropropagation of the species. Meanwhile, different pH values and sucrose concentrations do not influence the multiplication rate significantly. Based on these results, the optimized protocol can be used for large scale clonal propagation of the species for commercial and conservation purposes.

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