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In vitro propagation of an endangered plant *Saussurea esthonica*

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Abstract

In vitro culture is a one of the methods for *ex situ* conservation of rare and endangered plant species. The aim of the present study was to examine the main aspects of *in vitro* culture initiation and propagation of *Saussurea esthonica*, an endangered wild plant species in Latvia. The effect of the cytokinins kinetin, 6-(γ,γ-dimethylallylamino) purine and 6-benzilaminopurine on the micropropagation of *S. esthonica* was analyzed. Successful initiation of tissue culture of *S. esthonica* was achieved from seeds. The best results on shoot proliferation were obtained using 6-benzilaminopurine at concentrations 0.5 to 1 mg L^{-1}. Addition of 6-(γ,γ-dimethylallylamino) purine promoted shoot elongation and root formation, but it was not effective on multiplication. The multiplication rate using kinetin at concentrations 0.5 to 2 mg L^{-1} was invariable without production of tissue swelling. About 30% of explants were rooted. The present study confirms that micropropagation of *S. esthonica* can be efficiently performed by using cytokinins at low concentrations.

Key words: endangered plants, micropropagation, *Saussurea esthonica*.
Abbreviations: 2-iP, 6-(γ,γ-dimethylallylamino) purine; BAP, 6-benzylaminopurine; MS medium, Murashige and Skoog medium.

Introduction

Saussurea esthonica Baer ex Rupr. is an endangered perennial wild plant species of Latvia, with only two populations identified at present. *S. esthonica* is a neoendemic species of the Baltic Region, characteristic of grass fens, originating from wetlands of the late glacial period (Ingelög et al. 1993). The species was considered extinct in Latvia since 1930, but was rediscovered in 1991 (Andrušaitis 2003).

To ensure preservation of biological and genetic diversity of wild plants outside their natural range, *ex situ* field collections, seed banks and *in vitro* culture collections need to be established. Previously, *in vitro* methods have been applied for conservation of threatened plant species in Latvia (Kļaviņa et al. 2004). Seeds and shoot apex explants from various rare and endangered plant species have been used for culture initiation, and multiplication achieved on hormone-free medium or supplemented with 6-benzylaminopurine (BAP). However, the initial establishment of tissue culture and multiplication of *Saussurea esthonica* has not reported (Klaviņa et al. 2004).

Species of genus *Saussurea* can be propagated *in vitro* through vegetative rootstocks and seeds (Joshi, Dhar 2003). However, culture initiation through vegetative shoots is problematic due to high contamination risk, while poor viability of seeds results in low germination rates. Reports of plant regeneration through tissue culture are available for some species of the genus *Saussurea* (Arora, Bhojwani 1989; Dhar, Joshi 2005; Guo et al. 2007), but these methods included plant regeneration through callus, which has not been recommended for rare plant conservation (Fay 1992). For rare plant conservation, plantlets should be propagated from a comprehensive sampling of the population genetic diversity and changes in genes or gene expression during culture should be avoided by using direct micropropagation methods with low levels of growth regulators to promote phenotypically stable axillary growth without callus production (Edson et al. 1997).

Cytokinins are plant growth regulators widely used in tissue culture for stimulating cell division and affecting of shoot and root morphogenesis. Cytokinins reduce primary root growth and lateral root density (Laplaze et al. 2007). Root length and diameter are important characteristics to be considered when describing and comparing root systems (Bouma et al. 2000), and are useful tools for comparing the effect of various growth regulators. At comparatively high concentrations some cytokinins can create somaclonal variation in leaf colour (Chuenboonngarm et al. 2001). To avoid these changes and maintain genetic integrity, appropriate cytokinin concentrations should be determined for the efficient propagation of rare plant species.

The present investigation was a part of study on applying *in vitro* methods for conservation of rare and threatened plant species. The aim of the paper was to

perform *in vitro* culture initiation and to study the effect of cytokinins and macronutrients at various concentrations on micropropagation of *Saussurea esthonica*.

Materials and methods

In vitro *culture initiation*

Culture initiation was performed from seeds or vegetative shoots with only a limited amount of initial material in order to protect the endangered natural populations.

Seeds were collected from natural populations in the vicinity of Apšuciems and Pope, Latvia, twice during a vegetation season – at the end of July and in September. In total 121 seeds collected in July and 68 seeds collected in September were used. A portion of the seeds were dried and stored at 4 °C for 12 weeks prior to germination. The remaining seeds were kept at room temperature for one week to several months. Seeds were sterilized in bleach (4.5% of active substance) : distilled water 1 : 1 (v/v) for 7 to 15 min followed by three rinses of sterile distilled water, placed in test tubes with 10 mL hormone-free medium containing half-strength MS (Murashige, Skoog 1962) macronutrients supplemented with 3% sucrose, 0.6% agar (pH 5.9) and incubated in a growth chamber at 24 ± 2 °C under a 16 h photoperiod regime, photon flux density of 40 μmol s^{-1} m^{-2}.

Shoots from a single plant transplanted from a natural population were cut in April and May, washed with soap, soaked in 0.2% $KMnO_4$ solution for 40 min, sterilized in bleach : distilled water (1 : 1) for 6 to 10 min followed by three rinses of sterile distilled water and placed on ¾ MS medium supplemented with 0.25 mg L^{-1} 6-benzylaminopurine (BAP), 3% sucrose, 0.6% agar (pH 5.9). Cultures were incubated in a growth chamber at 24 ± 2 °C under a 16 h photoperiod regime, photon flux density of 40 μmol s^{-1} m^{-2}.

Determination of cytokinin effect

Micropropagated shoots from *in vitro* germinated seeds were used as explants. *In vitro* shoots were cultivated on hormone-free MS medium for 2 weeks and then transferred onto media supplemented with ½ or full MS macronutrients, 3% sucrose, 0.6% agar, and cytokinin. As a cytokinin, kinetin at concentrations 0.5, 1, 1.5 and 2 mg L^{-1}; 6- (γ,γ-dimethylallylamino) purine (2-iP) – 0.25, 0.5, 0.75 and 1 mg L^{-1} or BAP – 0.25, 0.5, 0.75 and 1 mg L^{-1} were used. Media supplemented with ½ or full MS macronutrients and without plant growth regulators were included as controls. The pH was adjusted to 5.9 prior to autoclaving. Shoots were cultured in tissue culture jars (300 mL) containing 50 mL of the medium.

Each treatment consisted of 30 microshoots in three replicates. After four weeks of cultivation, percentage of explants producing shoots, number of shoots and roots were counted. All *in vitro* cultures were incubated in a growth chamber at 24 ± 2 °C under a 16 h photoperiod regime, photon flux density of 40 μmol s^{-1} m^{-2}.

Images of roots formed in media supplemented with kinetin and 2-iP were produced with a scanner Epson Perfection V750 PRO and analyzed using the image analysis system WinRHIZO 2008 to evaluate root morphology. Total root length per plant, surface area, average diameter and number of tips were measured.

Data are presented as means from three replicates ± SE. The level of significance of differences between the means was determined by ANOVA single-factor analysis.

Results

In vitro *culture initiation*

Sterilization with solution of bleach in distilled water for 7 to 15 min was appropriate for obtaining sterile seeds of *Saussurea esthonica*, but the seeds showed poor germination (only 4 to 8% of seeds germinated *in vitro*). The germination period ranged from 14 to 285 days. No seeds collected in September germinated. The best germination was obtained with seeds collected at the end of July, and stored at room temperature for one week or several months after collecting (Table 1). In addition, seed storage at 4 °C before germination positively affected the germination rate.

Shoot sterilization was not successful. Only one sterile shoot (12%) was obtained, which was used for propagation. However, after some time a secondary infection developed and shoots were transferred to *ex vitro* conditions. Therefore, further experiments were performed with explants obtained from germinated seedlings.

Shoot multiplication

The effect of half- and full strength MS macronutrient concentrations and varying levels of BAP, kinetin or 2-iP on shoot formation is shown in Table 2. The shoots on medium without cytokinins (control) failed to multiply. The percentage of explants producing shoots and number of shoots per explant were higher in cultures supplemented with BAP than with kinetin or 2-iP. The only statistically significant difference between the effect of growth regulator concentration was found for shoot number on media with full strength MS macronutrients supplemented with BAP ($p < 0.05$).

Treatments with full strength MS macronutrients

Table 1. Effect of seed storage conditions on *in vitro* germination of *Saussurea esthonica* seeds collected in July

Time and conditions of seed storage	Germination (%)
1 day (room temperature)	4
1 week (room temperature)	11
9 weeks (room temperature)	20
12 weeks (room temperature)	12
14 weeks (12 weeks at 4 °C)	21

Table 2. Effect of different cytokinins and MS mediummacronutrient concentration on shoot multiplication of *Saussurea esthonica* explants after four weeks of cultivation

MS macronutrient concentration (%)	Cytokinin concentration (mg L^{-1})	Explants producing shoots (%)	Shoots per explant (number)	Shoot length (cm)
50	–	0	0	0
	BAP 0.25	85.4 ± 7.3	2.7 ± 0.6	2.1 ± 0.4
	BAP 0.50	93.1 ± 3.3	3.5 ± 1.0	1.8 ± 0.2
	BAP 0.75	99.4 ± 0.6	4.8 ± 0.3	1.8 ± 0.2
	BAP 1.00	100 ± 0	5.3 ± 0.2	1.4 ± 0.3
	Kinetin 0.50	89.3 ± 1.8	2.0 ± 0.2	1.5 ± 0.3
	Kinetin 1.00	82.7 ± 2.3	2.4 ± 0.4	1.8 ± 0.3
	Kinetin 1.50	76.8 ± 10.8	2.0 ± 0.5	1.4 ± 0.1
	Kinetin 2.00	63.4 ± 13.3	2.2 ± 0.6	1.6 ± 0.3
	2-iP 0.25	23.8 ± 17.7	1.5 ± 0.4	3.3 ± 0.3
	2-iP 0.50	26.6 ± 19.0	1.6 ± 0.5	2.1 ± 0.7
	2-iP 0.75	5.1 ± 2.6	0.7 ± 0.3	1.0 ± 0.03
	2-iP 1.00	13.8 ± 9.2	1.1 ± 0.1	1.8 ± 0.3
100	–	0	0	0
	BAP 0.25	95.7±2.7	2.3 ± 0.3	2.6 ± 0.4
	BAP 0.50	90.3 ± 5.0	3.7 ± 0.9	2.1 ± 0.03
	BAP 0.75	100 ± 0	6.0 ± 0.5	2.1 ± 0.2
	BAP 1.00	100 ± 0	6.8 ± 0.3	1.5 ± 0.1
	Kinetin 0.50	68.8 ± 3.6	1.9 ± 0.2	1.8 ± 0.1
	Kinetin 1.00	87.5 ± 4.6	2.4 ± 0.5	2.0 ± 0.2
	Kinetin 1.50	88.1 ± 6.7	2.4 ± 0.5	2.1 ± 0.3
	Kinetin 2.00	76.3 ± 11.5	2.4 ± 0.5	1.6 ± 0.1
	2-iP 0.25	25.4 ± 18.2	1.0 ± 0.5	0.8 ± 0.2
	2-iP 0.50	23.5 ± 20.5	1.0 ± 0.5	2.0 ± 0.6
	2-iP 0.75	26.6 ± 15.0	0.9 ± 0.5	1.3 ± 0.3
	2-iP 1.00	11.0 ± 5.7	0.9 ± 0.5	1.6 ± 0.2

tended to increase the percentage of explants producing shoots as well as number of shoots per explant. However, the only significant difference between the treatments with ½ and full MS macronutrients was found for media supplemented with 1 mg L^{-1} BAP ($p < 0.05$).

Shoot length ranged between 0.8 and 3.3 cm, but growth regulator type and concentration did not significantly affect shoot length. However, there was a tendency for shoot length to decrease with increasing BAP level.

When comparing different cytokinins, there were significant differences between the effect of all three growth regulator treatments on percentage of explants producing shoots and number of shoots per explant at ½ MS and full MS concentrations. Kinetin in all applied concentrations showed similar results without production of tissue swelling, but the percentage of explants producing shoots was smaller than with BAP. Media supplemented with 2-iP promoted explant elongation, but for shoot formation 2-iP seemed to be inappropriate, as less than 30% of explants produced shoots.

Shoot length was not as much influenced by the type of cytokinin. However, increasing BAP levels negatively influenced shoot length.

Root formation

The best results in respect to root formation were obtained in cytokinin-free medium and media with 2-iP (Table 3). Less than 30% of explants cultivated on media with BAP produced roots, and the obtained roots were short and thick. Higher concentrations of BAP decreased the percentage of root formation as well as number of roots. Using BAP at concentration of 1 mg L^{-1}, rooting was close to zero. Significant differences in percentage of explants producing roots and number of roots per explant were found between all growth substance treatments at both ½ MS and full MS concentrations, except for kinetin and 2-iP at full MS.

Total root length, surface area, average diameter and number of root tips are shown in Table 4. All root parameters for explants grown on ½ MS was significantly different between kinetin and 2-iP treatments, however,

Table 3. Effect of different cytokinins and MS macronutrient concentration on root formation on *Saussurea esthonica* explants after four weeks of cultivation

MS macronutrient concentration (%)	Cytokinin concentration (mg L^{-1})	Explants producing roots (%)	Roots per explant (number)
50	–	87.2 ± 5.0	3.1 ± 0.2
	BAP 0.25	30.1 ± 9.8	1.4 ± 0.3
	BAP 0.50	27.3 ± 10.7	1.2 ± 0.1
	BAP 0.75	16.5 ± 9.0	1.1 ± 0.1
	BAP 1.00	6.3 ± 3.1	0.8 ± 0.4
	Kinetin 0.50	36.9 ± 5.6	2.3 ± 0.4
	Kinetin 1.00	34.8 ± 7.2	2.2 ± 0.5
	Kinetin 1.50	43.3 ± 14.2	1.9 ± 0.3
	Kinetin 2.00	34.8 ± 1.8	1.9 ± 0.4
	2-iP 0.25	81.5 ± 6.0	2.9 ± 0.6
	2-iP 0.50	83.8 ± 6.0	3.0 ± 0.8
	2-iP 0.75	82.5 ± 2.6	2.6 ± 0.3
	2-iP 1.00	73.4 ± 0.9	3.0 ± 0.3
100	–	87.4 ± 3.2	2.9 ± 0.1
	BAP 0.25	34.7 ± 13.6	1.5 ± 0.2
	BAP 0.50	28.5 ± 11.2	1.5 ± 0.3
	BAP 0.75	15.5 ± 8.9	1.2 ± 0.1
	BAP 1.00	0	0
	Kinetin 0.50	50.6 ± 5.9	2.8 ± 0.2
	Kinetin 1.00	52.5 ± 20.7	2.0 ± 0.6
	Kinetin 1.50	63.1 ± 10.7	2.1 ± 0.1
	Kinetin 2.00	60.9 ± 1.8	1.8 ± 0.1
	2-iP 0.25	88.0 ± 2.9	2.7 ± 0.2
	2-iP 0.50	87.8 ± 9.1	2.7 ± 0.3
	2-iP 0.75	64.7 ± 11.9	2.4 ± 0.6
	2-iP 1.00	73.0 ± 9.6	2.8 ± 0.4

neither cytokinin treatment significantly differed from the control. With full MS media, no significant differences were found between growth substance treatment and the control. Root average diameter stayed invariable at different kinetin and 2-iP concentrations, but treatments with kinetin tended to have thinner roots than treatments with 2-iP. Root length and surface area tended to decrease with increasing cytokinin concentration. Treatment with 2-iP at a concentration of 0.25 mg L^{-1} showed root length increase, in comparison with the control. This relationship was independent of MS macronutrient concentration. Number of root tips indicated the degree of root branching. Treatments with ½ MS macronutrients promoted branching and treatments with 2-iP increased the number of axillary roots in comparison with the control.

Discussion

Initiation of *in vitro* cultures of *Saussurea esthonica* was problematic due to the low germination rate of seeds and high contamination rate when using shoots. Studies on other *Saussurea* species have reported better results for seed germination. the germination rate for *S. lappa* was up to 30% (Johnson et al. 1997). Arora and Bhojwani (1989) reported that fresh seeds of *S. lappa* had a germination frequency of 30% with a period of germination three to four days. Dhar and Joshi (2005) used seeds of *S. obvallata* stored at 4 °C before culture initiation. Our results showed only insignificant improvement in germination after cold pre-treatment. However, the number of seeds used in these experiments was very low, due to the limited opportunities to collect seed material from wild *Saussurea esthonica* populations in Latvia. Therefore, future investigations should be performed on possible factors promoting seed germination.

As contamination risk is one of main problems in establishment of *in vitro* cultures, we consider that the most appropriate way for culture initiation for endangered plants is by seeds. Initiation via shoots is acceptable only in cases when seeds are not accessible. Fay (1992) preferred seeds

Table 4. Effect of different cytokinins and MS macronutrient concentration on root morphology of *Saussurea esthonica* explants after four weeks of cultivation

MS macronutrient concentration (%)	Cytokinin concentration (mg L^{-1})	Root length (cm)	Surface area (cm^3)	Average diameter (mm)	Root tips (number)
50	–	13.7 ± 2.3	2.7 ± 0.5	0.7 ± 0.1	8.9 ± 1.7
	Kinetin 0.50	9.7 ± 2.8	1.9 ± 0.6	0.6 ± 0	5.6 ± 2.6
	Kinetin 1.00	8.7 ± 3.7	1.6 ± 0.7	0.6 ± 0	6.1 ± 2.5
	Kinetin 1.50	7.3 ± 4.2	1.4 ± 0.8	0.6 ± 0	3.7 ± 1.2
	Kinetin 2.00	6.0 ± 1.1	1.2 ± 0.1	0.7 ± 0.1	3.6 ± 0.2
	2-iP 0.25	15.4 ± 2.3	3.0 ± 0.4	0.7 ± 0	10.3 ± 1.3
	2-iP 0.50	11.7 ± 1.9	2.5 ± 0.4	0.7 ± 0	8.0 ± 0.3
	2-iP 0.75	12.5 ± 0.7	2.7 ± 0.2	0.7 ± 0	8.0 ± 0.6
	2-iP 1.00	11.7 ± 0.8	2.5 ± 0.2	0.7 ± 0	7.7 ± 1.4
100	–	8.9 ± 0.4	1.8 ± 0	0.7 ± 0.1	6.4 ± 0.1
	Kinetin 0.50	8.9 ± 1.4	1.7 ± 0.2	0.6 ± 0	7.9 ± 0.9
	Kinetin 1.00	7.4 ± 5.6	1.6 ± 1.2	0.7 ± 0	5.5 ± 1.9
	Kinetin 1.50	5.7 ± 3.4	1.2 ± 0.7	0.7 ± 0.1	4.8 ± 0.5
	Kinetin 2.00	3.4 ± 0.1	0.9 ± 0	0.7 ± 0	4.6 ± 0
	2-iP 0.25	12.6 ± 1.5	2.5 ± 0.3	0.7 ± 0	7.0 ± 1.1
	2-iP 0.50	10.7 ± 1.8	2.1 ± 0.4	0.7 ± 0	6.6 ± 0.7
	2-iP 0.75	11.1 ± 5.6	2.3 ± 1.1	0.7 ± 0	7.0 ± 2.5
	2-iP 1.00	8.7 ± 4.7	1.8 ± 0.9	0.7 ± 0	6.5 ± 1.9

for rare and endangered plant culture initiation because they represent a wider range of genetic diversity.

In this study we investigated the effect of three cytokinins (BAP, kinetin and 2-iP) on shoot formation and root development. BAP has been used widely for shoot regeneration in protocols developed for other *Saussurea* species (Guo et al. 2007). Johnson et al. (1997) concluded that thidiazuron is more effective than BAP in shoot induction for *S. lappa*. Our results showed that BAP is most appropriate for shoot multiplication of *S. esthonica*, but it strongly inhibits root formation.

Treatment with kinetin was less effective on shoot formation than BAP, and the applied concentrations did not result in significantly different number of obtained shoots. Joshi and Dhar (2003) concluded that kinetin alone was less favourable for shoot multiplication of *S. obvallata* than BAP. There was a steady decrease in shoot length with a progressive increase in kinetin concentration, and a gradual increase in BAP concentration significantly increased shoot number. Kinetin in combination with naphthaleneacetic acid was most effective for shoot multiplication of subsequent subcultures. Our results showed that, at the concentrations applied, kinetin is most useful for promotion of rooting, with lower kinetin concentrations stimulating root elongation.

Root formation for *in vitro* cultures is usually achieved by using auxins: indole-3-acetic acid, indole-3-butyric acid and naphthaleneacetic acid. As root induction by auxins alone is problematic for some species, a combination of auxins with cytokinins is used. Root induction of *Corylus avellana* was achieved by using indole-3-butyric acid and kinetin (Gonzalez et al. 1991). Auxins promoted root induction for *S. obvallata* and *S. involucrata*, but for these species, rooting was possible without auxins (Joshi, Dhar 2003; Guo et al. 2007). Therefore, auxins were not used in the present study.

Our results showed that 2-iP at concentration 2.5 mg L^{-1} promoted root formation, but higher concentrations decreased root length and surface area. Chuenboonngarm et al. (2001) concluded that treatment with 2-iP at concentrations 2.5 to 10 mg L^{-1} gave multiple shoots (one to five) for *Gardenia jasminoides*, but resulted in somaclonal variation. However, in the present investigation, supplementation of 2-iP at 10 times lower concentration produced the same result, but somaclonal variations were not observed. Obviously this cytokinin is not appropriate for shoot proliferation of *S. esthonica*, but is useful for shoot elongation and root formation. 2-iP at concentration 2.5 mg L^{-1} promoted root formation in comparison with controls. Consequently, higher concentrations of 2-iP are not necessary.

Conclusions

Culture initiation of *S. esthonica* was successful by using seeds. However, the germination rate was low. Therefore, for conservation purposes, caution should be used to ensure that sufficient genetic diversity is represented in *in vitro*

cultures.

BAP promotes propagation, and at concentrations 0.5 to 1 mg L^{-1} it can be a useful for achieving fast propagation rates. At lower concentrations it can be appropriate for culture maintenance without frequent sub-culturing.

Kinetin and 2-iP are not useful for proliferation, but better suited for conservation purposes. Shoot formation is independent of kinetin concentration starting from 0.5 mg L^{-1}. Therefore, an unnecessary increase of concentration can be avoided. Higher concentrations of kinetin promoted rooting but decreased root length. Treatment with 2-iP is suitable for root formation and at low concentration promotes rooting.

Effects of different macronutrient concentrations are generally low, but there is a trend that better proliferation can be achieved at full strength MS macronutrients. In contrast, the most efficient rooting is at half-strength MS.

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