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Full Length Article

In Vitro Micropropagation of Ornamental Rare Species *Sibiraea altaiensis:* An Endemic of Altai

Vitaliy Kirillov*, Mariya Serafimovich, Tamara Stikhareva and Bolat Mukanov
Department of Breeding, Kazakh Research Institute of Forestry and Agroforestry, Shchuchinsk 021704, Kazakhstan
*For correspondence: vitaliy.kirillov.82@mail.ru

Abstract

Sibiraea altaiensis (Laxm.) Schneid. is an endemic plant species of Altai, having great ornamental importance. In this study, efficient protocol for *in vitro* micropropagation of this rare plant was developed for the first time. Lateral buds with part of stem, excised from 1–2 years old shoots of mature trees of *S. altaiensis* were used as explants. Explants after sterilization were cultured on modified Murashige and Skoog's (*m*MS) medium, in which concentration of nitrate and ammonia nitrogen was reduced by 4 times and concentration of vitamins was increased. Concentration of agar in the medium was 0.35% (w/v). The shoot bud proliferation was the best on *m*MS medium supplemented with 6-benzylaminopurine (6-BAP) at 1.0 mg L^{-1} after 3 weeks of culture. Further, subculture on *m*MS medium without use of plant growth regulators (PGRs) for 3 weeks stimulated regeneration of 2–3 axillary shoots at explants. The shoot multiplication (5.83 ± 0.2) was the best on *m*MS medium supplemented with 1.0 mg L^{-1} 6-BAP after 3 weeks of culture. A large number of multiple shoots (28 shoots/explant) was observed by the tenth subculture on multiplication medium (alternation of 5 subcultures with 1.0 mg L^{-1} 6-BAP for 3 weeks and 5 subcultures without PGRs for 3 weeks). Shoots were best rooted *in vitro* on *m*MS medium without PGRs within 4 weeks. The plantlets with roots were effectively acclimatized in growth substrate - peat: sand: vermiculite (1:1:1) within 2 months (83.3%) and in the open ground within 3 months (50.0%). This protocol can be used effectively for rapid propagation and conservation of *S. altaiensis* at commercial scale. © 2019 Friends Science Publishers

Keywords: 6-Benzylaminopurine; Endemic; *In vitro;* Lateral buds; Micropropagation; *Sibiraea altaiensis*

Introduction

The genus *Sibiraea* Maxim. belongs to the subfamily Spiraeoideae Agardh. of the family Rosaceae Juss. The subfamily Spiraeoideae is considered to be the most primitive in the family Rosaceae and the origin of other subfamilies is associated with it. All of them are shrubs; flowers with apocarpous gynoecium from many carpels and superior ovary; fruit is multifollicles (Koropachinskiy and Vstovskaya, 2002).

The genus *Sibiraea* Maxim. was derived from *Spiraea* L. by Maximowicz in 1879 and contains five species. It grows in the European part of Southern Russia, Siberia, Southeastern Europe and Western China. *S. altaiensis* (Laxm.) Schneid. is an endemic of Altai. *S. croatica* Degen is spread in Croatia and Bosnia and Herzegovina (Eastern Alps). *S. tianshanica* (Krassn.) A. Pojark. is an endemic of Tien Shan. *S. tomentosa* Diels is in central China (Tutin *et al*., 1968; Ballian *et al.*, 2006; Syeva and Ailchiyeva, 2014). *S. angustata* (Rehd.) Hand. Mazz. grows in the western part of the Himalayan plateau (in China), creates specific plant communities and is used for production of biomass (Wu, 1998). There are 2 species - *S. tianshanica* and *S. altaiensis* growing in Kazakhstan (Pavlov, 1961).

The species of *S. altaiensis*, which is registered in the Red Data Book of Kazakhstan, is interesting and important for our research (Red Data Book of Kazakhstan, 2014), earlier it has been registered in the Red Data Book of the Kazakh SSR (Red Data Book of Kazakh SSR, 1981). *S. altaiensis* is a shrub plant up to 60–150 cm with thick branches and a dark-brown cortex. The leaves are sessile, entire, gradually narrowed to the base, roundish to the top, a little acuminate on the tip. The flowers are in long racemes bunched in a panicle. Axes of inflorescences and hypanthium are glabrous or barely pubescent. It is in blossom in May–June; it fruits in July–August (Pavlov, 1961).

S. altaiensis grows only in Altai, where it occupies an area of about 74 thousand sq. km (Koropachinskiy and Vstovskaya, 2002). The plant grows on open mountain valleys and slopes of the middle belt of mountains (Pavlov, 1961). In Kazakhstan it is found only in Kazakhstani Altai (Ivanovo, Uba, Narym, Southern Altai ranges). It grows in small groups or solitary (Red Data Book of Kazakhstan, 2014).

S. altaiensis contains traces of tanning agents, alkaloids, quinones, flavonoids and hydrocyanic acid. Ursolic acid was detected in the leaves. *S. altaiensis* is a source of food (the leaves are used as tea substitute) and an

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industrial raw material. Branches are used for medicinal purposes. Decoction of branches is used for stroke, fever and hepatitis (jaundice) in Altai. *S. altaiensis* extract is included in day cream for sensitive facial skin of Natura Siberica Company (Sokolov, 1987; Kirillov *et al.*, 2016).

S. altaiensis has great ornamental importance. It is recommended for the use in live fences, borders and strip plantations on the slopes, subject to water erosion, in the alpine rock garden as well as for interblock planting of trees and shrubs in large parks. It forms beautiful groups with larches in landscape plantings. Single plantings and group plantings are recommended. It is well cut and can be used for creation of live fences. It is a winter-hardy, shade-tolerant plant, but on light it reaches a bigger decorative effect (Tutin *et al.*, 1968; Koropachinskiy and Vstovskaya, 2002; Ballian *et al.*, 2006; Syeva and Ailchiyeva, 2014). However, the number of the species is reducing under the influence of economic activity in habitats and an intensive pasture of the cattle (the shoots perish). In natural conditions it propagates only by seeds that demands a lot of time. The seedlings develop very slowly and at early stages of development most of them perishes, very few plants live up to generative condition. In the conditions of Kazakhstani Altai there are no data on vegetative reproduction of *S. altaiensis*. *In vitro* propagation can be alternative method to produce large number of *S. altaiensis* plants and for conservation of this endemic species. At present, no information is available about *in vitro* propagation of *S. altaiensis*. Therefore, the aim of this research work was to develop an efficient *in vitro* multiplication method for *S. altaiensis* plant.

Materials and Methods

Plant Collection, Explant Preparation and Sterilization

Shoots of *S. altaiensis* were collected in April, 2016 from the arboretum of Kazakh Research Institute of Forestry and Agroforestry (Fig. 1a). *S. altaiensis* was derived from the seeds, which were transferred from Omsk (Russia) to Shchuchinsk and plants were placed in the arboretum in 1966. These plants have been used as the source of explants in our study. The shoots were preliminary kept in the distilled water at the room temperature before the emergence of the leaves. Lateral buds with a part of the stem, excised from 1–2 years old shoots of mature trees of *S. altaiensis*, were used as explants. Before the sterilization, the lateral buds were cleaned from scales and 2–3 topmost leaves. These explants were immersed into 2.5% aqueous solution of household soap for 5 min and after in 2% aqueous solution of a commercial bleaching agent, sodium hypochlorite (Belizna) (LLC "HimProm", Russia) for 5 min and then washed under running tap water for 5 min. The explants were externally disinfected by 0.025% thimerosal (merthiolate) ($C_9H_9HgNaO_2S$) (AppliChem, Germany) (w/v) in combination with 7% aqueous solution of a commercial bleaching agent, sodium hypochlorite (Belizna) in the laminar flow cabinet for 5 min. The explants were then washed with sterile distilled water (3 times) for 5–6 min.

Culture Medium and Culture Conditions

Modified Murashige and Skoog's (*m*MS) medium (macronutrients - 475 mg L^{-1} KNO_3, 400 mg L^{-1} NH_4NO_3, 170 mg L^{-1} KH_2PO_4, 370 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 110 mg L^{-1} $CaCl_2 \cdot 2H_2O$; micronutrients - 22.3 mg L^{-1} $MnSO_4 \cdot H_2O$, 0.75 mg L^{-1} KI, 6.2 mg L^{-1} H_3BO_3, 8.6 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.025 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 0.25 mg L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 0.025 mg L^{-1} $CoCl_2 \cdot 6H_2O$; Fe sources - 27.8 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 37.3 mg L^{-1} Na_2-EDTA$\cdot 2H_2O$; vitamins - 3.0 mg L^{-1} thiamine HCl, 1.0 mg L^{-1} nicotinic acid, 1.0 mg L^{-1} pyridoxine; aminoacids – 2.0 mg L^{-1} glycine, 25.0 mg L^{-1} *L*-glutamine) was used for shoot bud proliferation, shoot multiplication, *in vitro* rooting. The culture media contained 3% (*w/v*) sucrose, meso-inositol (100 mg L^{-1}) (Sigma-Aldrich, Germany), polyvinylpyrrolidone (20.0 mg L^{-1}) (Sigma-Aldrich, Germany) and 0.35% (w/v) agar (AppliChem, Germany). The pH of medium was adjusted to 5.7–5.8 using 0.1 N HCl or 0.1 N NaOH before adding agar. PGRs (auxins and cytokinins) were added into the medium after autoclaving in the laminar flow cabinet.

The culture tubes (150 × 25 mm; 25 mL/culture tube) plugged with cotton plugs wrapped in a double layer of cheese cloth were sterilized by autoclaving at 1.03 kg cm^{-2} and 121°C for 40 min. All of the cultures were incubated in a culture room maintained at 24 ± 2°C, under a 16/8 h light/dark cycle with a light intensity of 35–55 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Progress, Russia), with 55–60% relative humidity.

Shoot Bud Proliferation

There are two stages for shoot bud proliferation. The first, sterilized explants (Fig. 1b) were cultured on *m*MS medium supplemented with 6-benzylaminopurine (6-BAP) (Sigma-Aldrich, Germany), kinetin (KN) (Sigma-Aldrich, Germany) or thidiazuron (TDZ) (Sigma-Aldrich, Germany) at various concentrations (0.5, 1.0, 1.5 and 2.0 mg L^{-1}) alone and in combination with auxins, 0.5 mg L^{-1} α-naphthalene acetic acid (NAA) (Sigma-Aldrich, Germany), or 0.5 mg L^{-1} indole-3-acetic acid (IAA) (Sigma-Aldrich, Germany) for 3 weeks. After that the explants were cultured on *m*MS medium without PGRs for 3 weeks. The need of PGRs is determined by the average number of explants forming shoots, average shoot height and average number of shoots per explant.

Shoot Multiplication

Shoots (axillary branches) excised from the initial explants and proliferated from the shoot buds on cytokinin supplemented medium were further multiplied on *m*MS

medium supplemented with 6-BAP, or KN at different concentrations (0.5, 1.0, 1.5 and 2.0 mg L^{-1}) for 3 weeks. After 3 weeks of culturing axillary branches were formed and excised and then further used for re-multiplication or *in vitro* rooting. After 3 weeks, shoot multiplication was assessed by the multiplication rate (the total number of shoots produced from each initial explant) and the length of the longest shoot per culture.

In vitro Rooting

Multiple shoots (about 1.0–2.0 cm) were excised from the shoot conglomerate and transferred to rooting medium consisting of *m*MS medium supplemented with IAA, NAA, or indole-3-butyric acid (IBA) (Sigma-Aldrich, Germany) at different concentrations (0.5, 1.0 and 1.5 mg L^{-1}) for 1, 2 or 3 weeks and then on *m*MS medium without PGRs. *m*MS medium without PGRs was used as a control one. Data on percentage of rooting and number of roots per microshoot were recorded after 4 weeks of culture. The influence of PGRs and their concentrations were determined according to the number of microshoots rooted *in vitro* and the number of roots per explant and the length of the roots.

Acclimatization

Rooted plantlets were removed from the cultures, rinsed with the solution of potassium permanganate ($KMnO_4$) of a pale pink color to remove the adhering agar and planted in nursery pots containing peat (Garden Retail Service, Russia) mixed with sand and vermiculite [1:1:1, 1:1:0, 1:0:1, 0:1:1 (w/w/w)]. Nursery pots with the plants were covered with a plastic cap, creating conditions of increased humidity until the appearance of the young leaves. The plants were irrigated with normal water to prevent them from drying. The plants were incubated in a culture room maintained at 24±2°C, under a 16/8 h light/dark cycle with light intensity of 35–55 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Progress, Russia). Adaptation of microshoots to unsterile conditions was defined by the degree of survival which was expressed by the relation of the number of the shoots survived on a certain substrate to the total number of the shoots placed on this type of the substrate. For assessment of the dynamics of growth of the adapted plants there were determined the length of shoots and roots and the quantity of the formed leaves. The hardened plantlets were transferred to the open ground of the dendrological park of Kazakh Research Institute of Forestry and Agroforestry.

Statistical Analysis

All the experiments were repeated three times with 30 replicates. The data were subjected to the statistical analysis with the use of XLSTAT software, version 2018.03.51059 (Addinsoft SARL, Paris, France). The data are presented as means ± SE. The effects of PGRs were tested by factorial analysis of variance (ANOVA) and one-way ANOVA. Confidence interval – 95%. Tolerance – 0.0001. Comparisons with a control – Dunnett (two sided). Pairwise comparisons – Duncan. Duncan's multiple range test was used for separation of means.

Results

Sterilization described in the methods has allowed receiving of 82.2% of viable aseptic explants. The *m*MS medium was experimentally defined as a nutrient medium in which there was reduced the concentration of nitrate and ammonia nitrogen in 4 times and the concentration of vitamins was increased as compared with MS medium (Murashige and Skoog, 1962). Optimal concentration of agar in the medium was 0.35% (w/v).

The optimal rate of shoot development was achieved when using cytokinin 6-BAP (Fig. 2). The highest rate of shoot development (76.7%) and shoot growth (5.2 ± 0.1 cm) was found with 1.0 mg L^{-1} 6-BAP (Table 1 and Fig. 1c). On the medium containing 1.0 mg L^{-1} KN, there formed shoots from the buds of a smaller length (3.6 ± 0.1 cm), than in 6-BAP. Reduction and increase of concentration of 6-BAP and KN on the nutrient medium lowered the rate of shoot development; the growth of shoots in length was less intensive too. TDZ was ineffective for shoot bud proliferation explants of *S. altaiensis* in all the tested concentrations. The increase of concentration of TDZ up to 1.5–2.0 mg L^{-1} led to the death of the explants. Cytokinin application in combination with auxins NAA and IAA reduced the positive effect of 6-BAP and KN on the development of the shoots.

Further subculture on *m*MS medium without PGRs for 3 weeks stimulated regeneration of 2–3 axillary shoots at the explants (Fig. 1d). Within 3 weeks with PGRs, multiplication and elongation of shoots were observed on axillary shoots of the explants which were isolated from the initial explants (Fig. 1e). Multiplication rates of shoot were significantly affected by 6-BAP concentration when up to 5.83 ± 0.2 shoots per explant were recorded using 1.0 mg L^{-1} 6-BAP (Table 2 and Fig. 1f). Increased concentration of 6-BAP up to 2.0 mg L^{-1} led to the decreasing of shoot multiplication rates. Multiple shoot cultures (microshoots conglomerate) were formed with adventitious shoots. Significant difference was observed between various concentrations of 6-BAP on number of shoots produced per explant. The highest length of shoot (1.95 ± 0.05 cm) was achieved on medium with 1.0 mg L^{-1} 6-BAP, which differed insignificantly from the treatment containing 1.5 and 2.0 mg L^{-1} 6-BAP. When concentration of 6-BAP was increased above 1.0 mg L^{-1}, the shoot length fell down to 1.78 ± 0.03 cm and 1.72 ± 0.05 cm at 1.5 mg L^{-1} and 2.0 mg L^{-1} respectively. The highest shoot lengths were observed in 1.0 mg L^{-1} 6-BAP. After 3 weeks of culture, the maximum number of shoots (2.27 ± 0.2) was obtained on the medium containing 1 mg L^{-1} KN which differed insignificantly from concentrations 1.5 and 2.0 mg L^{-1} KN with the number of shoots 2.13 ±

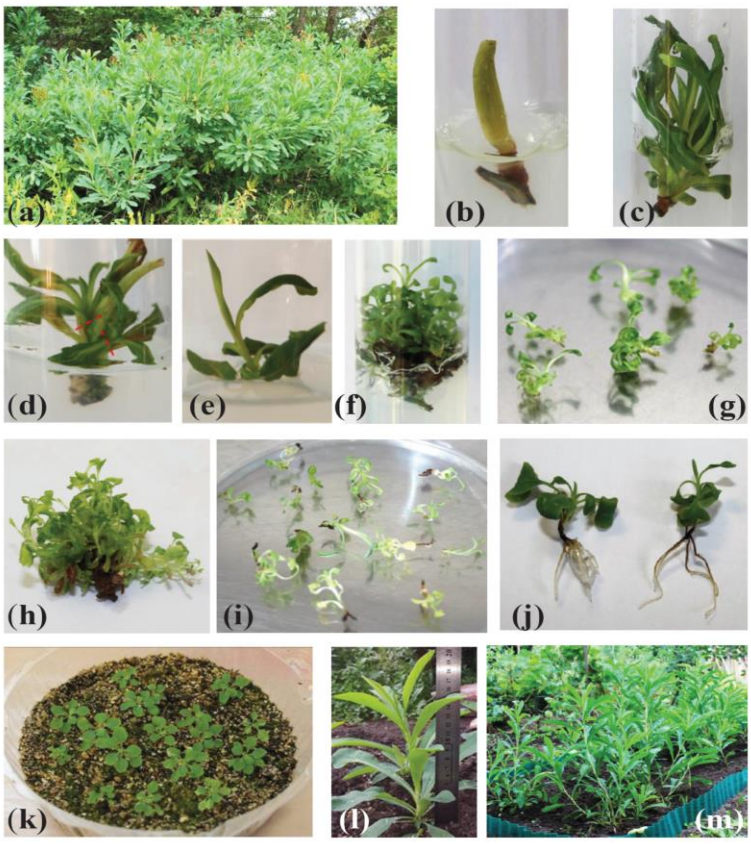

Fig. 1: *In vitro* micropropagation of *Sibiraea altaiensis*, (**a**) common view of *S. altaiensis* in the arboretum of Kazakh Research Institute of Forestry and Agroforestry, (**b**) initial explant (lateral bud), (**c**) shoot, formed from the lateral bud, on *m*MS with 1.0 mg L^{-1} 6-BAP after 3 weeks of culture, (**d**) regeneration of axillary shoots on initial explant on *m*MS without PGRs after 3 weeks of culture, (**e**) isolated axillary shoot from the initial explants, (**f**) shoot multiplication (microshoots conglomerate) on *m*MS with 1.0 mg L^{-1} 6-BAP after 3 weeks of culture, (**g**) separated conglomerate on 5-8 microshoots, (**h**) microshoot, forming conglomerate, on *m*MS without PGRs after 3 weeks of culture, (**i**) separated conglomerate on 8-15 microshoots, (**j**) *in vitro* rooting on *m*MS without PGRs after 4 weeks of culture, (**k**) acclimatized plants after 2 months in peat mixed with sand and vermiculite (1:1:1), (**l**) plant acclimatized to the open ground after 3 months, and (**m**) plants after winter in the following year

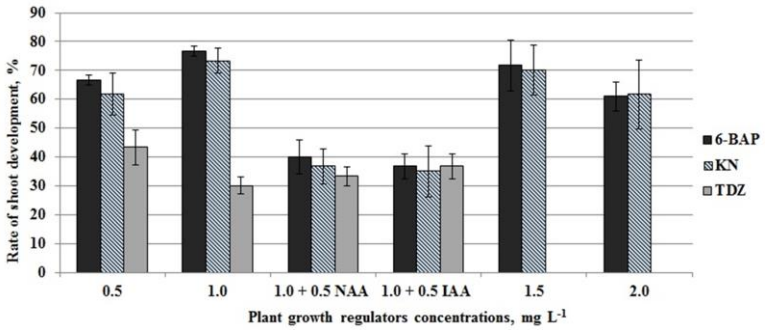

Fig. 2: Effect of plant growth regulator concentrations on shoot development from the initial explants of *S. altaiensis* (Results of 30 explants per treatment after 3 weeks of culture). 6-BAP = 6-benzylaminopurine, KN = kinetin, TDZ = thidiazuron, NAA = naphthalene acetic acid, IAA = indole-3-acetic acid. Vertical bars indicate ± SE of means

0.2 and 1.87 ± 0.2, respectively.

Separated microshoots from conglomerate (Fig. 1 g) formed again microshoots conglomerate on *m*MS medium without PGRs for 3 weeks culture (Fig. 1h). Shoot multiplication rates were 8–15 (Fig. 1i). A high number of multiple shoots (28 shoots/explant) was observed by the tenth subculture on multiplication medium (alternation of 5 subcultures with 1.0 mg L^{-1} 6-BAP for 3 weeks and 5

Table 1: Effects of plant growth regulators on the length of shoots (cm), formed from the lateral buds of *S. altaiensis* after 3 weeks of culture

PGRs	Concentration (mg L^{-1})					
	0.5*	1.0	1.0 + 0.5 NAA	1.0 + 0.5 IAA	1.5	2.0
6-BAP	3.5 ± 0.1 c	5.2 ± 0.1 a	2.9 ± 0.1 d	2.7 ± 0.1 de	4.0 ± 0.1 b	3.8 ± 0.1 bc
KN	2.9 ± 0.1 d	3.6 ± 0.1 c	2.6 ± 0.1 defg	2.7 ± 0.2 def	2.9 ± 0.1 d	2.5 ± 0.1 efg
TDZ	2.4 ± 0.1 efgh	2.3 ± 0.1 fgh	2.2 ± 0.1 gh	2.2 ± 0.1 h	0	0
Control	2.76 ± 0.2 de					

Mean values followed by the same letters, a–h in the same column, are not significantly different at the 0.0001 level of significance (Duncan's multiple range test. NAA = naphthalene acetic acid. IAA = indole-3-acetic acid. 6-BAP = 6-benzylaminopurine. KN = kinetin. TZD = thidiazuron. n = 30 explants per treatment. * = mean ± SD

Table 2: Effects of cytokinins on the shoot multiplication and shoot length of *S. altaiensis* after 3 weeks of culture

Cytokinin	Concentration (mg L^{-1})	Multiplication (%)	Shoots per explant (No.)*	Shoot length (cm)*
Control	-	33.3	1.23 ± 0.1 e	1.03 ± 0.03 g
6-BAP	0.5	36.7	2.17 ± 0.2 c	1.59 ± 0.06 c
	1.0	91.1	5.83 ± 0.2 a	1.95 ± 0.05 a
	1.5	75.6	4.43 ± 0.2 b	1.78 ± 0.03 b
	2.0	57.8	2.37 ± 0.2 c	1.72 ± 0.05 b
KN	0.5	26.7	1.43 ± 0.1 de	1.08 ± 0.02 fg
	1.0	68.9	2.27 ± 0.2 c	1.27 ± 0.04 d
	1.5	54.4	2.13 ± 0.2 c	1.22 ± 0.02 de
	2.0	47.8	1.87 ± 0.2 cd	1.14 ± 0.02 ef

Mean values followed by the same letters, a–g in the same column, are not significantly different at the 0.0001 level of significance (Duncan's multiple range test). 6-BAP = 6-benzylaminopurine, KN = kinetin, n = 30 explants per treatment. * = mean ± SD

Table 3: Effects of auxins on *in vitro* rooting of *S. altaiensis* shoots after 4 weeks of culture

Auxin	Concentration(mg L^{-1})	Rooting frequency (%)			Roots per explant (No.)*			Root length per explant (cm)*		
		1 W	2 W	3 W	1 W	2 W	3 W	1 W	2 W	3 W
Control	0	**97.8**			4.7 ± 0.2 a			1.8 ± 0.2 a		
IAA	0.5	9.1	27.3	47.8	1.5 ± 0.5 b	1.7 ± 0.3 c	1.6 ± 0.3 c	0.6 ± 0.1 bcd	0.6 ± 0.0 c	1.2 ± 0.1 b
	1.0	0	16.7	**70.4**	0 b	1.5 ± 0.5 c	2.1 ± 0.2 bc	0 d	0.6 ± 0.1 bc	1.2 ± 0.1 b
	1.5	0	29.2	47.8	0 b	1.7 ± 0.4 c	1.5 ± 0.2 c	0 d	0.9 ± 0.1 bc	1.0 ± 0.1 b
NAA	0.5	0	56.5	31.8	0 b	1.9 ± 0.3 c	1.6 ± 0.2 c	0 d	1.0 ± 0.1 bc	0.8 ± 0.1 b
	1.0	56.5	48.0	33.3	1.8 ± 0.2 b	2.3 ± 0.3 c	1.7 ± 0.2 c	0.5 ± 0.0 d	0.9 ± 0.1 bc	0.8 ± 0.1 b
	1.5	37.5	27.3	9.5	1.6 ± 0.2 b	1.3 ± 0.2 c	1.5 ± 0.5 c	0.6 ± 0.0 cd	0.4 ± 0.0 c	0.4 ± 0.0 b
IBA	0.5	28.6	**70.0**	30.0	4.2 ± 0.7 a	4.7 ± 0.2 a	2.3 ± 0.2 bc	1.1 ± 0.1 bc	1.1 ± 0.0 b	1.1 ± 0.1 b
	1.0	**60.0**	50.0	9.5	4.7 ± 0.5 a	3.8 ± 0.2 b	2.5 ± 0.5 bc	1.1 ± 0.0 b	1.0 ± 0.0 bc	0.8 ± 0.2 b
	1.5	**71.4**	40.9	20.0	4.7 ± 0.3 a	3.8 ± 0.3 b	3.0 ± 0.4 b	1.1 ± 0.0 b	1.0 ± 0.1 bc	0.7 ± 0.0 b

Mean values followed by the same letters, a–c in the same column, are not significantly different at the 0.0001 level of significance (Duncan's multiple range test). IAA = indole-3-acetic acid, NAA = naphthalene acetic acid, IBA = indole-3-butyric acid, n = 30 shoots per treatment. W = week. * = mean ± SD

Table 4: Effects of substrate composition on *ex vitro* acclimatization of *S. altaiensis* plantlets after 2 months

Substrate composition (peat:sand:vermiculite)	1:0:0 (Control)	1:1:1	1:1:0	1:0:1	0:1:1
Survival rate (%)	68.9	83.3	73.5	60.4	42.9
Shoot length (cm)*	1.30 ± 0.07 a	1.46 ± 0.09 a	1.38 ± 0.08 a	1.32 ± 0.08 a	1.26 ± 0.10 a
Leaves per shoot (No.)*	5.64 ± 0.29 ab	6.40 ± 0.33 a	6.11 ± 0.24 a	6.10 ± 0.28 a	4.86 ± 0.34 b
Root length per shoot (cm)*	7.21 ± 0.20 b	8.08 ± 0.24 a	7.70 ± 0.28 ab	7.56 ± 0.22 ab	5.35 ± 0.25 c
Roots per shoot (No.)*	3.10 ± 0.26 a	3.73 ± 0.30 a	3.47 ± 0.27 a	3.17 ± 0.21 a	2.19 ± 0.20 b

Mean values followed by the same letters, a–c in the same column, are not significantly different at the 0.0001 level of significance (Duncan's multiple range test). n = 30 plantlets per treatment. * = mean ± SD

subcultures without PGRs for 3 weeks).

Rooting was successfully induced within 4 weeks on *m*MS medium without PGRs (Fig. 1j). Rooting frequency, number of roots and root length per explant were 97.8%, 4.7 and 1.8 cm, respectively (Table 3). Also high rooting was achieved on *m*MS supplemented with 1.0 and 1.5 mg L^{-1} IBA after 1 week of culture, 0.5 mg L^{-1} IBA after 2 weeks of culture and 1.0 mg L^{-1} IAA after 3 weeks of culture. Rooting frequency on *m*MS medium supplemented with NAA in different concentrations was significantly lower (56.5% and below). Most of the shoots that were rooted on the medium with auxins had short thick roots and were not capable of further growth.

Healthy, rooted plantlets were transferred to *ex vitro* growth substrates in nursery pots. Table 4 shows the survival rate of these plantlets on the different *ex vitro* recovery media after 2 months. The maximum percentage of survival was 83.3% on growth substrate - peat: sand: vermiculite (1:1:1), 2 months after acclimatization (Table 4 and Fig. 1k). Sand and vermiculite proved to be the least

beneficial growth medium (42.9%).

These micropropagated plants were transferred into the open ground and the percentage of survival was 59.1% for 1 month and 50% for 3 months after transferring into the open ground. The average length of *S. altaiensis* after 3 months of adaptation formed 4.02 ± 0.25 cm (Fig. 1l). These plants got through winter. The following year the plants had good growth characteristics and morphology (Fig. 1m).

Discussion

Culture medium plays important role in process of micropropagation. Components of culture medium display a strong effect on morphogenesis. Murashige and Skoog's (MS) (Murashige and Skoog, 1962) medium was used as a basal medium for micropropagation of the genera of *Spiraea* L. (Lane, 1979) and *Physocarpus* Maxim. (Zhou, 2000; Wei *et al.*, 2006; Ilczuk *et al.*, 2013) which belong to the subfamily *Spiraeoideae*, like the genus *Sibiraea*. Culture medium optimization is essential because macro- and micronutrients source and concentration supplied to explants immediately influence on growth rates and development of plant. MS medium has high concentration of ammonia nitrogen and high quantity of total nitrogen in comparison with majority of other media. For *S. altaiensis*, the total amount of nitrogen was too high. It inhibited the growth of the explants *in vitro*. It can be explained that the plant family Rosaceae to which *S. altaiensis* is belong has tendency towards sensitivity to NH_4^+ toxicity (Britto and Kronzucker, 2002).

The quantity of agar, contained in the nutrient medium was an important growth factor of explants of *S. altaiensis* in the *in vitro* conditions. It is known that concentration of gelling agent cause effect on plants and tissue regeneration *in vitro* (Scholten and Pierik, 1998; Muthukrishnan *et al.*, 2013). Culture of explants in the nutrient medium with concentration of agar 0.5% (w/v) and more demonstrated inhibition of shoot growth and micropropagation and reduce the water availability in plants. It was noted by Selby *et al.* (1989). Our experiments showed that *S. altaiensis* shoots cultured *in vitro* grew faster on concentrations of agar 0.35% (w/v) in the medium. According to Casanova *et al.* (2008) low agar concentrations provide a poorly gelled medium that facilitates better diffusion of medium components leading to better growth and further rooting.

One of the most important factors in the *in vitro* micropropagation especially in proliferation stage is cytokinin hormones. It is well known that cytokinins regulate the shoot formation and multiplication and promote the cell division and expansion. TDZ was ineffective for shoot bud proliferation explants of *S. altaiensis*. Although TDZ belongs to the most active cytokinin-like compounds for woody plant tissue culture, sometimes it may inhibit shoot elongation of the buds (Meyer and Staden, 1988; Huetteman and Preece, 1993; Lu, 1993; Lyyra *et al.*, 2006). The rate of shoot development in the presence of this PGR in the nutrient medium was the smallest. The addition of cytokinins also was important for multiple shoot formation. The presence of 6-BAP as cytokinin in culture medium was important for shoot induction and multiplication of *S. altaiensis*. KN was less effective on shoot multiplication of *S. altaiensis* compared to 6-BAP. KN exhibited rather weak effects on multiplication.

Culture on the nutrient medium without PGRs was carried out with the purpose of prevention of negative effect of cytokinins, which can occur due to their gradual accumulation in plant tissue above the physiological level under long-lasting culture of explants in the mediums containing PGRs. Therefore, alternation of 3-week cycles of cultivation of *S. altaiensis* on the nutrient medium containing PGRs and the nutrient medium containing no PGRs was an obligatory condition for the receiving of the valuable microclones in the process of multiplication *in vitro*. Duration of culture had a significant influence on the efficiency of micropropagation of *S. altaiensis in vitro*. Shoot multiplication rate raised at the increase of the number of subcultures each time.

The rate of root formation on culture medium without auxins may be explained to the presence of endogenous auxin in the *in vitro* shootlets (Minocha, 1987). High concentrations of auxins can cause formation of short thick roots (Kongbangkerd *et al.*, 2005) and inhibition of root cell expansion and overall root growth (Chadwick and Burg, 1967; Sauer *et al.*, 2013). Substrate composition did not have a significant effect on shoot length, but influenced on leaves and root number per shoot, root length.

Conclusion

This is the first study that describes protocol of propagation *in vitro* for the rare endemic plant species of Altai - *S. altaiensis*. By using the above mentioned protocol one can receive hundreds of clonal plants. This protocol can be used to facilitate the rapid propagation of *S. altaiensis* for commercial and conservation purposes.

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