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EFFECTS OF GROWTH REGULATORS AND COMPOSITION OF NUTRIENT MEDIA ON *IN VITRO* REGENERATION OF *JUNIPERUS SABINA* L.

ВЛИЯНИЕ РЕГУЛЯТОРОВ РОСТА И СОСТАВА ПИТАТЕЛЬНЫХ СРЕД НА РЕГЕНЕРАЦИЮ IN VITRO *JUNIPERUS SABINA* L.

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Приведены результаты разработки метода регенерации экономически важного для Монголии растения — можжевельника казацкого (*Juniperius sabina* L.). Регенерация растений проводилась с использованием эксплантов листьев/побегов длиной 1,5–2,0 см, собранных летом. Реакция побегов на эксплантах зависела от типа питательной среды и концентрации регулятора роста 6-бензиламинопурина (БАП). Высокоэффективное побегообразование получено при выращивании на среде Мурасиге и Скуга (MS), среде Мурасиге и Скуга с витамином B5 Гамборга (MSB5) и базальной среде Шенка и Хильдебрандта (SH) с добавлением 0–1 мг/л 6-бензиламинопурина (БАП) длиной 0,5–2,6 см. Наибольшая скорость образования корней составила 75% на среде SH с добавлением 0,5 мг/л нафтилуксусной кислоты (НУК). Наибольшая длина корней наблюдалась на базальной среде Шенка и Хильдебрандта (SH) без регулятора роста.
Ключевые слова: Juniperus sabina, in vitro, регенерация, регулятор роста.

In this study we have to establish and optimize a regeneration for economically important Juniperius sabina L. in Mongolia. Plant regeneration were achieved with leaves/shoots explants collected in the summer with the length of 1.5–2.0 cm. Shoot responses on explants depended on nutrient medium types and the concentration of 6-benzilaminopurine (BAP) growth regulator. Highly efficient shoot formation was obtained when either on Murashige and Skoog (MS), Murashige and Skoog medium with Gamborg's B5 vitamin (MSB5) and Schenk and Hildebrandt Basal (SH) medium supplemented with 0–1 mg/l 6-benzilaminopurine (BAP) with the length of 0.5–2.6 cm. The highest root induction rate was 75% on SH medium supplemented with 0.5 mg/l naphthalene acetic acid (NAA). The longest root length observed Schenk and Hildebrandt Basal (SH) medium without growth regulator.
Keywords: *Juniperus sabina, in vitro,* regeneration, growth regulator.

INTRODUCTION

Juniper sp is well adapted to the dry and extreme climate of the Northern Hemisphere, and 68–80 species of plants grow widely [1]. In Mongolia, which has an extreme and harsh climate of Central Asia, is rich in genetic resources of natural wild plants. There are 3163 tuberous plants have been recorded in Mongolia, of which 6.9% vulnerable, 3.2% rare, 4.7% (very rare) endangered, 1.5% near threatened.

There are four *Juniper* species; *J. sabina*, *J. dahuricus*, *J. peudossabina* and *J. sibirica* distributed in mountainous regions of Khangai, Khentii, Altai and Gobi-Altai. *J. sabina* considered to endangered status and listed in the "Mongolian Red book" [16]. *Juniper* species develops low quality seed (empty seeds) with low germination and slow to coming out seed dormancy. For example, depending on the population, of its bush age and the weather of the year, 20–30% of the total seeds, and in some cases up to 4%, may contain embryos [14]. Therefore, in recent years, due to global climate change and excessive harvesting of plant resources for commercial purposes, the genetic resources of natural wild plants are decreasing. Therefore, it is necessary to investigate the possibilities of reproduction and natural resource restoration for the purpose of protecting the gene pool of Juniper plants, protecting resources, multiplying for the many purposes.

MATERIALS AND METHODS

In this study we used *J. sabina* leaves/shoots as an explant resources and had collected in July 2023 Mandal sum, Selenge province (latitude 48.85369, longitude 106.81295 a.s.m.l 1010 m).

In this experiment we used 3 types of medium; Murashige and Skoog medium (MS) [10] Murashige and Skoog medium with Gamborg vitamin (MSB5) [4] and Schenk and Hildebrandt Basal medium (SH) [15], either benzylaminopurine (BAP) or naphthalene acetic acid (NAA) growth regulator. The pH of all medium was adjusted to 5.8 with 1N NaOH or 1N HCl and autoclaved at 121°C for 15 min. The plant growth regulators were filter sterilized (0.22 μmol L^{-1} Millipors, USA) and added to cooled autoclaved medium. The experiment was performed in three replicated with 10–12 explants in each treatment.

Explant sterilization for *in vitro* initiation. Leaves/shoot cuttings collected in July, were selected as explants. To determine an efficient sterilization procedure shoot explants were washed with 0.1% soap water and 70% ethanol followed by soaking in 30, 40 and 50% commercial bleach, Clorox for 10 min and washed sterilized double distilled water with 3 times.

Explant preparation and *in vitro* regeneration. After surface sterilization, 1.5–2.0 cm length shoot cuttings; an explant were cultured on MS, MSB5 and SH medium with BAP concentration of 0, 0.5, 1.0, 2.0, 4.0 mg/l for direct shoot regeneration and with NAA of 0, 0.5, 1.0, 2.0, 4.0 mg/l for rooting. Shoot regeneration and rooting percent were calculated by comparing number of primary explants to regenerated shoots and rooted shoots. Newly initiated shoot and root from primary explants were measured by cm for length.

Analysis of variance (ANOVA) were evaluated using SAS software package (version 962, SAS Institute Inc., Cary, NC) and Duncan's Multiple Range Test were performed to analyze the means for significant difference at $p \leq 0.05$.

RESULTS

Enhancing effectiveness of explant sterilization. It is critical to use an effective sterilization method for obtaining explants source free of microbial contamination. The obtained results showed that sterilization of explants by 50% commercial bleach, Clorox for 10 min gives the best results in overcoming problems with internal bacterial infection. The contamination rate of the explant in 30% of commercial bleach was 48–56%, with 40% solution, it was 13–21%, and when it was sterilized with a 50% solution, there was not observed explant contamination. Thus it could represent a good sterilization method to obtain sufficient and healthy explants sources free of microbial contamination.

Effect of medium and BAP growth regulator concentration on the frequency of shoot growth from primary explants. For establishing an efficient plant regeneration of *J. sabina*, 1.5–2.0 cm length shoot explants cultured on three different medium, each medium supplemented with five different concentration of BAP were chosen for direct shoot regeneration and efficiency of shoot organogenesis as shown in table 1 and figure 1.

Table 1. Effects of BAP concentration in different medium on *in vitro* shoot regeneration of *Juniperus sabina* L.

BAP, mg/l	MS		MSB5		SH	
	Frequency of shoot regeneration, %	Shoot length, cm	Frequency of shoot regeneration, %	Shoot length, cm	Frequency of shoot regeneration, %	Shoot length , cm
0	100 ± 1.23^{a}	1.3	62.5 ± 1.54^{b}	1.8	100 ± 2.01^{a}	2.0
0.5	100 ± 2.14^{a}	1.2	100 ± 2.47^{a}	1.2	100 ± 3.4^{a}	2.8
1.0	83 ± 1.47^{ab}	2.6	100 ± 1.64^{a}	1.3	100 ± 1.6^{a}	1.0
2.0	1 ± 0.11^{d}	1.2	100 ± 2.35^{a}	1.8	25 ± 2.47^{c}	1.0
4.0	3 ± 1.2^{d}	0.5	3 ± 0.24^{d}	1.1	25 ± 2.12^{c}	1.1

The values are the mean of 3 replications ±SE. Different letters indicate statistically significant differences.

Shoot induction frequency was 83–100% in MS nutrient medium supplemented with 0–1 mg/l BAP, shoot length 0.5–2.6 cm, however shoot regeneration level low in MS medium supplemented with 2–4 mg/l BAP, shoot length 0.3–1.2 cm and shoot was not differentiated. Shoot induction frequency was 62.5–100%, shoot length 0.8–1.8 cm in MSB5 medium supplemented with 0–2 mg/l BAP, however shoot was not differentiated in MSB5 medium supplemented with 4 mg/l BAP. Shoot initiation observed in all treatment of SH medium supplemented with 0–4 mg/l BAP with 25–100%, shoot length 0.8–2.8 cm. Our experiment suggest that the MS, MSB5 and SH medium supplemented

with 0–1 mg/l BAP significantly inducing shoot initiation and formation of *J. sabina*, but the frequency of shoot regeneration decreased with the increase of kinetin concentration up to 4 mg/l (table 1, fig. 1). Based on the color, shape and growth of initiated shoot formation, BAP concentrations of 0.5 and 1.0 mg/L were suitable for shoot formation in this experiment and were selected for further study.

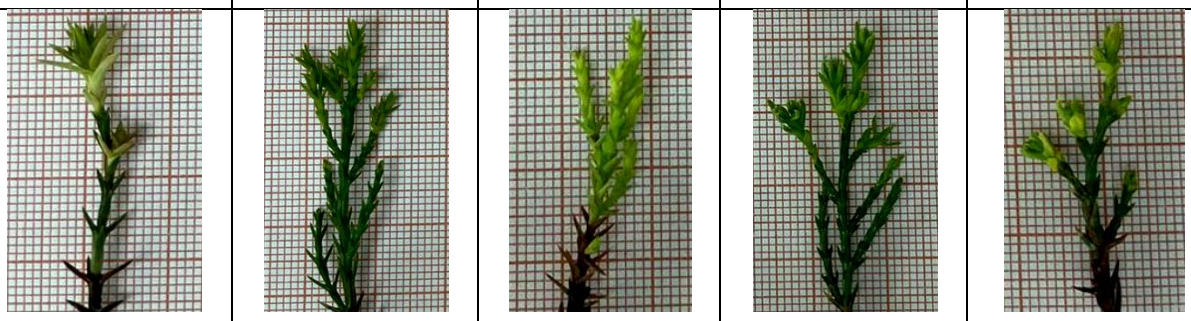
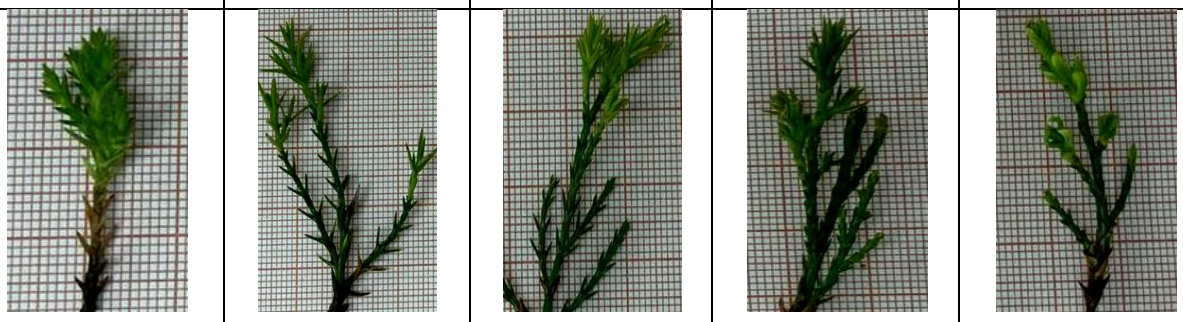
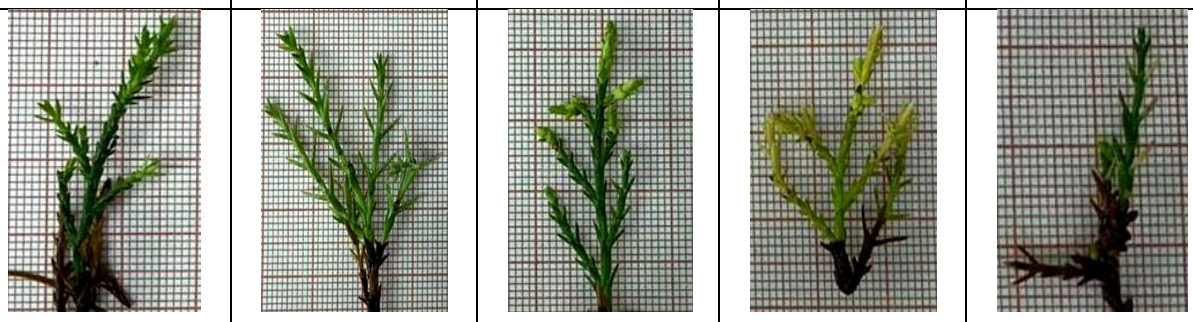
Medium	Concentration of BAP, mg/l				
	0	0.5	1.0	2.0	4.0
MS					
MSB5					
SH					

Figure 1. *J. sabina* shoot formation on MS, MSB5 and SH medium supplemented with different concentration of BAP growth regulator

Rooting of elongated shoots and recovery of whole plants. NAA was selected from the auxin-type growth substances for rooting medium preparation, and 0, 0.5, 1.0, 2.0, and 4.0 mg/L were added to MS, MSB5 and SH medium for rooting of propagated shoots (table 2).

Table 2. Effects of NAA concentration in medium type on rooting of *Juniperus sabina* L.

NAA, mg/l	MS		MSB5		SH	
	Rooting frequency, %	Root length, cm	Rooting frequency, %	Root length, cm	Rooting frequency, %	Root length, cm
0	50	7.5[d]	0	—	66.6	26.5[a]
0.5	50	7.3[d]	0	—	75	10.5[c]
1.0	40	7.5[d]	0	—	67	18.5[b]
2.0	25	7.5[d]	34	1.2[f]	34	6.2 [d]
4.0	0	1[f]	33	7[d]	34	21[ab]

The values are the mean of 3 replications. Different letters indicate statistically significant differences.

Rooting frequency was 25–50%, root length was 1–7.5 cm in MS medium. Rooting frequency was higher in 0–2 mg/l NAA in MS medium compared to MS medium added 4 mg/l NAA. There was no rooting observed in MSB5 medium added 0–1 mg/l NAA, however rooting frequency was 34–67%, root length 1.2–7 cm in MSB5 medium supplemented with 2–4 mg/l NAA. All of SH medium treatment showed good rooting frequency between 34–75%, with the length of 6.2–26.5 cm. Roots vigorously formed healthy on SH medium supplemented with 0–4 mg/l NAA (Fig.2).

DISCUSSION

To date, extensive research has been conducted on *Juniperus* species in many areas in the world, but relatively few research on tissue culture has been conducted on *J. sabina*. Most of the studies of *J. sabina* concentrate on chemical composition and its profile, biological and antidiabetic activities [12], [9] cytotoxic effect and against cancer cells [13]. In our study of tissue culture of *J. sabina*, the highest frequency of shoot initiation from primary shoot cutting explants recorded on MS medium supplemented with low concentration of BAP (up to 0.5 mg/l), MSB5 medium with middle concentration of BAP (0.5–2.0 mg/l) and SH medium with low concentration of BAP up to 1 mg/l. Comparing of the three media tested for shoot initiation, SH medium showed the best results in terms of shoot length and shape.

The application of 0.11 mg/l BAP to the SH medium resulted in the fastest rate of multiplication (Castro, 2011). The optimal BAP concentrate was 0.5 mg/l on the three medium that we used in this study for shoot initiation. The similar results showed on three juniper species (*J. excelsa*, *J. horizontalis*, and *J. chinensis*) shoots was 0.5 mg/l in woody plant medium [17]. BAP growth regulator at 1.0 mg/l without or with 0.02 mg/l NAA was essential for adventitious shoot development and the start of active morphogenic responses on MS medium for *J. excelsa* and *J. cedrus* [2].

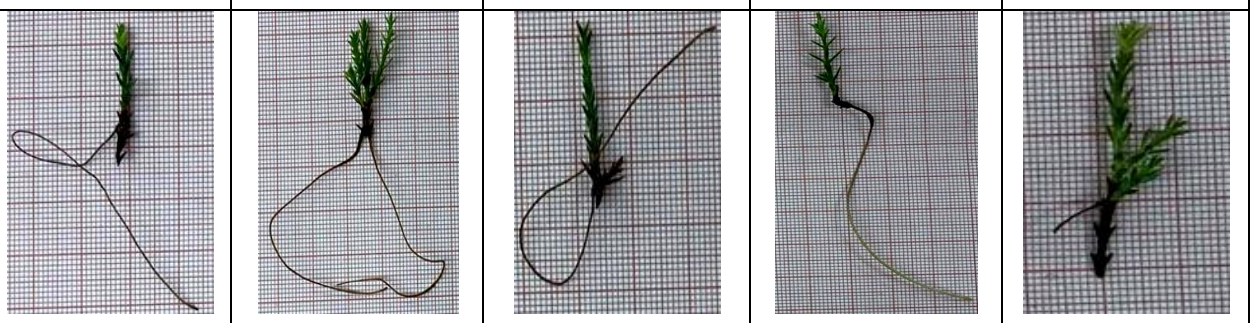
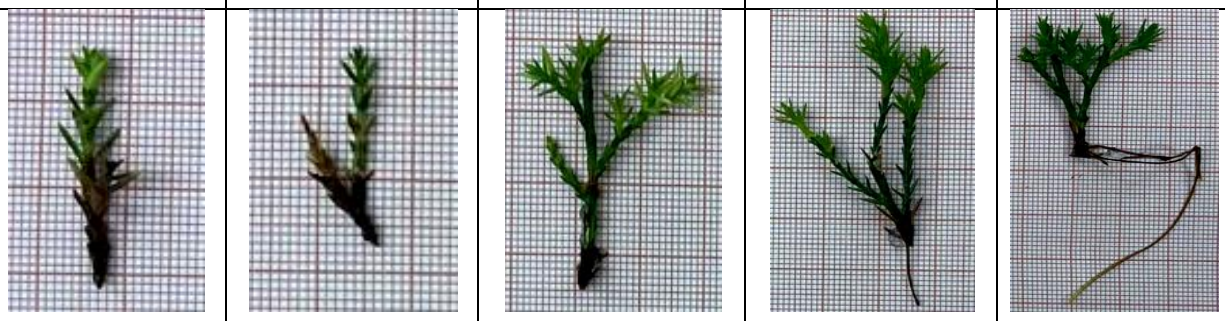
Medium	Concentration NAA, mg/l				
	0	0.5	1.0	2.0	4.0
MS					
MSB5					
SH					

Figure 2. Effect of NAA concentration on root regeneration from primary explants of *J. sabina* L.

For the rooting from primary explants, our result showed that low concentration (0–0.5 mg/l) of NAA in the MS medium rooting frequency was 50% with the length of 7.5 cm, while in SH medium rooting frequency was 75% with the length of 10.5–26.5 cm. High concentration (4 mg/l) of NAA does not affect root induction on MS medium while MSB5 and SH medium inducing root from primary explants with 33–34%. When

0.47 mg/l NAA and and 4% sucrose were added to modified SH medium, *J. oxycedrus* shoots were successfully rooted *in vitro* [5]. In general, most juniper species show good rooting when treated with IBA alone or in combination with NAA [8; 6; 17; 3; 7]. However, attempts to induce rooting in *J. polycarpos* have not been very successful, and it has been shown that this species has difficulty in *in vitro* rooting in media containing IBA and NAA [11]. *In vitro* rooting is viable in *J. chinensis* (87%), *J. phoenicea* (70%), *J. horizontalis* (68%), *J. thurifera* (up to 50%), *J. oxycedrus* (50%), and *J. excelsa* (42%) [8; 6; 17; 3; 7].

CONCLUSION

J. sabina L is one of the most important plant for Mongolian traditional medicinal plant and considered to endangered species in Mongolia. *Juniperus* regeneration protocol has been extensively practiced in the world, however genotype-independent and widely applicable protocol for efficient plant regeneration in *J. sabina* not yet available. In this study, an efficient *in vitro* regeneration protocol for *J. sabina* was established. In juniper tissue culture, primary explants: shoot cuttings are washed with a weak soap solution, rinsed with 70% ethanol for 5 minutes, treated with a weak sulfuric acid solution for 3 minutes, sterilized with 50% sodium hypochlorite solution for 15 minutes, and washed 3–4 times with sterile distilled water. For shoot initiation frequency from primary explants in MS medium supplemented with 0–1 mg/l BAP were 83–100%, and the length of shoot 0.5–2.6 cm, and in MSB5 medium supplemented with 0–2 mg/l BAP, shoot initiation frequency 62.5–100%, and the length of shoot were 0.8–1.8 cm. In MS and MSB5 medium supplemented with 2–4 mg/l BAP, shoots are deformed and grow in a distorted shape. All variants with BAP growth regulator added 0–4 mg/l to SH medium showed shoot initiation frequency ranging from 25–100% and shoot length ranging between 0.8 and 2.8 cm. The formation of roots from primary explants, all variants with 0–4 mg/l of NAA added to SH medium showed root formation, with root length ranging from 6.2–26.5 cm and root frequency ranged between 34 and 75%. In addition, roots formed in SH medium, and the root formation frequency and root length were the highest in SH medium among the three tested media.

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There is no conflict of interest.

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