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Influence of growth regulators on microclonal propagation of *Scrophularia umbrosa* Dumort under *in vitro* conditions

Naomi Asomani Antwi, Landysh Khusnetdinova, and Olga Timofeeva

Department of Botany and Plant Physiology, Institute of Fundamental Medicine and Biology, Kazan (Volga region) Federal University, ul. Kremlevskaya, 18, Kazan, 420008, Russian Federation

Address correspondence and requests for materials to Naomi Asomani Antwi, anasomani@stud.kpfu.ru

Abstract

The possibility of effective microclonal propagation of *Scrophularia umbrosa* Dumort for producing planting materials, aimed at preservation of the species has been shown. This will aid in the creation of artificial introduction populations of this endangered species in the Republic of Tatarstan, Russia. Optimal conditions for surface sterilization of seeds, development of microshoots, *in vitro* rooting and acclimatization have been developed. The most effective surface sterilization and germination was achieved with 10 % commercial bleach. Nodal explants were cultured in Murashige and Skoog's (MS) medium with different concentrations of 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA). The maximum number of microshoots was developed on MS medium containing 1.0 mg/l of BAP and 1.0 mg/l of IAA. Full strength MS medium with only IAA or together with (indole-3-butyric acid) IBA is optimal for rapid rooting of microshoots. The combination of soil: perlite (2 : 1) (v/v) was the best for *ex vitro* acclimatization of plantlets.

Keywords: micropropagation, *S. umbrosa*, surface sterilization, microshoot, morphogenesis, rhizogenesis, acclimatization

Introduction

Scrophularia umbrosa Dumort is a perennial herbaceous plant of the *Scrophularaeceae* family, used in folk medicine as an anti-inflammatory, diuretic and wound healing agent (Gubanov, 2004; Nikkhah et al., 2018). It grows along the banks of rivers and streams, in flowing swamps and is not so common on the territory of the Republic of Tatarstan. The changes in environmental conditions observed in recent years, as well as anthropogenic factors, have led to a sharp decline in the numbers of *S. umbrosa*. Currently, the species is endangered in the Republic of Tatarstan (Gamova, 2016). Various approaches are being implemented to preserve the species in the Republic of Tatarstan, including both the conservation of the natural environment and the cultivation of planting material for *ex situ* conservation in specially protected areas (Gamova, 2016).

Propagation using *in vitro* methods makes it possible to quickly restore the populations of endangered plant species (Jaramillo and Baena, 2007; Engelmann, 2011; Vetchinkina, Shirnina, Shirnin, and Molkanova, 2012) and provides a cost-effective, space- and time-saving method for large-scale production of planting material, which is especially important when seed availability is limited.

Studies have been conducted on the *in vitro* cultivation of some *Scrophularia* species (Maistrenko and Krasnoborov, 2009; Manivannan, Soundararajan, Park, and Jeong, 2015; Sivanesan, Lim, and Jeong, 2018). Maistrenko and Krasnoborov (2009) showed that MS medium containing 6-benzylaminopurine (BAP) in combination with 1-naphthylacetic acid (NAA) or kinetin was useful for successful microclonal

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Authors' information: Naomi Asomani Antwi, PhD Student, orcid.org/0000-0002-9540-5654; Landysh Khusnetdinova, PhD, Researcher, orcid.org/0000-0002-7867-2013; Olga Timofeeva, Dr. of Sci. in Biology, Head of Department, orcid.org/0000-0003-4921-458X

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propagation of *S. umbrosa* grown from the seeds collected from the Altai reproduction. Nevertheless, it is known that seeds of populations of different geographical origin may differ in germination rate, weight, and size (Tokhtar and Volobueva, 2012), which is most likely due to the differences in the endogenous level of phytohormones. In the literature, cases are often described when the same hormone exerts a completely opposite morphogenetic response (Catapan et al., 2002; Kalidass and Mohan, 2009; Nikule et al., 2020), which can be explained, among other things, by differences in the content of the hormone. This fact was taken into account when the work on clonal micropropagation of *S. umbrosa* from seeds collected under conditions of the Republic of Tatarstan was carried out. Moreover, the effect of BAP and indole-3-acetic acid (IAA) on the *in vitro* growth of *S. umbrosa* microshoots had not been studied before. We also studied the effect of rooting of the obtained microshoots on IAA and indole-3-butyric acid (IBA) under *in vitro* conditions and further acclimatization on various substrates for field reintroduction.

The aim of this study was to develop an effective method for *in vitro* propagation of *S. umbrosa* using BAP, IAA and IBA as growth regulators to obtain planting material for species restoration in the Republic of Tatarstan.

Materials and methods

One of the key steps in growing plants using tissue culture methods is both the selection of the explant and the selection of sterilization conditions. Seeds collected from *S. umbrosa* plants in the places of their natural growth on the territory of the Republic of Tatarstan were used as primary explants. Before sterilization, the seeds were immersed in a soapy solution for five minutes and then rinsed under running water. Further surface sterilization was carried out under aseptic conditions using a double sterilization procedure. First, the seeds were treated with 70 % ethyl alcohol for 30 seconds, followed by sterilization in a solution of commercial bleach "Belizna" (5.25 % sodium hypochlorite (NaOCl)) in various concentrations (10, 15 and 20 %) containing a drop of Tween 80 for 10 minutes. The seeds were then washed at least five times with sterile distilled water and placed on the surface of a hormone-free Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The condition of the sprouted plants was assessed visually during the three weeks of the experiment. Data on the percentage of contaminated cultures and seed germination after surface sterilization was recorded and analyzed.

Another important step in the process of *in vitro* propagation is the creation of optimal conditions for the rapid multiplication of plants. Nodal explants from shoots regenerated from seeds under *in vitro* conditions were subcultured onto MS medium for the production of mass planting material. To initiate morphogenetic growth processes, 1 cm long nodal explants were cultured on MS medium containing BAP alone at 0.5–2.0 mg/l concentrations; IAA alone at 0.1 to 2.0 mg/l concentrations, as well as BAP and IAA in combination with the same range of concentrations. After the cultivation of explants for 4 weeks, the regenerative ability of the explants was assessed. Data on the number of microshoots developed per explant, the rate of shoot induction, the length of shoots, the number of leaves per explant, the number of roots per explant was recorded.

Further studies were carried out on the rooting of microshoots under *in vitro* conditions. About 3–4 cm long single microshoots, separated from clusters of multiple microshoots, were cultivated on half and full strength MS media containing 20 g/l of sucrose, 8 g/l of agar as well as IAA and IBA at concentrations of 0; 0.5; 1.0 and 2.0 mg/L alone and in combination. Data on the length and number of developed roots were recorded after 2 weeks.

Microshoots that were successfully rooted under *in vitro* conditions were transferred to 100 ml plastic containers with three different sterilized substrates: soil: vermiculite: perlite (2 : 1 : 1); soil: vermiculite (2 : 1) and soil: perlite (2 : 1) in a volume by volume ratio (v/v) for acclimatization under *ex vitro* conditions. All containers with transferred plantlets were placed in mini plastic greenhouse containers for a period of 8 weeks. The seedlings that survived the acclimatization stage were planted on the territory of the nursery of rare plant species and the 'Russian-German Switzerland Nature Park'.

All cultures were grown in a "LiA-3" brand phytotron at a temperature of +26 ± 2 °C, a relative humidity of at least 70 %, a 16-hour photoperiod with an illumination of 3000 lux. Experiments to study the effectiveness of sterilization were carried out with 12 seed in three repetitions. To study the morphogenetic growth and rooting of plants *in vitro*, 12 nodal explants and microshoots each were used in five repetitions. For acclimatization in *ex vitro* conditions, 10 seedlings were used and the experiment was replicated three times.

All experiments were conducted in a complete randomized design. All experimental data were statistically analyzed using ANOVA at a significance level of $p < 0.05$ using GraphPad Prism 8.4.3. In cases where statistically significant differences were observed, the mean values were separated using Tukey's HSD test.

Results

With the sequential sterilization of seeds in a 70 % ethyl alcohol solution for 30 seconds and in different concentrations of commercial bleach solution (10, 15 and 20 %) for 10 minutes, no contamination was observed in initiated seed cultures after 21 days (100 %). At the same time, the seeds germinated actively. There were no statistically

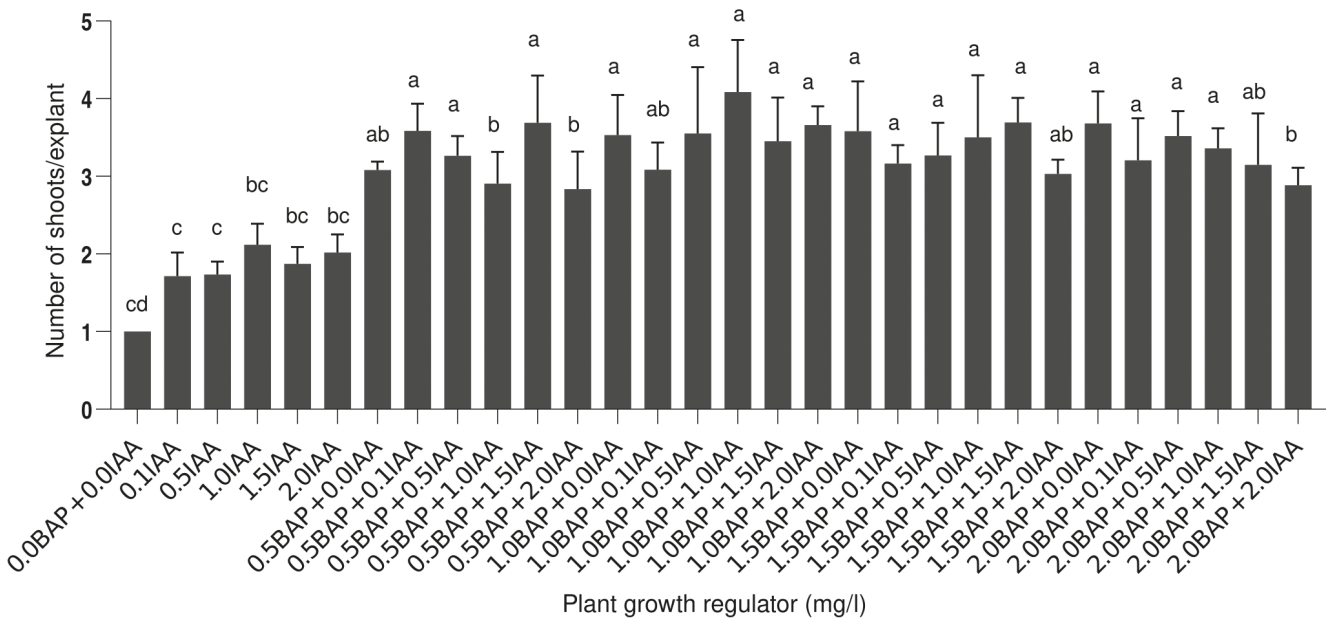

Fig. 1. Effect of BAP and IAA on microshoots regeneration in *S. umbrosa*. Each column is the mean of five replicates. Means with different alphabets are statistically different. The standard deviation of each mean is represented by error bars.

significant differences between the average percentages of germinated seeds for the entire period of observation (Table 1). The largest number of germinated seeds on both the 7th and 21st days was observed when the explants were treated with 10 % commercial bleach. In this variant, 10.67 and 76.0 % of *S. umbrosa* seeds germinated in the first 7 days and 21 days, respectively (Table 1).

The rate of microshoot induction ranged from 90 to 100 % (Table 2). There was no statistically significant difference in the effect of the composition of culture medium on the rate of microshoot induction.

Multiple microshoots were formed in all variants of media used, with the exception of MS medium without growth regulators (Fig. 1). Differences in the effect of nutrient medium composition on the average number of microshoots developed on one explant were statistically significant. The average number of microshoots developed per explant ranged from 1.0 to 4.10. The

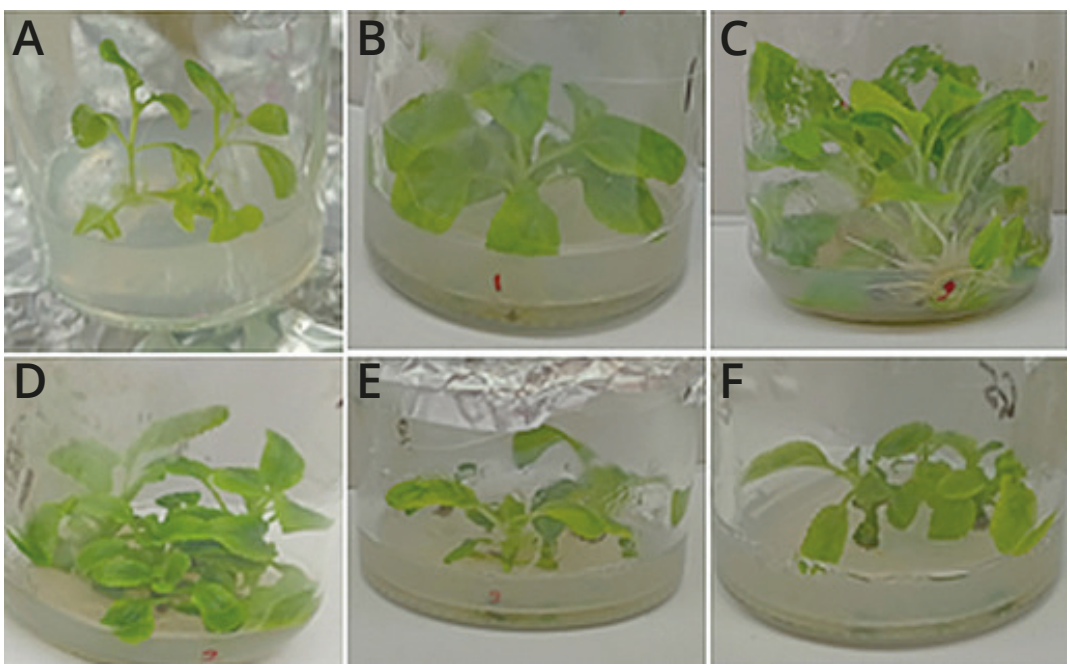

Fig. 2. *In vitro* seed germination and microshoot multiplacation of *S. umbrosa*. A — germinated seedlings after surface sterilization; Shoot development; B — on hormone-free MS medium; C — on 0.5 mg/l BAP; D — on 1.0 mg/l BAP and 1.0 mg/l IAA; E — on 1.0 mg/l IAA; F— on 2.0 mg/l BAP and 2.0 mg/l IAA.

Table 1. Effect of surface sterilization procedure on *S. umbrosa* seed germination

Variants	Germinated seeds, %	
	Day 7	Day 21
70 % ethyl alcohol + 10 % bleach	10.67 ± 2.31	76.00 ± 17.44
70 % ethyl alcohol + 15 % bleach	6.67 ± 4.62	72.00 ± 18.33
70 % ethyl alcohol + 20 % bleach	2.67 ± 2.31	73.33 ± 1617

Each value is the mean and standard deviation of three replicates.

combination of growth regulators at 1.0 mg/l BAP and 1.0 mg/l IAA contributed to the formation of the maximum number of microshoots (Fig. 1). Despite the fact that multiple microshoots were formed on the MS medium with only IAA, they were less in quantity compared to the media containing BAP as the sole plant growth regulators or in combination with IAA. Simultaneous development of roots and microshoots was observed in all variants of MS medium used in the study (Fig. 2).

Table 2. Effect of BAP and IAA concentration on morphogenetic growth of *S. umbrosa*

Plant growth regulators, mg/l		Induction of microshoots, %	Number of leaves/explant	Number of roots/explant
BAP	IAA			
0	0	100 ± 0	7.08 ± 0.19 c	7.95 ± 0.90 cd
–	0.1	93.32 ± 9.15	11.03 ± 0.66 b	13.9 ± 1.69 c
–	0.5	100 ± 0	11.40 ± 0.64 b	17.00 ± 1.59 b
–	1	90.0 ± 9.15	13.27 ± 0.90 b	19.71 ± 1.86 a
–	1.5	93.32 ± 9.15	12.97 ± 2.0 b	17.20 ± 2.01 b
–	2	96.66 ± 7.47	13.60 ± 1.09 b	20.37 ± 2.97 a
0.5	–	100.0 ± 0	14.0 ± 1.96 b	13.52 ± 0.99 c
1	–	93.32 ± 9.15	19.42 ± 2.33 a	8.21 ± 0.63 cd
1.5	–	93.32 ± 9.15	16.39 ± 1.26 ab	13.81 ± 0.63 c
2	–	96.66 ± 7.47	17.43 ± 2.34 ab	2.1 ± 0.74 d
0.5	0.1	96.66 ± 7.47	18.13 ± 1.44 ab	16.68 ± 1.01 b
0.5	0.5	90.0 ± 9.15	17.63 ± 2.97 ab	13.93 ± 2.94 c
0.5	1	96.66 ± 7.47	14.65 ± 1.12 ab	9.0 ± 1.0 cd
0.5	1.5	93.40 ± 14.76	15.51 ± 3.29 ab	5.40 ± 3.30 cd
0.5	2	93.32 ± 9.15	16.23 ± 2.46 ab	12.09 ± 2.13 c
1	0.1	90.0 ± 9.15	14.84 ± 1.65 ab	8.86 ± 0.92 cd
1	0.5	93.40 ± 14.76	17.40 ± 2.31 ab	13.10 ± 0.74 c
1	1	100.0 ± 0	19.43 ± 1.98 a	14.52 ± 1.28 b
1	1.5	90.06 ± 14.78	16.57 ± 3.23 ab	7.41 ± 0.96 cd
1	2	96.66 ± 7.47	17.73 ± 1.79 a	2.80 ± 0.84 d
1.5	0.1	90.0 ± 9.15	16.19 ± 1.49 ab	5.50 ± 1.22 cd
1.5	0.5	93.32 ± 9.15	15.63 ± 2.73 ab	8.64 ± 0.98 cd
1.5	1	96.66 ± 7.47	15.66 ± 1.31 ab	6.30 ± 0.78 cd
1.5	1.5	96.66 ± 7.47	17.0 ± 2.03 ab	2.80 ± 0.84 d
1.5	2	96.66 ± 7.47	17.33 ± 3.18 ab	3.80 ± 0.84 d
2	0.1	100.0 ± 0	13.80 ± 1.20 b	3.85 ± 0.71 cd
2	0.5	100.0 ± 0	13.67 ± 1.35 b	5.91 ± 1.14 cd
2	1	96.66 ± 7.47	17.93 ± 3.96 ab	6.76 ± 1.11 cd
2	1.5	90.06 ± 14.78	17.29 ± 1.92 ab	6.12 ±0.76 cd
2	2	93.32 ± 9.15	14.21 ± 1.28 b	4.07 ± 0.24 d

Each value is the mean and standard deviation of five replicates. Means with different alphabets are statistically different.

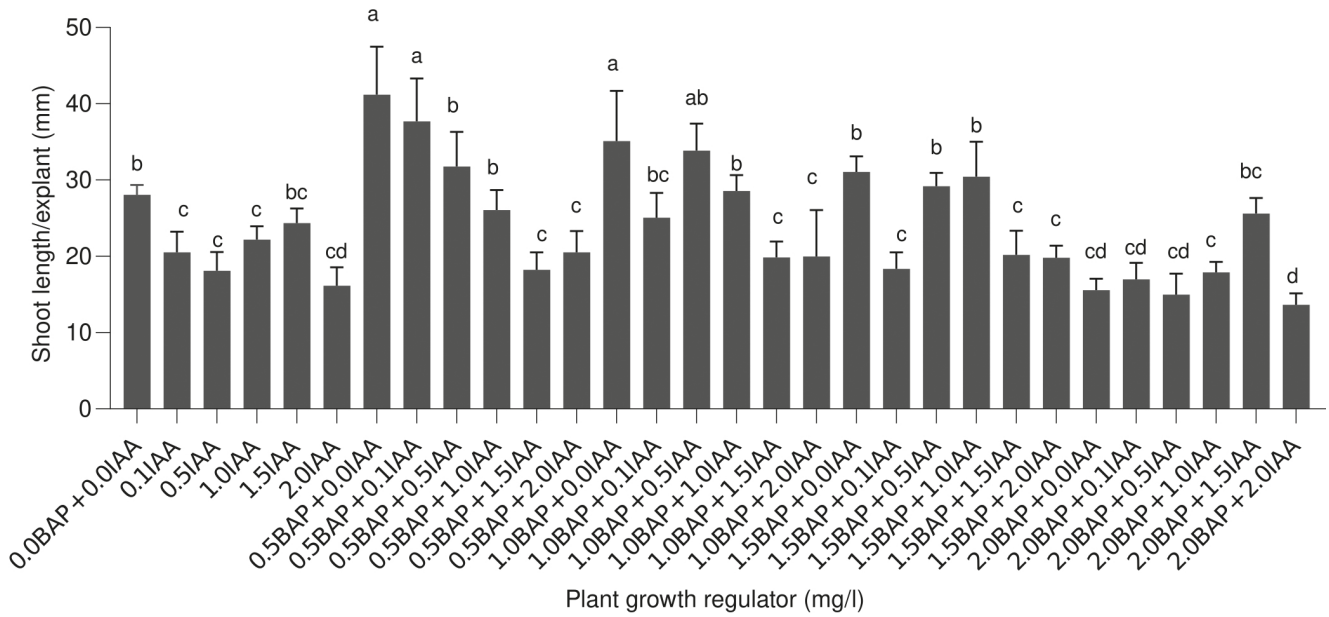

Fig. 3. Effect of BAP and IAA on shoot length of *S. umbrosa*. Each column is the mean of five replicates. Means with different alphabets are statistically different. The standard deviation of each mean is represented by error bars.

However, the best rooting was observed in the presence of IAA alone in the culture medium at concentrations of 0.5–2.0 mg/l (Table 2). The average number of developed leaves per explant ranged from 7.08 to 19.43 with the least developed leaves on MS media without hormones (Table 2). The combination of 1.0 mg/l of BAP and 1.0 mg/l of IAA contributed to the formation of the maximum number of leaves per explant. The average shoot length ranged from 13.67 to 41.20 mm. The longest shoot length was observed on the MS medium with a 0.5 mg/l BAP (Fig. 3).

The effect of rooting hormones and MS medium (half or full strength) on the number of roots developed and root length was statistically significant. The mean number of roots, developed by each microshoot, ranged from 7.25 to 31.0 on half strength MS medium and from 14.5 to 44.75 on full strength MS medium regardless of the rooting hormone used (Fig. 4A). The largest number of roots was formed on full strength MS medium with 0.5 mg/l IAA in combination with 1.0 mg/l IBA. On half strength MS medium, the largest number of roots was formed on 2.0 mg/l IAA in combination with 1.0–2.0 mg/l of IBA. In the cases where IAA or IBA were the only rooting hormones used, the highest number of roots was observed on the media with IAA on both half and full strength MS media. In cases where both root-

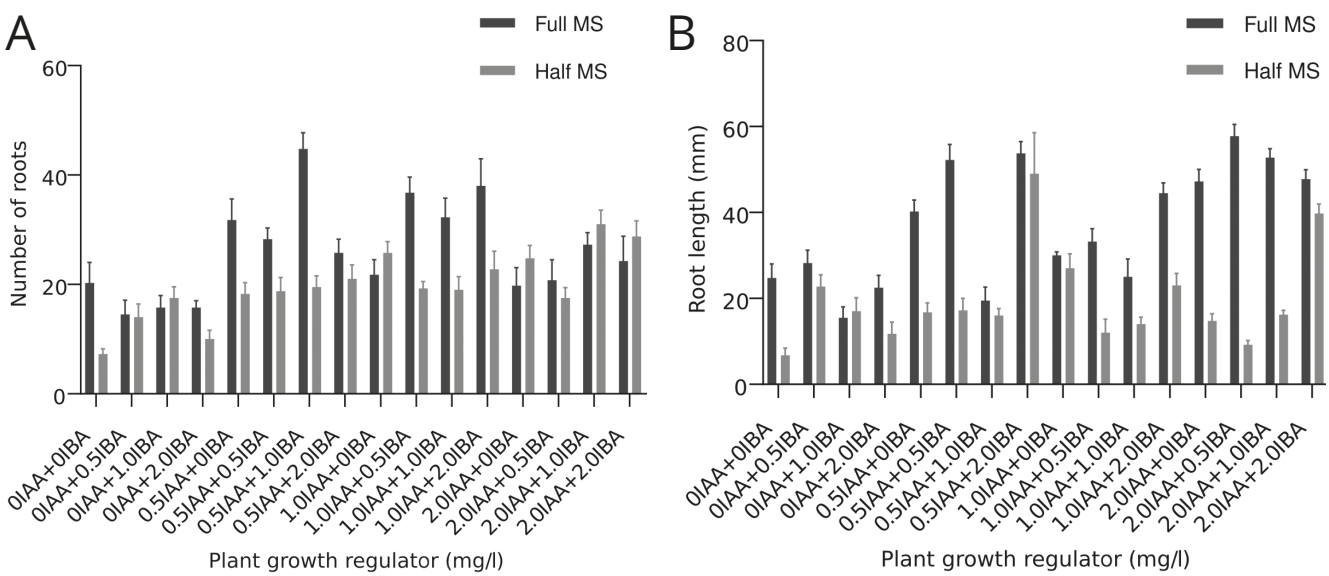

Fig. 4. Effect of plant growth regulator concentration on the number of roots developed per microshoot (A) and root length (B). Each column is the mean of four replicates. The standard deviation of each mean is represented by error bars.

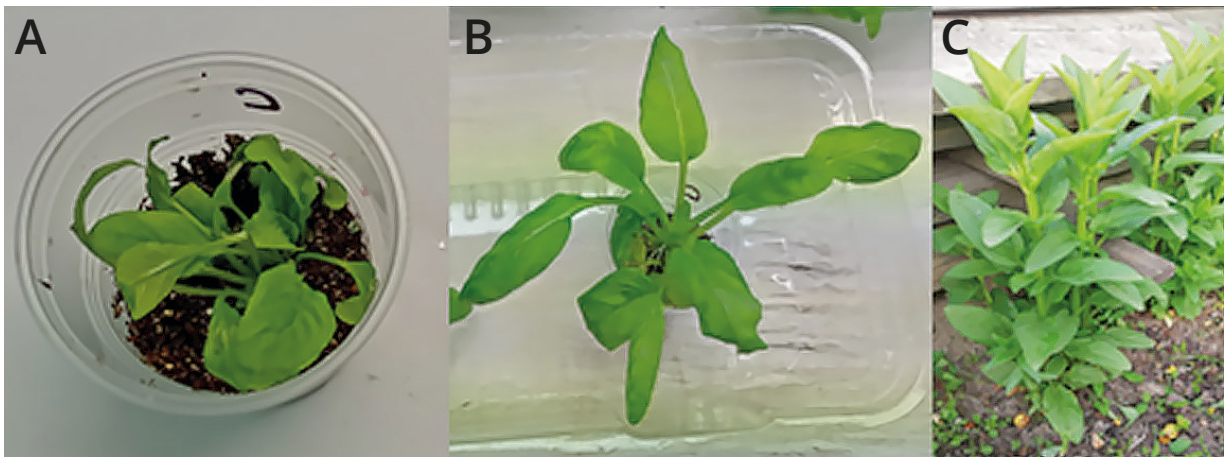

Fig. 5. *Ex vitro* acclimatization and field planting of *S. umbrosa*: A — *in vitro* plants transferred to a potting medium; B — acclimatized plants at 4 weeks old; C — two-year old plants.

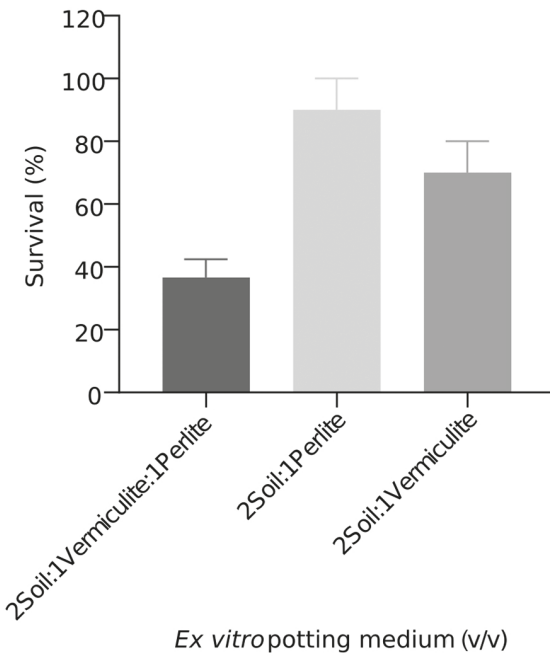

Fig. 6. Effect of the potting medium on survival of *S. umbrosa* plantlets during acclimatization. Each column is the mean of three replicates. The standard deviation of each mean is represented by error bars.

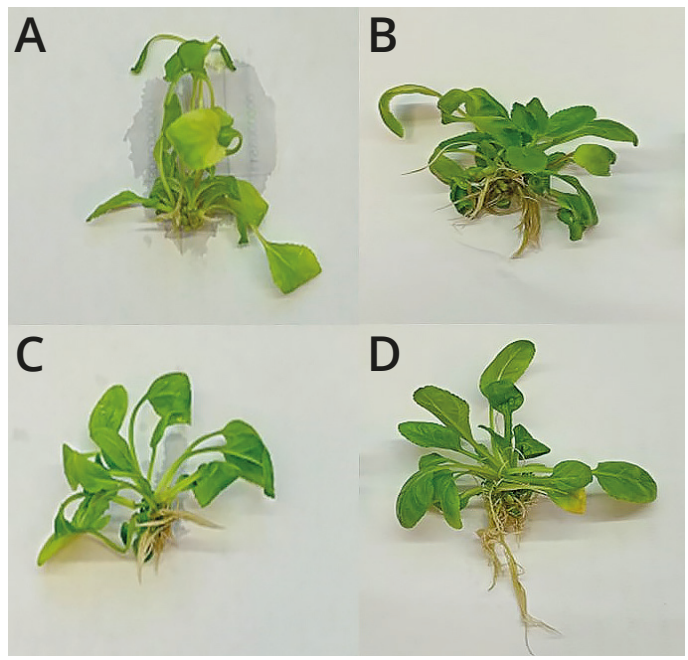

Fig. 7. Effect of IAA and IBA concentration on rhizogenesis of *S. umbrosa* under *in vitro* conditions. Half-strength (A) and full-strength (B) MS medium without IAA and IBA; half-strength (C) and full-strength (D) MS medium with 0.5 mg/l IAA and 0.5 mg/l IBA.

ing hormones were present in the culture media, IAA concentrations in the range of 0.5 to 1.0 mg/l yielded the highest number of roots on full strength MS medium and 2.0 mg/l on half strength MS medium.

The mean root length ranged from 6.25 to 49.0 mm on half strength MS medium and from 15.5 to 57.75 mm on full strength MS medium, regardless of the composition and concentration of hormones (Fig. 4B). The longest roots were formed by combining 0.5 mg/l IAA with 2.0 mg/l IBA, as well as 2.0 mg/l IAA with 0.5 mg/l IBA on half and full strength MS media respectively. The shortest roots were observed on hormone-free half strength MS medium and on full strength MS medium at 1.0 mg/l IBA and on 0.5 mg/l IAA with 1.0 mg/l IBA respectively. On the media containing only one of the hormones, the longest roots formed in the presence of IAA.

At the final stage of microclonal propagation of plants, the obtained microshoots were adapted to non-sterile soil conditions *ex vitro* (Figs 5, 6). The combination of soil and perlite in a ratio of 2 : 1 (v/v) gave a 91 % survival rate at acclimatization after 28 days. Then the plants were planted in the open ground — 20 plants on the territory of the nursery of rare plant species of the Kazan Federal University and 20 specimens in the natural conditions of the "Russian-German Switzerland national park" in Kazan.

Discussion

Surface sterilization of explants is essential for the cultivation of plant tissue cultures, as this process ensures the production of aseptic cultures by killing microbes. As a result of the studies, a high degree of seed germination was observed (more than 70 %) at all sterilization regimes (sequential sterilization of seeds in a 70 % solution of ethyl alcohol and in a solution of commercial bleach "Belizna") after 21 days, with no negative impact on the viability of seeds. Maistrenko and Krasnoborov (2009) showed that the use of only 15 % Domestos bleach within 15 minutes of surface sterilization of *S. umbrosa* seeds offers the fastest and most reliable sterilization. In our studies, pre-treatment of seeds with ethyl alcohol reduced the sterilization time in bleach to 10 minutes. Other authors (Mihaljević et al., 2013; Felek, Mekibib and Admassu, 2015; Firoz et al., 2016; Davoudpour et al., 2020) indicated that the application of 10–20 % concentration of commercial bleach and 70 % ethyl alcohol yields positive results in surface sterilization and *in vitro* regeneration of crops of various plant species, although compared to our studies, a certain level of contamination was observed.

Plant growth regulators play an important role in controlling the physiological growth processes during growth under *in vitro* conditions. In our work, the effect of the ratio of exogenous growth regulators (BAP and IAA) on the process of morphogenesis was studied. Induction of microshoots was largely successful in all media studied. Induction of microshoots from nodal explants of *S. umbrosa* did not depend on the presence of BAP or IAA in culture medium, since 100 % shoot formation was observed even on a hormone-free MS medium (Table 2). The differences in shoot formation rates observed in our experiments may be related to the location of explants on the original donor plant. There is evidence that the position of meristems on the source plants in relation to the apical meristem has a significant impact on the regenerative capacity of nodal explants prepared for *in vitro* cultivation (Kumar et al., 2022). Nodal explants with meristems farther from the apical meristem have less strength and may not stimulate the growth of new shoots.

Despite the fact that the induction of single shoots was observed even on a hormone-free MS medium, the number of these shoots was highly dependent on the presence of hormones in the culture medium. Induction of multiple microshoots is desirable in micropropagation as a means of rapidly increasing the amount of planting material. BAP is known to stimulate the formation of multiple microshoots in *Scrophularia* species, as well as in many other plant species under *in vitro* conditions (George, Hall, and Deklerk, 2008; Maistrenko and Krasnoborov, 2009; Manivannan, Soundararajan, Park, and Jeong, 2015; Sivanesan, Lim and Jeong, 2018). A study on *S. umbrosa* showed the most effective formation of microclones at BAP concentrations in the range of 0.2–1 mg/l alone or in combination with 0.25 mg/l kinetin on MS medium. The best rates of development of *S. umbrosa* microshoots were achieved on MS medium in the presence of 0.5 mg/l of BAP in combination with 0.1 mg/l of NAA (Maistrenko and Krasnoborov, 2009). Nodal explants of *Scrophularia kakudensis* formed more microshoots at BAP concentrations ranging from 0.5 to 2.0 mg/L (Manivannan, Soundararajan, Park, and Jeong, 2015). In another study, nodal explants of *Scrophularia takeimensis* Nakai also produced more shoots on MS medium with BAP alone and in combination with IAA at the same concentrations (Sivanesan, Hwang, and Jeong, 2008) as in our studies. In this study, the optimal nutrient medium for the formation of multiple adventitious shoots was MS medium containing 1.0 mg/l of BAP and 1.0 mg/l of IAA. The formation of fewer microshoots in the media containing only IAA compared to the media containing BAP can be explained by the key function of auxins in stimulating root formation (George, Hall, and Deklerk, 2008). On the other hand, the presence of only IAA better stimulated the process of rhizogenesis, as evidenced by an increase in the number of roots on the media with only IAA at concentrations of 0.5 to 2.0 mg/l.

The smallest number of leaves (Table 2) observed on MS medium without hormones correlates with the formation of single shoots on the medium (Fig. 1). The longest shoots were observed on the medium with 0.5 mg/l of BAP (Fig. 2), which is economically beneficial for micropropagation, since the need for a separate stage of shoot growth is already provided.

Effective rooting of seedlings under *in vitro* conditions is necessary for survival at the acclimatization stage during micropropagation (Mali and Chavan, 2016). *In vitro* plantlets with a well-developed root system are well equipped for quick access to nutrients needed for further growth in *ex vitro* conditions. In this study, the concentration of MS basal medium played an important role in determining the overall rooting effect in *S. umbrosa*. In our study, full strength MS medium showed the best rooting effect on the number and length of roots developed within two weeks (Fig. 7). Better rooting observed on full strength MS medium as in our study was noted for other plants (Zayova, Petrova, Nikolova, and Dimitrova, 2016; Chen et al., 2020). On the other hand, in *S. kakudensis*, the highest number and length of roots were observed on half strength MS medium (Manivannan, Soundararajan, Park, and Jeong, 2015). Many authors have also reported that half strength MS medium produced more favorable results when plants were rooted under *in vitro* conditions (Hariprasath, Jegadeesh, Arjun, and Raaman, 2015; Okello et al., 2021; Amiri and Mohammadi, 2021). The best rooting of *S. umbrosa* on full strength MS medium can be attributed to a prefer-

ence for a richer nutrient supply, which promoted rapid and vigorous root development (Kumar and Reddy, 2011). Longer roots also formed on full strength MS medium (Fig. 4B).

In our studies, the best effect of auxins on root development was also observed on full strength MS medium. The combined action of IAA and IBA was effective for root development in *S. umbrosa*. IAA concentration in the range of 0.5–1.0 mg/l in combination with 0.5–2.0 mg/l IBA was optimal for stimulating root formation in our study on full strength MS medium (Fig. 4).

There is conflicting data in the literature on the role of auxins on the rooting processes. Our results showed that the use of IAA alone was ideal for root development compared to an IBA and auxin-free culture medium. Similar results were observed in *S. kakudensis*, where the best root length and numbers were obtained at 0.5 mg/l IAA and 1.0 mg/l IAA, respectively (Manivannan, Soundararajan, Park, and Jeong, 2015). In contrast, Maistrenko and Krasnoborov (2009) reported that hormone-free MS medium produced a better rooting effect under *in vitro* conditions. This difference in *S. umbrosa* rhizogenesis results in our study may be due to the genotypic effect of the different *S. umbrosa* samples used, as well as differences in endogenous hormone levels (Kumar and Reddy, 2011).

The highest survival rate on soil and perlite mixtures may be due to perlite's ability to loosen the soil and provide greater aeration (Bar-Tal, Saha, Raviv, and Tuller, 2019). It has been shown that *S. umbrosa* seedlings obtained *in vitro* survive best on loose soil during acclimatization to field conditions (Maistrenko and Krasnoborov, 2009).

Conclusion

Thus, for sterilization of *S. umbrosa* seeds under *in vitro* conditions, 10 % commercial bleach "Belizna" solution in combination with 70 % ethyl alcohol, which showed the best antiseptic effect and turned out to be the most economical in sterilization of seeds, can be recommended. Induction of multiple microshoots with elongated shoots at minimum concentrations of phytohormones provides the most economical and genetically stable method of clonal propagation of *S. umbrosa* under *in vitro* conditions. Therefore, optimal for clonal micropropagation of *S. umbrosa* is MS basal medium containing only BAP at a concentration of 0.5–1.5 mg/l or 0.5–1.0 mg/l BAP in combination with IAA at a concentration of 0.1–1.0 mg/l based on all available growth parameters. However, taking into account the fact that for mass propagation of plants it is necessary to minimize costs, and, first of all, for phytohormones as the most expensive components of the nutrient medium, it is advisable to use MS medium, containing only 0.5 mg/l BAP or a combination of 0.5 mg/l BAP with 0.1 mg/l IAA.

Full strength MS medium containing auxins in the combinations of 0.5–1 mg/l IAA and 0.5–2 mg/l IBA is optimal for rapid rooting of microshoots *in vitro*. However, for cost-effectiveness, a complete MS medium containing 0.5 mg/l IAA or a combination of 0.5 mg/l IAA and 0.5 mg/l IBA should be recommended. The combination of soil : perlite (2 : 1) is suitable as a substrate for the *ex vitro* acclimatization of *S. umbrosa*.

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