The creation of a Taraxacum kok-saghyz high frequency regeneration system

A A Verbitskaya¹, A S Ivanova¹, ³, N G Konkova² and A K Gaponenko¹

¹ Institute of Developmental Biology named after A.K. Koltsov of the Russian Academy of Sciences, 26 Vavilov Street, Moscow, 119334, Russia
² All-Russian Institute of Plant Genetic Resources named after N.I. Vavilov, 42-44 Bolshaya Morskaya Street, Saint Petersburg, 190000, Russia
³ Russian Potato Research Center named after A.G. Lorkh, 23 v Lorkh Street, Kraskovo village, Lyubertsy, Moscow region, 140051, Russia

E-mail: timoshenko.alekseevna@gmail.com

Abstract. The aim of this research was to study the morphogenetic ability of Taraxacum kok-saghyz root tissues and to optimize the culture medium for the subsequent genetic transformation of plants. The effects of exogenous hormone exposure on survival, in vitro shoot induction, and root formation were studied by using root tissues. For the cultivation the samples of kok-saghyz, the Murashige-Skuga nutrient medium was used as the basis, supplemented with sucrose 20 g/l, vitamins B5 1 mg/l, and also containing agar 5.5 g/l. The pH value is 5.7. For the plant regeneration induction, growth regulators, auxins and cytokinins were added in culture media. In this study, the roots of D 1 mm and two media variants were used for comparison: variant 1. MS + 6-BAP 1 mg/l; variant 2. MS + 6-BAP 1 mg/l + IAA 0.2 mg/l. An effective protocol for the regeneration of kok-saghyz explants was developed. There was a high percentage of regeneration of 87.6% on the medium containing a combination of cytokinin and auxin, as well as a high percentage of direct shoot formation, which was 65.1%, the degree of rooting was 100%, the resulting cultured plant tissues grew well and had a high survival rate after transplantation.

1. Introduction
Taraxacum kok-saghyz, better known as Russian dandelion, is a perennial rubber-bearing plant of the Aster family (Astraceae) [1]. This plant contains a significant amount of high-quality rubber (up to 27% by dry weight), which is comparable to Hevea brasiliensis rubber.

Recently, Taraxacum kok-saghyz has regained popularity and many countries have started research activities to improve its agronomic characteristics, which is a prerequisite for its commercial use as an alternative source for the production of natural rubber. With the help of new methods of genetics, breeding, and molecular biology, it is possible to improve the quality and increase the quantity of this biopolymer. The current method used at kok-saghyz is genetic transformation, which can be very effective for improving the agronomic value and metabolism of this plant, as well as for changing properties such as the content and quality of rubber in the roots and optimizing the yield of natural rubber.
The efficiency of plant tissue transformation is considered a probabilistic process. It consists of the product of the frequencies of all events that determine the creation of a transgenic plant. The improvement of all processes is of great value when creating a transgenic plant with new characteristics and further using it as a commercial product. Since, out of dozens of plants subjected to transformation, only a few transformants will undergo selection and will have the necessary number of integrated copies of the gene, contain the integration region and a high level of its expression [2]. One of the most important stages that affects the efficiency of transformation is the process of regeneration of the obtained transformants. To do this, it is necessary to choose the optimal composition of the nutrient medium, as well as to choose explants from which it will be possible to propagate transgenic plants in the future.

For the cultivation of kok-saghyz, various explants (seeds, parts of roots, leaf plates) are used, in most works, the Murashige-Skuga (MS) nutrient medium is used as the basis [3, 4, 5]. To stimulate regeneration, the nutrient medium is modified with phytohormones, the final result depends on their concentration and combination. There are two possible ways of plant regeneration - direct regeneration, when shoots develop from an explant planted on a nutrient medium, bypassing the callus stage. Or through the stage of formation of callus. In this case, when the growth regulators are introduced into the medium, the explant cells in the in vitro culture begin to dedifferentiate, divide unorganized, and form a callus. Then, when removing or introducing the necessary auxins and cytokinins, as well as changing the type of phytohormones, the cells can differentiate and form meristematic centers in which the further development of shoots, embryos or roots occurs, which is determined by the type of phytohormones and physiological conditions.

The first cultivation of explants from the roots of Taraxacum officinale (a related plant of kok-saghyz) was carried out in 1970 [6]. In the literature, there are many studies on the effect of hormones on the regeneration and induction of various kok-saghyz explants. Some of them present data on the low frequency of callus formation from root explants, as well as on morphological changes in tissues [7, 8].

In this study, the task was to develop an effective protocol for inducing regeneration and obtaining a high frequency of callus formation and shoots with the selection of optimal root explants.

2. Materials and methods

2.1. Plant material
In this work, kok-saghyz plants (in vitro) obtained in the All-Russian Institute of Plant Genetic Resources named after N.I. Vavilov were used as explants. The obtained samples of Taraxacum kok-saghyz were grown under aseptic conditions (in vitro) on MS medium. Two types of root fragments were used: with a diameter $D = 1$ mm and $D < 1$ mm.

2.2. Composition of media and cultivation conditions
For the cultivation of kok-saghyz, the MS nutrient medium was used as the basis, supplemented with sucrose 20 g/l, vitamins B5 1 mg/l, and also containing agar 5.5 g/l. The pH value is 5.7. For the plant regeneration induction, growth regulators, auxins and cytokinins were added in culture media. In this study, the roots of $D = 1$ mm and two media variants were used for comparison: Variant 1. MS + 6-Benzylaminopurine (6-BAP) 1 mg/l; Variant 2. MS + 6-BAP 1 mg/l + indole acetic acid (IAA) 0.2 mg/l. Plant roots from test tubes were cut into segments of 1-2 cm and transferred to Petri dishes. The material was cultured in a climate chamber at the temperature of 25 °C and a 16-hour photoperiod. After the appearance of the first foci of regeneration, all explants were transferred to MS medium without growth regulators. After the shoots were formed, the explants were cultured for $\frac{1}{2}$ MS.

3. Results and discussions
To successfully create a transformation system, as well as for further microclonal reproduction of transgenic plants, it is necessary to choose the optimal type of explants and the composition of the medium for tissue regeneration.
Figure 1. Comparison of culture media for regeneration of Taraxacum kok-saghyz root explants (variant 1: MS + 6-BAP 1 mg/l; variant 2: MS + 6-BAP 1 mg/l + IAA 0.2 mg/l).

Regeneration foci on root explants were observed 7 days after planting on MS + BAP and MS + BAP + IAA. After 3 weeks, the number of regenerating explants, callus, and full-fledged shoots was examined and counted. After statistical processing of the data, callus formation was higher in the culture medium in variant 1 than in variant 2 (Figure 1).

This may be due to the absence of auxins in the culture medium of variant 1, which leads to dedifferentiation of tissues (Figure 2 b, c).

Figure 2. Regeneration of root explants: a - cultivation of explants on the nutrient medium; b - formation of callus on the wound surfaces of the root; c - organogenesis; d - regeneration of the shoot from the root.
The resulting callus is light green in color with a dense structure. In the future, the rudiments of leaves and roots developed from the callus, and full-fledged shoots were formed.

In the second variant, the percentage of regeneration is significantly higher than in the first one. This result could be influenced by the combination of auxin and cytokinin in the nutrient medium. The presence of auxins in the medium led to the formation of embryoids and shoots from root explants. The number of shoots formed in the second variant is 32.4% higher than in the first variant. The shoots are light green in color, arranged in a group. In most cases, shoots are formed on the root tissue, bypassing the callus stage (Figure 2 d).

Based on the obtained average values in the experiment and the constructed diagram, it can be argued that the differences between the environments in the first and in the second variant are reliable. A higher percentage of regeneration and shoot formation in the second variant compared to the first is confirmed at a significance level of $p=0.05$. The resulting difference in the average values as the result of comparing the percentage of callus formation is not accidental and can be repeated in the following experiments.

A culture medium containing BAP-6 with a concentration of 1 mg/l (variant 1) is suitable for the rapid production of callus from kok-saghyz root explants, since it showed the best result between the compared variants.

For shoots direct regeneration from root explants, a medium containing BAP-6 and indole acetic acid with concentrations of 1 mg/l and 0.2 mg/l, respectively, was more effective (variant 2). Shoots obtained on this medium, after a while, showed the rudiments of roots and then the development of a powerful, long root was observed. This type of medium was used in further experiments to compare the regeneration efficiency as a function of the root diameter (Figure 3).

The efficiency of plant regeneration was calculated by dividing the number of regenerated plants by the number of original root fragments. Already at the stage of direct regeneration of shoots, the difference between the variants was visible by ~15 times, the variant with a root diameter of less than 1 mm showed the inexpediency of using it in experiments to create a transformation system due to the low frequency of regeneration of both shoots and callus formation.

![Figure 3. Regeneration efficiency as a function of root diameter.](image)

The resulting plants (Figure 4) from the root segments did not differ morphologically from the original forms, there was no elongation of the shoots and changes in the shape of the leaves.
4. Conclusion
An effective system of plant regeneration in vitro from root explants for further genetic transformation was developed for the kok-sagyz population. In this study, it is shown that the segments of the roots can regenerate into a full-fledged plant on the environment and Skoog medium with the addition of growth regulators. From root explants on MS medium with the addition of cytokinin 6-BAP and auxin 1 mg/l and indole acetic acid 0.2 mg/l, embryoids and shoots were formed with a frequency of 87.6% and 65.1%, respectively. On the MS medium with cytokinin 6-BAP 1 mg/l, regeneration and formation of morphogenic callus was observed with a frequency of 85.2% and 61.7%. We have shown the efficiency of plant regeneration from root fragments with a diameter 1 mm. This protocol can be used not only for the microclonal reproduction of kok-sagyz populations, but also for the regeneration and reproduction of transgenic plants. As the result of the conducted research, full-fledged kok-sagyz plants were obtained from the root segments.

5. Acknowledgments
The work was carried out within the financial support of the State Task of the IDB RAS No. 0108-2019-0001.

References
[1] Rodin L E 1993 Taxonomic description of Taraxacum kok-saghyz Actanstituti Botanici Academiae Scientiarum 1 187–189
[2] Turasheva S K and Boguspaev K K 2013 Screening of morphogenetic ability of wild tau-saghyz Bulletin of KazNU 3 193-197
[3] Turasheva S K and Boguspaev K K 2014 Obtaining a cell suspension culture of wild forms of tau-saghyz Bulletin of KazNU 3 28-34
[4] Uteulin K, Mukhambetzhanov S and Rakhimbaev I 2014 Recovering Taraxacum kok-saghyz Rodin. Via Seed and Callus Culture Engineering and Technology Int. J. of Bioengineering and Life Sciences 4 385-387
[5] Bowes B G 1970 Preliminary observations on organogenesis Taraxacum officinale tissue cultures Protoplasma 71(1-2) 197-202
[6] Bowes B G 1971 The occurrence of shoot teratomata in tissue cultures of Taraxacum officinale Planta 100(3) 272-276
[7] Booth A and Satchuthananthavale R 1974 Regeneration in root cuttings Taraxacum officinale II. Effects of exogenous hormones on root segments and root callus cultures New Phytologist 73(3) 453-460
[8] Kuluev B R, Minchenkov N D and Gumerova G R 2019 Russian dandelion (Taraxacum kok-saghyz Rodin): rubber extraction methods and prospects for biotechnological methods application Plant Biotechnology and Breeding 2(2) 33-43