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Aseptic Germination and *Agrobacterium rhizogenes*-mediated Transformation of *Taraxacum hybernum* Steven

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Abstract

Taraxacum hybernum Steven, also known as krym-saghyz, is one of the potential sources of natural rubber. The authors studied the aseptic germination of the *T. hybernum* seeds using various sterilizing agents and their effect on contamination. *In vitro T. hybernum* plants were used for *Agrobacterium rhizogenes*-mediated transformation. A4 and 15834 strains of *A. rhizogenes* showed similar effectiveness after inoculation by injection into the hypocotyls. Authors generated the hairy roots of *T. hybernum* and the transgenic forms of this plant using *A. rhizogenes*-mediated transformation.

Introduction

Growing demand for rubber compels scientists to search alternative sources of natural rubber, instead of potentially vulnerable systems of *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg (Para rubber tree). Experience in the production of natural rubber in the USSR shows that the most economically viable alternative to *H. brasiliensis* is kok-saghyz, *Taraxacum kok-saghyz* L.E. Rodin, sometimes referred to as Russian dandelion (McAssey et al. 2016). Another alternative source of natural rubber is *Taraxacum hybernum* Steven or krym-saghyz – perennial herbaceous plant of the genus *Taraxacum* (Asteraceae). This is a relatively poorly studied species that grows on the southern coast and in the foothills of the Crimean peninsula, where it was discovered as a rubber plant by

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Soviet botanists in 1931 (Bondarenko 1941). The species is also present in Bulgaria and Turkey (Kirschner et al. 2007). However, in those countries, *T. hybernum* remains virtually unexplored.

In the conditions of natural habitat in the Crimea, *T. hybernum* is characterized by a double vegetative period, in spring and autumn, and a stage of summer dormancy in the dry season. *T. hybernum* roots, containing up to 7% of latex, can be a source of natural rubber. The quality of the krym-saghyz rubber is high, while the resin content in the latex is relatively low. However, the quantitative content of rubber, specially its accumulation in the first growing season was significantly less compared to kok-saghyz (Lapin 1935, Il'in and Yakimov 1950). Difficulties in cultivation of this ecologically specialized species and a lower content of rubber makes it less attractive for the industrial cultivation in comparison with kok-saghyz (Kirschner et al. 2013). Rubber accumulates in krym-saghyz roots in large amounts only during the second year of cultivation, and therefore the plant should survive in winter. However, the frost resistance of *T. hybernum* is quite low, that makes its cultivation in most territory of Russia is very problematic.

Since the rubber of *T. hybernum* is in the roots, one way to avoid difficulties with cultivation of this plant in the field is to cultivate isolated roots. The *rol* genes of *Agrobacterium rhizogenes* can be transferred to the culture of isolated roots for making their growth more active. Expression of *rol* genes promotes rapid and unlimited growth of isolated root cultures on hormone-free media (Kuzovkina et al. 2004). Therefore, there is a need to develop protocols for the genetic transformation of *T. hybernum* for applied research, such as metabolic engineering. Methods of genetic engineering can be used to improve the agronomic properties and metabolic activity in krym-saghyz roots.

One of the methods of plant genetic modification is *A. rhizogenes*-mediated transformation. These agrobacteria contain the natural bacterial *rol* genes, which are often co-transformed with target genes, located in a separate binary vector. These *rol* genes alter the endogenous concentrations of plant hormones, enhancing the growth of hairy roots (Pavlova et al. 2014). *A. rhizogenes*-mediated transformation methods have been developed for some species of dandelions (Mahesh and Jeyachandran 2011), including for kok-saghyz (Zhang et al. 2015). At the same time, methods of working with cultures *in vitro* have not been developed for *T. hybernum*. Therefore, the purpose of our study was to determine the optimal methods of sterilization and seed germination of krym-saghyz. Also, the task was to develop an effective protocol of *A. rhizogenes*-mediated transformation, producing hairy roots and generating from them transgenic plants of *T. hybernum*.

Materials and Methods

Mature achenes of *T. hybernum* were collected in the Crimea in 2015 and were reproduced at the greenhouse of Institute of Biochemistry and Genetics of Ufa Scientific Center of RAS in 2016.

To determine the best germination method, achenes previously cleared from pappus were washed with water containing 0.1% Tween 20 detergent, rinsed with distilled water, and left in water until fully swollen (2 hours). Floating achenes were removed. Then, the achenes were transferred to Petri dishes with filter paper moistened with a liquid medium with half concentration of the MS salts without sucrose. The half of these Petri dishes was placed directly into the Binder climatic chamber (Germany) without cold treatment (method A). The other half of the Petri dishes was held at 5°C for three days and then transferred to a climatic chamber (method B). Each variation of experiment included 90 achenes.

For sterilization, the achenes were treated in 70% ethanol for 3 minutes. Then the achenes were immersed in different concentrations of sodium hypochlorite (NaClO) solution, or 0.1% $HgCl_2$ solution, with addition of Tween 20 (0.1%). Aseptic achenes were kept in sterile water until they are swollen up (for 2 hours). Floating achenes were removed. After swelling, the achenes were dried on sterile filter paper and placed on half-strength MS with 1% sucrose and 0.6% gelrite in aseptic conditions. Petri dishes were kept in darkness at +5°C for **3** days, and then transferred to the Binder climatic chamber, equipped with fluorescent grow lamps Fluora, at 24°C under 50 μmol m^{-2} sec^{-1} photon flux density and photoperiod of 16/8 hours (day/night). Germination and contamination rate was expressed as percentage of plated achenes. The mean value was counted for three replications.

The authors used A4 and 15834 strains of *A. rhizogenes*, transformed with binary vector pCambia 1301 that contains reporter *GUS* gene with catalase intron and selective *hpt* gene of hygromycin phosphotransferase, encoding tolerance for the antibiotic hygromycin (http://www.cambia.org). Bacterial cultures were grown in Petri dishes on solid Lysogeny broth (LB) medium (Sambrook 2001) with addition of 100 mg/l rifampicin and 50 mg/l kanamycin, or in liquid LB medium (120 rpm, 28ºC, 20 hours) with the same antibiotics. Bacterial cultures were centrifuged at 3500 g for 15 minutes and then resuspended in liquid half-strength MS with the addition of 100 μmol of acetosyringone. Three weeks old sterile plants were used for transformation. It was performed using three methods. In the first method, petiole explants of *T. hybernum*, grown *in vitro*, were kept in agrobacterial suspension for 30 minutes, blotted dry on the sterile filter paper and then transferred to half-strength MS for co-cultivation in

darkness. In the second method, intact sterile plants were injected in the stem area with a needle, covered with *A. rhizogenes*, taken from a Petri dish. For the third method, the roots were dissected and *A. rhizogenes*, taken from a Petri dish, were put on the underside of separated stems. The plants were dipped vertically in solid half-strength MS. Plants used as a control were treated the same way, but were not inoculated with *A. rhizogenes*. After 3 days of incubation, explants and inoculated plants were transferred to hormone-free basal half-strength MS with addition of 400 mg/l of cefotaxime in order to achieve adventive roots. In the case of the injection method, primary roots were cut off 4 weeks after the development of adventive roots. Transformation efficiency was expressed as ratio of transformed explants or seedlings to their total amount. In the experiments, 30 - 40 explants (seedlings in methods 2 and 3) were used in each variant. Mean values were counted for each experiment separately, and then overall value for all experiments was evaluated. These numbers with a standard error are presented. T-test was performed to determine the statistical difference in transformation efficiency between two treatments using the Instat 2.03 statistical package (GraphPad Software, San Diego, USA).

To identify the *GUS* activity in hairy roots, we dissected lateral roots and incubated them in histochemical reagent X-Gluc ("Fermentas") at 37ºC for one to 24 hours. To prepare histochemical reagent 15 mg of X-Gluc were suspended in 20% methanol, containing 0.08% Triton X-100, 0.16% potassium ferrocyanide, 8 mM Na_2EDTA and 80 mM Na_2HPO_4, pH 7.0. After the incubation, dissected roots were soaked in 50% glycerol-water solution, and then observed with a microscope.

We used the PCR method to prove the integration of *A. rhizogenes* T-DNA into the genome of *T. hybernum*. Extraction of DNA from hairy roots was carried out using the cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich 1985). Once the DNA had been extracted, the polymerase chain reaction (PCR) analysis was performed to verify the presence of *rolB* gene. 5'-TTCAGATTTACTATAGCAGGC-3' and 5'-GCAAGTACCTTGTTCATTCA-3' primers were used to identify *rolB* gene. The size of the amplicon was 266 bp, with optimal annealing temperature 54ºC. Nucleotide sequence of the *rolB* gene is available from X64255.1. To avoid the risk of contamination of hairy roots with agrobacteria, we performed PCR analysis for chromosomal gene of *A. rhizogenes* (WP_034523040) using primers 5'-CCCGCACCCGATCCAAGACAAACTCA-3' and 5'-CGCCCGAAGCCTCACCCACGAAC-3'. The size of the amplicon was 476 bp, with optimal annealing temperature 62ºC. All primer pairs were selected using PrimerSelect program of Lasergene package (DNAStar, Inc., USA). The electrophoresis of the PCR products was performed on 1.0% agarose gel under a

constant voltage of 80 V. The gel was subsequently stained with ethidium bromide solution and examined under UV light.

Results and Discussion

As the first result of our research, it was shown that the germination of *T. hybernum* is a gradual and rather long process. The first seedlings started to appear after a few days, while the others appeared consecutively for a few weeks. Therefore, it was important to develop methods for seed stratification of krym-saghyz. In order to stimulate the seed germination, disturbance of their rest is necessary. Therefore, the achenes of krym-saghyz were placed in different conditions of germination, and the dynamics of their germination was investigated.

The degree of germination of seeds with both germination methods (A and B) was similar (Fig. 1). However, the rates of seed germination were significantly different. Without cold treatment, the rate of seed germination was low, and all living seeds germinated only 11 days after sowing. After cold treating of achenes for three days, the total seed germination rate increased dramatically between four and eight days after sowing the achenes (Fig. 1). Thus, for krym-saghyz, as well as for a number of other cultures it is characteristic that stratification for several days improves synchronicity and germination rate of seeds, although it does not significantly affect the percentage of seed germination.

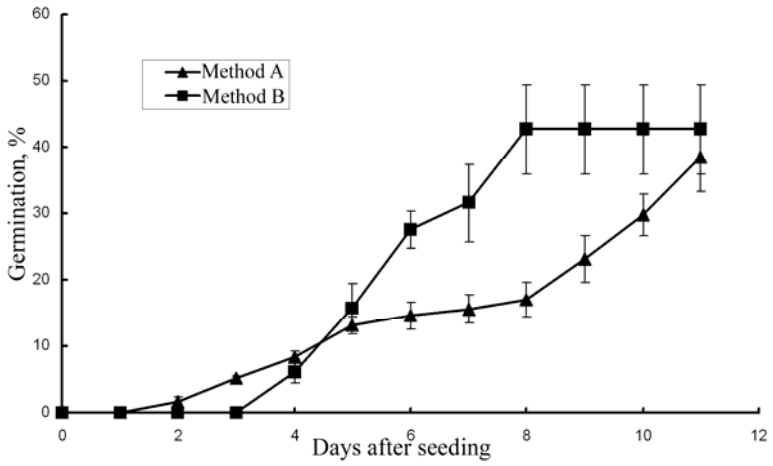

Fig. 1. The total germination rate of seeds sown in two different methods within 11 days after sowing of the achenes. Method A - the achenes are sown on half MS in a climatic chamber without stratification. Method B - the achenes are sown on half MS and stratified at 5°C for three days and then transferred to the climatic chamber. Bars indicate standard error (SE).

The use of NaClO solution as a sterilizing agent gave much better results, although it was impossible to obtain completely sterile seeds and seedlings with a high germination rate. Increasing the concentration of NaClO to 2.5% of its content in the sterilizing solution markedly reduced contamination, but not greatly affected seed germination rate. We believe that the best way to sterilize the krym-saghyz achenes is immersion in a 2.0% solution of sodium hypochlorite for 13 - 15 minutes or in its 2.5% solution for 11 - 13 minutes. A further increase of the concentration of sodium hypochlorite with an extension of the sterilization time to 20 minutes resulted in a sharp decrease in germination, although the contamination of seeds and seedlings also decreased. Next we produced sterile plants of *T. hybernum*, which were used in *A. rhizogenes*-mediated transformation experiments.

There is information that the roots are the best explants for the transformation of plants of the genus *Taraxacum*, and the production of transgenic seedlings, as they have a high capacity for regeneration (Lee et al. 2004, Zhang et al. 2015). However, we believe that it is inconvenient plant material to produce the hairy roots. For efficient transformation and regeneration of transgenic plants the roots with diameter larger than 1 mm are required (Zhang et al. 2015), that is difficult to reach in a sterile culture. Our preliminary experiments showed that during the cultivation of the root sections, most of them become necrotic after one week of cultivation. To overcome this problem, certain modifications of the media used for cultivation are required.

The use of leaf petioles as the explants for transformation was ineffective regardless of *A. rhizogenes* strain. The petiole explants, after co-incubation with agrobacteria, quickly turned brown and died, without leading to the emergence of hairy roots. The use of various modifications of the basic medium with the addition of ascorbic acid (1 - 5 mg/l) and PVP (600 - 800 mg/l) was also not effective.

At the injection and cutting sites, the adventitious roots of krym-saghyz usually appeared on the 7 - 10th day. It did not depend on the *A. rhizogenes* inoculation and it was the normal response of the *T. hybernum* seedlings to the removal of their apical root meristem. The features of root formation and their growth rate did not differ in inoculated and control plants (Fig. 2a).

In the selection of roots, physiological markers of transformation were their active growth, lateral branching and plagiotropism. The main criterion of transformation efficiency was the analysis of *GUS* activity. Using histochemical analysis, we identified co-transformed hairy roots carrying the T-DNA of the binary vector pCambia 1301.

A. rhizogenes-mediated transformation using intact *T. hybernum* plants (methods 2 and 3) was quite effective. Among the adventitious roots growing from the site of injection 12 - 14 days after inoculation, when the samples were incubated in a histochemical reagent, roots with characteristic blue staining were detected (Fig. 2b). It indicates the introduction and expression of the reporter *GUS* gene in the cells.

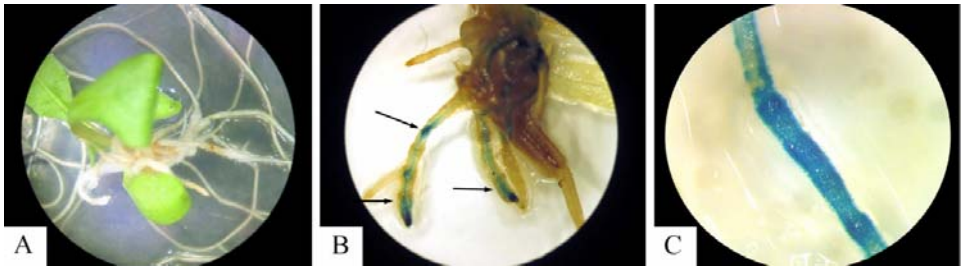

Fig. 2. Generating of *T. hybernum* hairy roots. A. Appearance of adventitious roots in inoculated *T. hybernum* plants generated by method 2. B. Co-transformed hairy roots of krym-saghyz three weeks after the inoculation of *A. rhizogenes* carrying the binary vector pCambia 1301. C. Expression of the reporter *GUS* gene in isolated hairy roots of *T. hybernum* (4 weeks after inoculation). The arrows show a blue staining indicating the expression of the *GUS* gene.

When using the transformation method 2 different *Agrobacterium* strains (A4 and 15834) had similar efficiency (81.7 and 83.3%, respectively). When using the method 3 strain A4 was significantly more effective than 15834 strain. After the emergence of adventitious roots, the seedlings were transferred to a medium with a reduced concentration of cefotaxime (200 mg/l) until the bacteria were completely eliminated. At the first two or three transplantations, part of the shoots was left to maintain the root growth activity. In the future, we obtained isolated root lines, separating them from the shoot and cultivating them in a liquid MS. For further work we selected only those roots that differ by characteristic blue staining after histochemical analysis (Fig. 2c).

As is well known, *A. rhizogenes* differs from disarmed *A. tumefaciens* strains in that it contains natural bacterial *rol* genes that are often transformed together with target genes located in a separate introduced plasmid (Pavlova et al. 2011). Based on these considerations, we performed a PCR analysis of well-growing *GUS*-positive hairy root lines of *T. hybernum* for the presence of the *rolB* gene. Of the 12 analyzed lines of *T. hybernum* hairy roots, the *rolB* gene was detected in 11 lines (Fig. 3a). However, the presence of *rolB* gene is not a proof of successful agrobacterial transformation, since it is impossible to exclude contamination of root cultures with *A. rhizogenes*. Therefore, we conducted a PCR analysis to

search for one of the chromosome genes of agrobacteria (WP_034523040). It was shown that root lines numbered 6 and 9 contain the chromosomal *A. rhizogenes* gene (Fig. 3b). This might be due to the contamination of the roots with agrobacteria. Therefore, lines 6 and 9 of the hairy roots were not investigated further.

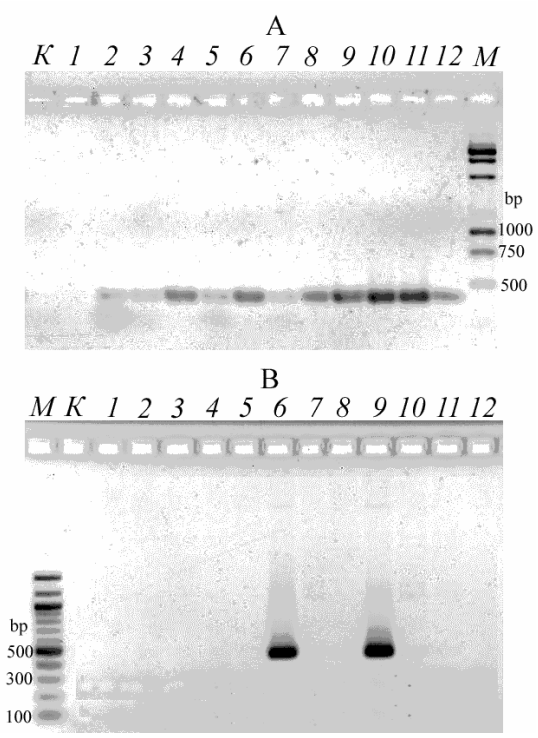

Fig. 3. Electrophoregrams of the results of PCR analyses. A. Results of PCR analysis of *rolB* gene. B. Results of PCR analysis of the chromosome *A. rhizogenes* gene. K - wild-type roots of *T. hybernum*, M - molecular weight markers, 1 - 12 - lines of isolated growing *GUS*-positive adventitious roots of *T. hybernum*.

Spontaneous regeneration of shoots from the root explants on non-hormonal medium appears to be a characteristic feature of morphogenesis in tissue culture of *Taraxacum* species (Uteulin 2015). The endogenous hormone level of the plant itself might be sufficient to induce regeneration. We observed spontaneous development of the shoots from hairy roots of krym-saghyz (Fig. 4a), including the ones expressing reporter *GUS* gene, which manifested in the blue coloring of the leaves of generated plants (Fig. 4b). It seems that regeneration of the shoots from transgenic roots of krym-saghyz can be enhanced by using hormonal media. This is a field for further studies.

We generated 9 hairy root lines of krym-saghyz, containing both the *rol* genes of *A. rhizogenes* and the T-DNA of the binary vector pCambia 1301. Of the three used methods, the method of injecting into hypocotyls is more effective while preserving the intact root. In this case up to 80% of the original inoculated plants can form transgenic roots expressing the reporter *GUS* gene, as well as target genes. When using the method 2 strains A4 and 15834 of *A. rhizogenes* had similar efficiency in *Agrobacterium*-mediated transformation of *T. hybernum*.

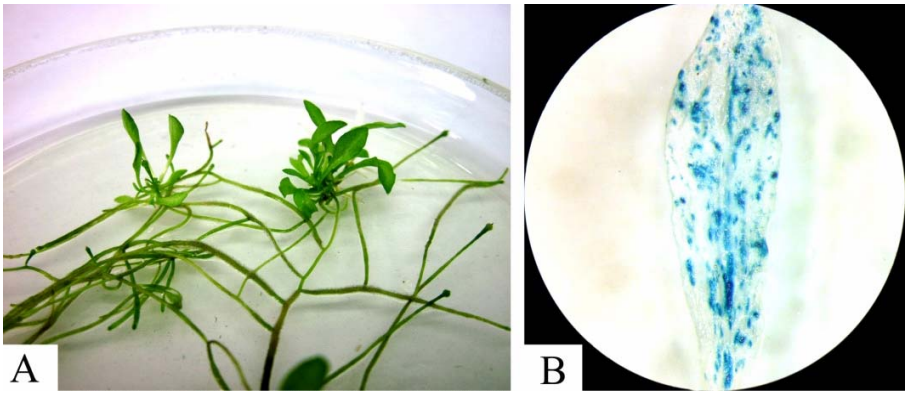

Fig. 4. The regeneration of transgenic shoots of *T. hybernum*. A. Spontaneous induction of the shoots on the isolated hairy root culture of *T. hybernum* on the half-strength non-hormonal MS medium. B. Histochemical analysis of reporter *GUS* gene expression in the leaf of regenerated shoots.

Authors have developed the simple protocol for generating of hairy roots and transgenic plants of *T. hybernum*. The protocol of agrobacterial transformation of *T. hybernum* can be used for targeted change of its properties by methods of genetic engineering and genome editing. It should be noted that in the world there is a growing interest in the genetic engineering of kok-saghyz, but there is no such research for krym-saghyz. For example, a recent work has been published on the CRISPR/Cas9 genome editing of kok-saghyz using *A. rhizogenes* (Iaffaldano et al. 2016). In that study, hairy roots of *T. kok-saghyz* were obtained with broken of *1-FFT* gene without selective antibiotic and from these hairy roots were produced regenerated plants of *T. kok-saghyz*. In that study, as in Zhang et al. (2015), root segments were used for *Agrobacterium*-mediated transformation. We have shown that the greatest effectiveness of agrobacterial transformation is achieved using whole plants of krym-saghyz.

Preliminary analysis showed that the young hairy roots of *T. hybernum* do not contain visually detectable rubber latex. Perhaps the hairy roots of krym-saghyz can produce rubber as a result of changing cultivation conditions. We

suggest that for this purpose it is necessary to induce a secondary growth of hairy roots. Moreover, according to our observations the secondary growth of krym-saghyz hairy roots can be induced spontaneously after several months of cultivation without transplantation. However, to analyze the rubber content in hairy roots of *T. hybernum*, additional studies are required.

So, we have created model systems of hairy roots and transgenic plants of *T. hybernum*, which can be used in the study of rubber production by this less-studied species of dandelions.

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References

Bondarenko PV (1941) Krym-saghyz. Priyemy vyrashchivaniya v sredney Azii. Uzfan, Tashkent. pp. 20 (In Russian).

Il'in MM and Yakimov PA (1950) Kauchukonosy i guttaperchenosy SSSR. Rastitel'noye syr'ye SSSR. **1**: 61-142 (In Russian).

Kirschner J, Stepanek J, Cerny T, Heer PD and van Dijk PJ (2013) Available *ex situ* germplasm of the potential rubber crop *Taraxacum kok-saghyz* belongs to a poor rubber producer, *T. brevicorniculatum* (Compositae-Crepidinae). Genet. Resour. Crop Evol. **60**: 455-471. doi: 10.1007/s10722-012-9848-0.

Kirschner J, Stepanek J and Greuter W (2017) Taraxacum // *In:* W. Greuter, E. von Raab-Straube (ed.). Compositae. Euro+Med Plantbase - the information resource for Euro-Mediterranean plant diversity. Berlin: Botanic Garden and Botanical Museum Berlin-Dahlem. http://ww2.bgbm.org/EuroPlusMed/ (Accessed 25.04.2017).

Kuzovkina IN, Al'terman IE and Karandashov VE (2004) Genetically transformed plant roots as a model for studying specific metabolism and symbiotic contacts of the root system. Biology Bulletin **31**: 255-261. doi: 10.1023/B:BIBU.0000030146.87748.f0

Iaffaldano B, Zhang Y and Cornish K (2016) CRISPR/Cas9 genome editing of rubber producing dandelion *Taraxacum kok-saghyz* using *Agrobacterium rhizogenes* without selection. Ind. Crops Prod. **30**: 356-362. doi: 10.1016/j.indcrop.2016.05.029

Mahesh A and Jeyachandran R (2011) *Agrobacterium rhizogenes*-mediated hairy root induction in *Taraxacum officinale* and analysis of sesquiterpene lactones. Plant Biosyst. **145**: 620-626 doi: 10.1080/11263504.2011.584702

Lapin AK (1935) Kul'tura kauchukonosov. Sel'skoye khozyaystvo SSSR. Yezhegodnik. Sel'khozgiz, Moscow. pp. 65-73 (In Russian).

Lee MH, Yoon ES, Jeong JH and Choi YE (2004) *Agrobacterium rhizogenes*-mediated transformation of *Taraxacum platycarpum* and changes of morphological characters. Plant Cell Rep. **22**: 822-827. doi: 10.1007/s00299-004-0763-5.

Pavlova OA, Matveyeva TV and Lutova LA (2014) *ROL*-genes of *Agrobacterium rhizogenes*. Russ. J. Genetics Appl. Res. **4**: 137-145. doi: 10.1134/S2079059714020063.

Rogers SO and Bendich AJ (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol. Biol. 5: 69-76. doi: 10.1007/BF00020088.

Sambrook J and Russell DW (2001) Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbour Laboratory Press, Cold Spring Harbour. pp. 2100.

Uteulin K, Yesbolayeva B, Mukhambetzhanov S, Iskakova A, Bari G, Zheksenbai A, Piven V, Zhabykbaev Ch, Zhambakin K and Rakhimbaev I (2015) Introduction to *in vitro* culture of isolated *Taraxacum kok-saghyz* roots. KzNaU Bulletin. Biology series **65**: 364-367.

Zhang Y, Iaffaldano BJ, Xie W, Blakeslee JJ and Cornish K (2015) Rapid and hormone-free *Agrobacterium rhizogenes*-mediated transformation in rubber producing dandelions *Taraxacum kok-saghyz* and *T. brevicorniculatum*. Ind. Crops Prod. **66**: 110-118. doi: 10.1016/j.indcrop.2014.12.013.