Rapid Rubber Extraction and NMR Spectroscopy of Rubber, Extracted from the Endemic Species *Scorzonera Tau-Saghyz*

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Abstract

Scorzonera tau-saghyz Lipsch. et G.G. Bosse is an endemic rubber producing plant, growing in mountain regions in South Kazakhstan. The rubber content in plants and the quality of biopolymer has an important impact on industrial rubber production. The results of this study showed that the amount of rubber in *S. tau-saghyz* roots fluctuates between 7.74% and 38.75%. The amount of synthesized and deposited rubber biopolymer particles depends on various factors such as physiological age of plant, origin, temperature, moisture and environmental conditions. We optimized the extraction method of natural rubber by using n-hexane as a solvent for direct extraction. This method allows extracting the maximum amount of rubber from 3–4-year-old plants. NMR results show structural links of natural isoprene rubber in the root extract sample. There is a clear relationship between methyl, methine and methylene protons which corresponds to isoprene rubber structure. The samples having strongly marked singlets that are inherent for rubber functional groups confirms the stereospecific structure of rubber. Good solubility of the root extract in deuterated chloroform can characterize the low molecular weight of the polymer. NMR characterization of rubber, extracted from *S. tau-saghyz* roots, is reported for the first time. Regeneration *in vitro* provides an important opportunity for endemic preservation by rapidly increasing the number of plants. The best regeneration of adventitious shoots was obtained on MS medium containing 5.5 μM kinetin and 0.5 μM NAA. The plants were successfully acclimatized in a glasshouse with 75% of *S. tau-saghyz plantlets*, respectively surviving after transfer to e*x vitro* conditions.

General abbreviations

NMR – Nuclear Magnetic Resonance
PGR – Plant Growth Regulators
NAA – 1-naphthaleneacetic acid
MS – Murashige and Skoog medium
BA – 6-benzylaminopurine
IAA – Indole-3-acetic acid
GA_3 – Gibberellic acid

1. Introduction

Rubber has been found in over 2500 plant species, however, only *Parthenium argentatum* Gray, *Taraxacum koksaghyz* and the rubber tree *Hevea brasiliensis* Muell Arg. synthesize plenty of quantity of high molecular weight rubber [1–5]. *Scorzonera tau-saghyz* Lipsch. & G.G. Bosse rubber producing species grow naturally in South Kazakhstan. The origin of the *Scorzonera* taxa belonging to *Compositae* family is the Central Asia region. It is rubber semi-shrub, with laticifer cells produced and accumulated in roots, achieving up to 35–38% of latex like produced by the Pará rubber tree (*Hevea brasiliensis*). *S. tau-saghyz* is an economically significant species used as an alternative source of natural rubber. After its discovery in 1934 by Lipschits S. in its native habitat, the high valleys

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of the Karatau Mountains of Kazakhstan, it was found to have a high rubber content in its roots [6, 7]. The potential of the perennial was investigated and it was developed to serve as a strategic source of natural rubber in the Soviet Union for over two decades until the 1950s [8–10, 4]. *S. tau-saghyz* produces natural rubber that can have physical properties and applications equivalent to those of Hevea (*Hevea brasiliensis*) rubber [11, 12].

The standard extraction methods are drying moist raw plant material and grinding using a pebble mill used for isolation of biopolymer [3]. Isoprene units of polyisoprene (rubber) can be quantified by NMR [14]. NMR spectroscopy is one of the methods used to characterize organic material since the NMR spectrum can show the chemical structure of individual atoms. One-dimensional (1D) NMR spectroscopy used to determine the primary structure of biopolymer. For instance, signals in ^{1}H- and ^{13}C-NMR spectra are assigned to a primary structure based on chemical shift values estimated for the plausible structural units of organic material [15, 16]. Two-dimensional (2D) NMR spectroscopy are used to investigate correlations between homo- and hetero-nuclei (i.e., ^{1}H-^{1}H, ^{1}H-^{13}C and ^{13}C-^{13}C). Various pulse sequences i.e., heteronuclear single-quantum coherence (HSQC), heteronuclear multiple-quantum coherence (HMQC), heteronuclear multiple-bond coherence (HMBC) are indispensable in detecting the primary structure of rubber [17].

During recent years, the anthropogenic influence on the mountain environment has increased and disturbance is the greatest threat to endemic plants, growing in these regions [18]. Conventional propagation is difficult in *Scorzonera* due to low seed viability and germination rate. Protected areas with specific conservation measures are an efficient way to protect biodiversity, but uncertainty persists, owing to a range of different climate-change driven effects. More than half the endemic species growing within Central Asia might soon lose suitable habitat for their survival. Hence, it will be necessary to prepare alternative *ex-situ* measures, of which the *in vitro* propagation presented in this paper is claimed to be the most efficient. Biotechnology, especially *in vitro* regeneration studies, is very important to preserve genetic resources. A balance between auxin and cytokinin determines the in vitro regeneration of plants grown in artificial medium [19–21]. Type of explants, media composition, growth conditions, genotypes and physiological condition of the explants affect callus induction and plant regeneration [22–25]. Therefore, optimised growth conditions, suitable for explants and different genotypes are needed to identify the best route for large-scale utilization in biotechnology and propagation. However, an efficient protocol for the large-scale propagation of endangered endemic rubber producing species like *S. tau-saghyz* Lipsch. & G.G. Bosse has not been developed yet.

The goal of this investigation was to optimize and simplify solvent extraction methods to determine the quality of rubber, accumulated in 2–5-year-old plant roots using NMR. In this article, NMR characterization of rubber, extracted from *S. tau-saghyz* roots, is reported for the first time. We applied 2D-NMR spectroscopy together with Distortionless Enhancement by Polarization Transfer (DEPT), and correlation spectroscopy (COSY) measurements for the structural analysis of rubber from *S. tau-saghyz* species. Also, this research aimed to develop a protocol for *in vitro* regeneration of the rubber producing semi-shrub *S. tau-saghyz* to increase the number of species in population and improve the efficiency of natural rubber production from roots.

2. Experimental

2.1. Plant material preparation and extraction

Wild-type plants *S. tau-saghyz* Lipsch. et G.G. Bosse were harvested on the mountains areal of the Karatau National Natural Park in South Kazakhstan (Central Asia) (Fig. 1). Shoot tips and leaves of one-two monthly plants were used for *in vitro* propagation. Roots of 2–5-year-old plants were used to determine the quality and quantity of rubber.

The production of rubber takes place in *S. tau-saghyz* largely in the root. After harvesting, roots of *S. tau-saghyz* were separated from the green vegetative mass, washed from the soil and dried in the shade. Roots were cut into pieces and grounded at the mill to powder. This is an important stage of the process as barks of roots are almost fully grounded at the mill. This simplifies the process and removes the necessity to use alkali for the destruction of wood followed by washing rubber threads (fibrils). All stages were arranged at room temperature (+22…+26 °C). The dry weight of the plant root was then determined.

Four grams of shredded roots (size 1–3 mm) were selected and placed into Erlenmeyer flask.

Fig. 1. Rubber-producing species *S. tau-saghyz* Lipsch. et G.G. Bosse: (a) – plants in the natural habitat; (b) – typical *S. tau-saghyz* plant.

50 ml of n-hexane was added to the flask and it was connected to the extraction system with a reverse fridge. Rubber was extracted for 2 days using gentle agitation with a constant speed of 40 rpm (revolutions per minute). After extraction, the flask with suspension is installed in a rotary evaporator. When removing solvent several rotor evaporators are used which helps to save time. The temperature of water-bath must be no more than 30 °C. It is left for 20–30 min. After that, the remainder is dried in vacuum drying equipment at 24 °C for 22–24 h. This allows materials to dry in a short period of time while maintaining their properties. The resultant rubber samples are weighed on analytical or technical weights. All stages of work were arranged at room temperature (+22…+26 °C). When using this method, the time to get a natural rubber from rubber-containing plant material of *S. tau-saghyz* is 2–3 days. The obtained samples are then weighed and the amount of rubber from the initial dry weight of roots is calculated.

The following equipment was used for extraction process: chemical purified n-hexane "ТУ 2631-158-44493179-13" ("Ecos-1" company, Russia), rotor evaporator IKA RV05 basic (IKA Werke GmbH & Co. KG, Germany) and water bath of rotor evaporator IKA HP4 (IKA Werke Gmbh & Co. KG, Germany), vacuum drying cabinet VD115 (BINDER GmbH, Germany), analytical weights AS 220/X (RADWAG Wagi Elektroniczne, Poland).

2.2. NMR Spectroscopy

For carrying research on NMR analysis, samples of plants were selected on previously discovered regions of Karatau mountains (at an altitude of 1500 m), where the population with the highest (38.75%) amount of rubber in roots is located. The rubber was extracted and purified in accordance with a simplified method that is described above.

In the present study, 1D- and 2D-NMR measurements were performed to analyze the structure of rubber, that accumulated in roots of *S. tau-saghyz*. One-dimensional ^{1}H, ^{13}C, DEPT NMR spectra and two-dimensional homonuclear correlation spectra COSY ^{1}H-^{1}H and heteronuclear correlation HMQC ^{1}H-^{13}C and HMBC ^{1}H-^{13}C of biopolymer were obtained on the JNM-ECA JEOL 400 spectrometer (JEOL, Tokyo, Japan) at 399.78 and 100.53 MHz on protons and carbon atoms respectively using a solvent of $CDCl_3$.

Chemical shifts are measured relative to the residual protons or carbon atoms of the deuterated solvent. To obtain NMR spectra 60 mg of the biopolymer sample was dissolved in 0.6 ml of deuterated chloroform and placed in a 5 mm glass ampoule. Spectra were obtained at 25 °C.

2.3. Explant source and culture condition

Leaf and shoot tips from a one-two-month-old seedling were surface sterilized with 70% ethanol (Sigma Aldrich) for 10 sec and then soaked in 0.5% commercial bleach 5% NaOCl, with a drop of Tween 80 (Merck) for 15 min and rinsed several times with sterile deionized water. Surface sterilized leaves of plants were cut into small pieces (0.5–1 cm) and cultivated on Murashige and Skoog (MS) standard medium [26] added PGRs, 3% (w/v) sucrose, 0.8% (w/v) agar (Sigma Aldrich). Shoot tips survived after surface sterilization were cut into 0.1–0.3 cm and cultured on solid MS medium, supplemented with NAA/IAA at 0.5 μM and

their combination with 5.5 μM kinetin/BA: 5.5 μM kinetin + 0.5 μM IAA (MR_1 medium); 5.5 μM kinetin + 0.5 μM NAA (MR_2 medium); 5.5 μM BA + 0.5 μM IAA (MR_3 medium); 5.5 μM BA + 0.5 μM NAA (MR4 medium).

For shoot regeneration, callus derived from both types of explants were cultured on MS fortified with various concentrations and combinations of auxines and cytokinins: 1 μM kinetin + 0.5 μM NAA + 0.5 μM GA_3; 1 μM BA + 0.5 μM IAA + 0.5 μM GA_3; 5.5 μM kinetin + 0.5 μM NAA + 0.5 μM GA_3; MS + 5.5 μM BA + 0.5 μM NAA + 0.5 μM GA_3. Callus was initiated more from leaf explants, than from shoot tips. Data was recorded as the percent of the leaf explants giving rise to callus induction.

Explants were cultivated under aseptic conditions in chambers for growth (16 h light/8 h dark, 25 °C, Percival Scientific, model I-66HILQ, Perry, IA, USA). After 6 weeks of being cultured, the extent of multiple shoot formation and the number of shoots per explant were recorded. Rooting was achieved after the transfer of the shoots to an MS medium added 0.5 μM GA_3 and 3% sucrose. Rooted plants were transferred to the prepared substrate containing a mixture of sand:potting soil:vermiculite (10:2:2) with a pH of 7.5–8.0, or in a mixture of limestone sand:potting soil:vermiculite (Vermit Group) (10:1:2) with a pH of 7.5–8.5, and transferred to greenhouse conditions.

Mean values were analyzed using a one-way analysis of variance. Significant differences among treatments were detected using multiple range tests (MRT) at the 0.05 level of probability. The statistical package SPSS® 21.0 was used for data analysis. All experiments were repeated three times.

3. Results and Discussion

3.1. Rubber extraction from plant material

The method of obtaining a natural rubber from rubber-containing plant material was simplified, particularly on the example of plant *S. tau-saghyz*. The optimization of the method is achieved by direct extraction of rubber from the ground in mill dry roots of *S. tau-saghyz* using n-hexane as a solvent.

We incubated 4 g of powdered roots from 2–5-year-old *S. tau-saghyz* plants in 50 mL of n-hexane for 24 h at 22 °C with gentle agitation. The solvent was removed by using rotor evaporator IKA RV05 (IKA Werke GmbH & Co. KG, Germany), then a sample was dried in vacuum condition. The usage of hexane as the solvent is suitable and expedient as it is not insoluble in water and has a high volatility. It is also less expensive and less toxic than acetone and cyclohexane which are usually used as the solvent [27].

A natural rubber was fully extracted when we use this optimized method. Moreover, the data about the amount of rubber in *S. tau-saghyz* samples were collected in serial experiments (Table 1). Experimental data from Table 1 shows the comparative mean amount of rubber per gram dry weight of root tissue at different ages for various samples. The rubber content increased with increasing plant age. This is in agreement with studies carried out by previous researchers who stated that the amount of rubber of the rubber-producing plant tends to increase slightly with age [28, 29].

Obtained results showed that the amount of rubber in roots fluctuates between 7.74% and 38.75%, because the amount of synthesized rubber depends on various factors (physiological age of plant, origin, climate condition etc.). The rubber producing capacity appears to increase in second year plants up to the time of seed formation. In the first year of plant development, a rubber content appears to increase most rapidly in the last month of the growing season, that is, after seed production.

For example, the maximum amount of rubber was indicated in 3–4-year-old plants. However, the climate condition in 2014 was better, than in 2013, therefore, the rubber quantity was 3 times greater. Plants with a large root and relatively large rosettes had the highest rubber yields. The form and size of roots appear to vary in plants of different genetic constitution as well as in response to environmental factors. On average 4-year-old plants have large amounts of biomass and accumulated enough rubber quantity in their roots for industrial production of latex, resin and other rubber products. Fresh rubber is white to creamy in color, sometimes having a pinkish tinge and weathering to a light straw or light brown color. Rubber extracted from *S. tau-saghyz* Lipsch. et G.G. Bosse and *Taraxacum koksaghyz* Rodin is not inferior in quality to *Hevea* rubber. Generally, greater amounts of rubber are found in much more branched roots than in taproots of equal weight.

Compared to other methods of rubber extraction, such as andacidic methods, alkaline pretreatment of raw material and enzimatic hydrolysis, the extraction with n-hexane proves to be cost-effective and least energy intensive. For example, alkaline pretreatment of biopolymer results in partial solubilization of organic carbon and proteins, reduc-

Table 1

Rubber Content of *S. tau-saghyz* in dry root samples (on the North-East mountains areal of the Karatau National Natural Park, Dzhelagan-Ata)

#	Age of plants (in years)	Dry weight of roots sample, g	Year of harvesting	Weight of raw material, g	The amount of rubber, %	The amount of rubber, g
1	4	9.6	2013	4	14.75	0.590
2	4-5	68.6	2013	4	11.48	0.450
3	3-4	53.8	2014	4	38.75	1.550
4	4-5	76.8	2014	4	34.00	1.359
5	3-4	47.8	2014	4	14.18	0.567
6	2-3	59.3	2014	4	11.92	0.477
7	2-3	38.2	2015	4	13.75	0.550
8	3-4	44.7	2015	4	7.74	0.309
9	4-5	57.2	2015	4	12.00	0.480

ing fiber content and thereby releasing rubber from root tissues [30]. However, in this case, the yield of rubber is lower than the extraction of natural rubber from *Taraxacum kok-saghyz* by means of commercial hydrolytic enzymes (45 mg/g from dry roots) [31].

The aqueous extraction method of natural rubber from Russian dandelion and Guayule shrub has one disadvantage associated with recalcitration and contamination with tightly bound lignocellulosic debris [32]. The additional process for rubber purification may increase processing cost. This is not efficient biotechnological procedures for producing rubber products from *Taraxacum kok-saghyz* and *Parthenium argentatum* (Guayule). The data of the rubber content in *S. tau-saghyz* samples did not differ from the results of previously known more expensive methods of rubber obtaining.

3.2. Analysis of NMR spectra for root extract sample from S. tau-saghyz Lipsch. et G.G. Bosse

The primary structure of rubber in root extracts was determined by NMR spectroscopy. NMR measurements were carried out using JEOL ECA-400 NMR spectrometer operating at 399.78 and 100.53 MHz for ^{1}H and ^{13}C, respectively (Fig. 2). The polymer was dissolved into chloroform-*d* without tetramethylsilane (TMS).

In ^{1}H NMR spectrum of analyzed sample 3 singlet signals were found at 1.67 (a), 2.02 (c) and 5.10 (в) ppm. Proton integral intensity of discovered signals is related as 3H:4H:1H respectively. Singlet at 1.67 ppm can be classified into methyl groups with a double bond. More intensive singlet at 2.02 ppm corresponds to methylene protons on different sides of the sample's double bond. Signal

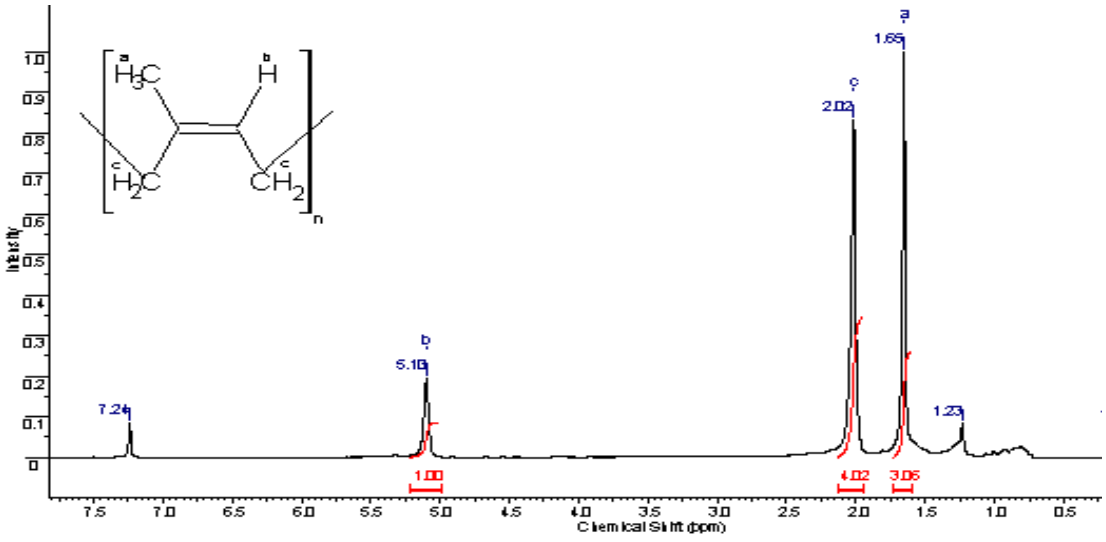

Fig. 2. ^{1}H NMR spectra of rubber in root extracts of *S. tau-saghyz* Lipsch. et G.G. Bosse plants.

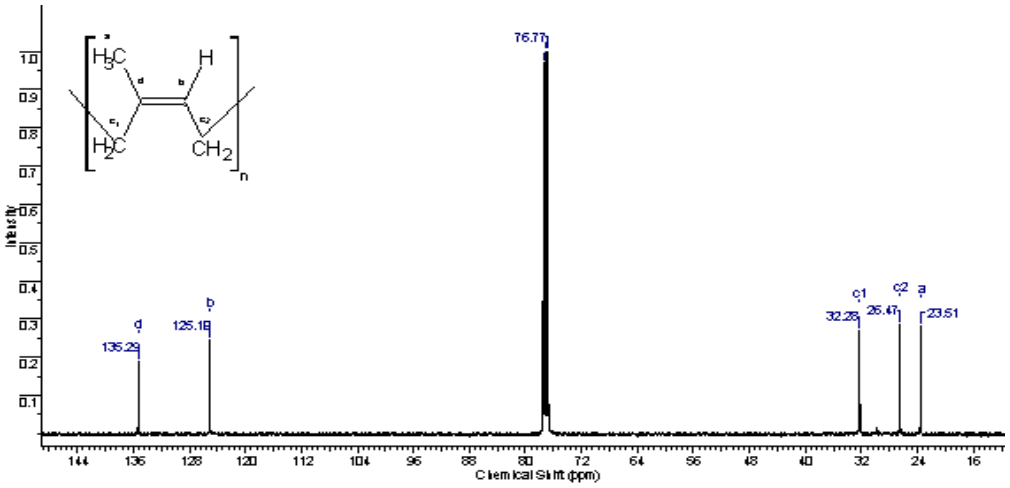

Fig. 3. ^{13}C NMR spectra of root extracts from *Scorzonera tau-saghyz* Lipsch. et G.G. Bosse.

at 5.10 ppm, which is located at the weakest field, informs about protons located at a double bond. Proton identification of the sample shows the presence of methylene protons located at the different sides of unsaturated bond, unsaturated proton and the methyl group at double bond (Fig. 2).

As shown in Fig. 3 in ^{13}C NMR spectrum of analyzed sample signals of carbon atoms were found at 23.51 (a), 26.47 (c_2), 32.28 (c_1), 125.10 (в) and 135.29 (d) ppm (Fig. 3).

It should be noted that the areas of the appearance of the spectral signals of ^{1}H NMR and their intensity, as well as ^{13}C NMR, allows identifying this biopolymer as polyisoprene rubber. A comparison of the obtained spectral data with the literature confirmed the correctness of the assignment of the spectral signals of the biopolymer [17, 33].

The correctness of the assignment of the ^{13}C NMR spectra was confirmed by the data of the DEPT spectra. Chemical shift of ^{13}C NMR biopolymer at 23.51 ppm corresponds to the methyl carbon atom, chemical shifts at 26.47 and 32.28 ppm corresponds to methylene carbon atoms and the presence of a chemical shift at 125.10 ppm shows the presence of the CH-carbon atom in the spectrum. No apparent chemical shift in the DEPT spectrum at 135.29 ppm shows the presence of a quaternary carbon atom in the biopolymer (Fig. 4).

Two-dimensional COSY and HMBC measurements were made to collect two-dimensional hyper complex data. The structure of the biopolymer was also confirmed by the methods of two-dimensional NMR spectroscopy of COSY ^{1}H-^{1}H and HMQC ^{1}H-^{13}C, which makes it possible to establish spin-spin interactions of homo- and heteronuclear nature.

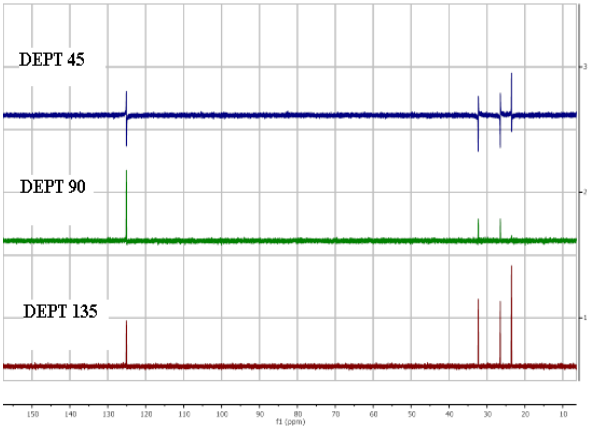

Fig. 4. DEPT spectra of root extracts from *S. tau-saghyz* Lipsch. et G.G. Bosse.

In the spectra of the ^{1}H-^{1}H COSY NMR biopolymer, spin-spin correlations through the three bonds of neighboring methine and methylene protons of H^b-H^c with cross-peaks with coordinates at 5.10, 2.03 and 2.02, 5.10 (Fig. 5) are observed.

Heteronuclear interactions of protons with carbon atoms through a single bond were established by spectroscopy of ^{1}H-^{13}C HMQC for couples present in the biopolymer: H^a-C^a (1.66, 23.73), H^c-C^{c2} (2.03, 26.81), H^c-C^{c1} (2.03, 32.52) and H^b-C^b (5.09, 125.28) (Fig. 6).

In the NMR spectra of the HMBC ^{1}H-^{13}C biopolymer sample, heteronuclear interactions appear through the 2 and 3 bonds of H^a protons with the carbon atoms C^{c2}, C^b and C^d; H^c with C^a, C^b and C^d, and also H^b with C^a, C^{c1} and C^{c2} (Fig. 7).

Received results show that there are structural links of natural rubber (isoprene rubber) in the root sample. There is a clear relationship between

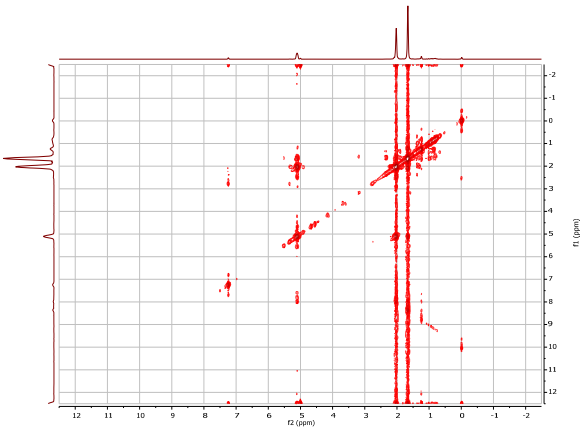

Fig. 5. COSY ^{1}H-^{1}H NMR spectra of biopolymer from root extracts of *S. tau-saghyz.*

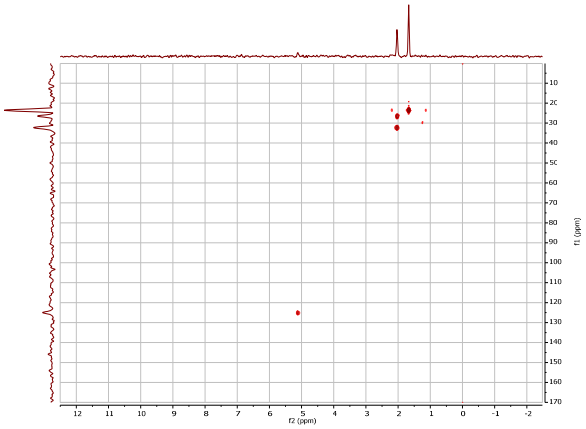

Fig. 6. HMQC ^{1}H-^{13}C NMR spectra of root extracts from rubber producing plant *S. tau-saghyz.*

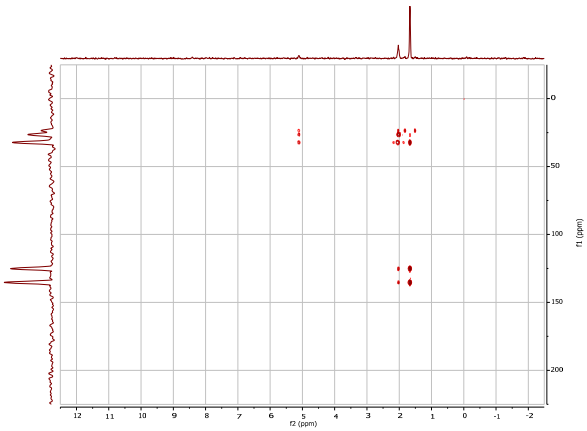

Fig. 7. HMBC ^{1}H-^{13}C NMR spectra of root extracts from *S. tau-saghyz* Lipsch. et G.G. Bosse.

methyl, methine and methylene protons which corresponds to isoprene rubber structure. Having strongly marked singlets that are inherent for rubber functional groups, confirms the stereospecific structure of the rubber. Good solubility of the sample in deuterated chloroform can characterize the low molecular weight of the polymer.

3.3. Effect of Plant Growth Regulators (PGRs) on multiple shoot formation

During the past several years, the anthropogenic influence on the mountains environment has increased and disturbance is the greatest threat to endemic plants, growing in mountains regions. The endemic rubber producing plant species growing within Central Asia might lose suitable habitats in these areas. Hence, it will be necessary to prepare alternative *ex situ* measures, of which the *in vitro* regeneration and propagation presented in this paper is claimed to be the most efficient. For this aim, we tested various combinations and concentrations of plant growth regulators to determine their effect on plant regeneration.

The formation of multiple shoots was detected after 6 weeks of being cultured on modified MS medium containing high concentration cytokinins. Between 10% and 25% of the explants cultured on MR_1–MR_4 mediums supplemented with 5.5 µM BA and 5.5 µM kinetin produced shoots. The results of the experiment are presented in Table 2. The best proliferation of axillary shoots was obtained on MS medium with 5.5 µM kinetin and 0.5 µM NAA and 3% sucrose.

In this study synthetic auxin α-naphthalene acetic acid (NAA) in low concentration induces callus formation in leaf explants, meanwhile when β-Indole-3-acetic acid (IAA) is used in low concentration, root initiation occurs. Low levels of auxin are also required for root elongation. Both β-Indole-3-acetic acid and gibberellic acid induce and regulate elongation shoots and roots. Gibberellic acid (GA_3) is mostly used for internode elongation and meristem growth [34, 35]. In the present study, the number of multiple shoots produced in the presence of 5.5 µM BA and 0.5 µM IAA after being cultured for 4 weeks in modified MS medium was small with senescence of the leaves, suggesting that 6-benzyladenine might inhibit normal shoot formation in this plant.

Many reports have demonstrated the positive effect of cytokinins on micropropagation in plant tissue culture [20, 36, 37]. In this study, the addition of 5.5 µM kinetin in the growing medium was successful in promoting shoot multiplication in all the explants with an average number of 3–4 shoots per explant. In addition, a combination of kinetin and gibberellic acid, lead to the promotion

Table 2
Effect of PGR on the multiple shoot formation of *S. tau-saghyz* Lipsch. et G.G. Bosse from cultured shoot tip explants raised in a modified MS medium for 6 weeks

Medium	Concentration of PGR [μM]	Frequency of shoot formation, [%]
MR_1	5.5 μM kinetin + 0.5 μM IAA	12.5 ± 3.3
MR_2	5.5 μM kinetin + 0.5 μM NAA	25.0 ± 4.3a*
MR_3	5.5 μM BA + 0.5 μM IAA	10.0 ± 3.1
MR_4	5.5 μM BA + 0.5 μM NAA	22.5 ± 4.1a*

of organogenesis and rhyzogenesis. Cytokinins 6-benzyladenine and kinetin initiate cell proliferation in rubber plant cells when they are cultured on a medium that also contains a low concentration auxin α-Naphthalene acetic acid. Kinetin at concentration 5.5 μM promotes cell division and stimulate the growth of shoots in vitro. In the study, when cytokinin is used in high concentration, kinetin (without NAA and GA) inhibits root formation and induces adventitious shoot formation. Rooting was achieved after the transfer of the shoots to an MS medium with 0.5 μM GA_3 and 3% sucrose. The ratio of auxin and cytokinin in the culture decides morphogenesis. Kinetin modifies apical dominance by promoting axillary shoot formation. In the present study high ratio kinetin: NAA (50:1) leads to shoot formation. Whereas the ratio of kinetin: NAA (10:1) isn't high, however, it gives rise to axillary and shoot proliferation, callus initiation, and embryogenesis.

Successful micropropagation of plants that can survive under the natural environmental conditions depends on the acclimatization process. The acclimatization process carries out while the plants are still under in vitro condition. A few days before the process was to be carried out, the cover of the test tube was removed. For the first 3 weeks, the regenerated plants were maintained in the culture room at 25±2 °C. When regenerated plants reached 4–5 cm they were transferred into pots of mixed soil. After transplanting, the plantlets were watered regularly to prevent them from drying.

The type of substrate or medium used for germination is important to seedling establishment. In general, a substrate should be light and porous to provide adequate oxygen, yet retain moisture and allow for proper drainage. Most commercial germination mixes contain a blend of peat moss, vermiculite, perlite, and sand. In this study the rooted plants were acclimatized on a mixture of sand, potting soil and vermiculite in a ratio of 10:2:2, with pH in the range of 7.5–8.0. The plants were successfully acclimatized in the greenhouse with 75% of plantlets, respectively surviving after transfer to ex vitro conditions.

4. Conclusions

Rubber is a strategically important natural material, a biopolymer with useful properties, such as high elasticity, resilience, and thermal stability, which cannot be matched by synthetic alternatives. The semi-shrub *S. tau-saghyz* Lipsch. et G.G. Bosse, growing in mountain regions in Kazakhstan is a fast-growing plant, producing large amounts of biomass with a high concentration of rubber. It's one of the very few rubber producing plants to have ever been used commercially for high-quality natural rubber/latex production. Our simplified method of obtaining rubber from plant material allows for the optimal extraction of rubber from roots. The optimization is achieved by direct extraction of rubber from milled dry roots using n-hexane as a solvent.

The structure of the extracted rubber was identified by the 1D NMR spectra of ^{1}H, ^{13}C and DEPT and the two-dimensional homonuclear correlation spectra of COSY ^{1}H-^{1}H, the heteronuclear correlation of HMQC ^{1}H-^{13}C and HMBC ^{1}H-^{13}C. This analysis showed the polyisoprenoid nature of the samples and their full compliance with the natural rubbers described in the literature.

S. tau-saghyz such as other endemic species that persist in small, isolated populations with low genetic diversity has limited ability to adapt to new climate conditions. The present work reports the first protocol for the in vitro propagation of an endangered endemic rubber plant from Central Asia. The present study suggests that it is possible to improve the frequency of shoot organogenesis in *S. tau-saghyz* Lipsch. et G.G. Bosse by supplementing MS medium with 5.5 μM kinetin and 0.5 μM α-Naphthalene acetic acid. This protocol for in vitro regeneration provides an important opportunity for the propagation and preservation of rubber producing endangered species *S. tau-saghyz* by rapidly increasing the number of plants, without disturbing the wild population. The clones, obtained by the clonal propagation method can be used for renewal

and strengthening the natural population, for collections in botanical gardens and for fundamental studies on the ecology and biology of species.

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