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Research Article

An Efficient Method for Micropropagation of Red-List Herbaceous Plant Species (*Hedysarum cretaceum*)

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Abstract

Hedysarum cretaceum Fisch is a critically endangered herbaceous species with high ornamental and phytomeliorative properties; thus, it needs urgent conservation and protection methods to conserve. In the present study, an efficient method for microtonal propagation of *H. cretaceum* is developed. The seed sterilization was optimized as treatment with 96% alcohol (2 min) and burned (1 sec) through a burner flame. Murashige and Skoog (MS) medium with the addition of 0.5 mg/L 6-Benzylaminopurine (BAP) (shoots per explant

– 18.0) and 1.0 mg/L BAP and 0.1 mg/L Indole-3-acetic acid (IAA) (shoots per explant – 18.4) for the shoot and ⅓ MS supplemented with 0.1 mg/L IAA (rooting indicator – 62%) for roots were identified best composition for the proliferation and regeneration. Additionally, the medium for maintaining callus culture was determined – DKW with the addition of 2.5 mg/L BAP and 0.1 mg/L IAA, the possibility of direct organogenesis *in vitro* in the studied plant was also standardized. In addition, seed germination after various pre-sowing treatments was studied. It is shown that scarification makes achieving 80% germination of seeds possible. Also, sulfuric acid was used as a pre-sowing seed treatment, germination was 45%. Thus, using the protocols we developed, it is possible to maintain the *H. cretaceum* culture *in vitro* and increase seed germination using pre-sowing treatment.

Keywords
Fabaceae; red-list plants; rare plant species; micropropagation; *in vitro*; *Hedysarum cretaceum*

1. Introduction

Hedysarum cretaceum Fisch is a perennial herbaceous obligate chalk, insect-pollinated plant with the characteristics of xeromesophyte (a plant of dry habitats), heliophyte (adapted to life in open, well-lit places), and pteridophyte (living mainly on rocks, scree, and other stony substrates) [1-3]. Seeds are used to propagate *H. cretaceum*, which grows rapidly with the help of root offspring. The endemic spread of *H. cretaceum* to the steppe part of the Don and Volga basins is noted [3, 4]. Besides, *H. cretaceum* is an important breeding species and a prospective phytomeliorant with ornamental, fodder, and phyto meliorative characteristics [4, 5]. However, the natural habitat of *H. cretaceum* has lately been diminished and recognized as an 'endangered species' [4], thereby, the possibility of extinction was assessed, and the species was placed on the red list [4, 6, 7].

Preserving the gene pool of living organisms is considered one of biology's most streamlined operations nowadays. Because several plant species serve as the foundation for most terrestrial biocenoses, special attention is paid to the gene pools of these organisms [8]. In many instances, plants cannot efficiently maintain their population levels over time. Diverse factors including anthropogenic impact in the form of habitat degradation, regional climate change, relict species, fragmented and isolated regions, and issues with seed self-reproduction, *etc.,* may play an important role. Further, reducing the number of plant populations eventually results in the extinction of entire species. This is the cause of degradation, destruction, and disappearance of certain phytochromes or biocenoses [9, 10]. As a result, actions to conserve the gene pool of plants and manage and restore the quantity of their populations, are vital [4].

Microclonal propagation is a modern biotechnology-based tool for obtaining plants due to their potential to regenerate cells and tissues. Also, this method is a reliable and highly prolific strategy to reproduce protected plant species because of its numerous advantages [11, 12]. Many endangered and economically important plant species, for example, *Pulsatilla vulgaris*, *Guldenstaedtia monophylla* [13], *Crataegus pojarkovae* [14], *Amomum subulatum* [15], *Tricholepis*

role [16] have been protected and expanded by micro clonal propagation technique. To date, to the author's knowledge, no study has been reported for *in vitro* regeneration of this red-listed, valuable, and rare species of *H. cretaceum*. The limiting factors of this species include the following: "narrow ecological amplitude, low competitiveness, a small number of populations and their spatial separation, habitat destruction (industrial and local chalk mining, forest reclamation and terracing of slopes), anthropogenic habitat disturbances (excessive grazing, recreational load, etc.)," as well as low seed productivity and field germination, and reproduction occurs exclusively by seeds ([4], p. 1001), then for this plant it is necessary to develop protocols for micropropagation.

Therefore, the habitat of the *H. cretaceum* is being diminished as a result of anthropogenic human action, thereby, it is crucial to devise methods for preserving this species using *in vitro* culturing. This study aims to develop a technology for the microclonal reproduction of *H. cretaceum* under the aseptic condition to preserve the gene pool of the population of this species. It may support expanding its plantation further to meet various objectives.

2. Materials and Methods

2.1 Plant Material

The seeds were collected from the Botanical Garden of the Southern Federal University's Collection of rare and endangered plant species of the Rostov region (47.2389°N, 39.6437°E), Russia. Rostov region is in an area of moderately continental and arid climate. The average annual precipitation is 548 mm, most of which falls during the frost-free period. Summer is hot; the maximum temperature can reach 40°C. In general, the winters are mild to moderately cold. The average temperature in January is -5°C, the average absolute minimum air temperature is -20°C to -25°C, and the absolute minimum temperature is -32°C. The growing season lasts 216 days (from 1 April to 4 November), and the frost-free period is 258 days on average [17].

2.2 Analysis of Seed Reproduction

The appearance of seeds was described by following the methods described in the Atlas of descriptive morphology of higher plants [18]. “Considering the external signs of the seeds, the following indicators are of great importance: the size, shape, nature of the surface and color of the seed; the shape, size and location of the seed hilum and raphe, as well as various kinds of appendages” ([18], p.18).

Morphometric indicators of seeds were determined using an eyepiece-micrometer (MOB-1-15x, LOMO, Russia). The average values were measured by $\bar{x} = \Sigma x_i/n$, coefficient of variation (V), % of the length and % width of seeds by $Cv = Sx/\bar{x} \times 100\%$ [19]. The weight of 1000 seeds was determined using analytical scales. To determine the mass of 1000 seeds, 4 samples of 100 seeds were taken, weighed, and recalculated by 1000 pieces, and average results were used [20].

2.3 Experimental Design

2.3.1 Pre-Sowing Treatment (PT) Techniques for Seeds of *H. cretaceum*

1. Scarification. *H. cretaceum* Seeds have a physical type of rest period, called seed dormancy. Scarification (56-60%), stratification (44.2%) and chemical treatment (38.7%) were used as PT methods for the related species of Gmelin's sweetvetch (*Hedysarum gmelinii*) [21]. Scarification of the seed hardcovers was carried out using sandpaper with a grain size of p500.
2. Impaction (collision). Impaction-light scarification, violation of the integrity of the seed coat by hitting sand or small stones on the seeds. The fifty seeds were placed in plastic containers with expanded clay and sand (1:1) and shaken for 1 h.
3. Treatment with 80% sulfuric acid for 1 h in a ceramic mortar.
4. Control samples (germination of seeds without pre-sowing treatment).

2.3.2 The Explants Were Sterilized Using Two Methods

1. Scarification of seeds with sandpaper followed by washing by ethyl alcohol (C_2H_5OH) and mercury (II) chloride ($HgCl_2$) for 3 and 2 min, respectively. Then the seeds were washed thrice with distilled water for 10 minutes.
2. Wash the seeds in distilled water for 15 minutes, followed by 96% ethyl alcohol (C_2H_5OH) treatment (2 min) and burning by burner flame (1 sec).

Germination was performed on agar medium at a concentration of 7 g/L without phytohormones for 7 days.

After germination, the plantlets were placed on MS medium [22, 23] and Driver-Kuniyuki Walnut (DKW) medium [24, 25]. For proliferation, BAP (Green Agrolab, Russia) was added to the media, as well as a combination of BAP and IAA (Green Agrolab, Russia). BAP was added in concentrations of 0.1, 0.5, 1.0 and 2.5 mg/L, and IAA in of 0.1 mg/L concentrations. We also made the passage on Gamborg and Eveleigh [26] medium without phytohormones. At the rooting stage of plant regeneration, IBA and IAA in concentrations of 0.1, 0.5, 0.7, and 1.0 mg/L were added to the media. Control samples were also laid.

The MS medium with ½ and ⅓ concentrations of macronutrients and the White medium [27], was utilized to determine the best environment for rooting in *H. cretaceum* plants. The modified nutritional media for shoot proliferation and histogenesis was studied with cultivation times of one and two months, respectively [11, 28].

Seeds and plantlet transfers were carried out in laminar airflow (Safe Fast Elite, France) with LAF velocity of 0.40 and BARR velocity of 0.53. Sterilization was carried out in an autoclave MLS-3771L (Sanyo, Japan) at 121°C for 20 min and a pressure of 1.5 atmospheres. The pH of the media was adjusted to 6.0. The explants were placed at room temperature (24-25°C) under 2800 Lux for a 16-hour photoperiod using fluorescent lamps T5 28 W (Elektrostandart, Russia).

2.4 Statistical Analysis

During the study regarding nutrient media selection, indices including shoots per explant (the average number of new shoots per explant), root formation percentage, and the average length of

shoots and roots were observed. The Student's t-test was used to compare the values of plant shoots per explant on different media at a 5% significance level [19]. The results of the experimental data were processed statistically using the computer programs Microsoft Office Excel 2016 and STATISTICA.

3. Results

3.1 Seed Reproduction

H. cretaceum has a relatively large seed curve, round, and flattened shape with a protruding embryo. The surface is bare and smooth; the color is matte or glossy, the color scheme is dominated by light and yellow-brown shades; the scar is small, located in the center of the abdominal concave part of the seed, rounded shape. The average length, width and weight of 1000 seeds were 3.03 ± 0.12 mm, (V = 12.29%), 2.48 ± 0.08 mm (V = 9.83%), 5.5 ± 0.2 g, respectively.

The histogram of germination indicators (Table 1, Figure 1, and Figure 2) shows that the most effective method of PT was a variant with the use of scarification, where the highest germination rate (80%) was obtained. The germination process of this type of seeds was characterized by a high degree of friendliness (the number of simultaneously sprung seeds from the entire sample), i.e., 48%. Statistically significant differences were observed with the indicators in the control. In this experiment, the complete removal of hard seeds was achieved.

Table 1 The indicators of germination of *H. cretaceum.*

Germination	Control	Scarification	Impaction	H_2SO_4
Overall germination rate, %	17 ± 3.76	80 ± 3.46	25 ± 4.33	45 ± 4.97
Germination energy, %	3 ± 1.71	48 ± 4.99	1 ± 0.99	12 ± 3.25

* the calculation was carried out with 50 seeds per experiment.

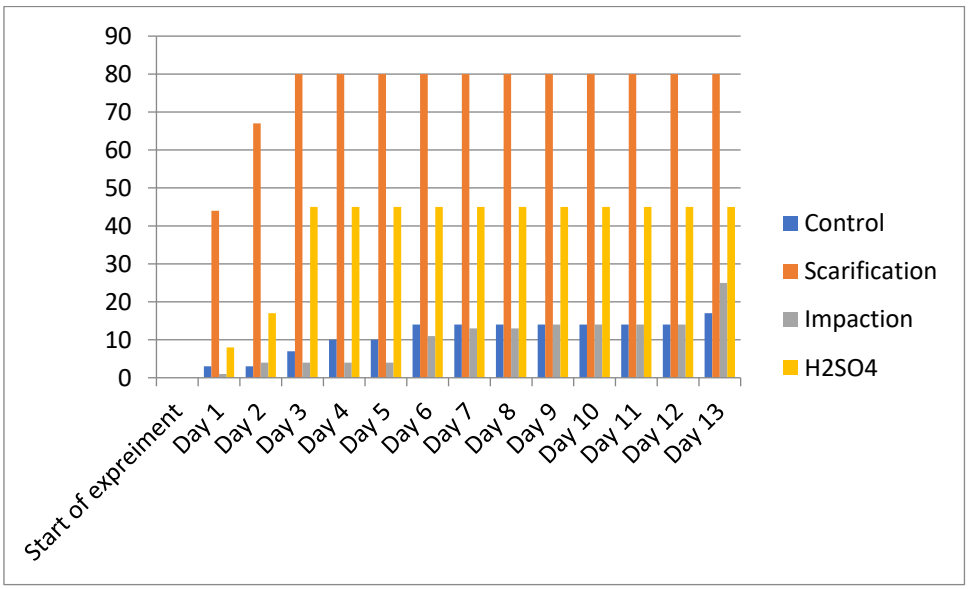

Figure 1 General germination of *H. cretaceum.* * the calculation was carried out with 50 seeds per experiment.

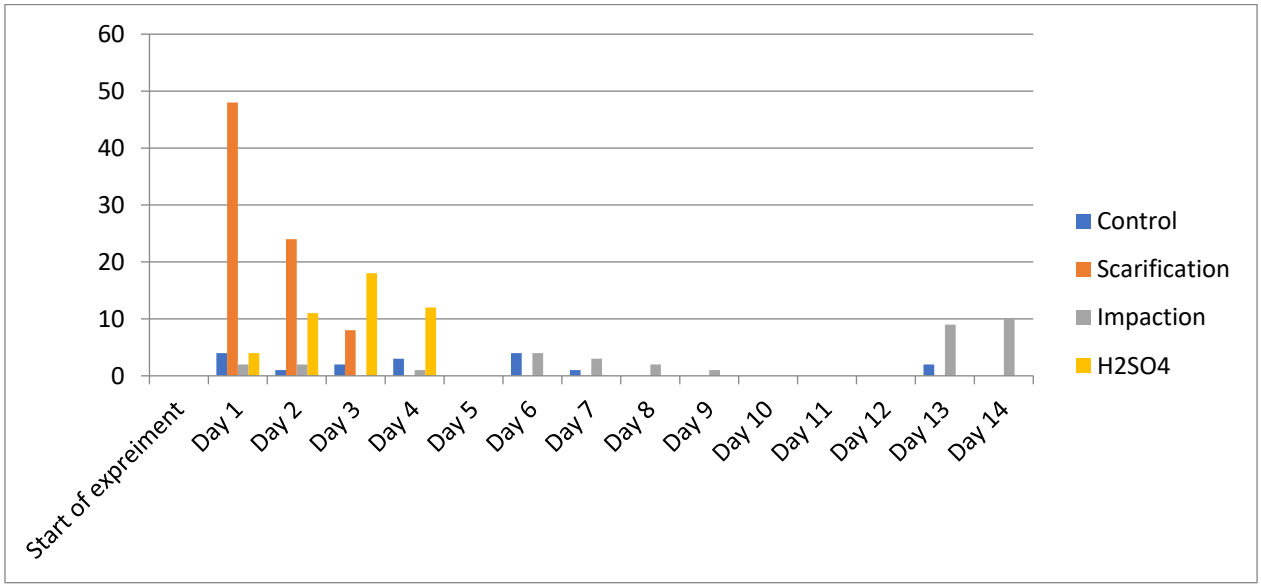

Figure 2 Dynamics germination of *H. cretaceum.* * the calculation was carried out with 50 seeds per experiment.

The possibility of utilizing H_2SO_4 as a PT was given special consideration. Here the germination rate was 45%, while the remaining seeds were infected (55%). The germination energy in this experiment was 12%, and the differences with the control for both indicators were significant (t = 6.24 for total germination, t = 2.45 for germination energy).

Seeds from control samples and treatment with impaction have lower germination rates, i.e., 17% and 25% (Figure 3). The proportion of affected seeds is quite high in control (83%) and impaction (62%). Solid and unaffected seeds were only in the impaction at the end of observations (13%). When comparing the results of these experiments, no significant differences were obtained.

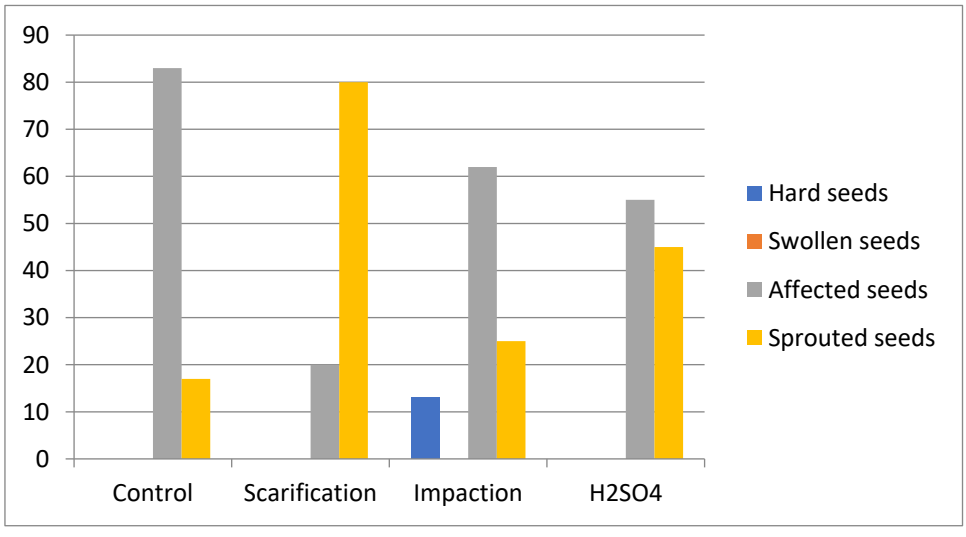

Figure 3 Germination indicators of *H. cretaceum.* * the calculation was carried out with 50 seeds per experiment.

3.2 Impact of Sterilization of Planting Material on Germination

As a result of using two methods of sterilization of planting material, it was found that washing the seeds in distilled water with subsequent treatment with alcohol and firing in a burner flame was the most effective method of their disinfection (Figure 4).

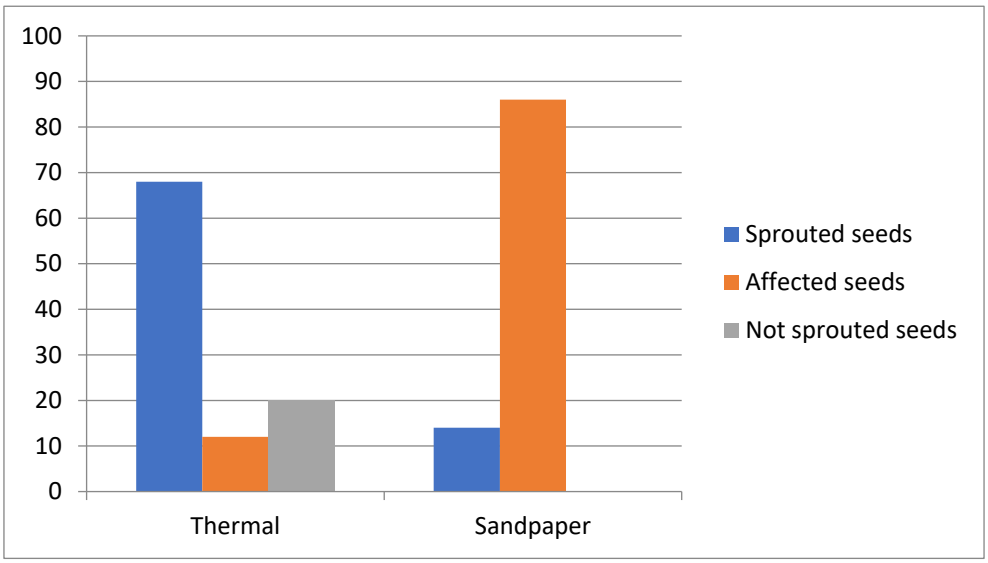

Figure 4 Histogram of distribution of results of seed sterilization of *H. cretaceum*. * a total of 200 seeds were analyzed.

Using this method, 87.5% of sterile seeds were obtained. During heat treatment, 20% of ungrown seeds were also observed. Another method of sterilization: scarification of seeds with sandpaper followed by washing them in ethyl alcohol (C_2H_5OH) and mercury (II) chloride ($HgCl_2$) for 3 and 2 min, respectively. Then the seeds were washed thrice with distilled water for 10 minutes, and observed a bacterial lesion of 86%. On the other hand, 14% of sprouted seeds were detected when using the sterilization technique.

3.3 Cloning and Proliferation

The shoots per explant, this is the number of individual new shoots (Table 2) and the average length of shoots (Table 3) on two media: MS and DKW were compared (Figure 5). Variants of the experiment with the use of BAP and IAA, as well as using only BAP, were put forward. The concentration of IAA in all variants of the experiment with its use was 0.1 mg/L.

Table 2 Average values of the shoots per explant of *H. cretaceum.*

Conc. BAP,	MS medium			DKW medium		
mg/L	Control	BAP	BAP + IAA	Control	BAP	BAP + IAA
0.1	8.4 ± 1.3	7.6 ± 1.4	17.8 ± 3.3	6.2 ± 1.2	6.8 ± 1.1	2.2 ± 0.8
0.5		18.0 ± 1.2	7.0 ± 0.8		2.6 ± 0.5	2.0 ± 0.5
1.0		18.0 ± 1.2	18.4 ± 3.3		4.0 ± 0.4	1.4 ± 0.4
2.5		8.6 ± 0.8	17.4 ± 2.7		5.4 ± 1.2	2.4 ± 0.4

* the calculation was carried out with 25 plants per experiment.

Table 3 Average values of the length shoots of *H. cretaceum.*

Conc. BAP,	MS medium			DKW medium		
mg/L	Control	BAP	BAP + IAA	Control	BAP	BAP + IAA
0.1	3.5 ± 0.6	3.1 ± 0.9	5.6 ± 0.4*	3.8 ± 0.3	4.3 ± 0.3	2.0 ± 0.7*
0.5		5.2 ± 0.3*	2.8 ± 0.5		1.9 ± 0.6*	1.2 ± 0.6*
1.0		4.4 ± 0.5	0.7 ± 0.1*		3.6 ± 0.3	0.5 ± 0.4*
2.5		4.2 ± 0.4	0.7 ± 0.2*		2.8 ± 0.6	1.8 ± 0.6*

* significant difference. ** the calculation was carried out with 25 plants per experiment.

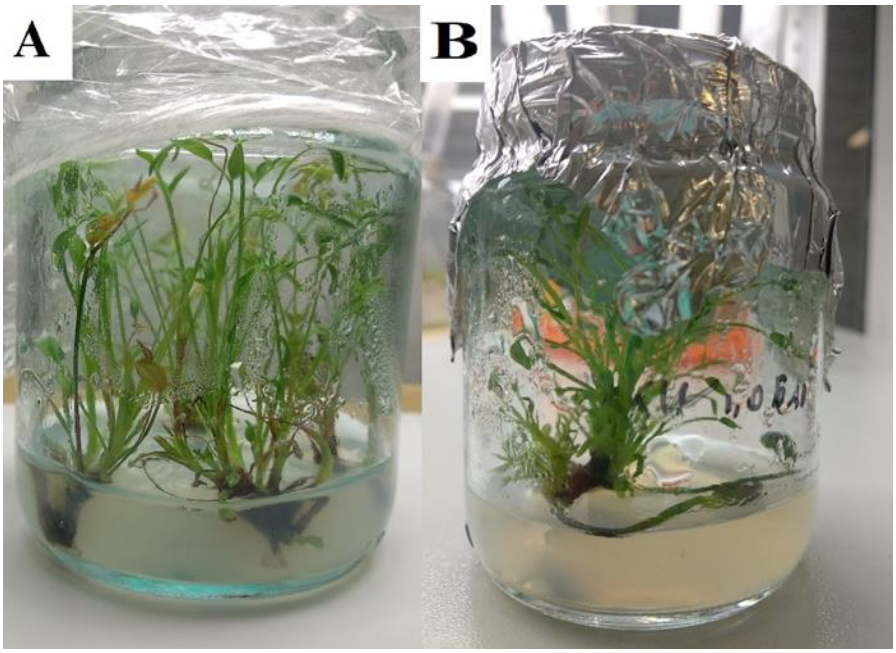

Figure 5 Proliferation of *H. cretaceum* on MS medium with the addition 0.5 mg/L BAP and 0.1 mg/L IAA (A) (2.8 ± 0.5) and DKW medium with the addition of 0.1 mg/L BAP (B) (4.3 ± 0.3).

It was found that the effective concentrations of hormones for the multiplication of shoots on the MS medium are only 0.5 mg/L separately (18.0 ± 1.2), as well as 1.0 mg/L in combination with 0.1 mg/L IAA (18.4 ± 3.3). For the DKW medium, the most effective experimental options were the use of BAP separately at a concentration of 0.1 mg/L (6.8 ± 1.1) and combined use with 0.1 mg/L IAA, where the concentration of BAP was 2.5 mg/L (2.4 ± 0.4).

In other experiments, vitrification of shoots and weak growth were observed. Intensive callus formation was grown in the DKW medium using BAP at a concentration of 2.5 mg/L and 0.1 mg/L IAA. This nutrient medium can produce the callus culture of *H. cretaceum.*

3.4 In Vitro Rooting

The histogenesis in different culture media was studied. The effect of ½ MS and DKW medium with adding auxins – IAA and IBA in concentrations of 0.1, 0.5, 0.7, and 1.0 mg/L were compared. As a result of the experiment, the process of active histogenesis was observed in a variant with a medium of ½ MS without adding phytohormones. The average root length was 6.9 ± 0.43 cm, and

the rooting coefficient was 100%. In other versions of the experiment, histogenesis was almost not observed.

At the same time, in all variants of the experiment, except for control in the medium of ½ MS, there was strong vitrification of plants.

Based on the results obtained, it was decided to experiment to study the rooting of *H. cretaceum* plants on media ½ MS, ⅓ MS, ½ DKW and White (Table 4, Figure 6).

Table 4 Values of *H. cretaceum* rooting indicators on MS, DKW, and White nutrient media.

Indicator	½ MS	⅓ MS	½ DKW	White
% rootings	59%	62%	32%	3%
The average length of the roots	5.2 ± 0.6	7.8 ± 0.5	2.1 ± 0.5	2.1 ± 0.9

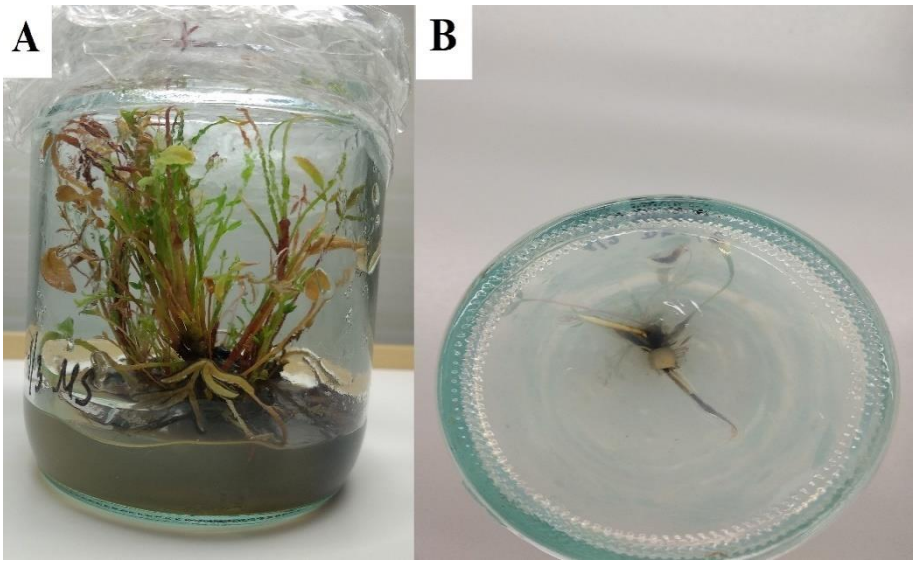

Figure 6 Rhizogenesis of *H. cretaceum* on the ⅓ MS medium (A) (5.2 ± 0.6) and White medium (B) (2.1 ± 0.9).

According to the results of this experiment, the most suitable environment for all the subjects was ⅓ MS, where the rooting percentage was the highest. The MS environment also showed a satisfactory result. The statistical comparison did not reveal significant differences between the values of the average root length in these media.

Unexpected results were noted during the observation of direct organogenesis, the production of roots from leaves was noted on MS and DKW media (Figure 7). This phenomenon was observed on MS and DKW media with the addition of 0.5 and 1.0 mg/L IAA and when using ½ MS and ⅓ MS.

Figure 7 Direct organogenesis of *H. cretaceum* on the MS medium with the addition of 0.5 and 1.0 mg/L IAA.

The DKW and White media showed less satisfactory values of rooting parameters. When cultivating regenerating plants on a medium of ½ DKW, the rooting rate was higher than white's medium (32% and 3%, respectively).

4. Discussion

As a result of washing the seeds in distilled water, followed by alcohol treatment and roasting in a burner flame, it became obvious that roasting seeds, in addition to the sterilizing effect, has a scarifying effect. During heat treatment, 20% of ungrown seeds were observed. Presumably, this is because too high flame temperatures damaged the embryos, since it is known that the seed coat of *H. cretaceum* is less thick than the seeds of many other representatives of the Fabaceae family [29]. The high percentage of infection (86%) with the first method sterilization method is because when scarification using sandpaper, infection of the seed germ tissues probably occurred.

The average values of the shoots per explant when using the DKW environment were lower than those for the MS. A significant excess of the shoots per explant when using the MS medium compared with the DKW medium. As a result of the combined action of BAP and IAA in MS and DKW media, the data obtained are consistent with the literature data that combinations of various growth regulators often have a high synergistic effect [30, 31]. In most cases, the average values of the length of *H. cretaceum* shoots on different media are consistent with the values of the shoots per explant. Thus, when utilizing the MS medium, it was observed that adding BAP separately at a concentration of 0.5 mg/L and employing IAA together, resulted in the maximum shoot length. The highest values of shoot length on the DKW medium were determined when using BAP at a concentration of 0.1 mg/L both separately and in conjunction with 0.1 mg/L IAA. At the same time, the concentration of BAP 2.5 mg/L, most likely, inhibited the growth of shoots in length. However, it stimulated their proliferation and callus formation.

A significant excess of the average length of shoots (compared with the control sample) was observed on the MS medium with the addition of BAP of 0.1 mg/L and 0.1 mg/L IAA. When using the DKW environment, the shoot length value was significantly less as compared to the control. When comparing the identical variants of the experiment on different media, samples using BAP 0.5 mg/L and BAP 0.1 mg/L together with 0.1 mg/L IAA showed a significant excess of MS media over DKW media (α = 5%, k = 5, t_{teor} = 2.57).

Thus, based on the evidence presented here, it is difficult to conclude with certainty that the DKW environment is more suitable to the proliferation of *H. cretaceum* shoots.

The experience with the B5 setting was not impactful because on the seventh day of the experiment, when developing *H. cretaceum* plants on this medium, strong vitrification of shoots was seen, and growth ceased. This is probably due to the lower calcium content in the B5 medium compared to MS and DKW, which is the most important component of mineral nutrition for the studied plants.

Microclonal propagation protocols have been developed for many species of the genus *Hedysarum* L., included in the regional red books and the red book of the Russian Federation [13, 32-34]. For example, Alieva et al. [34] showed that scarification positively affects seed germination of *H. daghestanicum* (68-75%). For elongation, MS medium was used with the addition of BAP at 2.5 mg/L and IBA at a concentration of 0.5 mg/L. Subsequently, this medium gave rise to callus formation in all nodal explants and leaf plates [34]. To induce the development of *H. argyrophyllum* shoots, an MS culture medium containing BAP at a concentration of 2.0 mg/L and IAA at a concentration of 0.1 mg/L was used [33]. On this medium, the growth rate of shoots ranged from 3 to 5. it is Interesting that in the experiment using kinetin at a concentration of 0.5 mg/L with the addition of IAA at a concentration of 0.2 mg/L; callogenesis is initiated, followed by morphogenesis of *H. argyrophyllum* explants [33].

In general, the optimal nutrient medium for the proliferation of shoots of species from the genus *Hedysarum* L. is the combined action of cytokinin (BAP) at concentrations from 1.0 mg/L (as shown in our study) to 2.0-2.5 mg/L [33, 34] and various auxins (IBA, IAA) at concentrations of 0.1-0.5 mg/L. But callogenesis runs in different environments. In our case, this is a DKW medium with the addition of 2.5 mg/L BAP and 0.1 mg/L IAA, and in the work of Alieva et al. [34], MS medium with the addition of 2.5 mg/L BAP and 0.5 mg/L IBA. And in work on *H. argyrophyllum* [33] to trigger callogenesis, a lower concentration of another cytokinin (kinetin) is necessary with the addition of a slightly increased concentration of auxin (IAA) up to 0.2 mg/L compared to our experiment.

For sterilization of seeds of *H. argyrophyllum* Ledeb. and *H. grandiflorum* Pall. Various active substances were used. As a result of the experiment, it turned out that mercury-containing sterilizing solutions are more effective than chlorine-containing ones [32]. *H. grandiflorum* is optimal for shoots was the nutrient medium MS with the addition of 1.0 mg/L BAP, the multiplication factor was 4.9 for *H. argyrophyllum* – BAP 2.0 mg/L + IAA 0.1 mg/L, the reproduction rate of 8.0. For rooting micro shoots *H. argyrophyllum* and *H. grandiflorum* used a medium of ½ MS with the addition of IBA at a concentration of 0.5 mg/L [32]. As shown, the medium 1/3 MS was used for the histogenesis of *H. cretaceum*. The presence of phytohormones, but the concentration of micro and macro elements may play an important role in the organogenesis of the genus *Hedysarum* L species.

For *H. theinum* Krasnob. Growing shoots from mother plants were used as explants. Thus, Novikova et al. [13] used an MS medium containing NAA at 0.1 mg/L and BAP at 2.0 mg/L for *in-vitro* culture in the first passage. The MS medium for differentiation in the second passage was supplemented with 1.0 mg/L BAP or a combination of two cytokinins: 1.0 mg/L BAP and 1.0 mg/L triacontanol (the latter was administered to shorten the internodes).

The use of the MS medium with a half concentration of macronutrients is explained by the fact that the concentration of ammonium nitrate in the whole MS is quite high compared to the DKW

medium. It is known that the high content of this salt in nutrient media can inhibit organogenesis, especially rhizogenesis. As a result of the experiment, the process of active rhizogenesis was observed in a variant with a medium of ½ MS without adding phytohormones. In other versions of the experiment, rhizogenesis was almost not observed. Most likely, such data can be associated with the fact that the addition of auxins in low concentrations significantly inhibits rhizogenesis, and the concentration of 1 mg/L of IBA and IAA has a weak stimulating effect on root formation.

The statistical analysis results do not allow us to conclude which of the two media (⅓ MS and MS) is better suited for stimulating the rhizogenesis of *H. cretaceum in vitro*. On this basis, we can conclude that the effects of certain auxin concentrations on the rhizogenesis of *H. cretaceum* were negligible. Growing the research object on MS medium with different concentrations of macronutrients is more efficient than using white and DKW medium. A higher rooting rate on a ½ DKW medium than White's medium (32% and 3%, respectively) is presumably because the white medium is more different in the content of macro-salts from MS than DKW. The average root length was small, and samples of rooted plants in these media were 8 and 2 samples, respectively.

Most likely, the influence of auxins in these concentrations has a stimulating effect on the direct organogenesis and regeneration of *H. cretaceum*. It is possible that this process was also influenced by BAP, which was used at the stage of shoot proliferation. It is known that the combined use of auxins and cytokinins stimulates organogenesis *in vitro* in many plants, and this process can be recorded starting from about 6 weeks of cultivation [35, 36]. In the present study, at 6 weeks of cultivation, the plants were already on the media for rhizogenesis. This may explain why direct organogenesis was obtained on hormone-free media. Further research is needed to understand the causes of direct organogenesis in *H. cretaceum*.

Thus, for the successful proliferation of plants of the genus *Hedysarum* L., the ratio of cytokinins and auxins in a certain concentration is necessary. In this case, cytokinins should be in higher concentrations than auxins. The standard cytokinin used in all experiments is BAP for all studied plants of the genus *Hedysarum* L.. This may be due to a milder effect on plant tissues. IBA, IAA, and NAA were used as auxins. The resulting protocols for the production of *H. cretaceum* callus will help in the future to obtain secondary metabolites [37, 38] for use in medicine or veterinary medicine [39].

5. Conclusions

This work developed the optimal protocols for *in vitro* conservation and reproduction of *Hedysarum cretaceum*, a rare Rostov region plant. However, the protocol for conserving a rare species is not fully completed, because work is still underway to adapt *H. cretaceum* to post-aseptic environmental conditions. Upon selecting optimal soil mixtures characteristic of the soil conditions of the natural habitat of this species, the protocol of cultivation of a rare representative of the genus *Hedysarum* will be completed. The authors expect that the collected data will be useful to scientists working in plant biotechnology, commercial applications and the protection of biodiversity.

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Author Contributions

The idea of manuscript was planned by CVA and BSD. The basic literature was collected and first draft was prepared by CVA, BSD, EOU edited the draft. Experimental part by BSD, SVV, AAS and BAA. Processing and analysis of results by CVA, BSD, DPA, VTV. RVD, KA, MEMM and BAA edited the final draft and CVA approved the final version of the manuscript. All the authors have read and approved the submission of manuscript.

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Competing Interests

The authors have declared that no competing interests exist.

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