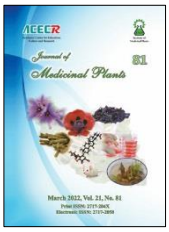
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Research Article

In vitro propagation and callus induction of medicinal endangered plant meadow rue (*Thalictrum minus* L.) for producing berberine

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ABSTRACT

Background: *Thalictrum minus* L. is a medicinal plant contains berberine, which is used for complementary treating of some diseases. Excessive harvesting of this species from rangelands to extract the alkaloid has endangered its survival. **Objective:** This study was aimed for *in vitro* propagation of this species and berberine production. **Methods:** The seeds were germinated at *in vitro* culture conditions. Then, the leaves were used as explants for callus induction in two MS and LS basal media supplemented with BA (0, 0.1, 0.5 and 1 mg/L) in combination with different concentrations of auxins, NAA, 2,4-D or IAA. **Results:** The results showed that MS medium with the plant growth regulator combination of 0.5 mg/L BA and 0.5 mg/L NAA had the highest callus formation and 100 % regeneration. Some calli were transferred to three liquid culture media (MS, LS, or ½ MS) with 1 mg/L BA + 0.1 mg/L NAA or without plant growth regulator to produce the berberine. The HPLC results identified 6 % of berberine in the liquid medium. This amount of berberine can be easily released into the culture medium, therefore, it will be easier to extract. **Conclusion:** This method can be successfully used for propagation and extraction of berberine without plant destruction.

1. Introduction

The side effects of chemical drugs and modern medicine's inability to treat some diseases have made people more interested in using plant-derived medicinal compounds. Plants are a rich source of natural biologically active compounds. They produce a wide range of secondary metabolites that enable them to adapt to their environment [1]. Alkaloids are one of these metabolites. Some alkaloids are employed in the pharmaceutical industry. For example, morphine is used as an analgesic [2] and vinblastine as an

Abbreviations: MS, Murashige and Skoo; LS, Linsmaier and Skoog Medium; BA, Benzyl Adenin; NAA, 1-Naphthaleneacetic acid; 2,4-D, 2,4-Dichlorophenoxyacetic acid; IAA, Indole Acetic Acid; GA3, Gibberellic Acid; HPLC, High Performance Liquid Chromatography; LSD, Least Significant Differenc

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anticancer drug [3]. Berberine is one of the isoquinoline alkaloids applied to treat cancer, cardiac arrhythmia, diabetes, and hyperlipidemia [4]. Also, its antibiotic, antiviral and antifungal activities have been proven [5]. Common berberine-producing species are *Coptis japonica* and *Thalictrum minus* from the Ranunculaceae family, *Phellodendron amurense* from the Rutaceae family, *Berberis aristata* from the Berberidaceae family [4]. Researchers attempted to produce berberine from different plants in different ways due to the medicinal importance of berberine, a long-term process for its production with high cost.

Tissue culture is one of the important technologies that can cause widespread changes in the improvement of berberine production. In this regard, it can be mentioned that callus induction has increased secondary metabolites in some medicinal plants [6]. By callus culture alkaloids secretion into the media and sprouts formation increase. The callus cells can also produce similar compounds found in whole plants [7]. Therefore it can be used as a tool to extract secondary metabolites, including berberine. *Thalictrum minus*, which is known by the generic name of meadow rue, is one of the large genera in Ranunculaceae [8]. It is a perennial, herbaceous plant with relatively thick rhizomes and clustered complex flowers living in the highlands and the germination rate is low that is a significant challenge for this plant species. Since berberine is used to formulate various drugs, it is essential to increase its production by culturing *T. minus* callus cells. For example, in a study [9] berberine produce from *T. minus* callus tissue. Also, in an experiment by adding spermidine to *T. minus* suspension culture they tried to increase berberine production [10]. In another study, the authors designed an immobilized cell culture system to produce berberine from *T. minus* cells [11]. In a study, hairy root culture used to increase berberine production in *Berberis aristata*, and after 28 days, the hairy roots started to produce berberine [6]. This is a comprehensive study to formulate an effective protocol for the regeneration process of *T. minus* using the explants of the seed-grown samples. Besides, a suspension culture was established for the quantification of berberine as an important alkaloid. Also, we investigated the ability of this plant to adapt to natural environmental conditions.

2. Materials and Methods

2.1. Plant material and surface sterilization

The seeds of *Thalictrum minus* were obtained from the Research Institute of Forest and Rangelands, Iran, with 70718 Herbarium code. The seeds were washed with water and Tween 20 under running tap water to remove tween 20 from the surface of the seeds. The seeds were surface sterilized with 70 % ethanol solution for 60 seconds and then for 25 minutes in 2.5 % sodium hypochlorite solution. Finally, they were washed three times with sterile distilled water under sterile conditions. After that, the excess water was removed by sterile filter paper.

2.2. Germination test

Seeds viability was assayed with a tetrazolium test according to the method presented by a study [12]. The seed coats were removed, then soaked in a 1 % tetrazolium solution for 24h. This method specified all the living tissues respiring and reducing a colorless chemical (tetrazolium) into a red-colored compound using a hydrogen cation transfer reaction catalyzed by the enzyme dehydrogenase. Two of the procedures used gibberellic acid (GA3) inorder to break the seeds dormancy phase. The first one was its application into ½ MS medium with a concentration of

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1 mg/L (i.e., ½ MS medium + 1 mg/L GA3). The other procedure was the immersion of the seed in 500 mg/L GA3 for 24 h, then culturing on the free ½ MS medium in Petri dishes. The cultures were restored to germinate in dark with a temperature of 4-5 °C [12] for 120 days. The germinated seeds then were subcultured in a plant growth regulator-free ½ MS medium.

2.3. Callus induction and regeneration

The leaves of sterile plants that grew from the last test were cut into 0.5 cm × 0.5 cm pieces under laminar airflow. They were cultured to form the callus in two culture media, MS and LS, supplemented in 9 different treatment with BA (0, 0.1, 0.5 or 1 mg/L) in combination with NAA (0 or 0.5 mg/L), 2, 4-D (0, 0.1, 1 or 2 mg/L) or IAA (0 or 0.5 mg/L) at 25 °C in 16/8 (light/ dark) condition. Forty-five days after transferring to the culture medium, the percentage of callus formation was evaluated. Three replications were considered for each treatment. The containers (30ml) with three samples have filtered caps to facilitate the exchange of gases for better callus formation (Fig. 1).

Almost 14 days after callus formation, explants began to regenerate (Fig. 1b). Thirty days after beginning of regeneration symptoms, the regeneration (%) was calculated. Repeatedly they were subcultured in the same medium, including MS and LS medium with the same plant growth regulator combinations at 25°C in 16/8 (light/ dark) condition, Except for LS culture medium containing 0.1 mg/L 2,4-D, removed from the treatments due to the lack of callus formation.

The regenerated plants (Fig. 1e) were transferred to pots containing the coco-peat and perlite (1:1) substrates (Fig. 1f). The pots were irrigated every eight days and fed with 20-20-20 N-P-K fertilizers every two weeks. Then the number of acclimatized plants was recorded after forty days and almost all of the plants were acclimatized and the percentage of acclimation was 91.66 %. Two weeks after the plants were transferred to pots with coco-peat perlite substrates, the leaves became more extensive. After three months, in the spring, the plants were transferred to the garden and the next spring, the seeds were collected (Fig. 1g).

2.4. Cell suspension culture

The calli obtained from leaves in the previous experiment were transferred to liquid culture media to secrete berberine better. The culture media examined including MS, ½ MS and LS containing 1 mg/L BA and 0.1 mg/L NAA or without any plant growth regulator. The culture glasses (30 mL) were placed in the dark at 25 °C on a 120 rpm shaker. Twenty days after culture, the media was evaluated for berberine content.

2.5. Qualitative and quantitative evaluation of berberine

A qualitative evaluation of berberine was performed by the color change in the culture medium and its fluorescence under UV light [13]. After confirming the presence of fluorescent berberine, samples were assayed by HPLC to determine the real amount of berberine in the culture medium. The standard solution of berberine was prepared in 0.01, 0.05 and 0.1 (mg/mL) concentrations and then U.V. absorption was read at 345 nm in HPLC, and its calibration curve was plotted. The liquid culture medium was injected into the HPLC without extraction and just by adding ionic buffer. U.V. absorption was read at 345 nm. The weight percentage of berberine in the culture medium was obtained with the aid of the linear equation of the calibration curve and the berberine sample's absorbance. The standard of berberine-HCl with the product no. of 83251 was purchased

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from Sigma-Aldrich Company. The standard solution was prepared using methanol [14]. Berberine was assayed in the culture medium with three replications by ion-pair reverse-phase HPLC with detection at 345 nm using a 250 mm × 10 mm Analytical column. The quantities were expressed as berberine-HCL equivalents.

2.6. Statistical analysis

All the experiments were performed in a completely randomized design, and at least three replications with ten explants were considered for each treatment. All the data in the present study were analyzed using a one-way analysis of variance (ANOVA), and means of the treatments were compared by LSD test ($P < 0.05$) using JMP 8.0 software.

3. Results

3.1. Seed germination

Among ten seeds placed in tetrazolium solution, 90 % turned red and had a firm tissue. However, 10 % of the seeds changed to browning and the tissues were softened, indicating the seeds could not germinate. Therefore, the seeds had 90 % viability, which is an acceptable percentage for seed viability. 120 days after initial sowing, the seed germination (%) was calculated. The results showed that the culture medium containing GA_3 at 1 mg/L had 40 % germination while the plant growth regulator-free culture medium with GA_3 preculture immersion had 83.3 % germination.

3.2. Callus induction and growth

The treatments responding to callus induction were significant statistically (Table 1) and considered in this experiment, as Table 2 displays them. In both MS and LS media, 0.5mg/L BA and 0.5mg/L NAA had the highest percentage of callus formation (Fig. 1a). A higher amount of BA (i.e., 1mg/L) in combination with NAA decreased the callus production slightly compared to the higher response. Also, BA with IAA or 2,4-D in both basal media adversely affected callus production as the percentage of callus production decreased significantly. In the treatment, even when 2,4-D was used alone in LS medium, the callus formation was not observed (Table 1).

Table 1. Effect of culture medium and plant growth regulators on callus percentage of *Thalictrum minus*

Medium	Plant growth regulator (mg/l)	Callus formation (%)
LS	(0.5) BA + (0.5) NAA	97.66^{ab}
	(0.1) BA + (1) 2,4-D	71.20^{c}
	(0.1) 2,4-D	0.00^{e}
MS	(0.5) BA + (0.5) NAA	98.00^{a}
	(1) BA + (0.5)NAA	81.66^{bc}
	(1) BA + (0.5) IAA	5.33^{e}
	(0.5) BA + (0.5) IAA	25.76^{d}
	(0.5) BA + (1) 2,4-D	34.33^{d}
	(1) BA + (2) 2,4-D	22.33^{d}

Table 2. Analysis of Variance of culture medium and plant growth regulators on callus percentage of *Thalictrum minus*

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	36302.187	4537.77	50.2738
Error	18	1624.700	90.26	**Prob > F**
C. Total	26	37926.887		< .0001*

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3.3. Plant regeneration

The plant regeneration percentage was calculated as follows, more than 20 plants in each treatment were considered 100 % (Fig. 1d) and the other explants (Fig. 1c) were compared against it and the data were significant statistically (Table 3). MS medium supplemented with 0.5 mg/L BA and 0.5 mg/L NAA showed the highest percentage of regeneration (100 %).

In this case, regeneration decreased significantly with increasing BA to 1 mg/L combined with NAA. Also, the application of 2,4-D with BA, achieved a high percentage of regeneration (98.33 and 82.66 %). However, LS medium in both plant growth regulator combinations (see Table 4) resulted in less percentage of regeneration compared to MS medium.

Table 3. Analysis of Variance of culture medium and plant growth regulators on regeneration percentage of *Thalictrum minus*

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	28805.923	3600.74	56.7026
Error	18	1143.040	63.50	**Prob > F**
C. Total	26	29948.963		< .0001*

Table 4. Effect of culture medium and plant growth regulators on regeneration percentage of *Thalictrum minus*

Culture Medium	Plant growth regulator (mg/l)	Regeneration (%)
LS	(0.5) BA + (0.5) NAA	61.66^{c}
	(0.1) BA + (1) 2,4-D	61.66^{c}
MS	(0.5) BA + (0.5) NAA	100.00^{a}
	(1) BA + (0.5) NAA	89.66^{ab}
	(1) BA + (0.5) IAA	19.33^{d}
	(0.5) BA + (0.5) IAA	60.00^{c}
	(0.5) BA + (1) 2,4-D	82.66^{b}
	(1) BA + (2) 2,4-D	98.33^{a}

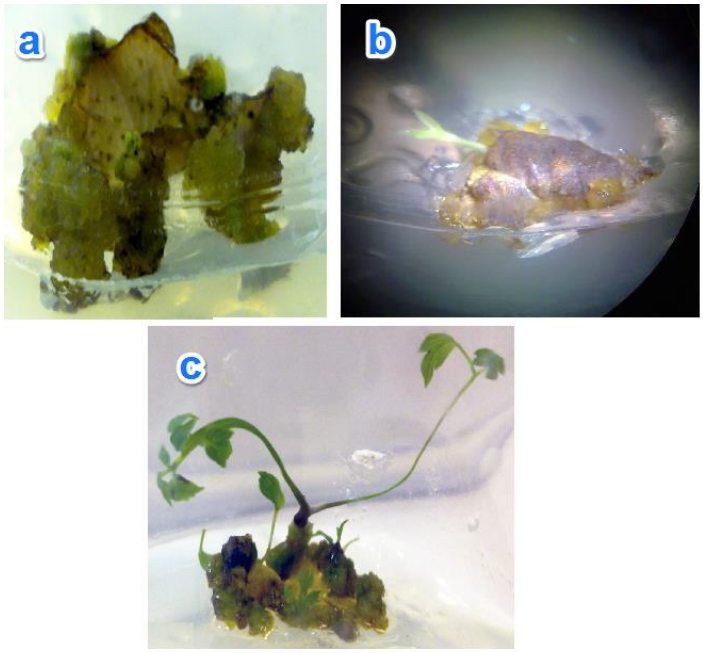


Fig. 1. In indirect micropropagation of *T. minus*. a) Callus formation on leaves. b) Bud formation on calluses with 10X resolution. c) Regenerate the calluses and plant formation.

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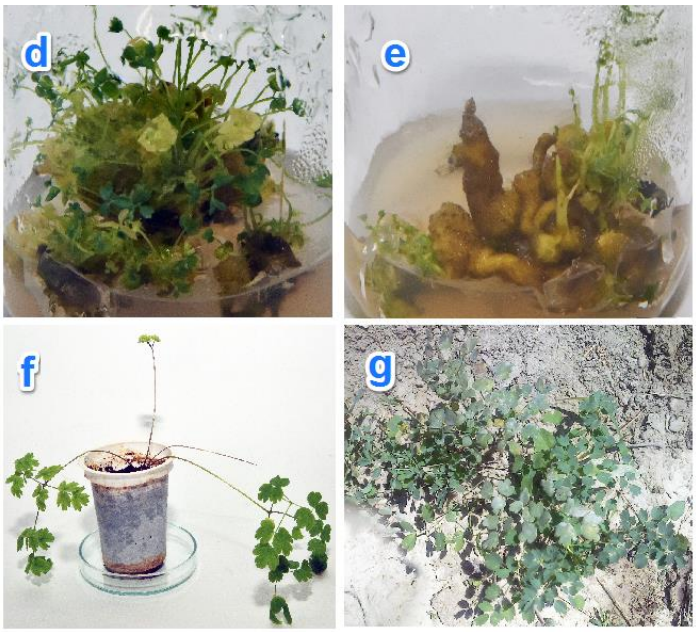


Fig. 1. In indirect micropropagation of *T. minus*. d) Stimulation of callus formation and plant regeneration. e) Rhizomes formed on calluses, and plant formed on rhizomes. f) Acclimatization of plant and g) Adapted plants in the garden.

3.4. Qualitative and quantitative evaluation of berberine

According to Nakagawa et al. (1984), berberine emits yellow fluorescence under UV in liquid culture [19]. A color conversion arose twenty days after callus culturing in a liquid medium. This conversion to yellowish was confirmed under UV light (Fig. 2). ½ MS Medium without any plant growth regulator produced the most yellow color and fluorescent signal compared to the other media, which can be seen in Fig. 2. BA with NAA in the medium also caused it to become yellow but it was less than plant growth regulator-free media. This issue may be due to nutrient stress, the plant overcomes stress by altering physiological processes which produce secondary metabolites such as berberine [3].

The standard curve of HPLC analysis illustrated a linear relationship given by, $y = 38336x + 6083.4$, ($R^2 = 0.9962$) (Fig. 3). The weight percentage of berberine in the culture medium was obtained from equation of this curve. This amount was equal to 6 mg in 100 mL of culture medium.

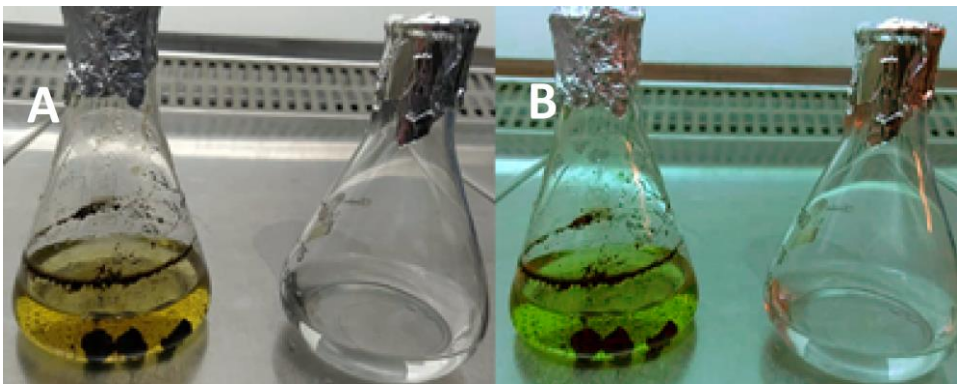


Fig. 2. Liquid culture of *T. minus* calli and without explant as fluorescent control in a) white light and b) U.V. light.

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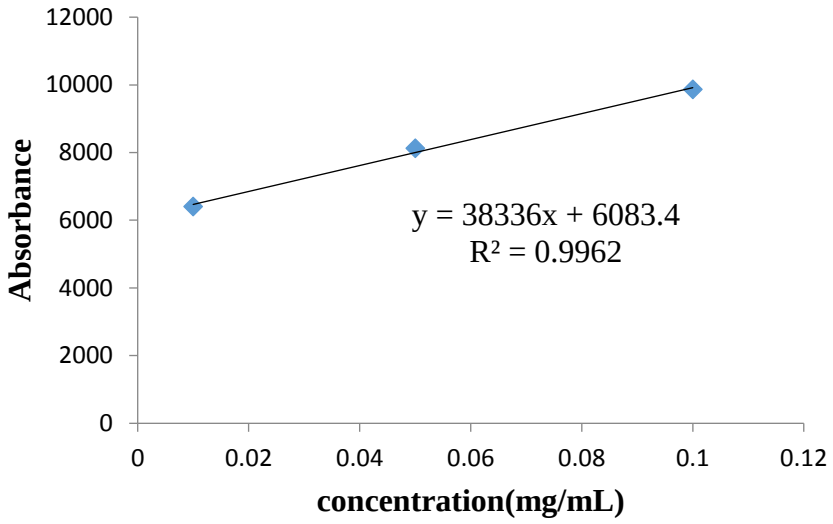


Fig. 3. Standard curve of berberine

4. Discussion

In a study, the results showed that *Thalictrum mirable* seeds germinated 100 % after 12 weeks in a 1 °C cold environment [15]. Also, in another study [12, 5] on germination of *T. cooleyi* showed that lowering the temperature is necessary for GA_3 activity, and GA_3 alone cannot lead to seed germination. According to a study [16], the idea of immersion in 100 mg/L GA_3 with 5 °C temperature for germination of *T. uchiyamae* seeds was found effective as 95 % of seeds were germinated. So, the previous studies confirm the findings of this study [16].

It was observed that BA is more effective for callus production than other cytokinins such as Kinetin, Zeatin, and isopentenylaminopurine in *Thalictrum minus* [17]. In a study, callus formation was induced successfully by using leaf explants in LS culture medium with BA and NAA in *Thalictrum minus* [18]. Also, Berberine production in *T. minus* callus culture is not completely inhibited by any auxin, including 2, 4-D combined with cytokinin but it is stimulated by a higher ratio of auxin to cytokinin [19]. However, in the present study, when the concentration of auxin (2,4-D) was used in the combination of BA, the callus production decreased. This issue was observed in both LS and MS media. However, it is known that in *T. minus*, NAA concentration shows a synergistic effect with BAP in the regulation of secondary metabolism [18].

In general, it was clearly observed that LS medium (in all plant growth regulator combinations) resulted in less percentage of regeneration compared to MS medium. In addition, results of an investigation indicated that 0.5 mg/L BA with 0.1 mg/L NAA was used to regenerate *T. foliolosum* [20]. They showed that regeneration with this combination was up to 85% more than other treatments. Similarly, the regeneration rate decreased with increasing concentrations of BA to 2 mg/l in *T. foliolosum* [17] *Heracleum candicans* [21], *Ceropegia bulbosa* [22], *Withania somnifera* [23], and *Bacopa monnieri* [24].

It was reported that in callus liquid culture of *Thalictrum minus*, over time, the color of culture medium turned to yellow and some crystals were formed in the liquid medium [19]. In their study, 87% of the total berberine was present in the medium culture. A yellow emission attained

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from fluorescent berberine observed in this study was consistent with previous studies [19].

Such amount of berberine in liquid culture, namely, 6 %, was in agreement with previous studies [19]. Berberine release into the culture medium is most likely due to secretory activity in living cells and is not due to cell autolysis as seen in *Coptis japonica* and *T. minus* because berberine production is almost correlated to cell growth [25]. *Coptis* usually stores berberine in its vacuoles, while *T. minus* excretes it exogenously into the Intracellular circumstance [26].

5. Conclusion

An efficient micropropagation protocol can lead to successful and rapid propagation of every endangered plant. With employing the method presented in this investigation, (i.e., MS + 0.5 mg/L BA + 0.5 mg/LNAA) the plant can be produced throughout the year regardless of seasonal restrictions and can assist its survival. The results of regeneration are evidence to introduce this procedure efficiently. Furthermore, this species is a source of berberine substance that can be used in the pharmaceutical industry. In this regard, the present study demonstrated that *T. minus* callus cells release large amounts of berberine (6 %) into a liquid culture medium that can be extracted as nitrate or chloride. It is an advantage of *Thalictrum minus* among plants containing berberine, which can produce more cost-effective berberine.

Author contributions

NM: Study supervision and data interpretation; AS: Experimental analysis and preparation of manuscript draft; AZ: Experimental analysis; AK: study supervision, data interpretation; ZSM: Original idea presentation, study design and revision of the manuscript.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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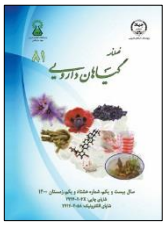
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مقاله تحقیقاتی

تکثیر درون شیشه‌ای و القای کالوس گیاه دارویی در حال انقراض برگ سدابی (*Thalictrum minus*) برای تولید بربرین

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اطلاعات مقاله

گل‌واژگان:
برگ سدابی
آلکالوئیدها
شرایط درون شیشه‌ای
متابولیت ثانویه

چکیده

مقدمه: برگ سدابی یک گیاه دارویی حاوی بربرین است که در درمان کمکی برخی از بیماری‌ها استفاده می‌شود. برداشت بیش از حد این گونه از مراتع برای استخراج آلکالوئید، بقای آن را به خطر انداخته است. **هدف:** این مطالعه به تکثیر در شرایط آزمایشگاهی این گونه و تولید بربرین پرداخته است. **روش بررسی:** این مطالعه با سه آزمایش جوانه‌زنی بذر، باززایی غیرمستقیم و در نهایت شناسایی بربرین از طریق کشت کالوس در محیط مایع انجام شد. بذرها در شرایط کشت درون شیشه‌ای جوانه زدند. سپس برگ‌ها به عنوان ریزنمونه برای القای کالوس در دو محیط پایه MS و LS حاوی (۰، ۰/۱، ۰/۵ و ۱ میلی‌گرم در لیتر BA) همراه با غلظت‌های مختلف اکسین، NAA، و 2,4-D یا IAA استفاده شدند. **نتایج:** نتایج نشان داد که محیط کشت MS با ترکیب تنظیم‌کننده رشد گیاهی ۰/۵ میلی‌گرم در لیتر BA و ۰/۵ میلی‌گرم در لیتر NAA، بیشترین کالوس‌زایی و ۱۰۰ درصد باززایی را داشت. برخی از کالوس‌ها به سه محیط کشت مایع (MS، LS یا ½MS) با ۱ میلی‌گرم در لیتر BA به همراه ۰/۱ میلی‌گرم در لیتر NAA یا بدون تنظیم‌کننده رشد، برای تولید بربرین منتقل شدند. نتایج HPLC حضور ۶ درصد بربرین را در محیط مایع شناسایی کرد. این مقدار بربرین را می‌توان به راحتی در محیط کشت آزاد کرد، بنابراین استخراج آن آسان تر خواهد بود. **نتیجه‌گیری:** بنابراین با این روش می‌توان این گیاه را با موفقیت تکثیر کرد و بربرین را بدون تخریب گیاه استخراج نمود.

مخففها: MS، محیط موراشیگ و اسکوگ؛ LS، محیط لینسمایر و اسکوگ؛ BA، بنزیل آدنین؛ NAA، نفتالن استیک اسید؛ 2,4-D، ۲،۴- دی کلروفنوکسی استیک اسید؛ IAA، ایندول استیک اسید؛ GA3، اسید جیبرلیک؛ HPLC، کروماتوگرافی مایع با کارایی بالا؛ LSD، حداقل اختلاف معنی داری

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