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Original Article

Successful Indirect Regeneration of *Arnebia pulchra* (Roemer and Schultes) as Medicinal Plant

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Abstract

Arnebia pulchra (Willd. ex Roem. & Schult.) Edm. is an endangered, perennial member of Boraginaceae family. Spectrophotometric and LC-Mass analysis confirmed the existence of shikalkin derivatives in its root skin and polyphenolic compounds in the inner part of its root. Studies on the regeneration of *A. pulchra* from its callus resulted in successful callus induction in the root explants excised from the young plantlets on MS medium amended with 2,4-D (1 $mg.l^{-1}$) and kinetin (0.5 $mg.l^{-1}$) at 25 °C in darkness. The highest frequency of shooting (15 shoots per callus) was achieved for the calli sub-cultured on MS medium supplemented with 2,4-D (0.1 $mg.l^{-1}$) and kinetin (2 $mg.l^{-1}$) in 2 weeks. The concentration and type of auxin were found critical for successful rooting. The best rooting conditions with a frequency rate of 80% were obtained for the regenerates which were sub-cultured on half-strength MS medium supplemented with IBA (1 $mg.l^{-1}$), NAA (1 $mg.l^{-1}$) and kinetin (0.2 $mg.l^{-1}$). The regeneration method developed in this work provides a basis for germplasm conservation of *A. pulchra*.

Key words: *Arnebia pulchra*, Callus, Rooting, Shikalkin, Shooting

Abbreviations: Murashing and Skoog (MS), 2,4-Dichlorophenoxyacetic acid (2,4-D), 3-Indoleacetic acid (IAA), N-(2-furanylmethyl)-1H-Purin-6-amine (kinetin), 3-Indolebutyric acid (IBA) and 3-Naphthaleneacetic acid (NAA), Gibberellic acid (GA_3).

Introduction

The Boraginaceae family embraces some important plants with medicinal properties. Some members of this family are experiencing the pressure of overexploitation and are ranked as critically endangered species [1]. Some genera of Boraginaceae such as *Alkanna* Tausch, *Lithospermum* L., *Echium* L., *Onosma* L., and *Arnebia* Forssk. are especially famous due to the red content of their roots known as shikalkin (a mixture of shikonin and alkannin) [2]. The pigment is used as dye in food additives and cosmetics [3]. It has also been used widely for treatment of burns, ulcers, and gynecological inflammations in Indian and Chinese traditional medicine [3]. Shikalkin derivatives have received considerable attention in contemporary pharmaceutical research too. Most of important research on their medicinal properties has been reviewed. These include such as anti-inflammatory, antiplatelet, antitumor, antimicrobial, antifungal, antiviral and contraceptive properties [4]. Large scale production of shikalkin by using a two-stage cell culture plan was reported in 1986 [5]. Since then, to address the growing demands for shikalkin pigment, the cell and tissue culture and regeneration of shikalkin

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producing plants are among the hot topics of plant biotechnology [4-6]. Although the cell culture of *Lithospermum erythrorhizon* Siebold & Zucc. was the first successful example in this field, further studies showed that other members of Boraginaceae, especially plants of *Arnebia* genus, are as important as *L. erythrorhizon* Siebold & Zucc. [5,7].

The genus *Arnebia* encompasses nearly twenty-five different species which include a variety of herbs, shrubs, and trees, mostly confined to Asia with a few species occurring in the drier parts of North Africa [4]. Roots of these plants usually contain various amounts of shikalkin and polyphenolic acids [8]. In a recent work, results of a study on the cell culture of *Arnebia euchroma* (Royle) I. M. Johnst. native to Dena altitudes in Iran were reported [9]. *Arnebia pulchra* (Willd. ex Roem. & Schult.) Edm. is also found in the North-West of Iran. However, because of over-exploitation and the fast progress of the industrial activities close to the natural habitat of these herbs, both species are now seriously endangered. Literature review shows that tissue culture of *A. euchroma* and *Arnebia hispidissima* (Lehm.) A. DC. were reported by Davydenkov *et al*. and Singh and Sharma, respectively [10,11], but no results have been reported on callus induction and regeneration of *A. pulchra*. Considering the increasing importance of shikalkin and polyphenolic acids in Biomedicinal Chemistry and Pharmaceutical Sciences, this research was devoted to the study of the in-vitro regeneration of *A. pulchra* on samples collected from the Khalkhal-Asalem region in the North-West of Iran.

Material and Methods

Plant samples and Preparation of medium

A. pulchra was collected from Almas altitudes (2500-3000 m) at Khalkhal-Asalem region in Ardebil Province at the end of June. Plant specimens were determined by the botanist of the scientific board of the Iran Research Institute of Forests and Rangelands. Seeds were dried at room temperature and stored at 4˚C until use. Inositol, 2,4-D, IAA, kinetin, IBA and NAA were purchased from Sigma-Aldrich Company (Germany). Other chemicals were taken from the authentic samples. MS medium was prepared according to the literature [12] and solidified by using agar (0.8% Wt.). To make half-strength MS medium, all the required ingredients were used at half concentrations. All culture media were supplemented with sucrose (3% Wt.).

Seed germination

A. pulchra seeds were sterilized by the conventional method using NaOCl solution [13]. Germination of the seeds was examined under different conditions (Table 1 a-d) using solid MS as the basal medium. The treated seeds were maintained under a 16-8 h light-dark photo-period at 25 °C. A light intensity of 500 lux was provided by cool white fluorescent tubes. The observations were recorded precisely for 4 weeks. The sprouted seeds were transferred to glass bottles containing hormone-free solid MS medium and maintained under the above mentioned conditions.

Callus Induction

To obtain callus, some explants were excised from the roots, leaflets, and stems of young plantlets (two weeks old) and transferred onto MS medium containing kinetin and 2,4-D. To stop browning, ascorbic acid (50 $mg.l^{-1}$) was also added to the induction medium and the explants were subcultured every week for 9 weeks. The most effective combination of hormones for the callus induction was 2,4-D (1 $mg.l^{-1}$) and kinetin (0.5 $mg.l^{-1}$) at 25 °C in the dark. The callus was proliferated on the same medium containing 2,4-D (2 $mg.l^{-1}$) and kinetin (2 $mg.l^{-1}$) under the same conditions. The resulting calli were sub-cultured every week.

Regeneration Experiments

To develop shoots, the calli were cultured on MS medium under the conditions introduced in Table 1 (A1-A5). Each treatment was in three replicates, each with three explants. The young shoots (regenerated calli) were transferred onto MS medium supplemented with GA_3 (1 $mg.l^{-1}$) to elongate.

For root induction, the shoots (2 or 3 leaves) were harvested and inoculated on full or half-strength MS medium supplemented with different concentrations of auxins as introduced in Table 1 (B1-B5) for two weeks. Then, the samples were transferred onto the hormones free MS medium and the results of the rooting were recorded during a 4-week period. A light intensity of 1000 lux in a 16-8 h light-dark photoperiod at 25 °C was used during both shooting and rooting steps.

Acclimatization

Well-rooted shoots of *A. pulchra* were removed from the culture tubes after 4 weeks and washed thoroughly with tap water to remove the adhering medium. The regenerated plants transferred to plastic pots containing a mixture of autoclaved soil, sand, and peat (1:1:1). Potted plantlets were initially kept in a growth chamber. Plantlets were covered with polythene bags for up to 45 days. To maintain a high humidity, the pots were irrigated with a quarter-strength MS basal salt solution every week with intermittent watering (Fig. 5.I).

Extraction and Sample Preparation for UV-Vis and LC-Mass Analysis

Roots of the collected samples were cleaned, washed with cold water and dried at 37 °C for 48 h. The dark-red skin of the *A. pulchra* root was dried and powdered. The powder (2.5 g) was subjected to soxhlet extraction by using CH_2Cl_2. The extracts were left to dry at room temperature in the dark. The dried residue was examined with Analytik-Jena spectrophotometer (Specord 210, Germany).

For LC-Mass analyses, samples were dissolved in the extraction solvent and centrifuged, then filtered through a 0.45 µm PTFE filter into testing vials. The samples were analyzed in series using an LC-MS instrument with a Waters 2690 HPLC system (Waters, MA, USA) equipped with a Micromass Triple Quadrupole Quattro-Ultima Mass Spectrometer (Micromass, Manchester, UK) consisting of HPLC quaternary pump, an auto-sampler and a vacuum degasser. The system was controlled by Micromass-MassLynx (ver 3.5) software. Chromatographic separations were carried out on a reverse phase XTerra MS C18 column (150×4.5 mm; particle size 5 µm) with a guard-column containing the same stationary phase. The mobile phase consisted of solvent A (0.01 M solution of NH_4CHO in water) and solvent B (0.01 M solution of NH_4 in methanol) at a flow rate of 0.3 ml min^{-1} in a linear-gradient mode. The solvent linear-gradient mode was: 30% (A); 5 min 10% (A); 7 min 30% (A); 13 min 30% (A); 15 min 90% (A); and 16 min 90% (A). The solute was inserted into the mass spectrometer using electro-spray ionization (ESI) probe in the negative mode. The specific LC-MS conditions for each analyte consisted of ESI capillary voltage = 3.5 kV, source temperature =150 °C, desolvation temperature = 300 °C, cone gas flow = 40 $l.h^{-1}$ and multiplier = 525 V.

Statistical Analysis

The results are an average of, at least, three replicates for each treatment. Data was analyzed by SPSS software to calculate the levels of significance. Multiple comparisons were carried out using one-way analysis of variance (ANOVA) followed by Duncan Test. P values of less than 0.05 were regarded as significant.

Results

Description of the plant and its pigment analysis

Both *A. euchroma* and *A. pulchra* bloom in mid-June and their flowers have similar structures. However, the colors of the petals may be dissimilar (Fig. 1.A and 1.B). The UV-Vis spectrum of the extract of the root skin of *A. pulchra* shows the characteristic features of shikalkin derivatives (Fig. 2.A) while the spectrum of the inner part of the root indicates the existence of polyphenolic acids (Fig. 2.B) Shikalkin pigment exhibits bumpy peaks in a range of 500 to 650 nm due to the existence of different derivatives while polyphenolic acids usually show λ_{max}(s) below 340 nm [13,14].

The LC-Mass analyses of this extract resulted in similar spectra to that of *A. euchroma* at retention times of 22-23 and 26-28 minutes. The mass spectra collected between retention times of 26 to 28 minutes are illustrated in Fig. 3. The methylene chloride extract of the dried powder of the *A. euchroma* root was a concentrated red solution (Fig. 2.C). This extract was fractioned by methanol before LC-Mass analyses. According to this, masses between 684 to 694 are assumed to be resulted from the homo-dimerization of propionylshikonin or hetero-dimerization of acetylshikonin with either isobutylshikonin or arnebin-6 (Fig. 4). In a similar way, masses between 610 to 618 could result from the hetero-dimerization of either shikonin with acetylshikonin or propionylshikonin with arnebin-7, alkannin or anhydroalkannin.

Seed Germination

A. pulchra showed poor seed germinability. Results in Fig. 6.A show that seeds without any treatment failed to germinate. But, out of the different treatments, a-d in Table 1, the highest rate of germination, 66.7%, was obtained by applying treatment d. Germination time was also variable. The shortest time of seedling (3-4 days) was also observed for the seeds treated under conditions d.

Callus induction, shooting and rooting

For the root explants, callus induction occurred 90.8% of the time after 35 days of inoculation on MS media containing 2,4-D (1 $mg.l^{-1}$) and kinetin (0.5 $mg.l^{-1}$) but the leaf and stem explants failed to form any callus even after 70 days on this medium (Fig. 5.A and 5.B).

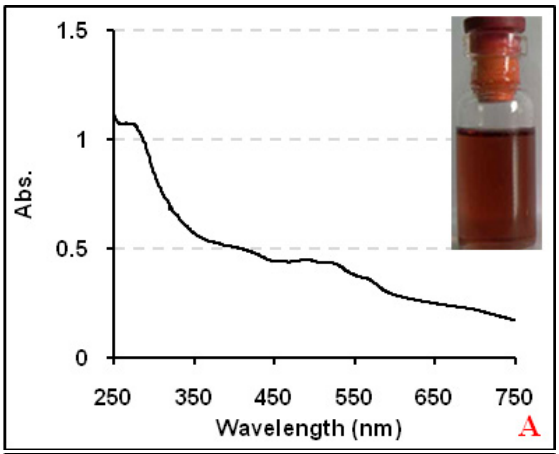
Treatments A1 to A5 (Table 1) were used for regeneration studies. Results of these experiments are illustrated in Fig. 6.B. Experiments confirmed that callus proliferation with limited regeneration occured on MS media containing higher concentrations of the auxin (2,4-D). Therefore, treatment A5 was selected for subculturing of the calli. The highest regeneration efficiency (15 shoots per callus) was obtained on MS medium supplemented with kinetin (2 $mg.l^{-1}$) and 2,4-D (0.1 $mg.l^{-1}$) in 2 weeks (Fig. 5.C-F).

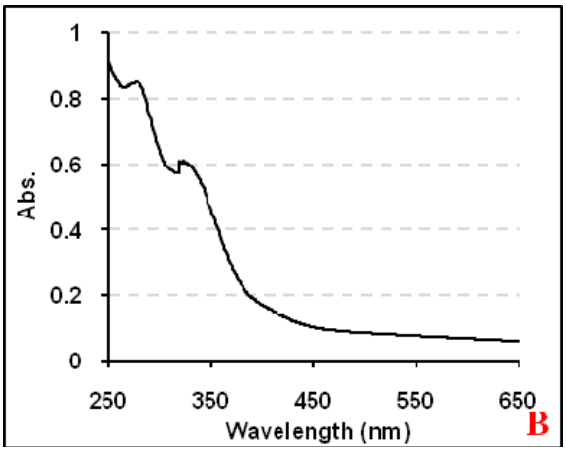
The regenerated shoots were harvested and placed on a rooting medium. Both MS and half-strength MS basal media with different combinations of IBA, NAA and kinetin Table 1(B1-B5) were used in these experiments. Results in Fig. 6C indicate that the best rooting (80%) was observed for the regenerates on the half-strength MS medium with B4 treatment (Fig. 5.G and 5.H).

Table 1 Treatments examined for the seeds germination (a-d), *in-vitro* shooting (A1-A5), and rooting (B1-B5) of *A. pulchra* (Willd. ex Roem. & Schult.) Edm. MS basal medium was used for germination and shooting experiments. Half-strength MS was used for rooting experiments.

Treatments	Pre-treatment	GA_3 ($mg.l^{-1}$)	kinetin ($mg.l^{-1}$)	2,4-D ($mg.l^{-1}$)	IBA ($mg.l^{-1}$)	NAA ($mg.l^{-1}$)
a	none	-	-	-	-	-
b	Scarification	-	-	-	-	-
c	none	1	-	-	-	-
d	Scarification	1	-	-	-	-
A1	-	-	0	0	-	-
A2	-	-	2	0.1	-	-
A3	-	-	2	0.2	-	-
A4	-	-	2	0.4	-	-
A5	-	-	2	2	-	-
B1	-	-	0	-	4	0
B2	-	-	0.2	-	4	0
B3	-	-	0	-	0	1
B4	-	-	0.2	-	1	1
B5	-	-	0	-	0	0.4

Fig. 1 Photos of A) *A. pulchra* (Willd. ex Roem. & Schult.) Edm., B) *A. euchroma* (Royle) I. M. Johnst., C) roots of *A. pulchra* (Willd. ex Roem. & Schult.) Edm.and D) roots of *A. euchroma* (Royle) I. M. Johnst.. Both plant samples were collected in Iran. See the experimental section for details.





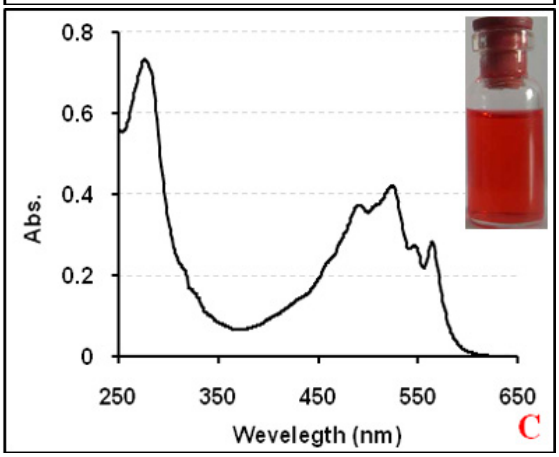
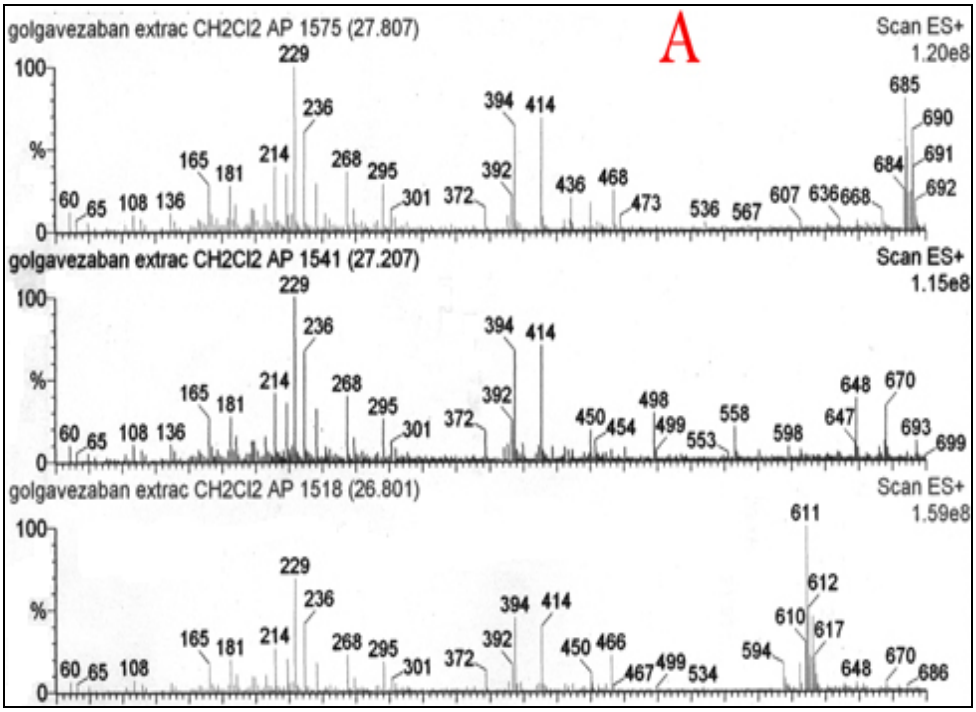


Fig. 2 The UV–Visible absorption spectra of the extracts of A) the outer skin of *A. pulchra* (Willd. ex Roem. & Schult.) Edm. root, B) the inner part of the *A. pulchra* (Willd. ex Roem. & Schult.) Edm. root and C) the *A. euchroma* (Royle) I. M. Johnst. root. See the Results section for more details.



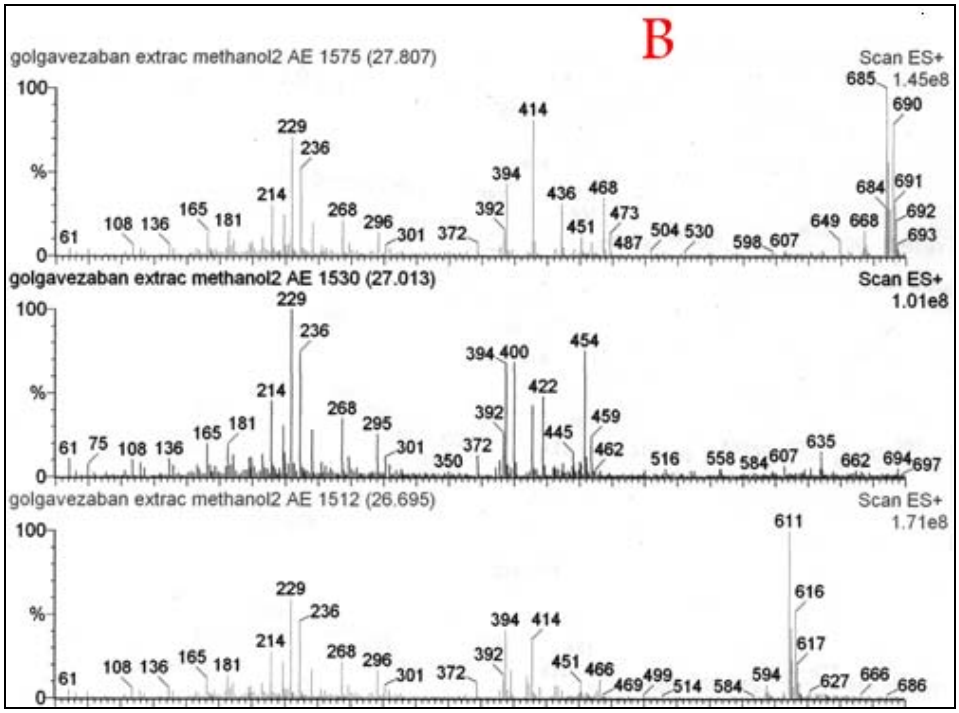


Fig. 3 LC-Mass spectra of the components found in the A) methylene chloride extract of the root skin of *A. pulchra* (Willd. ex Roem. & Schult.) Edm. and B) methanol fraction of the methylene chloride extract of *A. euchroma* (Royle) I. M. Johnst. root at retention time of 26 to 28 minutes.

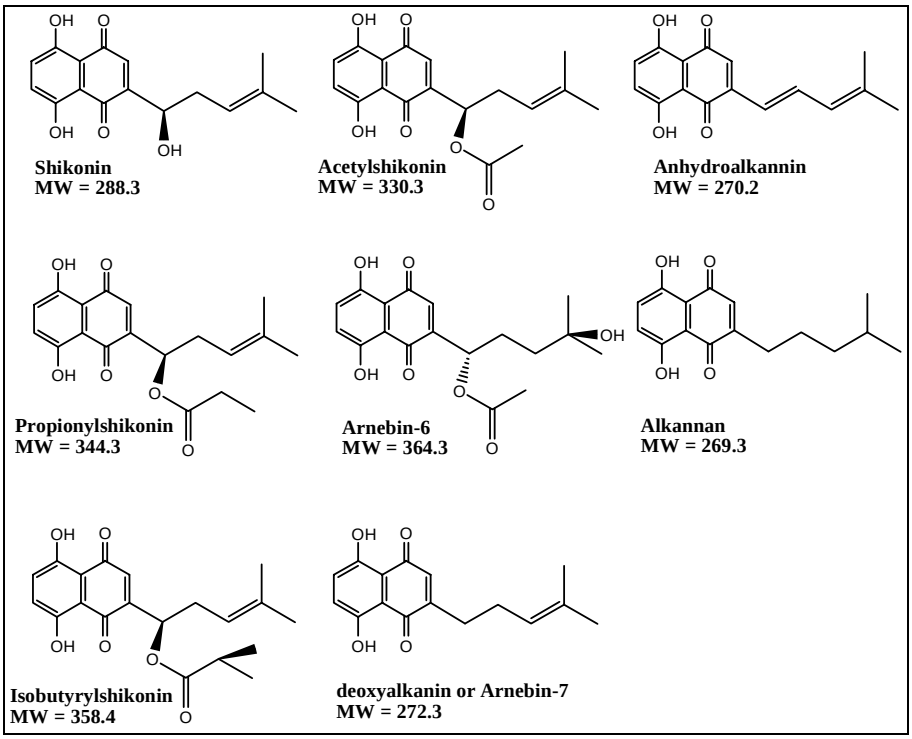


Fig. 4 Chemical structures of shikalkin derivatives which are assumed to be present in the methylene chloride extracts of *A. pulchra* (Willd. ex Roem. & Schult.) Edm. root skin and *A. euchroma* (Royle) I. M. Johnst. root.

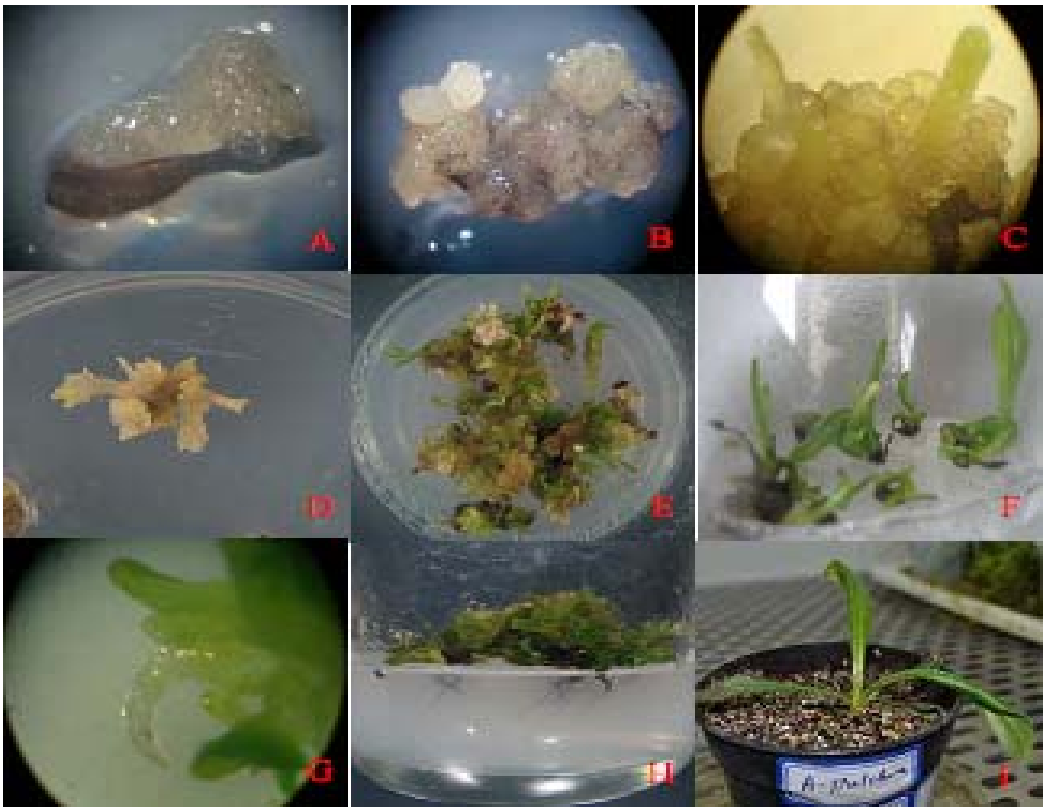


Fig. 5 Photos of different stages of indirect regeneration of *A. pulchra* (Willd. ex Roem. & Schult.) Edm. including A and B) callus induction in the root explants on MS medium containing kinetin (0.5 $mg.l^{-1}$) and 2,4-D (1 $mg.l^{-1}$), C) shooting on MS medium containing kinetin (2 $mg.l^{-1}$) and 2,4-D (0.1 $mg.l^{-1}$), D) growth of shoots on free hormone MS medium, E and F) elongation of shoots on MS medium containing GA_3 (1 $mg.l^{-1}$), G and H) rooting on half-strength MS containing IBA (1 $mg.l^{-1}$), NAA (1 $mg.l^{-1}$) and kinetin (0.2 $mg.l^{-1}$) and I) the potted plantlets in a growth chamber after 90 days.

Discussion

A. pulchra is perennial that grows at altitudes of 2500-3000 m. Just one kind of *A. pulchra* has been reported from Iran [15] and there is no local name for it. In contrast to *A. euchroma* which is found only on sandy slopes, *A. pulchra* samples were collected from a wet land on Almas altitudes. It grows up to 40 cm therefore; its stem is obviously taller than *A. euchroma*. Its acicular leaves are longer (> 10 cm) and softer than those of *A. euchroma*. There is one more important discernible point between *A. pulchra* and *A. euchroma*. While the roots of both species are strong, it is only the root of *A. euchroma* which is totally dark-red in color. *A. pulchra* root is composed of two distinct parts. The outer part (skin) is dark-red colored and the inner part is cream to dark-brown colored (Fig. 1.C and D).

To shed more light on the chemical composition of the red skin of the *A. pulchra* root, its dried powder was extracted using methylene chloride which produced a red-purple solution (Fig. 2.A). As seen in Fig. 3, the Mass spectra of both extracts of *A. euchroma* and *A. pulchra* show masses of dimer molecules of shikalkin derivatives. It has been suggested that the tendency of the naphthaquinonic compounds for participating in coupling reactions through Diels-Alder, radical and ionic mechanisms is the main driving force behind the formation of dimer molecules [16]. Assimopoulou and Papageorgiou suggested several dimerization mechanisms which could explain the formation of homo-dimers with molecular weights of 2MW ± 1, 2MW ± 2 and hetero-dimers with molecular weights of MW + MW' ± 1and MW + MW' ± 2 [16,17].

Among treatments used to increase germination, scarification ranked first. It enhanced germination up to 50% on half-strength MS medium. Similar results were obtained by He and Jia [18]. They found scarification as the most effective treatment to enhance the germination of *Anisodus tanguticus* (Maxim) Pascher, an endangered high altitude medicinal plant of China. In *A. tanguticus*, scarification enhanced germination up to 70% against 0% for the control [19].

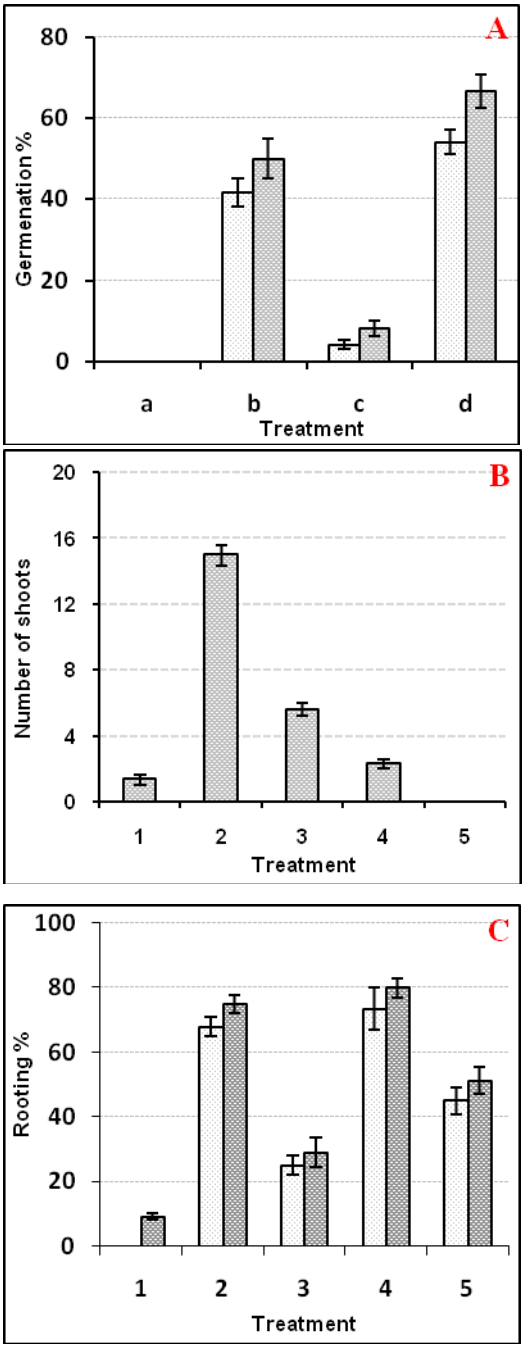


Fig. 6 Results of A) seeds germination of *A. pulchra* (Willd. ex Roem. & Schult.) Edm. under the conditions a-d (Table 1), B) shoot induction under the conditions A1-A5 (Table 1), C) root induction under the conditions B1-B5 (Table 1). Results are average of, at least, three replicates for each treatment. Multiple comparisons were carried out using one-way analysis of variance (ANOVA) followed by Duncan Test. P values less than 0.05 were regarded significant.

Successful callus induction in the root explants of *A. pulchra* was occurred on MS medium containing 2,4-D (1 mg.l^{-1}) and kinetin (0.5 mg.l^{-1}). Using the same medium and phytohormones concentrations, Chaudhury *et al.* achieved callus induction for the *A. hispidissma* root with a frequency of 80% [20]. In agreement with the literature [21-23], 2,4-D played a central role in the callus induction step and the right ratio of 2,4-D/kinetin facilitated the growth of the callus. The use of other auxins such as NAA failed to be effective (data is not shown).

The highest rate of shooting (15 shoots per callus) was observed for the *A. pulchra* callus which was subcultured on MS medium under Treatment A2 (Table 1). Mahipal *et al.* obtained 10.2 shoots per callus of *A. hispidissima* on MS medium supplemented by IAA (0.1 mg.l^{-1}) and kinetin (2 mg.l^{-1}) [24]. It is noteworthy to mention that using IAA caused darkening of the *A. pulchra* callus assumingly due to the induction of the phenolic compounds formation.

Relatively low-salt concentration medium is assumed to enhance rooting of shoots [25,26] and auxins, especially IBA is widely used to induce adventitious roots in many woody and herbaceous plant species [27,28]. Using half-strength MS medium, Mahipal *et al.* induced root in *A. hispidissima* by only one auxin, IBA (1 mg.l^{-1}) or NAA (1 mg.l^{-1}), and observed 85.2% and 51.2%, respectively, rooting [24]. With IBA (1 mg.l^{-1}), rooting was induced in *A. euchroma* with approximately 65% success [29]. In this study, half strength MS medium produced better results in the rooting experiments. However, IBA alone caused darkening of the regenerates without any root formation. A combination of NAA, IBA, and kinetin (Treatment B4, Table 1) was necessary to induce adventitious roots. This indicates that rooting of the *A. pulchra* regenerates is under control of a more complex hormonal regulation.

Conclusion

The regeneration method developed in this work provides a basis for germplasm conservation of *A. pulchra*, an endangered but medicinally important member of Boraginaceae. All the steps including seedling, callus induction, the growth of callus, shooting and rooting can be carried out under the conditions introduced in this paper. Experiments confirmed that rooting was more time consuming and needed more attention. Young plantlet can be acclimatized in the conventional growth chambers.

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