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In Vitro Propagation and Evaluation of Valtrate and Valereinic Acid in Valerina Officinalis L.

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Abstract

Valeriana officinalis L is the most cost-effective plant in Europe. Due to its cardiovascular, anxiolytic, sedative, and anticonvulsant properties, the root of Valeriana is extensively employed in herbal therapies. Seeds of Valeriana were germinated in vitro by soaking for 24 hours in 150 ppm Gibberellic acid (GA3) further, cultivated in half MS (Murashige and Skoog salts medium with vitamins) medium. Treatment of MS media with 5 ppm of Benzyl amino purine (BA) and 2,4-Dichlorophenoxy acetic acid (2,4-D) increased fresh and dry weights of calli produced from leaf explants. However, supplemented MS media with 3 ppm BA and 1 ppm 2,4-D enhanced the efficiency rate of regeneration. On the other hand, augmentation of MS-medium with 0.1 ppm Indole-3-butyric acid (IBA), achieved of rooting process, while acclimatization was accomplished by using peat moss:vermiculite (1:1). Valerenic acid and valtrate were analyzed qualitatively and quantitatively by HPLC in mother plant, callus, regenerated shoots, roots, and Dormival medication which used as a control. The findings showed that regenerated shoots had the greatest concentration of valerenic acid across various extracts (3.80±0.82mg/g DW), whereas in vitro-derived roots from leaf explants contained the highest concentration of valtrate (3.82±0.23mg/g DW).

Keywords: Valeriana officinalis L., Callus, Regeneration, Roots, Acclimatization, HPLC,Valerenic acid and Valtrate

1. Introduction

The Valeriana genus is a member of the Valerianaceae family, and has 419 species. Valeriana officinalis L. (Valerian), is indigenous to Europe and Asia and widely distributed in Chile, Brazil, South Africa, the United States, and subtropical Asia, and is regarded as the most commercially significant species [1,2].

The name Valeriana was taken from a Roman divergent of Valeria, a Roman monarch, or a man who first used it as a herbal remedy, or it was derived from the Latin word valere, which meant to be healthy [3]. Valerian is included in both the European and American pharmacopoeias [4]. There are at least 25 items in the United Kingdom and more than 400 products in Germany that include the crude extract of valerian [5].

Valerian is used to treat anxiety, sleeplessness, menstrual cramps, mood problems, and psychological stress disorders [6].

The dried root and rhizome contained valepotriates iridoids such as isovaltrateand valtrate as the primary active ingredients. Besides sesquiterpenes (valerenic acid, hydroxyl valerenic

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acid, and acetoxy valerenic acid. Furthermore, alkaloids and flavonoids [7-9]. Plant tissue culture is regarded as the essential foundation of plant biology, which is essential for conservation, mass multiplication, genetic modification, bioactive component generation, and plant improvement [10]. Some issues with the development and mass multiplication of valerian include poor seed production and a low germination rate. In addition, wild populations of this plant are presently threatened with eradication and extinction owing to irregular grazing and excessive harvesting by indigenous people for use in traditional medicine.

To retain and sustain the germplasm, the use of artificial propagation methods is required. Additionally, in vitro plant development by direct and indirect morphogenesis has several potential therapeutic plants uses [11].

More study is required on the present data on clonally propagated Valerian and the medium for in vitro regeneration of Valerian plantlet. Whereas Bhat and Sharma, 2015 [12] found that gibberellic acid pre-treatment for 72 hours and 24 hours of pre-chilling was most effective in stimulating seed germination. Benzyl amino purine (BA) and Indole-3-acetic acid (IAA) were also indicated for multiple shoot regeneration from shoot tips. Utilizing MS+5.0 mg/L BA+0.2 mg/L Naphthaline acetic acid (NAA) produced calli. However, regeneration was accomplished by using Indole butyric acid (IBA) and BA, which had favourable effects on plant regeneration and shoot induction [1]. However, utilizing BA (1mg/l) or (0.5mg/l) resulted in the longest shoots and roots were detected [13,14]. on the other hand, Zebarjadi et al., 2011 [15] recommended that IBA was an acceptable hormone for root growth in addition to employing pasteurized field soil, sand, and perlite for effective acclimatization. The determination of valerenic acid and the HPLC conditions was done by Sahiti et al., 2019. [16] As well as valtrate was determined by using HPLC technique as described by Bos et al., 2002 [17]. Moreover, Tousi et al., 2010 [13] stated quantification of valerenic acid and valtrate in different treatments of plant hormones in root induction and Ghaderi & Jafari, 2014 [11] reported HPLC quantification of valerenic acid and valtrate in regenerated and in callus explants.

This study aims to implement in vitro cultivation of Valeriana officinalis L. a European plant in Egypt to support the market with the valtrate and valerenic acid due to it is vital medicinal uses utilizing plant biotechnology techniques as a tool to achieve different in vitro culture methods from seed germination, and calli induction to explant regeneration using different plant growth regulators, in addition to rooting further acclimatization. As well as evaluation and determination of important biological plant secondary metabolites valtrate and valerenic acid viaquantitive and qualitative HPLC compared to Dormival drug in the Egyptian market.

2. Experimental

2.1. Chemical

Benzyl amino purine (Santa Cruz Biotechnology, USA), Indole- 3-acetic- Acid, 1-Naphthalen-eacetic acid, Gibberellic acid, Indole-3-butyric acid (Science Lab. The USA), and Murashige and Skoog salts with vitamins (Caisson Laboratories, USA). Valerenic acid and valtrate standards were purchased from PhytoLab GmbH & Co. KG Dutendorfer Str. 5-7, 91487, Vestenbergsgreuth, Germany. HPLC reagents were purchased from Merc.Dormival capsule were bought from El Ezaby Pharmacy in Egypt.

2.2. Plant roots and seeds

The plant roots of Valeriana officinalis L. were purchased from Kräuter Kühne,Peterspl. 8, 80331 München, Germanyand Seeds from Speril, Freckenhorster Str. 32, 48351 Everswinkel, Germany.

2.3. Seeds sterilization and in vitro culture

Seeds of Valeriana officinalis L. were surface sterilized using 70% of ethanol for 5 seconds, followed by washing three times with sterile distilled water. Then, they were immersed in different concentrations (10,20, and 30% v/v) of commercial sodium hypochlorite (Clorox)(NaOCl)for different times 10, 20, and 30 min., containing 5.25 Cl. Then, they were rinsed three times with sterile distilled water. The sterilized seeds were cultured under aseptic conditions on full or half salts strength of basal (free growth regulators) of Murashige and Skoog (MS) medium [18].

The following data were recorded after 28 days of incubation under light conditions at 25±1°C.

1-Percentage of seeds germination (%) was carried

out using the following equation:-

Number of germinated seeds/ total number of cultured seeds x100

2- Percentage of survival and development rate of germinated plantlets:

The record of this parameter was evaluated according to the following equation:-

Number of survival and developed plantlets/number of cultured seeds x100

Seeds were incubated under light conditions 16/8h at 25±1°C.

2.4. Enhancement of seeds germination

The percentage of seeds germination in previous experiments was very poor. Further, to enhance and achieve of seed germination ratio, pre-sterilized seeds in 70% (v/v) ethanol for 5 seconds and sterilized in a 20% (v/v) solution of sodium hypochlorite for 20 minutes. (Best results from the previous experiment) were soaked in different concentrations of gibberellic acid (0, 50, 100, 150, 200 ppm) and kept in a refrigerator at 4°C for 24 hours. In this experiment, half MS medium supplemented with vitamins, macro, and microelements, 3% sucrose, and 0.7% agar was used and the pH was adjusted to 5.8 with KOH (0.1 N) or Hcl (1 N) then, the media were autoclaved at 121 °C for 25 min. The percentage of seed germination was estimated within 10, 20, and 30 days. Cultures were incubated under the dark condition at 25±10C for 2 days unless the germinated seeds appeared. Further, germinated seeds were transferred to incubation under light conditions provided with white fluorescent lamps 1500 Lux for 28 days at 25±10C. The percentage of germinated and survival seeds was calculated for each treatment.

2.5. Callus induction

For callus induction sterilized leaf, stem, and root explants were excised from in vitro germinated plantlets (28 days of germinated seeds) and cultured on MS medium supplemented with different growth regulators as follow:

1- MS free growth regulator
2- MS +1ppm/l 2,4-D
3- MS+3ppm/l 2,4-D
4- MS+5ppm/l 2,4-D
5- MS+1ppm /lBA
6- MS+1ppm/l BA+1ppm/l 2,4-D
7- MS+1ppm/l BA+3ppm/l 2,4-D
8- MS+1ppm/l BA+5ppm/l 2,4-D
9- MS+3ppm/l BA
10- MS+3ppm/l BA+1ppm/l 2,4-D
11- MS+3ppm/l BA+3ppm/l 2,4-D
12- MS+3ppm/l BA+5ppm/l 2,4-D
13- MS+5ppm/l BA
14- MS+5ppm/l BA+1ppm/l 2,4-D
15- MS+5ppm/lBA+3ppm/l 2,4-D
16- MS+5ppm/lBA+5ppm/l 2,4-D

Cultures were incubated under light conditions for 28 days at 25±1°C.

The fresh and dry weights of calli cultures (mg/jar) were determined and recorded.

2.6. Indirect Regeneration

Indirect regeneration was derived from leaf calli cultures and cultured on an MS medium fortified with different concentrations of growth regulators as follows:

1- MS free growth regulator
2- MS+3ppm/l BA+1ppm/l NAA
3- MS+3ppm/l BA+1ppm/l 2,4-D
4- MS+3ppm/l BA+3ppm/l 2,4-D

All cultures were incubated under light conditions provided by white fluorescent lamps (1500 lux) at 25±1°C.

The following parameters were recorded as follows:

1- Percentage of regenerated shootlets (%).
2- Number of shoots/plantlet
3- Number of leaves/shoot
4- Length of plantlet (cm)
5- Length of the shoot (cm)

2.7. Root induction

In this experiment in vitro derived shootlets were re-cultured on the following media for root induction:

1- MS free growth regulators
2- MS + 0.1 ppm IAA
3- MS +0.1 ppm NAA
4- MS +0.1 ppm IBA

IAA: Indole- 3-acetic- Acid, 1-Naphthaleneacetic acid, NAA:1-Naphthaleneacetic acid.

The following parameters were recorded as follows:

1- Percentage of roots formation (%).
2- The number of formed roots /plant.
3- Length of root formation /plantlet (cm).

2.8. Acclimatization

For acclimatization, in vitro rooted plantlets were

gently washed with tap water and disinfected by soaking in Benlate solution (1g/l) for 20 min. Then they were transferred to plastic pots containing the following media:

1- Sand
2- Sand + peat moss (1:1).
3- Peatmoss + vermiculite (1:1)

The pots were covered with clear poly ethylene bags containing a few pores to allow gas exchange and sprayed with water to maintain high relative humidity. Covers were completely removed after four weeks, and the Percentage of acclimatized and survival plantlets were recorded.

2.9. Chemical analysis

High-performance liquid chromatography (HPLC, Waters LC 2695 – PDA detector) was performed using Column Kromasil C18 150×4.6mm,5µm, Mobile phase, A (0.1% orthophosphoric acid 80%:20% acetonitrile), and B (0.1% orthophosphoric acid 20%,80% acetonitrile) were the mode of elution: gradient Flow:1ml/min, temperature and ambient Wavelength 225 nm for valerenic acid and 254nm for valtrate as described by Bos el al., 1996 [19].

2.9.1. Standards authentic preparation

The standard solution of valerenic acid 1mg/1 ml of methanol was subsequently diluted to prepare different concentrations of valerenic acid (0.05, 0.1, 0.5, 1, 10, 50, 100, and 300 µg/ml). whereas valtrate standard solution of 1mg/ml valtrate were diluted to concentrations (0.1, 0.5, 1, 10, 50, 100, 200 µg/ml), then 50 µl was injected.

2.9.2. Samples Preparation

Five hundred milligrams (0.5 g) of mother plant roots (in vivo) and in vitro derived shootlets; calli cultures and root cultures were extracted using chloroform then sonicated for 15 min., filtered and the filtrate was evaporated using rotavapour then, diluted with methanol for HPLC analysis whereas commercial tablet of 300 mg of Dormival was dissolved in 50ml volumetric flask in methanol filtrated then 20µl was injected into HPLC and used as control treatment.

2.9.3.High-performance liquid chromatography (HPLC) condition

HPLC Waters LC 2695 – PDA detector analysis included Column Kromasil C18 150×4.6mm,5µm Mobile phase , Sol A (0.1% Orthophosphoric acid 80%:20% Acetonitrile) , Sol B (0.1% orthophosphoric acid 20% , 80% Acetonitrile) Mode of elution : Gradient Flow :1ml/min Temperature : Ambient Wavelength 220,254.

Time	Sol (A)%	Sol (B) %
0	55	45
5	55	45
19	0	100
21	0	100
23	55	45
28	55	45

2.9.4. Authentic evaluation

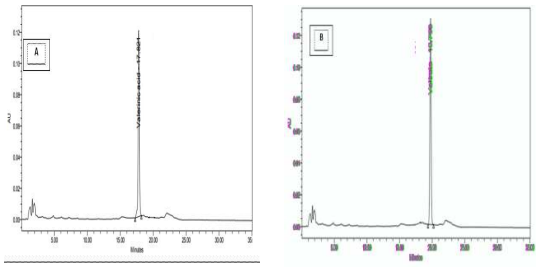


Fig. (1). Chromatogram of valerenic acid at 225nm (a) and valtrate at 254 nm standard using HPLC

2.9.4. Calibration Curve

Valerenic acid calibration curve was performed using concentrations (0.05, 0.1, 0.5, 1, 10, 50, 100, and 300 µg/ml) in addition to valtrate concentrations (0.1, 0.5, 1, 10, 50, 100, 200 µg/ml) using linear regression analysis of the peak area against respective concentrations of valerenic acid (Fig.2.a) and valtrate (Fig.2.b).

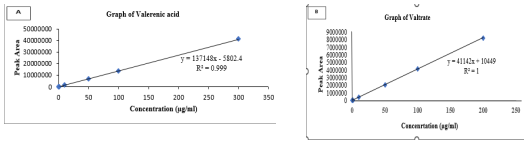


Fig. (2). Linearity calibration curve of valerenic (a) acid and valtrate (b)

Data Analysis

One-way analysis of variance (ANOVA) was applied and means were compared using Duncan's New Multiple Range test (DNMR) with a critical value of P-value 0.05.

All experiments were conducted as a complete randomized design in a factorial arrangement with three replications.

Results

1-Percentage of seeds germination

In this experiment, seeds of Valeriana officinalis L. were cultured in two salts strength of MS media (half and full strength). Illustrated data in Fig. (3) revealed significant differences within and between each group where, seeds treated with 20% of clorox, and incubated for 10 days showed the most achievable result (60±5.77a), (46.67±8.82a) for half and full-strength of MS medium, respectively. However, applying 30% of clorox and incubation for 30 days have shown the least percentage of seeds germination (3.33±3.33b, 0±0b) for half and full strength of MS media, respectively.

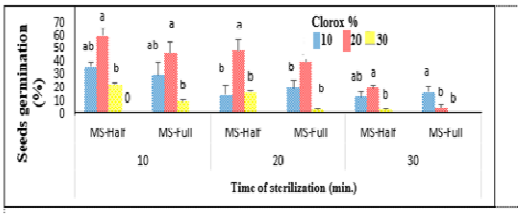


Fig. (3). Effect of different concentrations of clorox on the percentage of Valeriana officinalis L. seed germination using half and full strength of MS medium. Incubation was carried out under light conditions at 26 ±1°C. 10 seeds were cultured in each jar, the replicates were 3 jars for each treatment. The same letters in each column mean a non-significant difference; a different letter means a significant difference (P<0.05)

2-Percentage of survival and development rate of germinated plantlets

The germination of seeds is not enough, but the survival and development of germinated plantlets is very important. It has been shown obviously from Fig. (4) that the percentage of survival and development of Valereiana officinalis L. plantlets were elevated using half and full strength of MS medium. The high percentages of survival and development rates of germinated seeds (33.33±8.82a, 33.33±5.77a), were recorded by applying cloroxat 10 % for 10 min. for 10 days of incubation and these percentages were declined on utilizing 30% clorox for 30 days incubation.

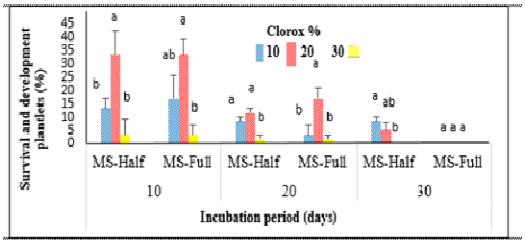


Fig. (4). Effect of different concentrations of clorox on percentage plantlets survival and development Seeds of Valeriana officinalis L. were germinated on half and full strength of MS medium. Incubation was carried out under light conditions at 26 ±1°C. 10 seeds were cultured in each jar; the replicates were 3 jars for each treatment. The same letters in each column mean a non-significant difference; a different letter means a significant difference (P<0.05).

3-Enhancement and achievement of seeds germination

Four concentrations 50, 100, 150, and 200 ppm of gibberellic acid in addition to the control treatment were supplemented to half salt strength of MS basal medium to investigate their efficiency on promoting and achieving of Valeriana officinalis L seeds germination. Data shown in Figs. (5,6) showed significant differences incubation days (10, 20, and 30) with different gibberellic acid concentrations. The highest percentage of seeds germination was noticed with 150 ppm of gibberellic acid on days 10, 20, and 30, respectively. The minimum percentage of seeds germination was recorded with 50 ppm gibberellic acid.

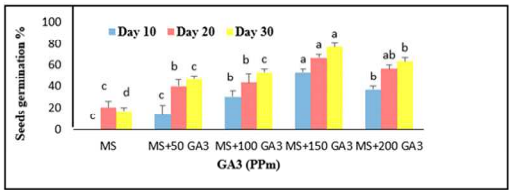


Fig. 5. Effect of different concentrations of gibberellic acid supplemented to ½ MS on in vitro Valeriana officinalis seeds germination. Cultures were incubated for 10, 20, and 30 days at 25 ºC under light conditions with different intervals with mean±SE.

Fig. 6. In vitro *V. Officinalis* seeds germinated on ½ MS medium supplemented with 150 ppm of gibberellic acid, and incubated in early-stage on darkness further in light condition 16/8 h for 30 days at 25 ±1 C°.

4-Calli fresh and dry weights (g/jar).

Data tabulated in Table (1) clearly show that calli fresh and dry weights of leaf, stem, and root explants of Valerina officinalis L. Data showed significant differences between the varied medium of different growth regulators concentrations, explants compared to the control medium. The tested leaf, stem, and root explants cultured on MS+5 ppm BA; MS+5 ppm BA+1ppm 2,4-D; MS+5ppm BA+3ppm 2,4-D, or MS+5 ppm BA+5 ppm 2,4-D showed significantly

different, and higher fresh and dry weights of calli compared to the control and other treatments. The greatest number of fresh weights 7.12±0.13, 6.4 ±0.48, 6.3±0.75 (g/jar) and dry weights 5.23 ±0.34, 4.89±0.55, 4.7±0.70 (g/jar) were recorded with derived calli from leaf, stem, and root explants, respectively. MS medium supplemented with 5 ppm BA and 5 ppm 2,4-D gave significant results of calli fresh and dry weights compared to control medium and other used media as shown in Fig. (7). On the other hand, fortified MS medium with different concentrations of 2,4-D alone gave the lowest amount of calli fresh and dry weights with different explants.

Table 1
Calli fresh and dry weights (g/jar) induced from leaf, stem, and root explants of *Valeriana officinalis* L. using different concentrations of BA and 2,4-D supplemented to MS medium and incubated under the dark condition at 25 ± 1 °C for 4 weeks

Treatments (ppm).	Calli fresh weights (g/jar)			Calli Dry weights (g/jar)		
	Leaf	Stem	Root	Leaf	Stem	Root
	Means±SE	Means±SE	Means±SE	Means±SE	Means±SE	Means±SE
MS free growth regulator	0±0h	0±0e	0±0a	0 ±0f	0±0e	0±0c
MS +1ppm 2,4-D	0.37±0.01gh	0.33 ±0.03de	0.4±0.06a	0.24 ±0.02df	0.19±0.05e	0.3±0.04c
MS+3ppm 2,4-D	0.36±0.03gh	0.52 ±0.05de	0.54±0.12a	0.25 ±0.07df	0. 32±0.05de	0.33±0.04c
MS+5ppm 2,4-D	0.42±0.01fg	0.38 ±0.07de	0.41±0.06a	0.27 ±0.03df	0.25±0.06e	0.24±0.05c
MS+1ppm BA	0.75±0.04efg	0.51 ±0.06de	0.34±0.06a	0.39 ±0.03df	0.34±0.04de	0.2±0.04c
MS+ppm BA+1ppm 2,4-D	0.61±0.05efg	0.59 ±0.09de	0.36±0.07a	0.41 ±0.02df	0.35±0.04de	0.43±0.07c
MS+1ppmBA+3 ppm 2,4-D	0.65±0.06efg	0.43 ±0.04de	0.35±0.08a	0.43 ±0.07df	0.35±0.08de	0.29±0.12c
MS+1ppm BA+5ppm 2,4-D	0.83±0.05ef	0.68 ±0.07de	0.33±0.04a	0.47 ±0.03df	0.36±0.05de	0.3±0.14c
MS+3ppm BA	0.69±0.07efg	0.67 ±0.10de	0.36±0.08a	0.35 ±0.03df	0.35±0.07de	0.19±0.08c
MS+3ppm BA+1ppm 2,4-D	0.9±0.04e	0.88 ±0.06d	0.74±0.09a	0.48 ±0.07df	0.4±0.02de	0.43±0.06c
MS+3ppm BA+3ppm 2,4-D	0.94±0.03e	0.91 ±0.03d	0.7±0.05a	0.53 ±0.04d	0.55±0.07de	0.22±0.12c
MS+3ppm BA+5ppm 2,4-D	0.94 ±0.02e	0.94 ±0.03d	0.76±0.01a	0.59 ±0.03cd	0.56±0.07de	0.42±0.05c
MS+5ppm BA	1.61±0.08d	1.75 ±0.03d	0.7±0.09a	1 ±0.13c	1.44±0.05cd	0.27±0.10c
MS+5ppm BA+1ppm 2,4-D	4.5 ±0.2b	4.25 ±0.76b	4.37±0.32b	3 ±0.12b	2.18±1.18bc	3.69±0.31b
MS+5ppm BA+3ppm 2,4-D	4.10±0.45c	4.81 ±0.03b	4.97±0.39b	2.85 ±0.41b	3.1±0.24b	3.33±0.40b
MS+5ppm BA+5ppm 2,4-D	7.12±0.13a	6.4 ±0.48a	6.3±0.75a	5.23 ±0.34a	4.89±0.55a	4.7±0.70a

The same letters in each column mean non-significant difference; different letter means significant difference (P<0.05)
Each value is the average of 3 replicates.

Fig. 7. Callus from leaf segments of *Valeriana officinalis* L. cultured on MS-medium supplemented with MS+5ppm BA+5ppm 2,4-D and incubated under dark Condition at 25 ±1 °C

5-Indirect regeneration

In this experiment, equal pieces (`250 mg/ jar) of leaf calli cultures were transferred to different media. The obtained results as shown in Figs. (8,9,and 10) shown that the best-regenerated media affected on length of the shoot (14.67 cm±0.77[a] cm), number of shoots (1.67±0.33[a]), number of leaves/plantlet (2.67±0.33[a]), length of plantlet (20.67±3.06[a]) and percentage of regeneration (32.33±3.38[a]) was MS medium supplemented with 3ppm BA in the presence of 1ppm 2,4-D. However, the last one was MS fortified with 3 ppm BA and 1 ppm NAA (3.57±0.6[c], 0.33±0.33[b], 9±1.53c, and 1±0[b], respectively).

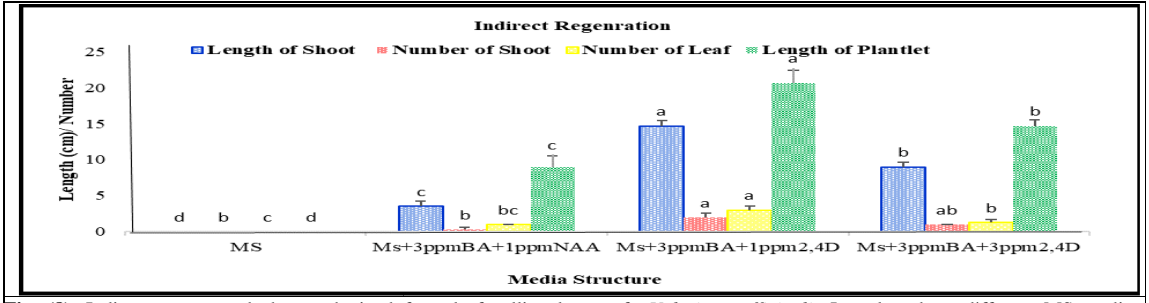


Fig. (8). Indirect regenerated shoots obtained from leaf calli cultures of *Valeriana officinalis* L. cultured on different MS media compositions

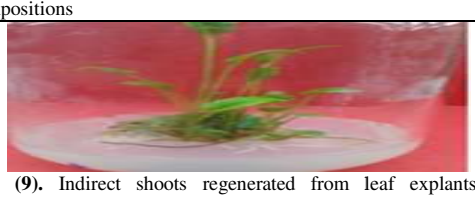


Fig. (9). Indirect shoots regenerated from leaf explants of *Valeriana officinalis*L.cultured on MS medium supplemented with 3 ppm BA +1 ppm 2,4-D and incubated under light condition 16/8 at 26±1°C for 4 weeks**.**

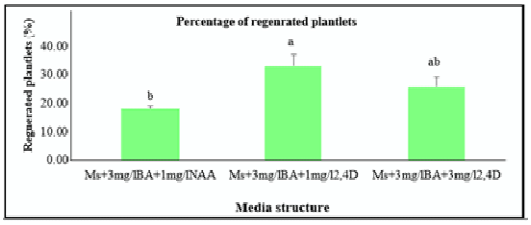


Fig. (10). Diagrammatic shown of *Valeriana officinalis* L. percentage of indirect regeneration with mean±SE.

Root induction

In this experiment, obtained shoots were transferred to rooting media as represented in Figs. (11, 12). The significant results clearly showed that the utilization of MS medium fortified with 0.1 ppm IBA for root induction was the most significant in bothpercentage of root formation (37.67±1.45[a]), lengths of root (4.67cm ± 1.20[a]), and number of roots (46±4.16[a]), and, where other cultures did not show any development of root system with 0.1 ppm IAA.

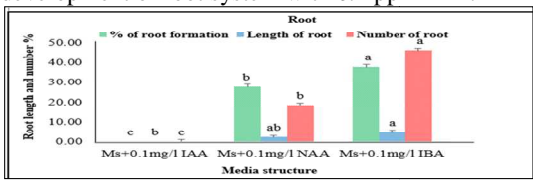


Fig. (11). Roots formation on *V. officinalis* shoot with mean±SE

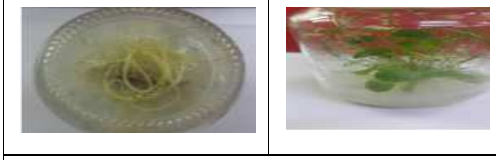


Fig. (12). Rooting for elongated shootlets induced with MS medium supplemented with 1 ppm of IBA and incubated under light condition 16/8 for 4 weeks at 26±1°C.

Hardening and Acclimatization

In this regard, the in *vitro*-derived plantlets were gently washed with tap water and disinfected by soaking in Benlate solution (1g/l) for 20 min. Then, plantlets were transferred to plastic pots containing sand; sand + peat moss (1:1) or peatmoss + vermiculite (1:1). Then, the pots were covered with clear poly ethylene bags containing a few pores to allow gas exchange and sprayed with water to maintain high relative humidity. Covers were completely removed after four weeks, and the percentage of acclimatized and survival plantlets were recorded. The obtained results clearly showed that the high percentage of acclimatized and survival plantlets 73.67±5.55[a] was recorded with culturing of *in vitro* derived plantlets in nutrient media containing peat-moss and vermiculite at 1:1 as shown Fig. (13, 14). However, the minimum number of acclimatized and survival plantlets 36.33±3.48[b]were recorded with culturing of in *vitro* derived plantlets on sand media.

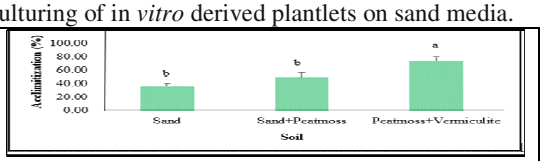


Fig. (13). Effect of different structures of soil for plantlet acclimatization and survival.

Fig. (14). *In vitro* derived *V. officinalis* plants cultured on Peatmoss and vermiculite (1:1) derived from regenerated leaf explants

Chemical analysis

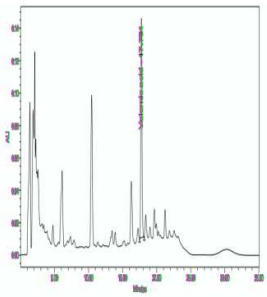
In this respect, valerenic acid and valtrate amounts (mg/g DW) were determined in *in vitro* derived

extracts of callus induced leaf, root induced leaf, regenerated shoots,original plant roots of *Valeriana officinalis* and Dormival (as control treatment) using HPLC as shown in Tables (2,3) amd Figs. (15,16).

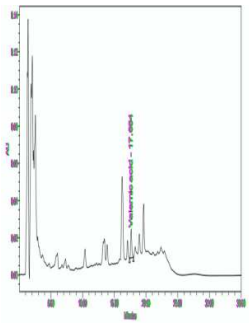
Table 2:
Estimation of valerenic acid(mg/g DW) in callus induced leaf,root induced leaf, regenerated shootsand in vivo roots of *Valeriana officinalis* L., compared to Dormival as a control sample using HPLC

	Sample ID	Retention time (min.)	Amount (mg/g DW)
1	Callus induced leaf	17.754	0.93±0.23
2	Root induced leaf	17.654	0.12±0.01
3	Regenerated shoots	17.748	4.15±0.97
4	Root of mother plant	17.800	3.80±0.82
5	Dormival	17.723	0.03±0.0

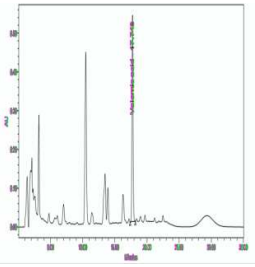
Each treatment is the average of 3replicates, Mean±S.E.
-Where: 1-Leaf callus/MS+5 ppm BA+3 ppm 2,4-D; 2- root induced leaf / MS+0.1 ppm IBA, 3- Regnerated shoots /MS+ 3 ppm BA+ 1ppm 2,4-D , 4- Root of mother plant ;5- Dormival.



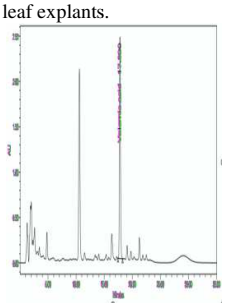
1-HPLC analysis of valerenic acid in leaf calli.



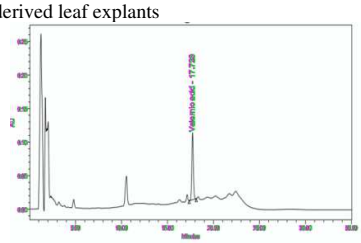
2- HPLC analysis of valerenic acid in roots derived leaf explants.



3-HPLC analysis of valerenic acid in regenerated shoots derived leaf explants



4-HPLC analysis of valerenic acid in mother plant roots.



5-HPLC analysis of valerenic acid in Dormival

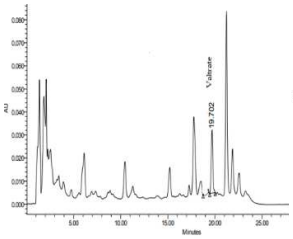
Fig. (15). HPLC analysis of valerenic acid in calli cultures(1), roots derived leaf explants (2), regenerated shoots from leaf explants(3) in mother plant roots (4) compared valarenic acid in Dormival (5)

Data in Table (2) reveal the quantity of valerenic acid (mg/g DW) using HPLC. The greatest amount of valerenic acid was found in regenerated shoots (4.15±0.97 mg/g DW), followed by mother plant root (3.80±0.82 mg/g DW), callus-derived leaf (0.93±0.23mg/g DW), and the least amount was thatroot-induced leaf explants (0.12±0.01 mg/g DW), whereas dormival drug contained 0.03±0.0 mg/g DW.

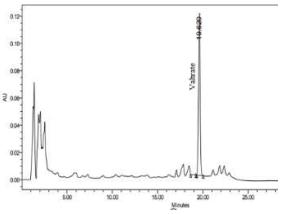
Table 3
Estimation of valtrate (mg/gDW) incallus induced leaf, root induced leaf, regenerated shootsand *in vivo* rootsof *Valeriana officinalis* L., compared to Dormival as a control sample using HPLC

	1. Sample ID	2. Retention time (min.)	3. Amount (mg/g DW)
1	Callus induced leaf	19.702	0.62±0.15
2	Root induced leaf	19.62	3.82±0.23
3	Regenerated shoots	19.692	3.01±0.29
4	Root of mother plant	19.760	2.38±0.34
5	4. Dormival	19.589	0.01±0.00

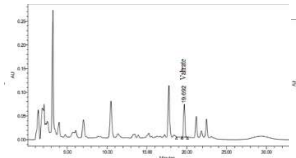
Each treatment is the average of 3 replicates, Mean±S.E.
Where: 1-Leaf callus/MS+5 ppm BA+3 ppm 2,4-D;2- root induced leaf / MS+0.1 ppm IBA, 3- Regnerated shoots /MS+ 3 ppm BA+ 1ppm 2,4-D, 4- Root of mother plant ;5- Dormival.



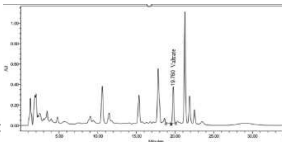
1-HPLC analysis of valtrate in leaf calli.



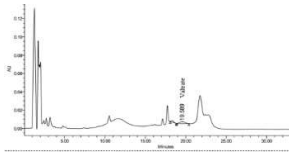
2- HPLC analysis of valtrate in roots derived leaf explants.



3-HPLC analysis of valtrate in regenerated shoots derived leaf explants



4-HPLC analysis of valtrate in mother plant roots



5-HPLC analysis of valtrate in Dormival

Fig. (16). HPLC analysis of valtrate in calli cultures(1), roots derived leaf explants (2), regenerated shoots from leaf explants(3) in mother plant roots (4) compared valarenic acid in Dormival (5)

Data in Table (3) compares the quantity of valtrate in mg/g DW incallus induced leaf, root induced leaf, regenerated shoots, mother plant leaf to the amount of valtrate in dormival medication. 3.82±0.23 mg/g DW,3.01±0.29 mg/g DW, 2.38±0.34mg/g DW, 0.62±0.15mg/g DW, and 0.01±0.00 mg/g DW were recorded with root-induced leaf, regenerated shoots, mother plant root, callus-induced leaf, and dormival medication as the control treatments, respectively.

Discussion

In this work, we explored a procedure for seed sterilization, germination, callus, regeneration, and root induction from *Valeriana officinalis* L., using various auxin and cytokinin as plant growth regulators. As well as the detection of valerenic acid and valtrate in different *in vitro*chloroformic extracts using HPLC analysis and compared with chloroformic extract of mother plant root. The experiment was effective in sterilizing seedlings; employing 70 percent ethanol for 5 sec. subsequently, 20 percent of Clorox for 20 min. On the contrary of Zebarjadi et al., 2011[15] sterilized the seeds using 70 percent of ethanol for 1 min, then followed by 2 percent of sodium hypochlorite solution for 15 min. Otherwise, Tansaz et al., 2014 [14]sterilized seeds by utilizing 0.1 percent Hg_2Cl_2 for 10 min. then submerged in 70 percent ethanol for 60 sec. and lastly, 20 percent sodium hypochlorite in addition to tween 0.05 percent for 10 min. However, Torkamani et al., 2013 [20] sterilized the seeds employing 70 percent ethanol for 1 min. followed by washing using sterilized water then submerged in 2 percent sodium hypochlorite solution for 10 min. On the other side, Yang et al., 2019 [21]disinfected leaf explants using 70 percent ethanol and 0.1 percent mercuric chloride.

For enhancement of *V. officinalis*L. seeds germination, it was found that soaking of seeds in 150 ppm of GA_3 for 24 hr. then germination of seeds on MS+ 3 ppm GA_3 under dark conditions for 3 days followed by transferring to light condition resulted in an elevated percentage of seeds germination. Noticed that Bhat & Sharma (2015) previously reported that germination of *V. officinalis* L. seeds was obtained by soaking seeds in GA_3 (200 ppm) at 4 $^{\circ}$C for 72 h and then exposed to light conditions. On the other hand, Torkamani et al., 2013 [20] used 800 ppm of gibberellic acid and 10 % of sulphuric acid for seed germination for 15 min. Furthermore, Torkamani et al., 2014 [22] suggested that germination rate was best achieved on pretreatment of seeds with sulfuric acid (10 %) for 15 min. followed washing with sterile distilled water then, rinsed and soaked in 70 % ethanol for 1 min. then washed and immersed in 2% sodium hypochlorite for 10 min, followed by three rinses with sterile distilled water. The obtained data of callus production from explants was significantly higher with MS+5ppm BA+5ppm 2,4-D from leaf explants, in this regard, Ghaderi & Jafari, 2014 and Zamini et al., 2016 [1,11] differently mentioned that 2,4-D and Kinetin(Kn) hormones developed callus from leaf explants cultured on MS media containing 2,4-D (1.5 mg/l) + KIN (1 mg/l) and respectively 9.30 µMKn and 2.26 µM 2,4-D and diversely to Yang et al., 2019 [21]who utilized BA and NAA as follows MS+6mg/l BA + 5.0mg/L+0.2mg/L NAA to achieve a high yield of callus from leaf explants. The obtained regenerated shoots derived from callus of leaf explant have shown that the best-regenerated media affected on a percentage of regenerated shootlets, length of shoot, several shoots, number of the leaf, and length of plantlets were MS+3ppm BA+1ppm 2,4-D (33 ± 4.04^{a},14.67 ± 0.77^{a}, 1.67 ± 0.33^{a}, 2.67 ± 0.33^{a}, 20.67 ± 1.76^{a}). Whereas Tansaz et al., 2014 [14]reported that BA (0.5 mg/l) +NAA (0.25mg/lon MB(half (MS+B5) salts and vitamins, 10 ml/l Fe EDTA (iron stock) + 2.5% sucrose) achieved of shoot length using MB with BA (1 mg/l) + (0.5 mg/l). Adversely to Ghaderi & Jafari 2014 [11]who have shown that media supplemented with Thidiazuron(TZD) and NAA have marked regeneration yield from petiole and leaf explants.

In vitro rooting was noticeable from leaf explants of *V. officinalis* L. using MS medium supplemented with 0.1 ppm IBA**,** while, Ghaderi & Jafari 2014 [11]suggested that rooting was best and achieved by using the IBA hormone at a concentration of 2.46 µM. In contrast to Tousi et al., 2010 [13] who developed roots from petiole using 1.25 µM and 0.625 µM indole-3-acetic acid.

Regarding, the hardening and acclimatization process it was noticed that the best structural was peat moss:vermiculite (1:1). In contrast to Syahid et al., 2021 [23] who transferred the sterile plantlets into media containing soil, compost, and husk. Moreover, peat-moss and perlite (5:1) revealed by Ghaderi & Jafari 2014 [11] yielded the best-acclimatized plantlets.

The condition of HPLC analysis was mostly achieved by Bos et al., 1996 **[**19]who used methanol for sample preparation before injection into HPLC. otherwise, chloroformic extraction for different samples was the most appropriate solvent for valtrate and valerenic acid detection and in obtaining the highest yield.

Regenerated plantlets had the greatest concentration of valerenic acid (3.80±0.82mg/g DW) compared to Dormival capsule (0.03±0.0 mg/gDW). In addition, the estimate of valtrate in root-induced leaf cultures was3.82±0.23mg/g DW. While the regenerated shoots had3.01±0.29 mg/g dry weight, the mother

plant contained 2.38±0.34mg/g dry weight. These outcomes were accomplished by using MS medium enriched with 3 ppm BA and 1 ppm 2,4-D. MS+5 ppm BA+1 ppm 2,4-D had the least amount of valtrate compared to the other samples. In this regard Tousi et al., 2010 [13] reported that valerenic acid and valtrate are present in roots in greater amounts than the control when IAA hormone is used, whereas the highest valerenic acid concentration was observed in regenerated plantlets and the highest valtrate concentration was found in root cultures. Ghaderi & Jafari, 2014 [11]observed that valereinic acid and valepotriates were more prevalent in regenerated plantlets than in wild populations and field-grown plants.

Conclusion

A successful approach for *in vitro* growth of *Valeriana officinalis* L. was developed. This technique is critical now owing to worldwide warnings of climate change and the economic relevance of *Valeriana officinalis* L.'s root carrying secondary metabolites as a medicinal plant. Seed germination was successful when half MS medium was enriched with 150 ppm of GA3. Furthermore, MS media mixed with 5ppm BA and 5ppm 2,4-D was the most effective for callus induction from leaf explants. The addition of 3 ppm BA and 1 ppm 2,4-D to MS media resulted in indirect shoot regeneration from leaf explants. While rooting was aided by adding 0.1 ppm of IBA to MS media. However, a 1:1 blend of Peat-moss and Vermiculite was shown to be more favorable for acclimatization. Furthermore, valerenic acid and valtrate measurement by HPLC demonstrated that regenerated shoots included a large quantity of valerenic acid, but roots-induced leaf explants contained the highest amount of valtrate. Furthermore, plant biotechnology strategies for *in vitro* synthesis of the targeted pharmaceutical substances valrerenic acid and valtrate from *Valeriana officinalis* L. were advised.

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