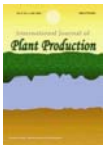

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Enhanced production of valerenic acids and valepotriates by *in vitro* cultures of *Valeriana officinalis* L.

S. Ekhteraei Tousi[a], T. Radjabian[b,*], H. Ebrahimzadeh[a], V. Niknam[a]

[a]*School of Biology, University College of Sciences, University of Tehran, Tehran, Iran.*
[b]*Department of Biology, Faculty of Sciences, Shahed University, Tehran, Iran.*
*Corresponding author. E-mail: rajabian@shahed.ac.ir

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Abstract

Valerian (*Valeriana officinalis* L.) is an important medicinal plant that is widely exploited for its roots and rhizomes which contain valepotriates and valerenic acids (with putative pharmacological activities). Thus root proliferation of valerian is very important. The aim of this study was to establish a practical tissue culture method for rapid and large-scale induction of *V. officinalis* L. roots with high capacity for production of valerian phytomedicine. Explants derived from leaves, petioles, and root segments (both basal and apical) of four months aged plantlets were cultured on MS basal medium supplemented with different concentrations (0.625-5 µM) of auxin and cytokinin hormones. Then accumulation of valerenic acids and valepotriates in developed root cultures was studied to find the best yielding conditions. Maximum valerenic acids (0.84%) and valepotriates contents (7.41%) were quantified in roots developed on petiole explants and in roots formed on root basal segments in medium supplemented with 1.25 µM and 0.625 µM indole-3-acetic acid, respectively. These values were significantly ($P<0.05$) higher than those in roots from basal and apical root segments without plant growth regulators as control. The highest average number (29.00) of directly formed roots developed on leaf explants in media supplemented with 5 µM α- naphthalene acetic acid. Also maximum number of indirectly developed roots (30.05) was observed on root apical segments in media containing 2.5 µM α- naphthalene acetic acid. These results suggest that besides the applied plant growth regulators, the type of primary explant is also relevant for biosynthetic capacity of these metabolites in root cultures.

Keywords: *Valeriana officinalis* L.; Valerenic acids; Valepotriates; Plant tissue culture.

Introduction

Plants within the Valerianaceae family have a long tradition in folk medicine (Becker and Chavadej, 1988). The subterranean organs of *Valeriana officinalis* L. (commonly called valerian) are used as mild sedatives (Bos et al., 1996). The roots of valerian contain several compounds with pharmacological activities. These include the essential oils and their sesquiterpenoid derivatives (valerenic acids), epoxy iridoid esters (valepotriates) and their decomposition products such as baldrinal and homobaldrinal, amino acids (arginine, GABA, glutamine, tyrosine), and alkaloids. Valerian also possesses small amounts of phenolic acids

and flavonoids, valerosidatum, chlorogenic acid, caffeic acid, choline, β-sitosterol, fatty acids, and various minerals (Upton et al., 1999). Among these constituents, valerenic acids (valerenic acid, acetoxyvalerenic acid and hydroxyvalerenic acid) and valepotriates (Figure 1) are often regarded as active principles in valerian commercial and medicinal preparations that display tranquilizing effects. Production of these resources is high in the root and scarce in the aboveground parts of *V. officinalis* plants (Violon et al., 1983). Plant cell and tissue culture may offer an alternative method for a controlled production procedure of these natural products. Often low quantities of the desired compounds are accumulated by undifferentiated cell cultures. The initiation of organ cultures stands a better chance in obtaining higher amounts of natural products (Pras, 2000). In this context a good example is the accumulation of valepotriates up to 0.83% in root cultures of *V. wallichii* DC. (Becker and Chavadej, 1988). In another practical method described for solid *in vitro* medium multiplication of *V. glechomifolia* Meyer, valepotriates content reached 3.54% in stems and 0.98% in leaves (Salles et al., 2002). Also, maximum valepotriate contents of acevaltrate (0.26%), valtrate (1.02%), and didrovaltrate (0.29%) were observed in liquid root cultures of *V. glechomifolia* after 7-8 weeks of culture (Maurmann et al., 2006). In addition, for profuse adventitious root development, hairy root cultures of some plants from Valerianaceae were established by infection of sterile plantlets with *Agrobacterium rhizogenes* in order to increase production of valepotriates (Gränicher et al., 1992; Gränicher et al., 1995; Banerjee et al., 1998). In this study we made an effort to establish a practical tissue culture method for rapid and large-scale production of valepotriates and valerenic acids in untransformed roots of *V. officinalis*.

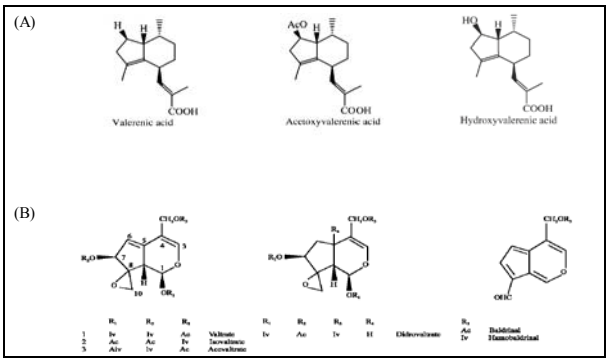

Figure 1. (A) Structure of valerenic acid and its derivatives (Dietz et al., 2005) and (B) some valepotriates (Bos et al., 2002). Abbreviations: Ac, Acetyl; Iv, Isovaleryl; Aiv, Acetoxyisovaleryl.

Materials and methods

Plant materials

V. officinalis seeds were collected from a field (Karadj city, Tehran province, Iran) at September 2006. The seeds were surface sterilized in 70% ethanol solution for 1 min, then soaked in 1% (w/v) sodium hypochlorite solution containing Tween-20 (two drops in 100 ml) for 15 min. Seeds were rinsed four times with sterile distilled water and left for germination on wet filter papers in Petri dishes. Seedlings were transferred on solid MS (Murashige and Skoog, 1962) basal medium containing 3% sucrose and 0.7% agar- agar and maintained

under a 16 h photoperiod provided by cool white fluorescent lamps (light source: Osram- L-Fluora 77R ;700 lux) at 25 ± 1 ºC. These environmentally controlled conditions were used in all of the experiments. The leaves (~10 mm^2), petioles (~10 mm), basal and apical segments of roots (~10 mm) obtained from four months old plantlets were used as explants.

Media and culture conditions for root culture development

Root induction was achieved by placing the mentioned explants on different root induction media, that was MS basal medium supplemented with different phytohormonal concentrations and combinations. These included a range of concentrations (0.625-5 µM) of indole-3-acetic acid (IAA) or α-naphthalene acetic acid (NAA) alone, as well as auxin: cytokinin combinations of IAA + kinetin (Kin) or NAA + Kin in concentration proportions of 5:1, 2.5: 1.25, 1.25: 2.5 and 1:5 (µM). Also different primary explants including leaf, petiole, basal segment of root (B.S.R.) and apical segment of root (A.S.R.) were inoculated on MS medium without plant growth regulators (PGRs) as control. The pH of the media was adjusted to 5.6 before autoclaving. All explants were subcultured to fresh medium every three weeks. The rooting response was evaluated after 60 days. For data recording of rooting, the average number of roots formed per explant was reported. Rooting in this study had two patterns: indirect rooting (development of root after callus formation) and direct rooting that didn't have dedifferentiation stage (Figure 2). Then mean numbers of roots developed on the explants were calculated (Table 1).

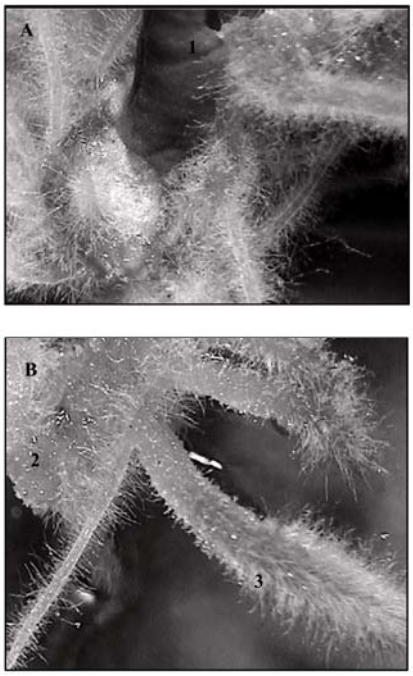

Figure 2. (A) Adventitious developed roots on leaf and (B) apical segment of root explants in MS medium supplemented with 5µM NAA and 2.5 µM NAA, respectively.1; Leaf explants, 2; callus induced on apical segment of root explants, 3; root developed indirectly from callus (magnification 6.2 x).

Table 1. Mean value contents ± SE of valerenic acids and some valepotriates (g/100g DW) in adventitious roots developed on different primary explants in MS basal medium supplemented with different concentrations of lAA, NAA and Kin. Abbreviations: HVA, hydroxy valerenic acid; AVA, acetoxy valerenic acid; VA, valerenic acid; TVA, total valerenic acid; DID, didrovaltrate; ACE, acevaltrate; VAL, valtrate; B.S.R., basal segment of root ; A.S.R., apical segment of root.

Explant	PGR Concentration(μM)		HVA	AVA	VA	TVA	DID	ACE + VAL	Direct roots (number)	Indirect roots (number)
Root from leaf	IAA	5	0.023±0.000	0.015±0.000	0.009±0.000	0.053±0.003	0.095±0.005	0.079±0.001	0.000±0.000	3.000±0.020
		2.5	0.003±0.000	0.004±0.000	0.002±0.000	0.009±0.000	0.236±0.004	0.246±0.004	0.000±0.000	0.650±0.483
		1.25	0.066±0.001	0.073±0.003	0.048±0.002	0.165±0.015	0.915±0.001	0.976±0.004	0.474±0.258	1.211±0.371
		0.625	0.011±0.000	0.014±0.000	0.009±0.000	0.023±0.002	0.537±0.003	0.485±0.005	0.667±0.371	0.889±0.442
	NAA	5	0.007±0.000	0.011±0.001	0.006±0.000	0.024±0.001	0.696±0.005	1.495±0.005	29.000±2.00	7.045±2.921
		2.5	0.018±0.000	0.015±0.002	0.008±0.000	0.034±0.001	0.821±0.001	1.410±0.008	0.000±0.000	3.000±0.637
		1.25	0.011±0.000	0.013±0.001	0.009±0.000	0.044±0.001	1.326±0.005	2.285±0.015	0.000±0.000	4.231±0.849
		0.625	0.012±0.000	0.014±0.001	0.010±0.000	0.048±0.001	1.394±0.006	1.326±0.024	2.700±0.637	4.500±2.035
	IAA/Kin	5+1	0.056±0.001	0.051±0.001	0.033±0.001	0.139±0.005	0.436±0.004	0.707±0.003	1.176±0.413	0.412±0.211
		2.5+1.25	0.045±0.008	0.057±0.009	0.041±0.006	0.093±0.014	1.025±0.006	1.497±0.005	1.944±0.659	5.500±1.456
		1.25 +2.5	0.089±0.022	0.116±0.029	0.080±0.020	0.270±0.050	0.797±0.003	0.748±0.006	1.375±0.632	0.188±0.136
		1+5	0.013±0.011	0.015±0.012	0.011±0.008	0.021±0.009	0.655±0.005	1.106±0.001	11.182±1.37	0.273±0.273
	NAA/Kin	5+1	0.005±0.003	0.006±0.004	0.004±0.002	0.006±0.000	0.260±0.010	0.345±0.005	28.12±2.347	0.000±0.000
		2.5+1.25	0.006±0.000	0.008±0.000	0.005±0.000	0.019±0.000	0.736±0.004	1.158±0.002	19.06±4.347	6.059±2.906
		1.25+2.5	0.007±0.000	0.009±0.000	0.006±0.000	0.019±0.000	0.535±0.005	0.710±0.010	10.61±1.372	0.000±0.000
		1+5	0.001±0.000	0.001±0.000	0.001±0.000	0.006±0.000	0.747±0.003	0.977±0.003	3.563±1.136	2.313±1.396

Continue Table 1. Mean value contents ± SE of valerenic acids and some valepotriates (g/100g DW) in adventitious roots developed on different primary explants in MS basal medium supplemented with different concentrations of IAA, NAA and Kin. Abbreviations: HVA, hydroxy valerenic acid; AVA, acetoxy valerenic acid; VA, valerenic acid; TVA, total valerenic acid; DID, didrovaltrate; ACE, acevaltrate; VAL, valtrate; B.S.R., basal segment of root ; A.S.R., apical segment of root.

Explant	PGR Concentration(μM)		HVA	AVA	VA	TVA	DID	ACE + VAL	Direct roots (number)	Indirect roots (number)
Root from petiole	IAA	5	0.021±0.000	0.027±0.002	0.017±0.001	0.046±0.002	1.469±0.005	2.168±0.001	0.056±0.030	0.400±0.050
		2.5	0.031±0.001	0.034±0.001	0.024±0.002	0.054±0.001	1.155±0.005	2.405±0.005	0.056±0.028	0.350±0.050
		1.25	0.439±0.002	0.551±0.001	0.385±0.005	0.839±0.001	0.403±0.001	0.276±0.001	0.180±0.010	0.210±0.020
		0.625	0.082±0.001	0.063±0.000	0.038±0.000	0.089±0.000	0.395±0.005	0.605±0.001	0.150±0.018	0.000±0.000
	NAA	5	0.010±0.000	0.012±0.000	0.009±0.000	0.030±0.001	1.186±0.004	1.476±0.025	0.000±0.000	2.400±0.150
		2.5	0.007±0.000	0.009±0.000	0.006±0.000	0.026±0.000	0.939±0.002	1.468±0.033	0.100±0.010	1.700±0.050
		1.25	0.037±0.001	0.051±0.000	0.036±0.001	0.130±0.000	1.768±0.033	2.754±0.046	0.000±0.000	0.800±0.010
		0.625	0.009±0.003	0.008±0.004	0.006±0.001	0.016±0.000	0.180±0.000	0.120±0.000	0.000±0.000	1.500±0.020
	IAA/Kin	5+1	0.015±0.002	0.026±0.005	0.012±0.001	0.036±0.005	0.841±0.005	3.581±0.001	0.150±0.010	0.160±0.020
		2.5+1.25	0.006±0.000	0.008±0.000	0.006±0.000	0.020±0.000	1.115±0.005	4.185±0.015	0.000±0.000	0.400±0.050
		1.25 +2.5	0.060±0.002	0.070±0.002	0.050±0.003	0.127±0.005	1.835±0.005	1.604±0.001	0.000±0.000	1.000±0.100
		1+5	0.019±0.005	0.024±0.005	0.016±0.004	0.059±0.006	0.373±0.017	0.565±0.023	0.143±0.097	1.300±0.020
	NAA/Kin	5+1	0.008±0.000	0.011±0.000	0.007±0.000	0.019±0.001	0.625±0.005	0.697±0.003	1.500±1.094	14.40±1.970
		2.5+1.25	0.018±0.000	0.019±0.000	0.016±0.000	0.025±0.001	0.905±0.001	1.468±0.033	0.000±0.000	4.000±0.739
		1.25+2.5	0.018±0.001	0.020±0.001	0.014±0.001	0.035±0.000	0.600±0.020	1.572±0.032	0.882±0.445	2.824±0.719
		1+5	0.012±0.000	0.013±0.000	0.009±0.000	0.022±0.000	1.040±0.000	1.368±0.033	0.000±0.000	2.190±0.466

Continue Table 1. Mean value contents ± SE of valerenic acids and some valepotriates (g/100g DW) in adventitious roots developed on different primary explants in MS basal medium supplemented with different concentrations of IAA, NAA and Kin. Abbreviations: HVA, hydroxy valerenic acid; AVA, acetoxy valerenic acid; VA, valerenic acid; TVA, total valerenic acid; DID, didrovaltrate; ACE, acevaltrate; VAL, valtrate; B.S.R., basal segment of root ; A.S.R., apical segment of root.

Explant	PGR	Concentration(μM)	HVA	AVA	VA	TVA	DID	ACE + VAL	Direct roots (number)	Indirect roots (number)
Root from B.S.R.	IAA	5	0.033±0.000	0.047±0.000	0.023±0.001	0.081±0.002	0.120±0.001	0.097±0.001	0.000±0.000	1.158±0.361
		2.5	0.029±0.001	0.026±0.000	0.018±0.000	0.089±0.000	0.185±0.001	0.124±0.001	0.000±0.000	7.500±0.020
		1.25	0.048±0.003	0.052±0.003	0.041±0.002	0.130±0.020	2.980±0.020	2.205±0.005	0.267±0.267	0.400±0.214
		0.625	0.013±0.001	0.013±0.006	0.010±0.003	0.055±0.019	3.122±0.002	4.289±0.011	0.000±0.000	15.667±2.21
	NAA	5	0.007±0.000	0.007±0.000	0.004±0.000	0.016±0.002	0.275±0.005	0.365±0.065	0.000±0.000	8.211±2.258
		2.5	0.007±0.000	0.007±0.000	0.005±0.000	0.018±0.000	1.468±0.033	2.770±0.030	0.000±0.000	18.00±2.025
		1.25	0.018±0.000	0.024±0.001	0.016±0.000	0.056±0.000	0.190±0.001	0.238±0.002	0.000±0.000	9.444±3.005
		0.625	0.025±0.000	0.033±0.001	0.024±0.001	0.077±0.000	0.926±0.004	2.254±0.046	0.000±0.000	19.46±2.401
	IAA/Kin	5+1	0.042±0.001	0.005±0.002	0.004±0.002	0.013±0.000	0.185±0.001	0.060±0.000	0.000±0.000	4.545±1.378
		2.5+1.25	0.005±0.000	0.006±0.000	0.005±0.000	0.016±0.000	0.277±0.003	0.639±0.002	0.000±0.000	11.50±0.060
		1.25 +2.5	0.101±0.001	0.120±0.000	0.091±0.009	0.300±0.000	0.573±0.002	0.849±0.001	0.000±0.000	1.200±0.374
		1+5	0.008±0.000	0.009±0.000	0.007±0.000	0.021±0.000	0.695±0.005	1.107±0.003	0.000±0.000	2.333±1.597
	NAA/Kin	5+1	0.005±0.000	0.007±0.000	0.005±0.000	0.024±0.001	0.608±0.003	0.937±0.004	0.000±0.000	26.06±1.745
		2.5+1.25	0.002±0.000	0.003±0.000	0.002±0.000	0.007±0.000	0.029±0.000	0.034±0.001	0.000±0.000	12.789±1.53
		1.25+2.5	0.009±0.000	0.013±0.000	0.009±0.000	0.030±0.000	0.207±0.003	0.268±0.003	8.571±2.173	6.571±2.206
		1+5	0.008±0.010	0.009±0.002	0.006±0.002	0.034±0.007	0.700±0.020	1.080±0.030	0.000±0.000	11.33±1.425

Continue Table 1. Mean value contents ± SE of valerenic acids and some valepotriates (g/100g DW) in adventitious roots developed on different primary explants in MS basal medium supplemented with different concentrations of IAA, NAA and Kin. Abbreviations: HVA, hydroxy valerenic acid; AVA, acetoxy valerenic acid; VA, valerenic acid; TVA, total valerenic acid; DID, didrovaltrate; ACE, acevaltrate; VAL, valtrate; B.S.R., basal segment of root ; A.S.R., apical segment of root.

Explant	PGR Concentration(μM)		HVA	AVA	VA	TVA	DID	ACE + VAL	Direct roots (number)	Indirect roots (number)
Root from A.S.R.	IAA	5	0.003±0.001	0.008±0.000	0.001±0.001	0.009±0.000	0.286±0.002	0.510±0.004	0.000±0.000	0.167±0.121
		2.5	0.004±0.000	0.005±0.001	0.003±0.000	0.019±0.000	0.410±0.001	0.560±0.001	0.000±0.000	18.563±3.24
		1.25	0.005±0.000	0.007±0.000	0.005±0.000	0.022±0.000	0.993±0.020	0.730±0.005	1.789±1.115	0.789±0.355
		0.625	0.020±0.003	0.027±0.006	0.019±0.005	0.068±0.006	1.299±0.020	1.701±0.020	0.000±0.000	10.688±1.15
	NAA	5	0.001±0.000	0.005±0.001	0.003±0.000	0.024±0.001	0.508±0.098	0.945±0.012	0.000±0.000	28.889±1.60
		2.5	0.006±0.003	0.008±0.003	0.005±0.003	0.041±0.004	1.100±0.080	1.900±0.043	0.000±0.000	30.045±2.33
		1.25	0.016±0.000	0.021±0.000	0.014±0.000	0.043±0.003	0.398±0.002	0.392±0.008	6.400±1.088	2.560±1.577
		0.625	0.001±0.000	0.001±0.000	0.001±0.000	0.003±0.001	0.665±0.005	1.400±0.001	0.000±0.000	17.167±2.58
	IAA/Kin	5+1	0.002±0.000	0.002±0.000	0.001±0.000	0.006±0.000	0.600±0.002	0.430±0.005	0.000±0.000	0.778±0.298
		2.5+1.25	0.002±0.000	0.002±0.000	0.001±0.000	0.007±0.001	0.278±0.003	0.490±0.001	0.000±0.000	4.400±0.500
		1.25 +2.5	0.046±0.000	0.051±0.001	0.033±0.001	0.125±0.005	0.738±0.003	0.459±0.002	0.000±0.000	1.045±0.276
		1+5	0.037±0.000	0.053±0.001	0.038±0.000	0.154±0.000	0.760±0.002	0.520±0.001	0.000±0.000	0.000±0.000
	NAA/Kin	5+1	0.003±0.000	0.004±0.000	0.003±0.000	0.009±0.000	0.165±0.005	0.308±0.002	0.000±0.000	14.211±1.92
		2.5+1.25	0.005±0.000	0.005±0.000	0.003±0.000	0.013±0.000	0.128±0.002	0.399±0.002	0.000±0.000	13.286±1.18
		1.25+2.5	0.008±0.000	0.009±0.000	0.006±0.000	0.028±0.000	0.153±0.003	0.193±0.001	10.364±1.22	0.000±0.000
		1+5	0.021±0.000	0.022±0.000	0.015±0.000	0.054±0.005	0.800±0.020	1.200±0.030	11.071±1.62	0.000±0.000

Sample preparation for valerenic acids quantification

The separated adventitious roots from the primary explants and also the explants themselves were dried at 20 ± 3 °C in a ventilated dryer for 12-15 hours. All samples were powdered and stored at -20 °C, until analysis. Each 0.2 g of powdered plant sample was first extracted with 5ml dichloromethane (DCM) and then evaporated. After volume adjustment to 1ml with DCM, organic acids were transferred into 2 ml 2% NaOH. The alkaline aqueous phase was acidified to pH~2 using 1% HCl, and the organic acids (including valerenic acid derivatives) retransferred into 1ml petroleum ether: diethyl ether (2:1) organic phase (Bos, 1997). The obtained organic phase was divided into two equal fractions and processed as follow:

- Fraction 1 was used to determine valerenic acid derivatives content using their respective aliquot extinction coefficients (ε) found in the literature (Bos, 1997). Thus a volume of 0.5 ml was dry evaporated then the residue collected in 1ml ethanol. After absorbance measurement at 212 nm (hydroxyvalerenic acid), 217 nm (acetoxyvalerenic acid) and 218 nm (valerenic acid), each valerenic acid derivatives content (Ci) was determined using the following formula:

$$C_i = \frac{Abs}{\varepsilon}$$

- Fraction 2 was used to determine the total valerenic acid derivatives (including valerenic acid, hydroxyvalerenic acid, and acetoxyvalerenic acid) content using a calibration curve. Each calibration curve point corresponded to a dilution obtained from the valerenic acid concentration stock solution. The stock solution was prepared using a valerenic acid standardized sample. Thus, a 0.5 ml of fraction 2 was dry evaporated then collected in 1ml methanol and its absorbance measured at 225 nm.

Sample preparation for valepotriates quantification

Each 0.2 g of powdered plant sample was first extracted with 5 ml DCM, and then dry evaporated. The residues were redisolved into 1 ml methanol. All obtained samples were used to determine the valepotriates content using a calibration curve at 207 nm (for didrovaltrate), and 254 nm (for valtrate + acevaltrate).

Calibration curve of valerenic acid and valepotriates

Stock solution (51 mg/l) of valerenic acid (HPLC grade, Fluka) was prepared in methanol and standard curve was obtained by using of 10 concentrations of the valerenic acid in the range of 2-50 mg/l.

For the quantitative determination of valepotriates, a standardized product, Valmane® (Lyssia GmbH c/o Solvay Arzneimittel) was used to plot two calibration curves. Stock solutions of valepotriates were prepared in methanol. One calibration curve for the determination of valtrate + acevaltrate amounts was in the concentration range of 5-156 mg/l.

The second calibration curve for didrovaltrate quantification was also linear in the range of 5-52 mg/l. All experiments have been performed during Autumn-Winter 2006 at the Plant Physiology laboratories of University of Tehran and Shahed University (Tehran, Iran).

Data analysis

To determine the efficiency of root induction of each medium, the mean number of adventitious roots per rooted explants was recorded after 2 months. Each type of the explants in each plant growth regulator (PGR) treatment consist of five replicates in which five explants were considered as one replicate. Data obtained from UV- spectrophotometry were subjected to one-way ANOVA by SPSS (version 13) software. Post hoc multiple comparisons between samples for valepotriates and valerenic acids contents were made using Tukey´s test. Significance level for comparison of mean differences was up to 0.05.

Results

Our quantitative analysis showed that acetoxyvalerenic acid was the dominant fraction in the most samples, compared to valerenic acid and hydroxyvalerenic acid (Figure 3). The survey of valerenic acids content in the studied samples showed the highest amounts of hydroxyvalerenic acid (0.44%), acetoxyvalerenic acid (0.55%), valerenic acid (0.39%) and valerenic acids (0.84%) developing roots on petiole in MS medium supplemented with 1.25 µM IAA. With attention to Tables 1 and 2, the maximum amounts of valerenic acids in mentioned sample (0.84%) was higher than the largest valerenic acids content in primary explants (leaf, 0.19%). Also roots developed on basal segments of root explants had considerable amount of didrovaltrate in MS media containing 0.625 µM IAA (3.12%) and 1.25 µM IAA (2.98%). Substantial amounts of acevaltrate + valtrate in roots developed on basal segments of root explants in media supplemented with 2.5 µM IAA + 1.25 µM Kin and 0.625 µM IAA, reaching 4.19% and 4.29%, respectively. Thus the highest overall valepotriates content (7.41%) was observed in roots formed on basal segments of root explants in MS medium supplemented with 0.625 µM IAA. Meanwhile, values of valerenic acids in basal and apical segments of root in four months aged *V. officinalis* L. plantlets grown on MS media without any PGR were 0.037% and 0.013%, respectively. Amounts of valepotriates in the basal and apical root segments were 0.68% and 0.65%, in that order (Table 2, Figure 3). The reported quantities of the mentioned compounds in root segments of plantlets were low in comparison with the highest amounts of valerenic acids and valepotriates evaluated in developed roots on primary explants (significant mean differences, $P<0.05$).

The highest average numbers of direct roots formed on leaf explants in MS media supplemented with 5 µM NAA + 1 µM Kin and 5 µM NAA were 28.12 and 29.00 (per explant), respectively. Also maximum numbers of formed indirect roots on root apical segments were observed in media containing 2.5 µM (30.05) and 5 µM NAA (28.89) (Table 1).

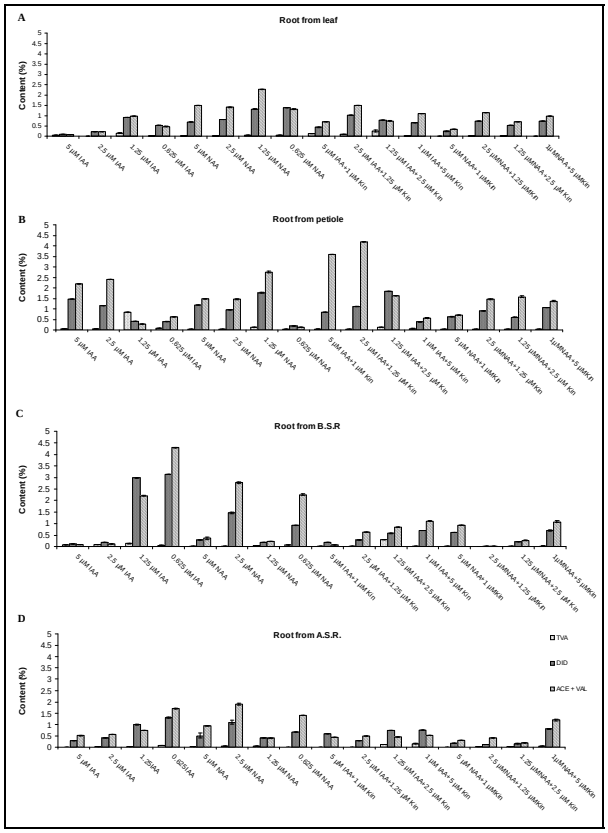

Figure 3. Comparative analysis of total valerenic acid (TVA), valepotriates including didrovaltrate (DID) and acevaltrate + valtrate (ACE +VAL) content in adventitious roots developed on primary explants A; leaf, B; petiole, C; basal segment of root (B.S.R.) and D; apical segment of root (A.S.R.) in MS basal medium supplemented with different concentrations of IAA, NAA and Kin.

Discussion

There are great interests for therapeutic uses of valepotriates and valerenic acids. For a long time, only *V. officinalis* with a rather low percentage of valepotriates was cultivated. All other therapeutically used plants came from field collections, which mean their supply was limited and uncertain. This makes it interesting to study the possibility of producing valepotriates through plant cell and tissue culture (Becker et al., 1977). In the 1990s, increases in yield of valepotriates in transformed roots by *A. rhizogenes* in Valerianaceae were reported frequently (Gränicher et al., 1992; Gränicher et al., 1995; Banerjee et al., 1998; Caetano et al., 1999). Gradually, investigators tend to increase valepotriates production without such bacterial infections. Thus the usefulness of exogenous PGRs like some auxins and cytokinins to improve valepotriates and valerenic acids production *in vitro* was examined. In *V. edulis* ssp. *procera* (Castillo et al., 2002), *V. glechomifolia* Meyer

(Salles et al., 2002; De Carvalho et al., 2004; Maurmann et al., 2006), *V. fauriei* var. *dasycarpa* Hara (Li et al., 2006), production enhancement of valepotriates and valerenic acids in plants exposed to some PGRs were reported. In cultured roots of *V. fauriei* var. *dasycarpa* Hara, the highest production of valerenic acids (0.48%) and valepotriates (0.37%) was in B5 medium. However, in the present study the most significant amounts of valerenic acids and valepotriates were 0.84% and 7.41% in MS medium, respectively.

The amounts of valerenic acids and valepotriates in wild plants of *V. officinalis* L. grown in field that studied in our previous researches were 0.09% and 6.99%, respectively (Eebrahimzadeh et al., 2008). Thus production of valerenic acids and valepotriates in developed roots on primary explants was higher than those of in roots from control plantlets (their contents mentioned in results section) and field grown plants. In the study carried out by Silva and colleagues (2002), the levels of valepotriates in the cultures were higher than those found in wild populations of *V. glechomifolia* as well.

In this survey, the highest content of valerenic acids was found in developed roots on primary explants in MS basal medium supplemented with IAA. The most value of total valerenic acid (0.84%) was analyzed in roots formed on petiole explants in medium containing 1.25 µM IAA. According to our results, IAA had more effect on valerenic acids synthesis enhancement than NAA. These data showed valerenic acids content increase was noticeable in medium including 1.25 µM IAA. The use of Kin as an exogenous cytokinin jointly with IAA didn't have markedly influence on valerenic acids content augment. Among applied media, the best concentration of NAA for valerenic acids content rising was 1.25 µM as well. In the majority of media in which NAA become concomitant with Kin, the value of valerenic acids was decreased. In both media including IAA or NAA, the most maximum values of valerenic acids were observed in adventitious developed roots on petiole explants. This comparison showed that although the types of PGRs and their different concentrations in media had distinct influences on valerenic acids content in developed roots, the kind of primary explants might affect the ability to synthesis and accumulation valerenic acids in developed roots as well. Moreover, similar to valerenic acids, the amounts of didrovaltrate in roots developed in media with low concentrations of IAA (0.625 and 1.25 µM) and NAA (0.625 µM) were greater.

In agreement with the study carried by Russowski and colleagues (2006), our results showed valtrate and acevaltrate were dominant valepotriates, followed by didrovaltrate. No significant relation was found among acevaltrate + valtrate contents in formed roots and applied PGRs. The highest amount of overall valepotriates (7.41%) was observed in roots developed on basal segments of root in media supplemented with 0.625 µM IAA. However, in the survey carried by De Carvalho and coworkers (2004) the best performance in valepotriates production in *V. glechomifolia* was in MS medium with 5.71 µM IAA. Similar to the results reported in the mentioned study, we have also shown that IAA is an important factor in valepotriates production and observed impact of auxins on valepotriates metabolism was not necessarily dependent on phytohormone-induced developmental changes in rooting. Our data support the observation by De Carvalho and coworkers (2004) that the benefits of auxin exposure were apparently correlated to auxin stability, since metabolically stable types of auxin, such as NAA, were not as beneficial for valepotriates yield. In spite of their study, we showed in our research, NAA was more efficient for rooting.

With attention to high values of valerenic acids and valepotriates in formed roots on petiole and basal segments of root and in the same primary explants (Tables 1, 2) we could demonstrate the main sources of valerenic acids and valepotriates biosynthesis are these organs, respectively. In addition, maybe leaves are the source for valerenic acids accumulation after synthesis in petiole (Table 2). Also potency of developed roots on each explant is possibly different from other explants in these compounds biosynthesis. According to the study of Caetano and colleagues (1999), it was unclear if valepotriates are at their location of biosynthesis or in the process of production transported from the sites of biosynthesis to storage sites in the more mature parts of the roots (root basal segment). Also, when the pericycle begins to form the lateral root primordium there is an absence of valepotriates in the region which may be due to a suppression of biosynthesis as the result of a change in function of the pericycle. Our investigation confirms their claim in where the highest rooting rate occurred in media containing 5 and 2.5 µM NAA, while valepotriates content were low in these media. Also in the report by Castillo and colleagues (2002), the data obtained support the hypothesis that valepotriates production in *V. edulis* ssp. *procera* is closely related to rhizome and root differentiation. We are currently carrying out additional studies such as the monitoring of the synthesis of these metabolites in different organs needed for clarifying these claims.

Table 2. Mean value contents ± SE of valerenic cids and some valepotriates (g/100g DW) in different primary explants in MS basal medium supplemented without any PGR. Abbreviations: HVA, hydroxy valerenic acid; AVA, acetoxy valerenic acid; VA, valerenic acid; TVA, total valerenic acid; DID, didrovaltrate; ACE, acevaltrate; VAL, valtrate; B.S.R., basal segment of root ; A.S.R., apical segment of root.

		HVA	AVA	VA	TVA	DID	ACE + VAL
Explants	Leaf	0.065±0.001	0.081±0.000	0.056±0.001	0.190±0.017	0.210±0.001	0.160±0.005
	Petiole	0.019±0.004	0.021±0.005	0.013±0.002	0.051±0.001	0.072±0.002	0.041±0.001
	B.S.R.	0.016±0.000	0.014±0.000	0.008±0.000	0.037±0.000	0.260±0.008	0.420±0.009
	A.S.R.	0.004±0.000	0.005±0.000	0.004±0.000	0.013±0.002	0.245±0.005	0.406±0.006

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