Influence of minerals on valerenic acid accumulation in hairy root cultures of *Valeriana officinalis* L.

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Abstract

The roots and rhizomes of *Valeriana* (*Valerianaofficinalis* L.) are rich with valuable metabolites such as valerenic acid and valepotriates are used as mild sedatives. The aim of the present study was to investigatethe effect of four levels of calcium and potassium compounds (half, full, 2 and 4-fold) of normal Murashige and Skoog (MS) media including KI and $CaCl_2$on *Valerianaofficinalis*L. hairy roots for scaling-up producing valerenic acid. Various explants of 42-day-old sterile seedlings derived from *V. officinalis*were used for a genetic transformation via*Agrobacterium rhizogenes*A13strain.Polymerase chain reaction (PCR) analysis and genes primers of*rol*B and *vir*Dwere conducted to confirm the transgenic nature of the roots and that the roots were bacteria-free. Then,the hairy root cultures of *V. officinalis*were maintained in the media for investigations of valerenic acid production ability. After 35 days the valerenic acid content in hairy roots was measured using high-performance liquid chromatography (HPLC) to determine the best yielding conditions.The highest valerenic acid (0.69±0.03 mg/g DW) accumulation was obtained from hairy roots culturedin2-fold of calcium media, which was1.92 times higher than normal culture (0.36± 0.01 mg/g DW). The results of this experiment also showed that application of double (880 mg/l) calcium in mediahad a positive effect on growth of transformed hairy roots.The results revealed that calcium concentration in the MS medium may be used for the intensification of the valerenic acid productionin*Valerianaofficinalis* hairy roots cultures.

Keywords: *Valeriana officinalis*; *Agrobacterium rhizogenes*; hairy roots; Valerenic acid

Abbreviations:
A: Agrobacterium; DW: Dry weight; MS: Murashige and Skoog; V: Valeriana

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Introduction

Valerianaofficinalis L. (*Valerianaceae*), with a long stem of 80-150 cm and a very short rhizome, is cultivated as a medicinal plant on a commercial scale in the northern parts of Europe and America (Cui et al., 2010). *Valerian*roots and rhizomes contain several compounds with pharmacological activities (Ekhteraei et al., 2010). These include essential oils, sesquiterpenoid derivatives (valerenic acid), iridoid esters

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(valepotriates) and alkaloids(Lei et al., 2012). Although all the constituents responsible for the beneficial health effects of valerian have not been fully elucidated, the valerenic acids, which have been shown to exhibit sedative activity, are often used as indicators of medicinal quality (Trauner et al., 2008).

Because secondary metabolism in the field-grown plants is highly regulated in time and space (as two major problems associated with climate), considerable efforts have been devoted to improve their productivity using optimization of growth and production media, elicitors, and *in vitro* technology (bioreactors) (Amdoun et al., 2009).Hairy roots produced by *A. rhizogenes* transformation are characterized by fast growth, a high degree of branching, and growth in hormone-free medium are advantages that make transformed roots efficient sources for secondary metabolites production (Samadi et al., 2012).

Improvement of the medium composition (Amdoun et al., 2009) and induction of secondary metabolite pathways by elicitors (Katrin et al., 2012) are major strategies to increase their productivity. Major minerals such as calcium (Ca^{2+}) (Katrin et al., 2012; Lei et al., 2012), potassium (K^{+}), and sulfur ($So4^{2-}$) are known to affect the growth and metabolism of hairy root cultures (Amdoun et al., 2009).

With references to the role of valerenic acid as an important chemical factor responsible for the sedative effect in human (Ekhteraei et al., 2010; Trauner et al., 2008) and the lack of report on production of valerenic acid through hairy root culture, in this study we made an effort to establish a hairy root culture for rapid and large-scale production of valerenic acid in *Valeriana officinalis* L. using different concentrations of calcium chloride ($CaCl_2$), and potassium iodid (KI).

Materials and Methods

Plant material and maintenance of tissue cultures

Seeds of *V. officinalis* (provided from a seed company, Isfahan, Iran) were used as the starting material for the hairy root cultures. In order to sterilize the seeds, 70% ethanol and 2% solution of sodium hypochlorite (commercial bleach) were used. Then the seeds were rinsed four times with sterile distilled water and left for germination on solid MS (Murashige and Skoog, 1997) basal medium containing 3% sucrose and 0.7% agar. The cultures were maintained in a growth chamber at 24 ± 2°C under8 h light/16 h dark photoperiod and the cool white fluorescent lamps (40 µmol/ms) used for the illumination. The roots (≈15mm^2), leaves (≈15 mm^2), and hypocotyls (≈15 mm^2) obtained from 42-day-old sterile seedlings were used as explants for inoculation by bacteria suspensions.

Establishment of hairy roots

The explants were used for transformation with strain 'A13' of *A. rhizogenes*. Maintenance conditions of hairy roots and their induction were explained in a previous study (Samadi et al., 2012). The extraction of DNA from both hairy roots and untransformed roots (control) was carried out using the CTAB method (Khan et al., 2007).Then PCR analysis was used for confirming the transgenic nature of the roots corresponding gene-specific primer pairs.

Experiments with two minerals

In order to investigate the effect of potassium and calcium ions on biomass and valerenic acid content, root tips (0.25 gr fresh weight) were maintained for 35 days in the media of following composition: normal MS medium, modified MS medium with 1/2($CaCl_2$= 220mg/l, KI = 0.42 mg/l), 2($CaCl_2$= 880 mg/l, KI= 1.66 mg/l) and 4-fold ($CaCl_2$= 1760 mg/l, KI= 3.32 mg/l) of their concentrations in the normal MS medium ($CaCl_2$= 440 mg/l, KI= 0.83 mg/l).

Hairy root cultures were grown in the dark on rotation shaker (100 rpm) at 25± 2°C in Petri dishes containing 25 ml of hormone free medium and sub-cultured every two weeks. Control and treated hairy roots were harvested on the 35th day of cultivation and analyzed for dry weight (DW) and valerenic acid content. All treatments were set up in a completely randomized design (CRD) with three replications.

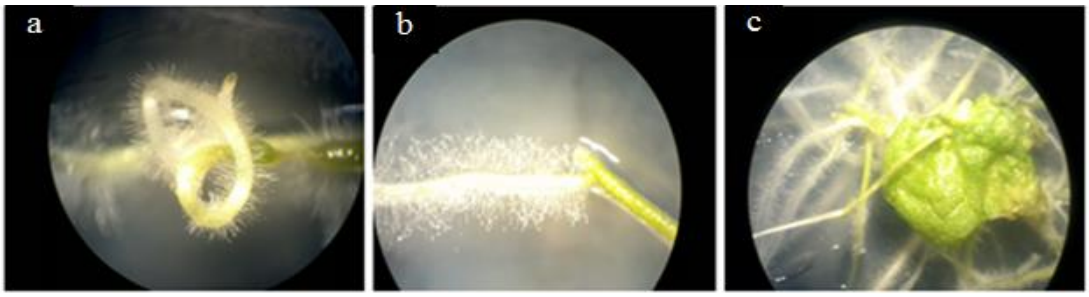

Fig. I. The hairy roots induced on explants by *A. Rhizogenes*strain 'A13'. a, b, c including root, hypocotyl, and leaf explants, respectively.

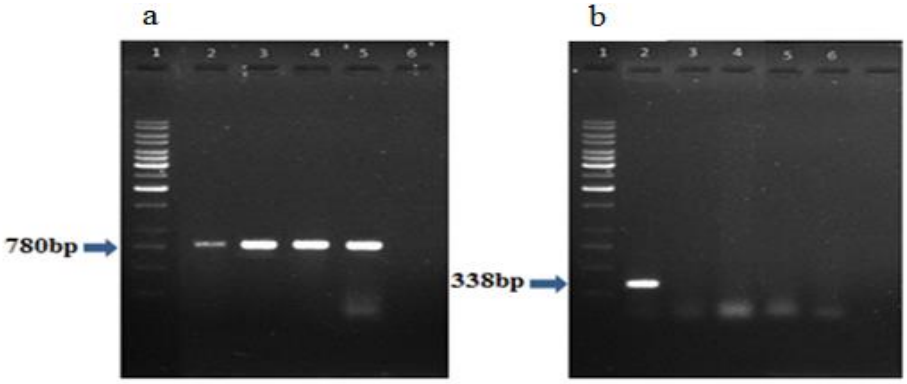

Fig. II. (a) PCR analysis for hairy roots of *V.officinalis* using the *rol*Bgenes specific primers. 1: 1Kb DNA Ladder, 2: Ri plasmid from *A. Rhizogenes*strain 'A13'as a positive control, 3- 5 : Transgenic hairy roots induced on hypocotyl, root, and leaf explants, 6: Wild plant root as negetive control. (b) PCR analysis for hairy roots of *V.officinalis* using the *Vir*Dgenes specific primers. 1: 1Kb DNA Ladder, 2: Ri plasmid from *A. Rhizogenes*strain 'A13'as a positive control, 3-5: Transgenic hairy roots induced on hypocotyl, root and leaf explants, 6: Wild plant root as negetive control.

Extraction and quantification of valerenic acid

Valerenic acid was extracted from lyophilized and powdered roots (approx. 0.2 gr) thrice with 5 ml of 70% MeOH using sonication for 10 min and then the solution was diluted to 15 ml with MeOH and mixed. All samples were filtered through a 0.45 µm micro-filter (Merck, Germany) and this solution was used for chemical analysis. HPLC was performed on the Knauer HPLC system (Germany) with a Spectra UV-K 2501 detector. The optimum HPLC condition for separation was a 0.5% phosphoric acid: methanol mixture (27:73) as mobile phase by a flow rate of 1.5 ml/min and a nucleosil column 100 (C18; 250×3 mm) with a pre-column (25×4.6 mm, particle size 5 µm). Detection wavelength was used at 225 nm and the injection volume was 10 µl. All chemicals were of a HPLC grade (Sigma-Aldrich Co.). The amount of valerenic acid was calculated from calibration curves. All experiments were conducted with 3 replicates.

Statistical Analysis

Data for root weight were set up in a completely randomized design (CRD) with three replicates per treatment. Variation within the treatments were evaluated and analyzed by one way ANOVA test using SPSS (version 16), and significant differences between the mean values was determined using Fisher's least significant differences (FLSD) at a 5% probability level.

Results

Hairy roots of *V. officinalis* grown in solid MS medium exhibited a typical transformed phenotype (plagiotropic growth and high degree of lateral branching) (Fig. I). PCR analysis showed the presence of *rol* B and *rol* D amplified products confirming the transgenic nature of the roots (Fig. II).

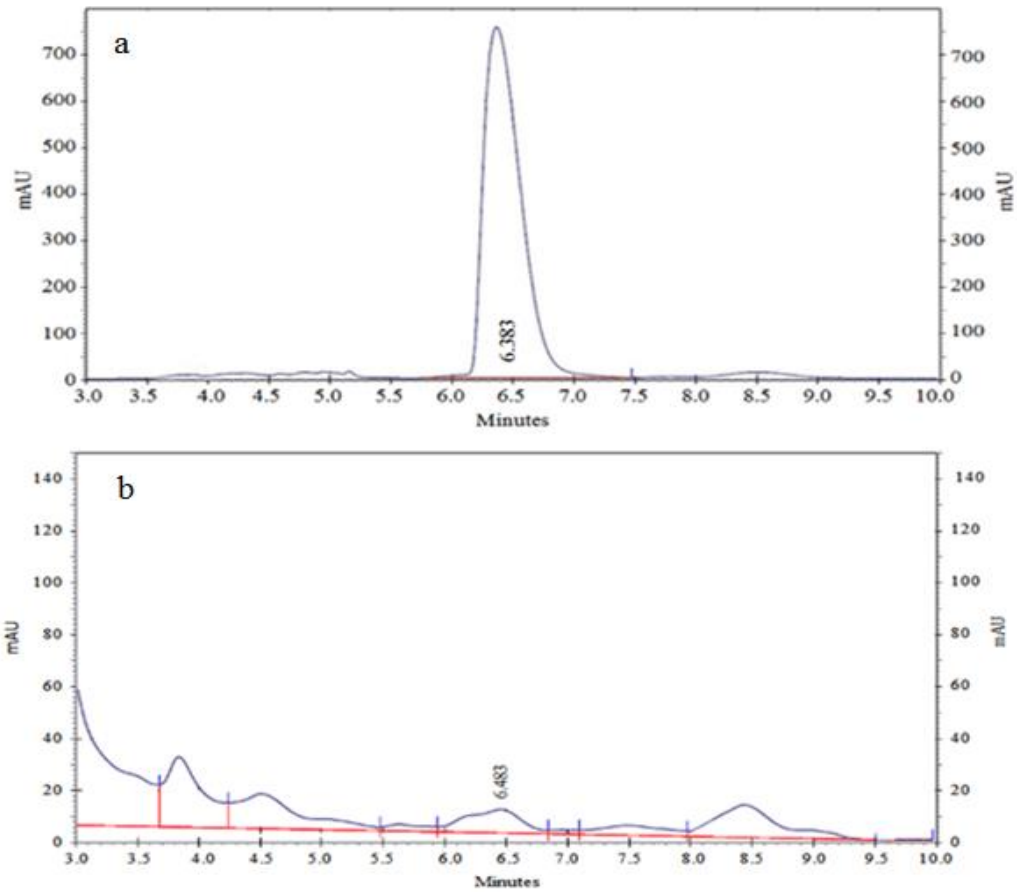

Fig. III. (a) HPLC chromatogram for valerenic acid standard.(b) Valerenic acid from hairy roots cultured under normal MS medium (control)

The effects of culture media were evaluated for biomass and for valerenic acid content. The growth of the hairy roots maintained in the media of various concentrations of calcium [Ca^{2+}] and potassium [K^{+}] was different. The highest biomass (229 mg/25ml DW) was obtained with hairy roots, which were grown into MS medium containing double calcium concentration ($CaCl_2$= 880 mg/l) (Fig. IV).The effect of different concentrations of calcium and potassium ions was also examined on the accumulation level of valerenic acid. The results showed that hairy root cultures grown at double calcium concentration ($CaCl_2$= 880 mg/l) led to higher valerenic acid (0.69± 0.03 mg/g DW) production. This level of valerenic acid was about 1.92 times more than that of control culture (0.36± 0.01 mg/g DW) (Figs. III b and V). But, further increase of calcium concentration (4-fold of that present in normal MS medium) inhibited valerenic acid accumulation (0.23 ± 0.01 mg/g DW) in comparison with control culture (significant mean differences, $p<0.05$).However, potassium at the concentration of 3.32 mg/l (4-fold the control concentration) decreased the biomass accumulation (Fig. IV), but resulted in higher production of valerenic acid, which was 1.2 fold the valerenic acid content of control roots (Fig. V). In contrast, both root growth and valerenic acid were reduced with double (1.66 mg/l) and halved (0.42 mg/l) potassium content (Figs. IV and V).

Discussion

For a long time, the Murashige and Skoog (MS) medium has been used to grow plant culture. Improvement of the medium composition (Katrin et al., 2012) and induction of secondary metabolite pathways by elicitors (Eskandari et al., 2012) are two major methods to increase secondary metabolites productivity. The results of this study showed for the first time that modification of the MS medium ionic content using calcium [Ca^{2+}] and potassium [K^{+}] is critical

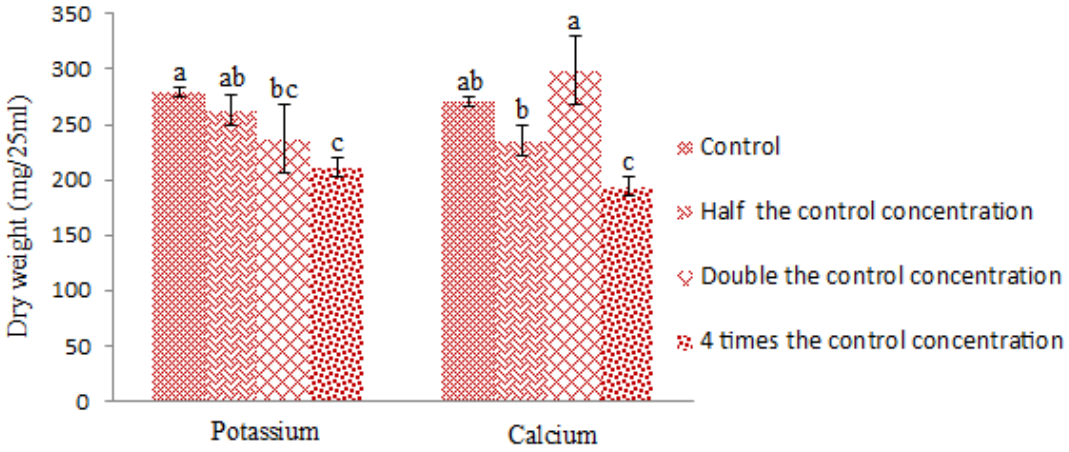

Fig. IV. Effects of various concentrations of Ca^{+2} and K^{+} on the dry weight. The bars represent means ± SE. Bars followed by different letters are significantly different ($p< 0.05$).

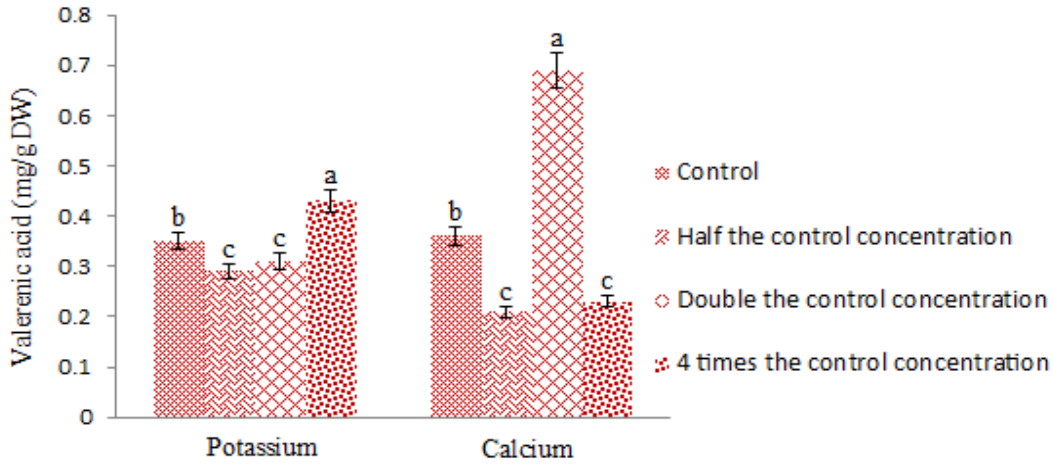

Fig.V.Valerenic acid accumulation in the hairy root cultures grown under the influence of various concentrations of Ca^{+2} and K^{+}; The bars represent means ± SE. Bars followed by different letters are significantly different ($p< 0.05$).

for the optimal valerenic acid production in *V. officinalis* hairy root cultures. The results showed that increasing concentration of KI in the MS medium tended to decrease the dry weight of roots. The reason for this might be that potassium was available by other medium compounds such as KNo_3. These results were in agreement with the study carried out by Moshtaghi et al. (2006).As already has been reported by Amdoun et al. (2009), potassium had a positive effect on alkaloids production by *Daturastramonium* L. Also the results of this study showed that high level (3.32 mg/l) of KI in the medium resulted in increased accumulation of valerenic acid. Potassium is vital to many plant processes such as enzyme activation, transport of sugars, water, and nutrient transport and starch synthesis. It plays many important regulatory roles in development and activates at least 60 different enzymes involved in plant growth (Moshtaghi et al., 2006). The effect of Potassium may be attributed to its role in activation of key enzymes in biosynthetic pathway.

The inclusion of 880 mg/l concentration of $CaCl_2$ in the medium (2-fold of what is present in normal MS medium) promoted the growth and valerenic acid accumulation in the hairy roots. In a recent study by Katrin et al. (2012) it was found that increase of the calcium content in the MS medium augments the scopolamine production in

Hyoscyamus niger root cultures, but the content of hyoscyamine decreased by increasing calcium concentration. Also treatment of hairy roots by high concentration of calcium caused a decrease in dry weight and valerenic acid production. This result is in agreement with the findings of Noor et al. (2013) who suggested that high calcium chloride in the growth medium caused a significant reduction in the relative growth rate of calluses and this was related to toxic effect of high Ca content on calluses growth. Lardet et al. (2007) showed that decrease in calcium concentration of the pre-cultured medium led to a drop in callus calcium content in *Heveabrasilinsis*. The result of this study is similar to previous studies that reported Ca/pectate interaction as a regulator of growth dominates the requirement for calcium ions, and as a factor involved in the control of cell growth (Noor et al., 2013). Also they concluded that increased growth rate of tissue culture may be the result of calcium effect on plant hormone activity that affected growth *in vitro.*

Calcium is needed for cell wall strengthening and provides protection against biotic and abiotic stress (Aranda et al., 2009). Root cultures of *Beta vulgaris* L. when exposed with high concentration of Ca^{+2} (2-6 folds of what is present in the MS basal medium) led to the enhancement of peroxidase (Thimmaraju et al., 2006). Enhancement of accumulation of secondary metabolites by Ca^{+2} could be attributed to its reported role as both nutrient and secondary signaling molecule in response to pathogen attacks (Lukasz et al., 2009). The present study showed that calcium at concentration of 3.32 mg/l augmented the valerenic acid, as an important metabolite in *V. officinalis* hairy roots. Thus this result can be utilized for the intensification of the valerenic acid production and manipulation of calcium content is recommended for MS medium optimization.

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