Journal of Medicinal Plants Research Vol. 5(20), pp. 4984-4992, 30 September, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

*Full Length Research Paper*

# Establishment of a practical tissue culture for producing hairy roots of *Valeriana officinalis* L. via *Agrobacterium rhizogenes*

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Accepted 3 March, 2011

**Valerian (*Valeriana officinalis* L.) is used in traditional medicine for its root or rhizome as a mild sedative and tranquilizer in many countries. In order to evaluate the potential production of hairy root of *V. officinalis* under *in vitro* conditions the regeneration of *V. officinalis* via indirect organogenesis and also Agrobacterium-mediated transformation was conducted. Leaves, stems and roots of *in vitro* plants were used as the explants for indirect organogenesis. The highest callus formation frequency was achieved on a medium supplemented with (0.1 mg/L Kin + 2 mg/L 2,4-D) for leaf and root explants (100%) and (0.1 mg/L Kin + 10 mg/L 2,4-D) for stem explants (100%). Furthermore, the highest percentage of shoot regeneration was obtained on a medium containing 0.5 mg/L NAA + 2 mg/L BAP (60.00%) in leaf-derived calli. Hairy roots of valerian were obtained following co-cultivation of leaf, stem and root-derived callus with *Agrobacterium rhizogenes* strains AR15834 and LBA 9402. As a result, the LBA9402 strain appeared to be better than the AR15834 strain in terms of both mean of hairy root formation (68.62%) and production of the respective hairy root (90%). The optimal growth of the hairy roots occurred on selective MS medium containing 20 mg/L rifampicin and 250 mg/L cefotaxime without any plant growth regulators. This study revealed that rapid growth of the hairy roots of *V. officinalis* may offer an attractive alternative to the exploitation of this valuable medicinal plant species via optimizing tissue culture technique.**

**Key words:** *Valeriana officinalis*, indirect organogenesis, *rolB* gene, *Agrobacterium rhizogenes,* hairy roots.

## INTRODUCTION

The genus Valeriana which encompasses nearly 200 perennial herb species throughout the world is mainly found in the temperate and cold regions. *Valeriana officinalis* is native to Europe and Asia. The genus also the America (*Valeriana edulis*) (Larsen, 1986). Valerian is widely used as a mild sedative and sleep aid for includes species used medicinally in India (*Valeriana wallichii*), Asia (*V. officinalis* var. *latifolia* - Kesso root) and insomnia, excitability, and exhaustion. It has depressant activities on the central nervous system and has been described as having equalizing effects, acting as a sedative in agitated states and a stimulant in fatigue (Fernandez et al., 2006). The long standing clinical use of valerian is as a sedative mainly attributed to valepotriates, their breakdown products baldrinals, valerenic acid, valerenal and valeranone, and other constituents in the essential oil (Nishiya et al., 1992; Bos et al., 1996). *V. officinalis* preparations are considered safe despite the known *in vitro* cytotoxic activity of valepotriates (Bos et al., 1998) and no acute side effects have been reported (Chan, 1998).

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**Abbreviations: 2, 4-D,** 2, 4-Dichlorophenoxyacetic acid; **BAP,** 6-benzylaminopurine; **IBA,** indole-3-butyricacid; **Kin,** kinetin; NAA, α-naphthaleneacetic acid; **PGR,** plant growth regulator(s); ***rolB*,** root locus B.

Biotechnological tools are important for multiplication and genetic improvement of the medicinal plants by adopting techniques such as *in vitro* regeneration and genetic transformations. Some researches are going for the application of plant transformation and genetic modification using *Agrobacterium rhizogenes*, in order to increase production of those secondary metabolites, which are naturally synthesized in the roots of the mother plant (Chilton et al., 1982; Tepfer, 1990). Hairy roots, transformed with *A. rhizogenes*, have been found to be suitable for the production of secondary metabolites with fast growth rate due to their stable and high productivity in hormone-free culture conditions (Giri and Narasu, 2000). In this context, many studies have been carried out in association with *in vitro* organogenesis of different species of Valeriana genus (Mathur et al., 1988; Enciso-Rodriguez, 1997; Kaur et al., 1999; Salles et al., 2002; Abdi and Khosh-Khui, 2007; Ekhteraei et al., 2010). Moreover, several successful reports have been presented in respect of genetic transformation of this valuable medicinal genus by *A. rhizogenes* method (Granicher et al., 1992, 1995; Banerjee et al., 1998; Caetano et al., 1999; Rahimi et al., 2008). In this research, we made an effort to optimize a practical tissue culture method and following genetic transformation by *A. rhizogenes* for evaluating potential production of hairy root of *V. officinalis* under *in vitro* conditions.

## MATERIALS AND METHODS

### Plant material

Seeds of *V. officinalis* were collected from a field (Isfahan province, Iran) at late August 2008. The seeds were surface-disinfected with 70% (v/v) ethanol for 1 min, treated with 2% (v/v) sodium hypochlorite solution (NaOCl) for 15 min, thoroughly rinsed with sterile distilled water 4 times, and were blot-dried inside a laminar hood. They were then germinated in sterile culture bottles each containing 25 ml of MS basal medium (Murashige and Skoog, 1962) with 0.7% (v/v) (g/L) agar and 3% (v/v) (g/L) sucrose and maintained at 25±1°C under the 16 h light/ 8 h dark photoperiod. The leaves (1 $cm^2$), stems (1 $cm^2$) and roots (1 $cm^2$) derived from 35 days old plantlets were used as explants.

### Media and culture condition for callus and shoot development

The culture media used throughout the experiments for optimizing of tissue culture consisted of full strength MS basal salts and $B_5$ vitamins supplemented with various concentrations and combinations of phytohormones. Callus induction was achieved by placing the mentioned explants on MS medium supplemented with 0.1 mg/L Kinetin (Kin) in combination with (0, 2, 5 and 10 mg/L) 2,4-dichlorophenoxyacetic acid (2,4-D). The obtained calli transferred to shoot regeneration medium composed of (0.1, 0.2 and 0.5 mg/L) α-naphthaleneacetic acid (NAA) either singly or in combination with (0.2, 0.5, 1 and 2 mg/L) 6-benzylaminopurine (BAP). The MS medium without plant growth regulators (PGRs) was used as control. The percentage of the explants producing calli and regenerated shoots (from calli) were determined after 15 days and 15 to 22 days onwards respectively. The efficiency of PGRs and their concentrations were recorded on the basis of visual observation, expressed as the percentage of callus formed and produced shoots from calli and the ineffective treatments were discontinued. The pH of all media adjusted to 5.7 before autoclaving at 121°C for 20 min. The cultures were kept at 25±1°C under cool-white light with a 16 h photoperiod (40 to 60 µmol/$m^2$/s) and subcultured on fresh media at 14 days interval.

### Root induction and acclimatization

Regenerated shoots were excised and transferred to the elongation medium containing hormone-free MS for 14 days (2 to 4 leaves stage).

For promoting development of the roots, the plantlets were transferred to MS medium supplemented with 2 mg/L indole-3-butyricacid (IBA). For acclimatization, rooted shoots were removed from culture, washed in water to remove agar residues and transplanted to pots filled with a mixture of pasteurized field soil, sand and perlite (1:1:1 by volume) in controlled conditions in a growth chamber set at 25±1°C and 70% relative humidity with a 16 h photoperiod (30 to 45 µmol/$m^2$/s) for 21 days.

### Statistical analysis

All experiments were laid out as a factorial experiment based on completely randomized design with three replications and each replicate were made by using 3 Petri dishes per medium each of which contained 10 explants. Also, for transformation experiment, each treatment consisted of 5 explants per Petri dish. Analysis of variance was calculated, and significance of differences between means was conducted using Duncan's multiple range test (Duncan, 1955) at P = 0.01. For data normalization, it given in percentages was subjected to arcsine ($\sqrt{X}$) transformation before statistical analysis.

### Bacterial strains

*A. rhizogenes* LBA9402 and AR15834 were used for plant transformation. These strains are resistant to rifampicin. Bacterium was grown in LB medium at appropriate temperatures (28°C) with shaking (180 rpm).

### Plant explants infection and hairy root multiplication

The leaf and stem segments (1 $cm^2$) and calli were immersed in a suspension of *A. rhizogenes* ($OD_{600}$ = 0.6-1) for 5 to 7 min with constant shaking, blotted with sterile filter paper to remove excess bacteria, and co-cultivated on MS solidified medium containing (0.1 mg/L NAA+1 mg/L BAP), (0.5 mg/L BAP+0.5 mg/L NAA+0.5 mg/L 2,4-D) and (hormone-free) for 2 days in darkness. Explants were then transferred onto selective medium containing 250 mg/L cefotaxime and 20 mg/L rifampicin, and subcultured onto fresh selective medium every 15 days. Un-inoculated explants were used as a control. After 28 days of culture, the number of produced roots on selective medium to the number of cultured explants was determined. For increasing the number of transformed roots, the above procedures were repeated two times. The elongated roots (1 cm) were excised and transferred to MS medium without PGRs for 15 days. All cultures were incubated at 25±1°C under a 16/8 h photoperiod.

### PCR analysis of transformants

Genomic DNA was extracted from young roots of putative

transgenic and non-transgenic (control) plants, by using the cetyl-trimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980). Integration of the *rolB* gene into the plant genome was confirmed by PCR analysis. The specific primers for extending 430 bp fragment of nucleotides *rolB* gene were designed and their homology checked through the BLAST search. The primers consisted of 20 nucleotides with the sequence of 5'-GCTCTTGCAGTGCTAGATTT-3' and 5'-GAAGGTGCAAGCTACCTCC-3' for the forward and reverse, respectively. PCR analysis was performed to confirm the presence of a 430 bp *rolB* gene fragment in the putative transformants. In order to amplify the *rolB* gene sequence, PCR was initiated by a hot start at 95°C for 5 min and amplified during 35 cycles at 95°C for 30 s, 56°C for 30 s, 72°C for 1.5 min and followed by a final extension step at 72°C for 5 to 10 min. The electrophoresis of the PCR products was performed on 1% agarose gel applying a constant voltage of 80 volts. The gel was subsequently stained in ethidium bromide solution and examined under UV light.

## RESULTS

### Callus induction and indirect shoot regeneration

After 14 to 21 days of culture initiation, most of the leaves, stem and roots became green and increased slightly in volume, and then compact callus was formed at the cut edges of the explants (Figure 1a, b and c). Significant and non-significant differences among main levels of Kin and 2, 4-D concentrations and their interactions for callus induction have been represented in Table 1. Usually when interaction effects are significant, we have ignored for mean comparison of mean effects (Table 1). Signifying interaction between medium × explant showed that, different explants have varying response to callus induction in different levels of 2,4-D concentrations ($P<0.01$). Moreover, the highest callus formation frequency was achieved on a medium supplemented with (0.1 mg/L Kin + 2 mg/L 2, 4-D) for leaf and root explants (100%) and (0.1 mg/L Kin + 10 mg/L 2, 4-D) for stem explants (100%) (Table 2). Overall, the best medium for callus induction was 10 mg/L 2, 4-D (94.47%).

According to Table 3, the response of calli to shoot regeneration were influenced by concentrations of NAA and BAP tested ($P<0.01$). The highest percentage of shoot regeneration was obtained on a medium containing 0.5 mg/L NAA + 2 mg/L BAP (60.00%) in leaf calli (Table 4). On the other hand, stem explants showed positive morphogenetic response and readily developed multiple shoots compared with other explants in response to BAP concentrations. Formation of initial primordia producing shoots were indirectly occurred from the calli within 15 days after culture in different concentrations of NAA and BAP and subsequently normal shoots were developed after 22 to 29 days (Figure 1d). Regenerated shoots were transferred to medium lacking growth regulators and then MS medium supplemented with 2 mg/L IBA when at 2 to 4 leaves stage. Rooting of regenerants was observed after 14 to 21 days of culture on this medium.

Most rooted plantlets survived after 21 days in the controlled conditions. Consequently, the plantlets were transferred to soil pots (Figure 1e, f, g and h).

### Analysis of putative transformed hairy roots

Regarding most of the explants showed necrosis symptoms in response to infection with *A. rhizogenes* strains, the calli were used for this purpose. Results of analysis of variance showed that, there was a statistical difference among calli, media and strains and their interactions except the medium×strain interaction ($P<0.01$) (Table 5). The highest percentage of putative transformed hairy roots was achieved on (MS+20 mg/L rifampicin and 250 mg/L cefotaxime) as 90 and 81.25% for LBA9402 and AR15834 strains, respectively. In this respect, the best explants responding transformation with LBA9402 and AR15834 strains were leaf and root calli, respectively (Table 6). In general, there was a significant difference between *A. rhizogenes* strains in order that, LBA9402 strain was more efficient for transferring of *rolB* gene to valerian than AR15834 strain. Also, the same pattern was observed in context of production of hairy roots for other factors including calli and media which was used in this study (Table 7). After 28 days, some of the explants produced hairy roots after two co-cultivations on the selective medium (Figure 2a, b, c and d). Emerging hairy roots was not observed for the non-transformed control plants in medium without any PGRs. Observation of hairy roots phenotype in putative transgenic plantlets can be initial approval on transformation, but although some of hairy roots are escaped and probably non-transformed. By reason, PCR analysis should be performed with specific primers to confirm transformation of produced hairy roots.

### PCR analysis

Genomic DNA of putative transgenic and non-transgenic (control) regenerated hairy roots were analyzed by PCR for presence of *rol*B gene, using specific primers. The *rol*B gene was amplified using specific primers yielded fragments of 430 bp, using DNA of transgenic hairy roots as template. The putative transgenic which was grown on selectable medium contain antibiotic, first check for presence of Ti plasmid (escaped bacteria) with PCR via *vir*C gene primers. Then, the putative lines which did not result in a positive band were screened and checked by PCR for specific *rol*B gene. However, no amplification was observed in the control hairy roots, with the primers (Figure 3a and b). Presence of sharp bands in positive control confirmed that the specific primers were annealed with our construct properly (Figure 3a and b Lane P). PCR analysis of four plantlets for both strains showed that, 2 plantlets contained the *rolB* gene with 430 bp fragment (Figure 3a and b and Lane 1 and 3).

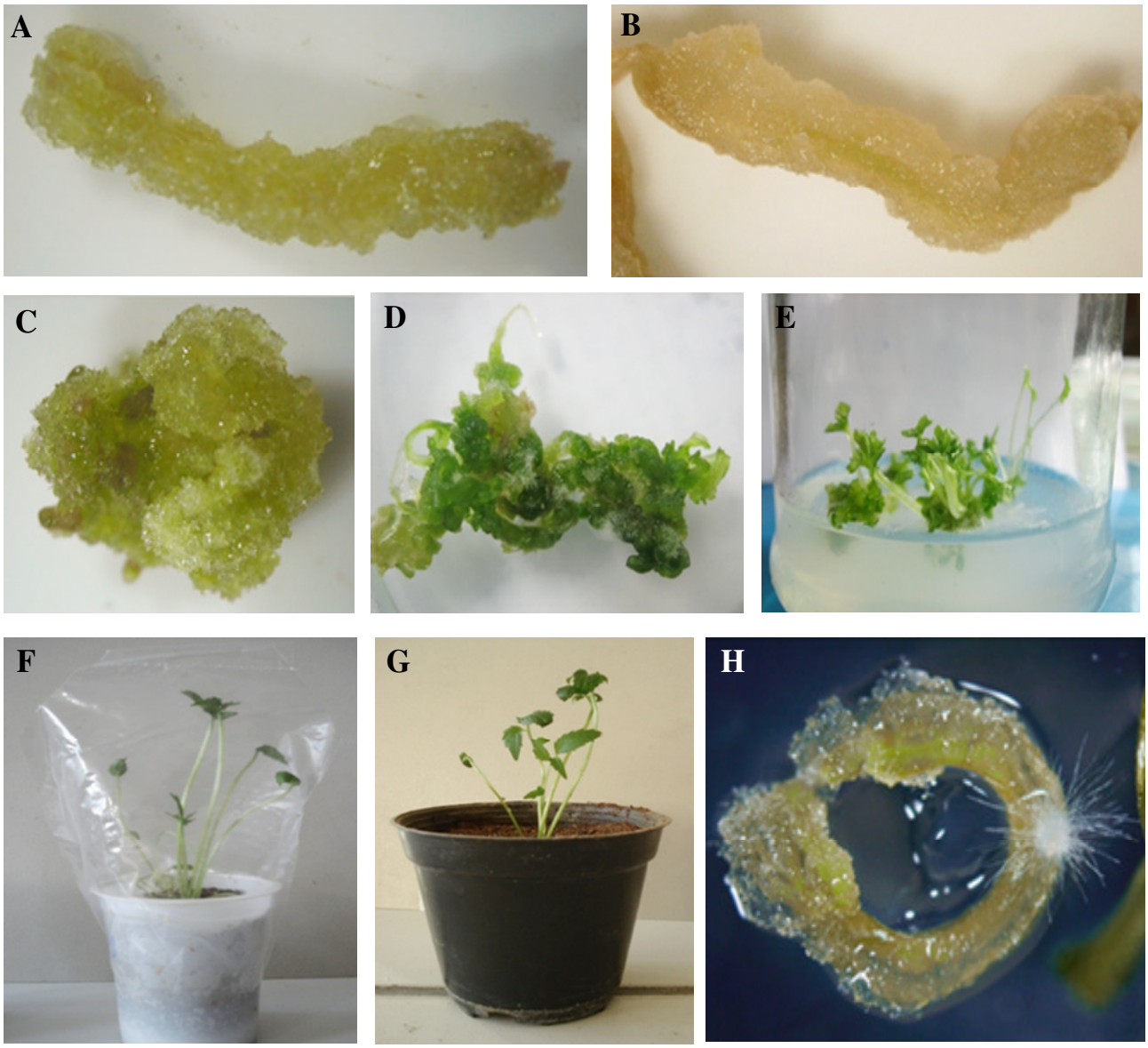


**Figure 1.** Different stages of indirect organogenesis in valerian. (A) Callus formation after 15 days of culture of valerian on the medium supplemented with 0.1 mg/l Kin and 2 mg/l 2,4-D concentrations from stem, (B) root and (C) leaf explants, (D) callus-derived regenerated shoots within 22 days after culture in 0.5 mg/l NAA and 2 mg/l BAP concentrations, (E) transferring of regenerated shoots to MS medium supplemented with 2 mg/l IBA for rooting, (F) and (G) transferring of rooted plantlets to soil pots under controlled conditions after 28 days, (H) emerging of roots in stem explant after 15 days.

**Table 1.** Analysis of variance for callus induction of *V. officinalis.*

| S. O. V | df | MS |
|---|---|---|
| Medium (A) | 3 | 7.9659 ** |
| Explant (B) | 2 | 0.0036 ** |
| AB | 6 | 5.17 ** |
| Error | 24 | 0.001 |
| CV (%) | | 1.5 |

ns: Non-significant, * and **: significant at 0.05 and 0.01 probability levels, respectively; S. O. V: source of variance, df: degree of freedom, MS: mean square CV: coefficient of variance.

**Table 2.** Effect of different concentrations of Kin and 2, 4-D on callus induction from leaf, stem and root explants of *V. officinalis* after 20 days of culture.

| Growth regulator concentrations (mg/L) | | Explant producing callus (%) | | |
|---|---|---|---|---|
| Kin | 2, 4-D | Leaf | Stem | Root |
| 0.1 | 0 | $0^{c}$ | $0^{c}$ | $0^{c}$ |
| 0.1 | 2 | $100^{a}$ | $83.3^{b}$ | $100^{a}$ |
| 0.1 | 5 | $100^{a}$ | $91.2^{b}$ | $100^{a}$ |
| 0.1 | 10 | $91.6^{b}$ | $100^{a}$ | $100^{a}$ |

Values within a column followed by different letters are significantly different at the 0.01 probability level, analyzed by Duncan's multiple range test.

**Table 3.** Analysis of variance for shoot regeneration of *V. officinalis.*

| S. O. V | df | MS |
|---|---|---|
| Explant (A) | 2 | 6.952** |
| NAA (B) | 2 | $0.034^{ns}$ |
| BAP (C) | 3 | 1.408** |
| AB | 4 | 0.807* |
| AC | 6 | 0.782* |
| BC | 6 | 1.711** |
| ABC | 12 | 2.342** |
| Error | 72 | 0.26 |
| CV (%) | | 23 |

ns: Non-significant, * and **: significant at 0.05 and 0.01 probability levels, respectively; S. O. V: source of variance, df: degree of freedom, MS: mean square CV: coefficient of variance.

**Table 4.** Effect of different concentrations of NAA and BAP on indirect shoot regeneration from leaf, stem and root calli of *V. officinalis* after 15 to 22 days of culture.

| Growth regulator concentrations (mg/L) | | Explant producing shoots (%) | | |
|---|---|---|---|---|
| NAA | BAP | Leaf | Stem | Root |
| 0.1 | 0.2 | $6.67^{h}$ | $0.00^{i}$ | $0.00^{i}$ |
| | 0.5 | $13.33^{g}$ | $13.33^{g}$ | $6.67^{h}$ |
| | 1 | $30.00^{e}$ | $46.67^{c}$ | $6.67^{h}$ |
| | 2 | $13.33^{g}$ | $6.67^{h}$ | $0.00^{i}$ |
| 0.2 | 0.2 | $13.33^{g}$ | $20.00^{f}$ | $0.00^{i}$ |
| | 0.5 | $13.33^{g}$ | $13.33^{g}$ | $0.00^{i}$ |
| | 1 | $0.00^{i}$ | $6.67^{h}$ | $0.00^{i}$ |
| | 2 | $30.00^{e}$ | $30.00^{e}$ | $6.67^{h}$ |
| 0.5 | 0.2 | $0.00^{i}$ | $13.33^{g}$ | $0.00^{i}$ |
| | 0.5 | $13.33^{g}$ | $0.00^{i}$ | $0.00^{i}$ |
| | 1 | $20.00^{f}$ | $40.00^{d}$ | $0.00^{i}$ |
| | 2 | $60.00^{a}$ | $50.00^{b}$ | $0.00^{i}$ |

Values within a column followed by different letters are significantly different at the 0.01 probability level, analyzed by Duncan's multiple range test

## DISCUSSION

Numerous factors are reported to influence the success of *in vitro* propagation of different medicinal plants and therefore, it is unwise to define any particular reason for the general micropropagation of medicinal plants

**Table 5.** Analysis of variance for transformed roots of valerian in response to transformation with *rolB* gene in selective medium.

| S.O.V | df | MS |
|---|---|---|
| Callus (A) | 2 | 0.148** |
| Medium (B) | 2 | 0.487** |
| Strain © | 1 | 0.2** |
| AB | 4 | 0.119** |
| AC | 2 | 0.048** |
| BC | 2 | 0.01 |
| ABC | 4 | 0.3** |
| Error | 36 | 0.007 |
| CV (%) | | 12.75 |

ns: non-significant, **: significant 0.01 probability level; S. O. V: source of variance, df: degree of freedom, MS: mean square, CV: coefficient of variance.

**Table 6.** Effect of different concentrations of NAA and BAP on transformed hairy roots of valerian.

| Growth regulator concentrations (mg/L) | | Strain | Explant producing hairy roots (%) | | |
|---|---|---|---|---|---|
| NAA | BAP | | Leaf | Stem | Root |
| 0 | 0 | $LBA_{9402}$ | $90^{a}$ | $85.92^{b}$ | $91.66^{a}$ |
| | | $AR_{15834}$ | $81.25^{b}$ | $75.00^{c}$ | $66.66^{d}$ |
| 0.1 | 1 | $LBA_{9402}$ | $79.16^{b}$ | $44.40^{f}$ | $66.66^{d}$ |
| | | $AR_{15834}$ | $63.05^{d}$ | $64.44^{d}$ | $45.45^{f}$ |
| NAA | BAP | Strain | Leaf | Stem | Root |
| 0.5 | 0.5 | $LBA_{9402}$ | $73.86^{c}$ | $40.00^{g}$ | $45.83^{f}$ |
| | | $AR_{15834}$ | $58.33^{e}$ | $36.10^{g}$ | $41.66^{f}$ |

Values within a column followed by different letters are significantly different at the 0.01 probability level, analyzed by Duncan's multiple range test.

**Table 7.** Response of studied factors to genetic transformation of valerian by *rolB* gene.

| Types of strains | Mean production of hairy roots |
|---|---|
| $LBA_{9402}$ | $68.62^{a}$ |
| $AR_{15834}$ | $59.23^{b}$ |
| Types of media | Mean production of hairy roots |
| MS free hormones | $81.75^{a}$ |
| MS+0.1 mg/L NAA+1 mg/L BAP | $60.50^{b}$ |
| MS+0.5 mg/L NAA+0.5 mg/L BAP+0.5 mg/L 2, 4-D | $49.30^{c}$ |
| Types of calli | Mean production of hairy roots |
| Leaf | $74.27^{a}$ |
| Root | $59.60^{b}$ |
| Stem | $57.8^{b}$ |

Values within a column followed by different letters are significantly different at the 0.01 probability level, analyzed by Duncan's multiple range test.

(Rout et al., 2000). The influence of PGRs and their interactions, as one of the most important factors, on tissue culture of different medicinal plant species have been described in detail. Rani et al. (2003) reported that the best medium for callus formation from hypocotyl, cotyledon and root explants of *Withania somnifera* L. achieved on MS medium supplemented with 0.2 mg/L Kin and 2 mg/L 2, 4-D as 100%. In another study, 2, 4-D (0.5

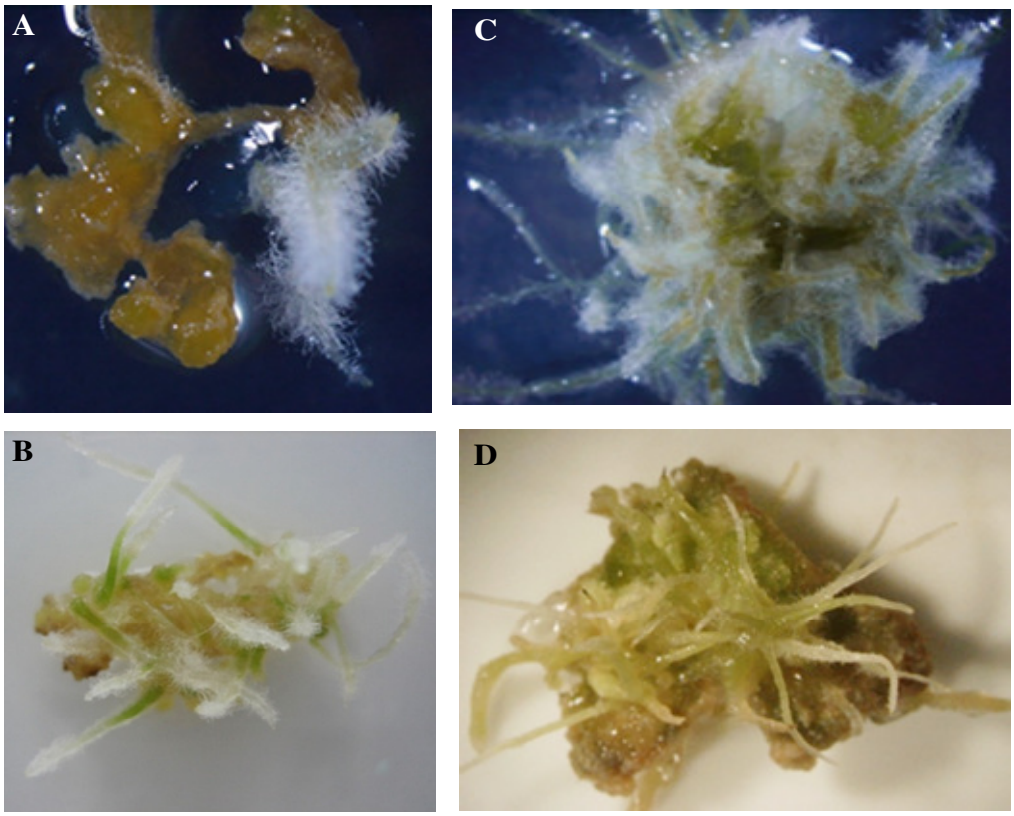


**Figure 2.** Putative transformed hairy roots of valerian that infected using LBA9402 and AR15834 strains of *Agrobacterium rhizogenes* on the selective medium containing 20 mg/L rifampicin. Hint: (A) and (B) production of putative hairy roots that presumably transformed infected using AR15834 strain; (C) and (D) many putative hairy roots using LBA9402 strain.

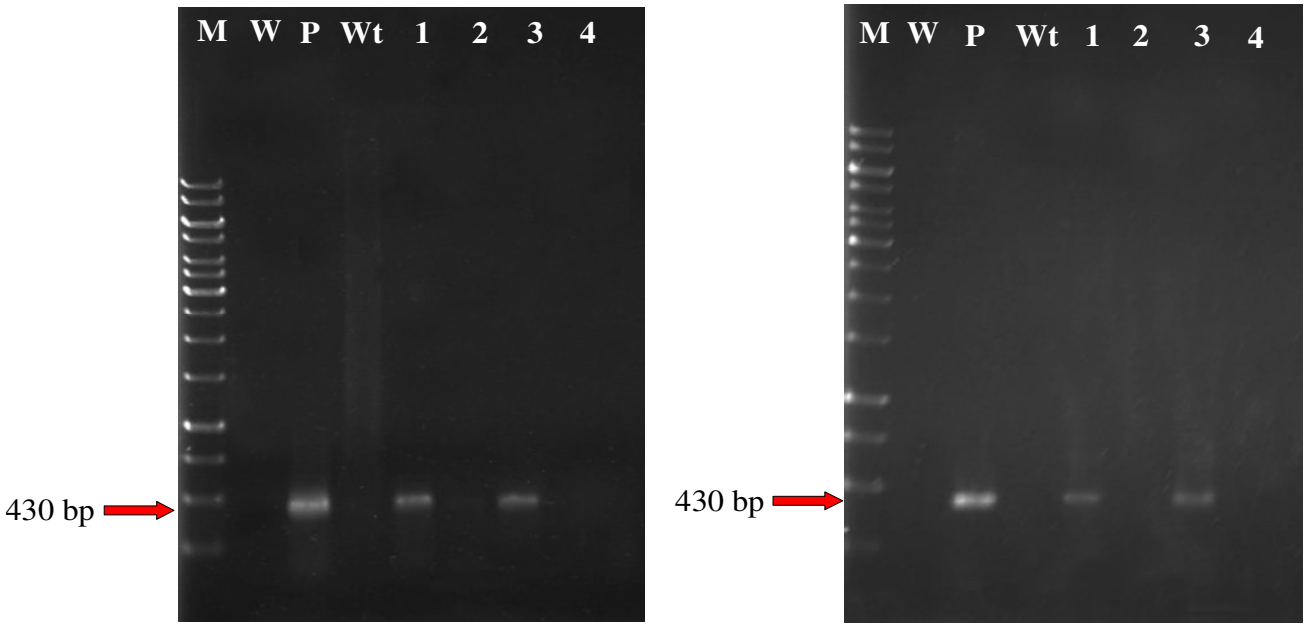


**Figure 3.** PCR analysis of genomic DNA from hairy roots of valerian on a 1% agarose gel (A), (B) Amplification of a 430 bp *rolB* gene fragments transformed with LBA9402 and AR15834 strains, respectively. Lane M: DNA size marker (100 bp ladder), Lane W: negative control (Water), Lane P: positive control (plasmid containing *rolB* gene), Lane Wt: negative control (DNA from non-transformed plant), Lanes 1 to 4: DNA from the plants that survived infection, respectively.

to 2 mg/L) introduced as one of the most important auxins in callus induction of *V. edulis* ssp. *procea* (Castillo et al., 2000) and *Leonurus heterophylus* (Yang et al., 2008). In the current study, combination of Kin (0.1 mg/L) and 2, 4-D (2 mg/L) for callus induction from leaf and root explants was more effective than other concentrations which was in agreement with these reports.

Besides, in this investigation the highest percentage of shoot regeneration was attained on a medium containing 0.5 mg/L NAA+2 mg/L BAP (60.00%) in leaf calli. It seems that, BAP phytohormone (1 to 2 mg/L) has a great potential for shoot regeneration of valerian compared with other cytokinins. Abdi and Khosh-Khui (2007) stated that, the maximum multiple shoot regeneration of *V. officinalis* was occurred on medium containing 1 mg/L BAP and also the numbers of shoots induced per explant were higher in the presence of 1 mg/L BAP in leaf explant. They found that among the cytokinins that they tested, BAP gave better result compared to Kin in shoot induction. The effective response of plantlets to 1 mg/L BAP during the micropropagation of other *Valerianaceae* species (Kaur et al., 1999) and other medicinal plants such as *Echinacea purpurea* L. (Koroch et al., 2002) has also been reported. In respect of rooting of shoots, we observed that rooting had the better response on MS medium without any PGRs compared to MS medium supplemented with 2 mg/L IBA after 21 days. It seems that rooting in absence of auxins may be attributed to endogenous auxin hormones in the plant. This results was reported previously too, by other researchers in detail for other medicinal plants (Sudhersan and Hussan, 2003; Lu, 2005).

As previously described, transformed roots are able to regenerate whole viable plants; hairy roots as well as the plants regenerated from hairy roots are genetically stable. Plants regenerated from Ri transformed roots display 'hairy root syndrome,' combined expression of the *rolABC* loci of the Ri plasmid is responsible for this expression. In addition, the growth medium has a significant effect on hairy root induction (Giri and Narasu, 2000). In present study, we observed superiority of LBA9402 strain for transferring of *rolB* gene to valerian compared with AR15834 strain in all explants. Similar observations in this context have been reported by the authors (Giri et al., 2001; Li and Leung, 2003). In contrast, in other *Valerianaceae* species such as *V. wallichii* LBA9402 strain was less efficient for hairy roots formation (Banerjee et al., 1998). Also, Ercan et al. (1999) reported that AR15834 strain had the highest percentage of hairy roots in hypocotyl explant of *Rubia tinctorum*. Giri et al. (1997) proposed that different strains of *A. rhizogenes* vary in their transforming ability and hairy root induction.

One of the important results of the current study was optimal emerging of hairy roots on the selective medium without adding PGRs. While, non-transformed hairy roots (control) did not grow in this medium. Many reports has been represented which supported accuracy of this result in other medicinal plants (Lu et al., 2008; Peng et al., 2008). Mehrotra et al. (2008) stated that application of auxins such as IAA in low concentrations (0.1 mg/L) to the medium promoted production of hairy roots of *Glycyrrhiza glabra*. On the other hand, it seems that applying higher concentrations of exogenous auxins in medium lead to increase of adventitious hairy roots by inducing cell division on surroundings area of the roots. It can be avoided activity of apical meristem due to high accumulation of auxins (Finlayson et al., 1996). PCR analysis is one of the current methods to confirm initial transformation of a plant. Regarding observation of hairy root phenotype in the calli, it can be said that *rolB* gene has been transferred into valerian genome and thus, a 430 bp fragment on the gel was expected to extend. However, additional analyses such as southern blot should be performed to determine copy numbers of the transgene. Regarding little attention was paid to the technical aspects of tissue culture and genetic transformation of valerian, a practical approach that established in the present research can be used for enhancing hairy roots as continuous source for the production of valuable secondary metabolites of the plant.

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