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Direct embryogenesis of Valerian (*Valeriana officinalis* L.) using leaf segments

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Abstract

A simple procedure for direct embryogenesis without an intervening callus production was developed in valerian (*Valeriana officinalis* L.), using the leaf segments. Direct somatic embryogenesis was induced using half-strength Murashige and Skoog (MS) medium supplemented with 2, 4 dichlorophenoxyacetic acid (0.5mg L^{-1}), glutamic acid (100mg L^{-1}), 4% sucrose and 8g L^{-1} agar. Embryo germination to form plantlets enhanced on MS medium supplemented with naphthalene acetic acid (0.1mg L^{-1}) and kinetin (2mg L^{-1}). Regenerated plants with well-developed root and shoot systems were successfully transferred to greenhouse.

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Introduction

Valerian (*Valeriana officinalis* L.) is an important medicinal plant which grows in several geographic areas of the world including Iran. The genus *Valeriana* encompasses nearly 250 species, found mainly in temperate zone regions. Plants of this genus have sedative, antispasmodic and ansiolitic properties mainly due to their production of Iridoidesters known as valepotriates, in the plant rhizomes (Hiller and Zetler 1996). *Valeriana officinalis* is the most commercially important species in the genus *Valeriana* for its secondary metabolites such as aromatic oil and valepotriates (Bos *et al*., 1998).

Considering its importance in the medicinal world (O'Hara *et al*., 1998) concentrated efforts are being made for its improvement using biotechnological approaches. In order to large-scale production, plantation and development of new varieties of *V. officinalis* with higher levels of secondary metabolites, establishment of an efficient induction and propagation system is necessary.

Plant regeneration has been described from shoot tip and axillary bud explants, from callus and from embryo-like structures derived from suspension cultures of *Valeriana wallichii* (Mathur *et al*., 1988; Mathur and Ahuia, 1991; Mathur, 1992), from adventitious shoots, seedlings, callus and also suspension cultures of *Valeriana edulis* ssp. *procera* (Enciso-Rodriguez, 1997; Castillo *et al*., 2000) and from shoot buds of *Valeriana jatamansi* (Kaur *et al*., 1999). Somatic embryogenesis is an efficient and high volume propagation system for the large number of plants within a short period. Successful genetic transformation attempts have mostly employed embryogenic callus or cell cultures as the target tissue in several medicinal plants (Tripathi and Tripathi, 2003). However, a major limitation of this callus system is the repeated subculture to select embryogenic callus portions among highly proliferating non-embryogenic tissue. This process is not highly producible and furthermore increases the chance of somaclonal variation.

As these limitations have become unavoidable, strategies to improve plant regeneration must necessarily include manipulation of the medium to embark upon new morphogenetic pathways (Pedroso and Pais, 1995). Direct somatic embryogenesis offers several advantages in medicinal plant improvement, as cost effective and large-scale clonal propagation is possible using bioreactors, ultimately leading to automation of somatic seed production and development of artificial seeds. Besides, such a system could provide a new source for use in genetic transformations.

The plant derived from direct somatic embryogenesis usually is unicellular in origin and hence genetically uniform (Pedroso and Pais, 1995). The leaf segments of valerian are an excellent source for the induction of indirect embryogenesis and the factors affecting this process had been studied (Castillo *et al.,* 2000) and direct somatic embryogenesis and factors controlling it have been studied in many plant species (Pedroso and Pais, 1995).

Although there are some report on shoot organogenesis and somatic embryogenesis of *Valeriana* genus (Chen *et al*., 2014), however, to the best of our knowledge there is no report about direct somatic embryogenesis in *Valeriana officinalis*.

The aim of this study was to establish a method for asexual multiplication of *Valeriana officinalis* through direct somatic embryogenesis.

Materials and methods

Plant material and cultures methods

Fresh leaves of valerian were collected from 4-month old greenhouse-grown plants (Fig. 1a). They were washed with tap water and a few drops of Rica (a commercial detergent). Then, they were surface sterilized in 70% ETOH for 1 min and rinsed twice with sterile distilled water. After that the leaves were immersed in a solution of 1.5% sodium hypochlorite for 10 min and rinsed four times with sterile distilled water. The leaves were cut into 7-8mm^2 segments and transferred to 150mL glass jars with 25ml of half-strength Murashige and Skoog (1962) (MS) medium.

After preliminary experiments, different MS media. Were supplemented with 0.5mg l^{-1} 2,4 dichlorop henoxyacetic acid (2,4-D), 0.5mg l^{-1} Naphthalene acetic acid (NAA), 100mg L^{-1} glutamic acid (Glu) or different concentrations of sucrose (3, 4, and 5%) (Table 1.). The pH of media was adjusted to 5.8 by 0.1 N HCl before.

Autoclaving for 15 min at 121°C and 1.5 kg cm^2 pressure. Cultures were placed initially under the dark condition for 2 weeks and thereafter they were maintained at 25±3°C under 16 h photoperiod provided by cool white fluorescent lamps (45 µmol m^{-2} S^{-1}) with relative humidity of 75-85%. Table 1.

Table 1. Different media used for induction of direct somatic embryogenesis in *Valeriana officinalis* L.

	PGR and additives (mg l^{-1})												
	M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8	M_9	M_{10}	M_{11}	M_{12}	M_{13}
NAA	-	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5
2,4-D	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5	-
Glutamic acid	-	-	-	100	100	-	-	100	100	-	-	100	100
Sucrose (%)	3	3	3	3	3	4	4	4	4	5	5	5	5

Embryo germination

For embryo germination 2 separate experiments were conducted. In the first experiment, the explants that formed embryo were divided to 4 segments (each fragment was about 0.2±0.03 g) and transferred to MS medium containing NAA (0.5, 1, 2, 5mg L^{-1}), gibberellic acid (GA_3) (0.5, 1 and 2mg L^{-1}), MS without growth regulators and different.

Combinations of NAA (0.1, 0.2 mg L^{-1}) and kinetin (1.5 and 2mg L^{-1}). In the second experiment, embryos were transferred individually to MS medium supplemented with NAA (0.1 and mg L^{-1}) and Kin (1.5 and 2mg L^{-1}) where the embryos were germinated and rootsandshoots were produced.

Acclimatization of regenerated plants

The 4-cm plantlets were transferred to small pots containing 1/3 vermiculite, 1/3 perlite and 1/3 sand (v/v). The pots were placed in transparency box and maintained under 25±5°C temperatures and 70% relative humidity for 4 weeks and then transferred to greenhouse.

Experiment design and data analysis

The experiment was conducted as a completely randomized design in a factorial arrangement with 4 replications and each replicate with 12 explants. The means were compared with Duncan new multiple range test (DNMRT) at 5% probability level. To determine the efficiency of embryo induction medium, responsive leaves that formed embryo was recorded after 4 weeks.

For both embryo germination experiments the percent of germinated embryo was recorded after 3 weeks and the number of plantlets was recorded 6 weeks after culture. Plantlets were recognized when they developed roots and shoots.

Results and discussion

Embryo induction media

The leaf segments showed swelling and initiation of small embryo-like structures in the 10-13 days after culture (Fig. 1b). In the following weeks, embryogenic clumps were visible at the cut end and surface of the explants (Fig. 1c and d).

Embryoied formation in cut edges of leaf explants was higher than other parts of leaves. Well-developed embryos were observed all over the cultured explants within four weeks of culture. Embryogenesis response in different media according to the media composition was different.

When glutamic acid and sucrose (4%) were added to medium, embryogenesis response increased. Maximum embryogenic response of leaf explants (57.21±2.7%) was observed on M_3 medium supplemented with 2, 4-D, 4% sucrose, and 100mg l^{-1} glutamic acid. Addition of NAA instead of 2, 4-D decreased percentage of explants response in M_9 (43.31±2.33%). Increasing sucrose concentration more than 4% reduced embryogenic response on M_{12} (25.28±1.24%), and M_{13} (13.39 ±2.28%) (Fig 1).

Fig. 1. Effects of different treatments on in vitro culture of valerian, (A) 4-month old greenhouse-grown plant of valerian, (B) Initiation of small embryo-like structures in the 10 to 13 day after culture in induction media, (C) and (D) Embryo formation in top and cut end of valerian leaf explant after 4 weeks. (E) Callus initiated in M2 medium with great potential for regeneration. (F) Germination of embryo mass in medium supplemented with 2 mg l-1 Kin and 0.1 mg l-1NAA.

The explants on M_2 and M_3 media did not exhibit any embryogenic response. However, explants on these media initially showed slight swelling and subsequently resulted to callus production.

M_2 showed higher. Percentage of callus proliferation compared to M_3 (data not shown). Callus initiated in M_2 medium had a great potential for regeneration (Fig. 1e). The explants on M_1 medium did not exhibit any response.

Embryo germination

In various supplements, somatic embryo germination response varied greatly (Table 2). MS without growth regulators showed low response to embryo germination. In this treatment, germination percentage per number of embryoid pieces and also number of plantlet per pieces was not considerable (Table 2). MS+NAA (0.5, 1, and 2mg l^{-1}) led to rhizogenesis of somatic embryos (Fig. 2A). The inclusion of GA_3 (0.5, 1 and 2 mg l^{-1}) in the germination medium increased the germination percentage and consequently plantlets with both well-developed shoots and roots. The combination of Kin-NAA enhanced somatic embryo germination percentage more than other treatments. Among Kin-NAA treatments, 2mg l^{-1} Kin and 0.1mg l^{-1} NAA showed high frequency of embryos and also plantlet formation. Embryo transferred individually to MS medium containing NAA (0.1mg l^{-1}) and Kin (2mg l^{-1}) showed considerable germination percentage and plantlet formation (Table 4) (Fig. 2b, c and d). Table 2, 3 and 4 here

Table 2. Effects of various supplements on germination of *Valeriana officinalis* L. embryos.

Media	Response
MS+ without growth regulators	Germination and plantlet production was low. Rhizogenesis.
MS+ NAA	Plantlets with both well-developed shoots and roots.
MS+ Kin and NAA	Plantlets with both well-developed shoots and roots
MS+GA_3	with vigorous growth of plantlets.

Table 3. Effects of various supplements on frequency and plantlet/embryo mass in *Valeriana officinalis* L.

PGR	Concentration (mgl^{-1})	Explants response (%)	Plantlet per each embryo mass
Without PGR	-	13.71g[z]	1.27+0.1e
GA_3 (mg l^{-1})	0.5	23.24f	2.71+0.01d
	1.0	38.00d	3.00+0.10c
	2.0	31.70e	2.71+0.11d
Kin+NAA (mg l^{-1})			
	1.5+0.1	43.37d	3.21+0.12c
	1.5+0.2	37.75d	4.00+0.02b
	2.0+0.1	61.32a	5.21+0.12a
	2.0+0.2	51.37b	4.27+0.13b

*Means in each column with similar letters are not significantly different at 5% level of probability using DNMRT.

Table 4. Effects of growth regulators on percentage of embryo germination and number of plantlets.

PGR (mgl^{-1}) Kin	NAA	Explant response (%)	Plantlet per each embryo mass
1.5	0.1	49+1.2c[z]	23bc
	0.2	41+1.73d	19c
2.0	0.1	78+3.21a	37a
	0.2	58+2.41b	25.6b

*Means in each column with similar letters are not significantly different at 5% level of probability using DNMRT.

Fig. 2. Effects of different treatments on in vitro culture of valerian (A) Rhizogenesis response of embryo in medium containing NAA. (B) Individual embryos in medium containing 2mg l^{-1} Kin and 0.1mg l^{-1} NAA. (C) Well germinated embryos in medium containing 2mg l^{-1} Kin and 0.1mg l^{-1} NAA. (D)Plantlets with developed shoots and roots obtained from embryo germination.

The induction and development of *in vitro* somatic embryos comprise complex processes. The composition of the basal culture medium, the type and levels of plant growth regulators (PGR), the carbon sources and the balance of organic and inorganic nitrogen sources are key factors. On the other hand, amino acids are primary nitrogen sources, including Glu, in order to stimulate the cellular growth and the communication between cells and tissues (Young *et al.*, 1999). The productions of direct somatic embryos on leaf explants of *V. officinalis* are possible by the addition of the Glu to the culture medium and increasing the sucrose to 4%. Absence of any callus formation indicated that the process of embryo development was direct. Glu is the main nitrogen source in the metabolism, being also precursor of Glutamine, and a nitrogen source for purins and pirimidins biosynthesis (Bohinski, 1991). This could explain the enhanced rate of induction and development of somatic embryos in the present investigation. Additionally, during the metabolism and protein synthesis the nitrogen originated from amino acids is quickly assimilated into carbonic skeletons (Lea, 1993). The somatic embryos in this study were formed more in the cut edges of leaf explants. Probably this may be due to increase in embryogenic competence of wounded tissue due to changes at the level of endogenous growth regulators (Ivanova *et al.*, 1994). This study also demonstrated that 2, 4-D is a suitable plant growth regulator than NAA for direct embryo induction in valerian. The conversion of somatic embryos to plantlets is a multi-step process. In many embryo genetic systems the transfer of somatic embryos to a culture medium free of PGR enhances the development of the somatic embryos and their conversion to plantlets.

One of the probable determinative factors for the low rates of somatic embryo conversion to plantlets is associated with the residual effects of 2, 4-D. Prolonged expositions to this PGR normally reduces the conversion and increases the number of abnormal somatic embryos (Cruz *et al*., 1990). In this study transferring the embryos to the MS medium without growth regulators did not lead to the embryo germination. When somatic embryos of *V. officinalis* were culture in medium supplemented with Kin-NAA and GA_3, the number of plantlets increased. The positive role of cytokinins may be related to reversion of negative effects caused by 2, 4-D to the cultures (Parrot *et al*., 1988). The role of the GA_3 in promoting the germination of somatic embryos is well documented in other embryogenic systems (Deng and Comu, 1992). Our results are in agreement with the findings of Castillo *et al.* (2000) who used combination of Kin-NAA for embryo germination in *Valeriana edulis* ssp Procera. In conclusion, the induction of direct somatic embryogenesis in valerian using leaf segments as described in this study could be useful in rapid propagation of the elite plant which has best characters for medicinal purposes. Furthermore, direct embryogenesis can be beneficial for gene transformation via particle bombardment or *Agrobacterium* in short time, avoiding somaclonal variation.

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