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Embryogenesis in Valerian (*Valeriana officinalis* L.) using leaf segments

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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Abstract: A protocol for direct embryogenesis induction without an intervening callus production was developed in valerian *(Valeriana officinalis* L.) using the leaf segments. Direct somatic embryogenesis was induced using by half-strength MS medium supplemented with 2,4-D (0.5 mg·l^{-1}), glutamic acid (100 mg·l^{-1}), 4% sucrose and 8 g·l^{-1} agar. The embryo germination and plantlet formation were enhanced on MS medium supplemented with NAA (0.1 mg·l^{-1}) and Kin (2 mg·l^{-1}). Regenerated plants with well-developed root and shoot systems were successfully (72%) transferred to the greenhouse.

1. Introduction

Valerian (*Valeriana officinalis* L.) is an herb belongs to Valerianaceae family which grows in several geographic areas of the world including Iran. The genus *Valeriana* encompasses nearly 250 species, found mainly in temperate zone regions. Some species of this genus have high economic demand in Iran and also, around the world. Plants of this genus have sedative, antispasmodic and anxiolytic properties mainly due to the production of iriod esters, known as valepotriates, in the plant rhizomes (Hiller and Zetler, 1996). The conventional methods of propagation of valerian are from seed and rhizome. Considering the importance of the genus *Valeriana* in the medicinal world (O'Hara *et al.,* 1998) concentrated efforts are being made to improve the propagation using biotechnological approaches. Plant regeneration has been described from shoot tip, axillary bud explants, via callus and embryo-like structures derived through suspension cultures of *V. wallichii* (Mathur *et al.*, 1988; Mathur and Ahuia, 1991; Mathur, 1992). Also, plant regeneration from adventitious shoots, seedlings, suspension cultures of *V. edulis* (Enciso-Rodriguez, 1997; Castillo-España *et al.,* 2000), shoot regeneration from leaf segments of *V. officinalis* (Abdi and Khosh-khui, 2007), shoot organogenesis and somatic embryogenesis from leaf explants of *V. jatamansi* (Rong *et al.*, 2014) and from shoot buds of *V. jatamansi* (Kaur *et al.,* 1999) and shoot

organogenesis and somatic embryogenesis from leaf explants of *Valeriana jatamansi* Jones have been described (Chen *et al.*, 2014). 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 (Leena and Jaindra, 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. 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-España *et al.,* 2000). Direct somatic embryogenesis and factors controlling it have been studied in many plant species (Pedroso and Pais, 1995; Chen *et al.,* 1999; Desai *et al.,* 2004; Kuo *et al.,* 2005; Quiroz-Figueroa *et al.,* 2006; Thengane *et al.*, 2006; Jayanthi *et al.,* 2011). Based on our knowledge, there is no report about direct somatic embryogenesis on *Valeriana officinalis.* The aim of this study was to establish a method for asexual multiplication of *Valeriana officinalis* through direct somatic embryogenesis.

2. Materials and Methods

Plant material and culture 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). They were surface sterilized by 70% Ethyle Alcohol for 1 min. and rinsed twice with sterile distilled water. 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 7-8 mm^2 segments and transferred to 150 ml glass jars with 25 ml of half-strength MS medium (Murashige and Skoog, 1962). The pH of media was adjusted to 5.8 by 0.1 N HC1 before autoclaving for 15 min at 121°C and 1.5 $kg \cdot cm^{-2}$ 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 $\mu mol \cdot m^{-2} \cdot S^{-1}$) with relative humidity of 75-85%.

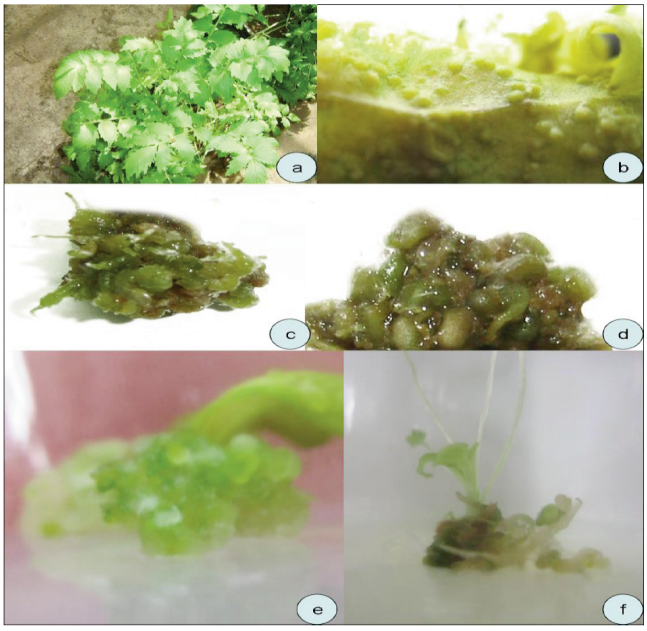

Fig. 1 - Effects of different treatments on *in vitro* culture of valerian, (A) 4-month old greenhouse-grown plant of valerian, (B) Initiation of small embryo-like structures in the 10 to 13 day after culture in induction media, (C) and (D) Embryo formation in top and cut end of valerian leaf explant 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^{-1} NAA.

Embryo induction and germination

For embryo induction, after preliminary experiments, MS media were supplemented with 0.5 $mg \cdot l^{-1}$ 2,4-D, 0.5 $mg \cdot l^{-1}$ Naphthalene acetic acid (NAA), 100 $mg \cdot l^{-1}$ glutamic acid (Glu) or different concentrations of sucrose (3, 4, and 5%) (Table 1). For embryo germination, two 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 and 5 $mg \cdot l^{-1}$), gibberellic acid (GA_3) (0.5, 1 and 2 $mg \cdot l^{-1}$), or MS added to different combinations of NAA (0.1 and 0.2 $mg \cdot l^{-1}$) and

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

PGR	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
Response (%)	-	-	-	12.4±0.3	11.21±1.1	5.28±0.1	3.4±0.03	57.21±2.7	43.31±2.33	9.8±0.15	8.8±0.1	25.28±1.24	13.39±2.28

kinetin (1.5 and 2 mg·l^{-1}). In the second experiment, embryos were transferred individually to MS medium supplemented with NAA (0.1 and 0.2 mg·l^{-1}) and kinetin (1.5 and 2 mg·l^{-1}) to stimulate the germination and roots and shoots development.

Acclimatization

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.

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's 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 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.

3. Results

Embryo induction

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). Embryoid 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. Different medium showed different embryogenesis response. When Glu and sucrose (4%) were added to medium, embryogenesis response increased. Maximum embryogenic response of leaf explants (57.21±2.7%) was observed on M_8 medium supplemented with 2, 4-D, 4% sucrose, and 100 mg·l^{-1} glutamic acid. NAA addition instead of 2, 4-D decreased percentage of explants response in M_9 (43.31±2.33%) medium. Increasing sucrose concentration more than 4% reduced embryogenic response on M_{12} (25.28±1.24%), and M_{13} (13.39±2.28%) (Table 1).

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

For embryo germination experiment, two kinds of explants were used. In the first experiment, the explants that formed embryo were divided to 4 segments (each fragment was about 0.2±0.03 g) and used as mass embryo. In the second experiment the embryos were transferred individually to MS medium supplemented with various supplements. Somatic embryo germination response varied greatly in various hormone supplements (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 2 mg·l^{-1}) led to rooting of somatic embryos (Fig. 2A). The inclusion of GA_{33} (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, 2 mg·l^{-1} Kin and 0.1 mg·l^{-1} NAA showed high frequency of embryos and also plantlet formation (Table 3). The somatic embryos were easily isolated with a pair of forceps, each developing into a single plant. The highest recovery

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
MS + NAA	Rhizogenesis
MS + Kin and NAA	Plantlets with both well-developed shoots and roots
MS +GA_3	Plantlets with both well-developed shoots and roots with vigorous growth of plantlets

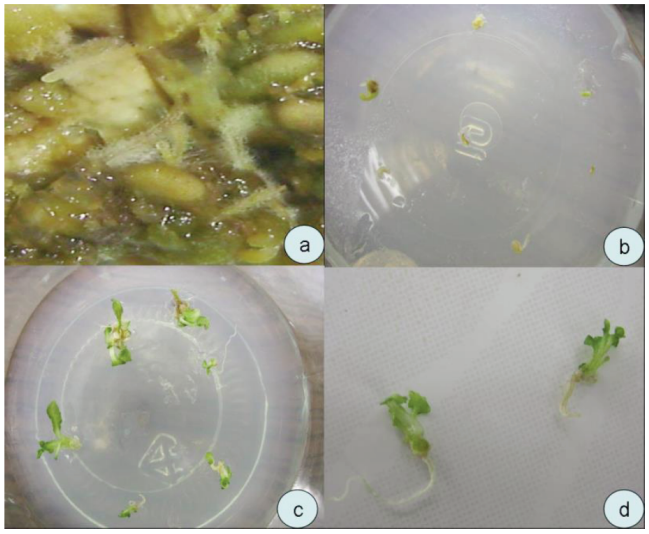

Fig. 2 - Effects of different treatments on *in vitro* culture of valerian (A) Rhizogenesis response of embryo in medium containing NAA. (B) Individual embryos in medium containing 2 mg l^{-1} Kin and 0.1 mg l^{-1} NAA. (C) Well germinated embryos in medium containing 2 mg l^{-1} Kin and 0.1 mg l^{-1}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

of somatic embryos (78+3.21%) was possible on MS medium supplemented with NAA (0.1 mg·l^{-1}) and Kin (2 mg·l^{-1}), this condition showed considerable germination percentage and plantlet formation while the fewest somatic embryos germination and developing into a single plant (41+1.73%) was observed in MS medium supplemented with a low concentration of NAA (0.1 mg·l^{-1}) and Kin (1.5 mg·l^{-1}) (Table 4).

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

Plant growth regulator	Explants response (%)	Plantlet per each embryo mass
PGR free	13.71 g	1.27+0.1 e
GA3 (mg·l^{-1})		
0.5	23.24 f	2.71+0.01 d
1.0	38.00 d	3.00+0.10 c
2.0	31.70 e	2.71+0.11 d
Kin+NAA (mg·l^{-1})		
1.5+0.1	43.37 d	3.21+0.12 c
1.5+0.2	37.75 d	4.00+0.02 b
2.0+0.1	61.32 a	5.21+0.12 a
2.0+0.2	51.37 b	4.27+0.13 b

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

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

4. Discussion and Conclusions

Using different plant tissue culture technique and plantlet regeneration have been reported in different species of Valeriana genus, such as *V. officinalis* (Abdi and Khosh-Khui, 2007; Abdi *et al.*, 2008; Reza *et al.*, 2009), *Valeriana wallichii* (Mathur *et al.*, 1988), *Valeriana edulis ssp. procera* (Enciso-Rodriguez, 1997), *V. jatamansi* (Kaur *et al.*, 1999; Das *et al.*, 2013), *Valeriana glechomifolia* (Salles *et al.*, 2002; Bello de Carvalho *et al.*, 2004), *Valeriana glechomifolia* (Bello de Carvalho *et al.*, 2004). However, direct somatic embryogenesis have never been reported from any explant of the species. In this study, the effects of different plant growth regulators (PGRs) and different supplement on the induction of direct somatic embryogenesis were investigated. We established an efficient embryo induction, somatic embryogenesis and plant regeneration system from leaf explants using various supplement and different types and concentrations of PGRs. The induction and development of *in vitro* somatic embryos comprise complex processes including cell division, differentiation, growth, and pattern formation (Capron *et al.*, 2009)., the composition of the basal culture medium, the type and levels of plant growth regulators (PGR), the level of carbon sources and the balance of organic and inorganic nitrogen sources are key factors for *in vitro* embryogenesis system. Primary nitrogen sources, including Glu, is very important in plant tissue culture in order to stimulate the cellular growth

and the connection 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 level to 4%. Absence of any callus formation indicated that the process of embryo development was direct. Glutamate occupied a central role in amino acid metabolism in plant. It can form by action of the glutamate synthase utilizing glutamine (Bohinski, 1991). Glutamine as a nitrogen source for purins and pirimidins biosynthesis, was significantly stimulated the direct embryogenesis in *Valeriana officinalis* in this study. This finding is in parallel with the results of Chowdhry *et al.* (1993), Gex *et al.* (2006) and Shahsavari (2011). 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). This stimulation suggested that organic nitrogen was a growth-limiting factor in Valerian cultures and the inclusion of glutamine decreased the culture lag phase, which indicated that glutamine was much more readily assailable than inorganic nitrogen. The somatic embryos in this study were formed more in the cut edges of leaf explants. Increasing in embryogenic competence of wounded tissue probably was related to the endogenous growth regulators changes in leaf tissue (Ivanova *et al.,* 1994). This study also demonstrated that 2, 4-D is more efficient than NAA to induce somatic embryos formation in valerian. Shoot organogenesis and somatic embryogenesis were also reported in cucumber in the presence of 2,4-D and NAA (Kuijpers *et al.*, 1996) and the same results were reported in Cassava leaf explants by Sofiari *et al.* (1997). Plant tissue studies on *V. edulis* revealed that 2,4-D induced somatic embryos while NAA induced shoots (Castillo-España *et al.,* 2000). Increasing the level of the sucrose from 3% to 4% showed a positive effect in embryo induction, while the induction response decreased at the presence of 5% sucrose in the culture medium. Similar results have been also reported for the other plant species (Luo *et al.,* 1996; Biahoua and Bonneau, 1999; Nakagawa *et al.,* 2001) Sucrose as common carbohydrate in the phloem sap of many plants have affects in plant tissue culture and the formation of somatic embryos in culture medium (Luo *et al.,* 1996; Nakagawa *et al.,* 2001) for this reason adding high concentration of sucrose can enhance the somatic embryos induction (Luo *et al.*, 1996; Nakagawa *et al.,* 2001). The positive effect of sucrose may related to the vital function of the sucrose such as controlling the several developmental processes in the cells (Gibson, 2000; Smeekens, 2000), nutritional function (serve as a carbon source during somatic embryogenesis) and osmotic regulator or cell osmolarity (Biahoua and Bonneau, 1999). The positive role of sucrose in the present study may be interpreted as both nutritional and osmotic regulatory functions of this carbohydrate. The conversion of somatic embryos to plantlets is a multi-step process. In many embryogenetic systems the transfer of somatic embryos into PGR free culture medium enhances the development of the somatic embryos and their conversion to plantlets. One of the 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). 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 Cornu, 1992). Our results are in agreement with the findings of Castillo-España *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. In addition, the identified protocol does not seem induce a proliferation of callus before the differentiation of somatic embryos. Regenerated plants with well-developed root and shoot systems were successfully (72%) transferred to greenhouse.

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