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Plant regeneration via somatic embryogenesis and organogenesis in *Verbascum speciosum* Schard.

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Abstract

Plant regeneration was achieved in *Verbascum speciosum* Schard. via organogenesis and somatic embryogenesis by culture of mature embryo explants. Two types of calli, embryogenic and non-embryogenic, were induced from mature embryo explants on Murashige and Skoog (MS) medium supplemented with different concentrations of benzyl adenine (BA) and α-naphthalene acetic acid (NAA). In order to further proliferate the somatic embryoids, the yellow and friable embryogenic calli were transferred on MS medium containing 0.5 mg^{-1} charchol and 0.1 or 1 mg^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) or into MS medium containing 60 g^{-1} sucrose, 50 mgl^{-1} casein hydrolysate (CH), 0.5 mg^{-1} kinetin (Kin), 5 mg^{-1} 2,4-D and 0.5 mg^{-1} charchol. Among the 3 tested media, MS medium containing 0.1 mg^{-1} 2,4-D and 0.5 mg^{-1} charchol was more effective for proliferation of embryonic calli. Somatic embryos were transferred to hormone free MS medium for maturation and shoot regeneration. In addition, shoots and roots regenerated from non-embryogenic calli in hormone free MS medium or containing NAA and BA. Shoot buds were obtained from non-embryogenic calli and they were transferred to MS medium supplemented with 1 mg^{-1} BA or Kin for further growth and multiplication. Regenerated plants then were potted and maintained in the greenhouse.

***Keywords*:** embryogenic calli, shoot and root regeneration, *Verbascum speciosum*

Introduction

The genus *Verbascum* L. belongs to the tribe Verbasceae (Valdes, 1987) of the family Scrophulariaceae and Turkey, Iran and Pakistan are the main centers of its diversity (Zohary, 1973; Huber-Morath, 1978; Valdes, 1987). It is the largest genus within the large family of Scrophulariaceae includes 360 species throughout the world (Judd et al., 2002) and 42 species in Iran (Sharifnia, 2007). Among the species distributed in Iran, 15 species are endemic (Sharifnia, 2007). As hybridization is a very frequent phenomenon within *Verbascum* (Huber-Morath, 1978; Karaveliogullari et al., 2004), a high frequency of morphological variations has been an intricate in delimiting the species. The *Verbascum* species are adapted to the various habitats and different regions of Iran including rocky mountains, open forests, road sides and the margins of the rivers. The genus *Verbascum* is known for the presence of variety of compounds and many investigations led to isolation of saponins (Hartleb and Seifert, 1994), alkaloids (Dranarov and Hais, 1997), flavonoids (Afifi et al., 1993) and phenylethanoids (Akdemir et al., 2004).

Plant tissue culture is one of the most important conservation techniques because it offers great potential for rapid cloning from a minimum amount of plant material. In addition, it plays a key role in the production of plant material required for different purposes such as breeding, genetic and biotechnological research, and the acquisition of industrial raw material (Endress, 1994; Sahai et al., 2010). Although there are a few reports on the organogenesis and plantlet formation from leaf, petiole and root explants in *Verbascum thapsus* L. (Caruso, 1971; Turker et al., 2001), to date there is no report on tissue culture of *V. speciosum* Schard. Here we report a protocol for organogenesis and somatic embryogenesis from mature embryo culture of *V. speciosum.* This protocol can enable us to produce enough plant material of the species required for different purposes such as breeding, genetic and biotechnological researches.

Materials and Methods

Callus induction

Plants of *Verbascum speciosum* were collected at fruiting phase from Hamedan Province, west of Iran in July to August 2011. Voucher specimens

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were deposited in the herbarium of Bu-Ali Sina University (BASU), Hamedan, Iran. Different explants including leaves, petioles and capsules were washed with tap water, surface disinfested in 70% (v/v) EtOH and 5% sodium hypochlorite for 15 min, and then rinsed three times in sterile distilled water. Then mature embryos were dissected from the capsules. Different explants were cultured on MS basal medium (Murashige and Skoog, 1962) supplemented with 0, 2.5 and 5 mgl^{-1} BA; and 0, 1.5 and 3 mgl^{-1} NAA along with 3% (w/v) sucrose and 1% (w/v) agar. The pH of all media was adjusted to 5.7-5.8 before sterilization by autoclaving at 121°C for 15 min. All cultures were kept at 5°C for 48 h and then incubated at 25 ± 2°C with 3000 lux light intensity from fluorescence cool tube light in culture room for 16 h. Reports of explants producing calli were recorded after 4 weeks and the calli were subcultured on fresh MS medium after 30 days of culture. All experiments were carried out in 3 replicates and 8 explants were used for each replication.

Embryogenic callus production

After 3 subcultures some embryonic regions produced on the calli in the media containing different concentrations of NAA and BA. Production of embryogenic regions was increased by further subcultures.

Embryogenic callus proliferation

In order to further multiplication the somatic embryoids, pieces of yellow and friable embryonic calli, produced in some concentrations of BA and NAA on the 30th day of culture, were transferred into 3 new media including 1) MS medium containing 0.1 mgl^{-1} 2,4-D along with 3% (w/v) sucrose and 0.5 mgl^{-1} charchol, 2) 1 mgl^{-1} 2,4-D along with 3% (w/v) sucrose and 0.5 mgl^{-1} charchol, and 3) MS medium containing 60 gl^{-1} sucrose, 50 mgl^{-1} CH, 0.5 mgl^{-1} Kin, 5 mgl^{-1} 2,4-D and 0.5 mgl^{-1} charchol. The data were recorded after 6 weeks of culture. Then, the embryonic calli with somatic embryos in earlier stages were transferred into hormone free MS medium for development of somatic embryos and shoot bud initiation.

Shoot and root multiplication

Shoot buds induced from non-embryogenic calli on MS medium containing different concentrations of BA and NAA, were transferred to MS medium supplemented with 1 mgl^{-1} BA or Kin for further growth and multiplication and data were recorded after 5 weeks. For root proliferation and plant regeneration, shoots along with thin roots were transferred to rooting medium comprises 3 mgl^{-1} NAA.

Plant acclimatization

For acclimatization, healthy plantlets with well-developed roots and shoots were chosen. They were removed from the culture media, washed in water to remove agar, and then transferred into the pots containing sterilized soil and perlite (1:1). The transplanted plants were covered with clear plastic bags to maintain humidity, placed in an acclimatization chamber, and watered with quarter strength MS mineral salt solution with 4 day intervals. After 2 weeks, the plastic bags were opened and the uncovered plants were maintained under natural day light conditions at 19-23°C in the laboratory. The survival frequency of the *in vitro* propagated plants was evaluated at the end of the 5th week of the acclimatization process.

Statistical analysis

Eight embryo explants or calli were used for each experiment in triplicates. The statistically significance of difference between means was estimated at the 5 percent level ($P < 0.05$) by the Completely Randomized Design.

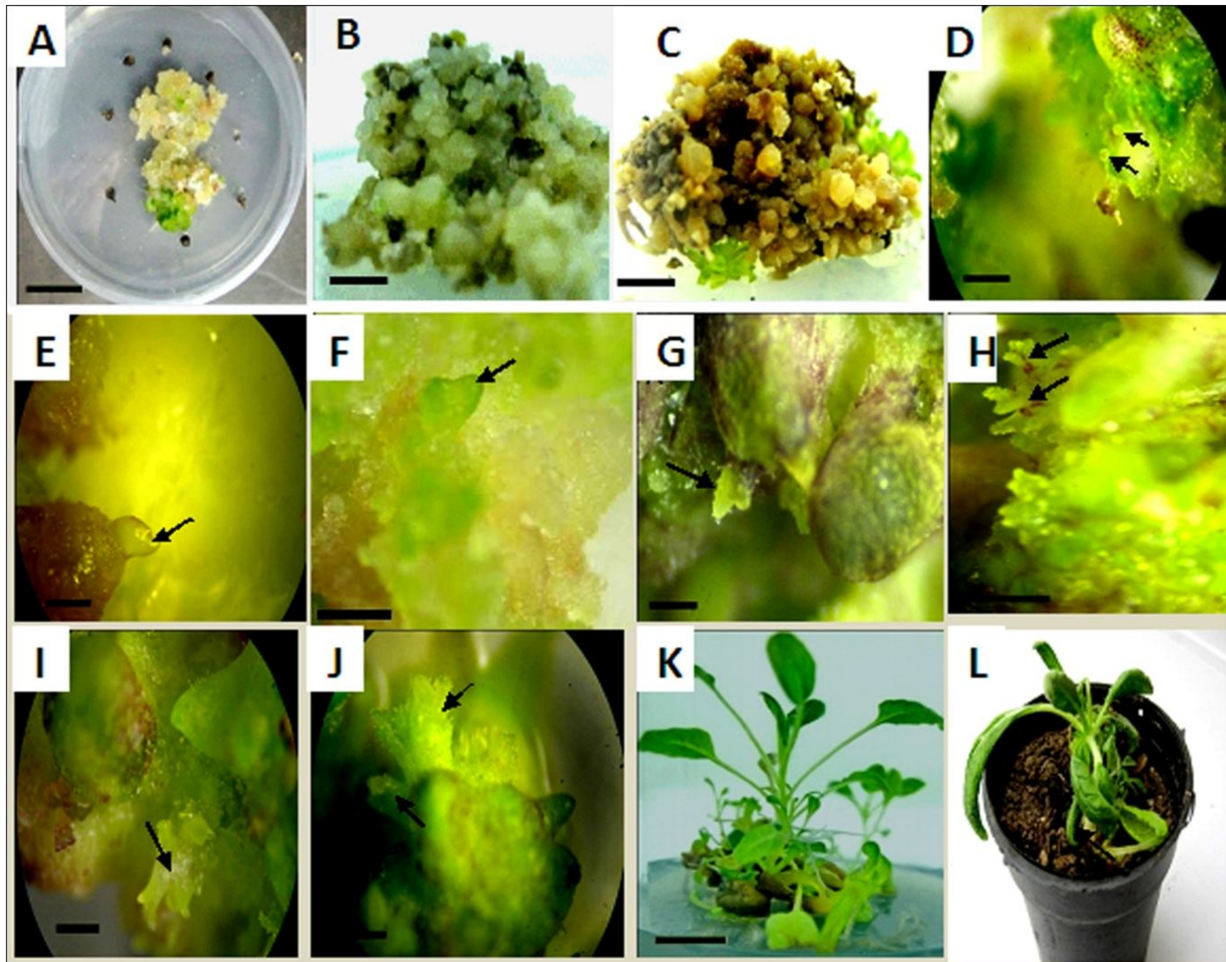

Figure 1. Somatic embryogenesis and plantlet regeneration from mature embryo explants in *Verbascum speciosum*. (A) Embryogenic callus induction in MS medium containing 2.5 mgl^{-1} BA without NAA after 6 weeks. (B) Embryogenic callus proliferation in MS medium containing 0.5 mgl^{-1} Kin, 5 mgl^{-1}, 2,4-D and 50 mgl^{-1} CH. (C) Embryogenic callus proliferation in MS medium containing 0.1 mgl^{-1} 2,4-D (Scale bar = 5 mm). (D) Embryogenic callus with globular somatic embryos in MS medium containing 0.1 mgl^{-1} 2,4-D (arrows). (E, F) Heart-shaped somatic embryos in hormone free MS medium (arrows). (G, H) Somatic embryos at torpedo stage in growth regulator free MS medium (arrows). (I, J) Somatic embryos at cotyledonary stage in hormone free MS medium (arrows). (K) Plant regeneration in hormone free MS medium (Scale bar = 10 mm). (L). Potted plant.

Results

Callus induction

Results showed that among 9 combinations of BA and NAA and different explants which were used, callus induction was successful only from mature embryo explants in 4 treatments. The highest frequency of non-embryogenic callus initiation was obtained in MS medium containing 1.5 mgl^{-1} NAA or 2.5 mgl^{-1} BA (table 1). However, the growth of calli formed at 1.5 mgl^{-1} NAA without BA was no longer promoted, but the produced adventitious roots vigorously. Calli which were induced on MS medium containing 2.5 mgl^{-1} BA, were heterogeneous and had morphogenetic potential. In addition, production of calli decreased by increasing BA. In the presence of only one growth regulator, NAA was more effective in root and BA in shoot regeneration. In MS medium without hormones both of shoots and roots were formed directly from the explants (table 2).

Embryogenic callus production

Two types of calli, embryogenic and non-embryogenic, were induced from mature embryo explants, which were distinguished well by their surface, color and texture. Non-embrogenic calli were soft, yellowish white and friable. Then, the calli turned green, hard, and compact with morphogenetic potential, while their peripheral portions remained yellowish white and friable as non-embryogenic calli after 10 weeks of culture. Embryogenic calli induction was observed only in MS medium supplemented with 2.5 mgl^{-1} BA without NAA (table 1). Somatic embryos were

observed on yellowish green embryonic calli after subculturing of the calli in the same fresh medium.

Embryogenic callus proliferation

The growth of yellow and embryonic friable calli was promoted by transferring them into new MS medium containing 0.1 or 1 mgl^{-1} 2,4-D and 0.5 mgl^{-1} charchol. Embryogenesis was increased significantly by 0.1 more than that by 1 mgl^{-1} 2,4-D (Table 3). The compact yellowish white calli spontaneously formed embryo-like structures (embryoids). However, no further differentiation was achieved in the embryos (figure 1).

In addition, transferring the embrogenic calli in new MS medium containing 60 gl^{-1} sucrose, 0.5 mgl^{-1} Kin, 5 mgl^{-1} 2,4-D, 50 mgl^{-1} CH and 0.5 mgl^{-1} charchol resulted in promotion of their growth, when they were transferred from MS medium supplemented with 2.5 mgl^{-1} BA without or 1.5 mgl^{-1} NAA (Table 3). The somatic embryos passed through each of the typical developmental stages in a growth regulator free MS medium after 8 weeks of culture, followed by conversion to plantlets (figure 1).

Shoot and root multiplication

Shoot formation was induced from non-embryogenic calli. Transferring of the shoot buds into MS medium supplemented with 1 mgl^{-1} BA or Kin resulted in further growth and proliferation of them after 5 weeks of culture (figure 2). Results showed that Kin was found to be more effective than BA in shoot proliferation and the number of shoots per explant was higher in the presence of Kin when compared to BA, whereas the nodular structures started to differentiate into an organized structure of shoots. In addition BA had no effect on root induction. The highest frequency of shoot induction in the presence of 1 mgl^{-1} Kin was obtained, when the shoot buds were transferred from MS medium supplemented with 1.5 mgl^{-1} NAA and 2.5 mgl^{-1} BA. Root formation and plantlet regeneration were also observed in the same medium (table 4).

Plant acclimatization

Well-developed and rooted plantlets were transferred to sterile soil with perlite for acclimatization and covered with plastic bags to ensure high humidity around the plants. After 2 weeks, the plastic bags were removed and the plants were transferred to greenhouse. In the 5th week of acclimatization, all of the plantlets survived and showed normal growth (figures 1 and 2).

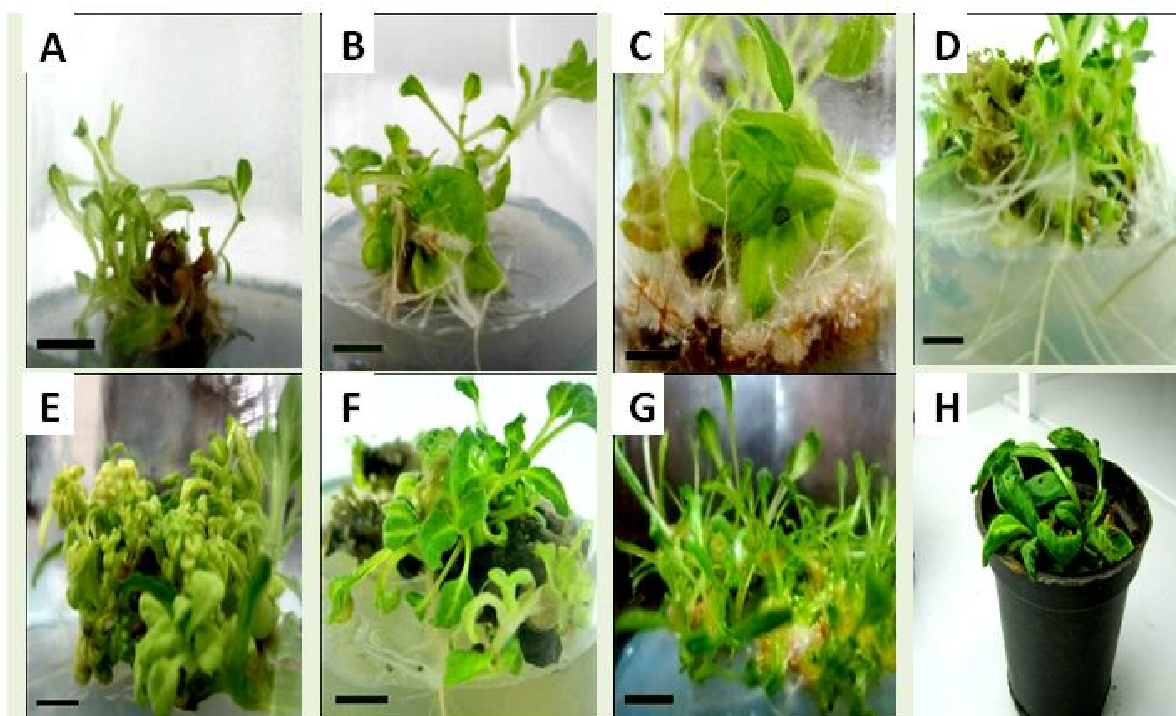

Figure 2. Shoot multiplication and plant regeneration in *Verbascum speciosium*. (A) Shoot regeneration on MS medium containing 1 mgl^{-1} each of BA and NAA after 6 weeks. (B, C) Root regeneration from the shoots on MS medium containing 3 mgl^{-1} NAA. (D, E) Shoot multiplication on MS medium containing 1 mgl^{-1} BA after 5 weeks. (F, G) Shoot multiplication on MS medium containing 1 mgl^{-1} Kin after 5 weeks (Scale bar = 10 mm). (H) Potted plant.

Table 1. Effect of various concentrations of BA and NAA on callus induction in *Verbascum speciosum* after 4 weeks.

Treatment (mgl^{-1})	Callus (%)	Embryogenic callus (%)
BA 0 + NAA 0	0^{c}	0^{b}
BA 0 + NAA 1.5	50^{a}	0^{b}
BA 0 + NAA 3	0^{c}	0^{b}
BA 2.5 + NAA 0	45.83^{ab}	16.67^{a}
BA 2.5 + NAA 1.5	41.66^{ab}	0^{b}
BA 2.5 + NAA 3	0^{c}	0^{b}
BA 5 + NAA 0	25^{b}	0^{b}
BA 5 + NAA 1.5	0^{c}	0^{b}
BA 5 + NAA 3	0^{c}	0^{b}

Each number is the mean of 3 experiments with 8 replicates per experiment. Means with the same letters within columns are not significantly different at $p < 0.05$.

Table 2. Effect of various concentrations of BA and NAA on shoot and root regeneration in *Verbascum speciosum* after 6 weeks.

Treatment (mgl^{-1})	Shoot (%)	Root (%)
BA 0 + NAA 0	37.5^{a}	37.5^{a}
BA 0 + NAA 1.5	0^{c}	50^{a}
BA 0 + NAA 3	0^{c}	0^{c}
BA 2.5 + NAA 0	29.16^{a}	0^{c}
BA 2.5 + NAA 1.5	20.83^{ab}	37.5^{a}
BA 2.5 + NAA 3	0^{c}	0^{c}
BA 5 + NAA 0	12.5^{ab}	0^{c}
BA 5 + NAA 1.5	0^{c}	0^{c}
BA 5 + NAA 3	0^{c}	0^{c}

Each number is the mean of 3 experiments with 8 replicates per experiment. Means with the same letters within columns are not significantly different at $p < 0.05$.

Table 3. Effect of 2,4-D, Kin and CH on embryogenic callus proliferation in *Verbascum speciosum* after 6 weeks.

Pretreatment (mgl^{-1})	1 mgl^{-1} 2,4-D	0.1 mgl^{-1} 2,4-D	0.5 mgl^{-1} Kin + 5 mgl^{-1} 2,4-D + 50 mgl^{-1} CH
BA 2.5 + NAA 0	65.33^{a}	66.44^{b}	66.7^{a}
BA 2.5 + NAA 1.5	66.44^{a}	88.89^{a}	77.7^{a}
BA 5 + NAA 0	0^{b}	100^{a}	0^{b}

Each number is the mean of 3 experiments with 8 replicates per experiment. Means with the same letters within columns are not significantly different at $p < 0.05$.

Table 4. Effect of 1 mgl^{-1} BA or Kin on shoot multiplication in *Verbascum speciosum* after 5 weeks.

Pretreatment	BA (1 mgl^{-1})			Kin (1 mgl^{-1})		
(mgl^{-1})	Shoot (%)	Root (%)	Plant (%)	Shoot (%)	Root (%)	Plant (%)
BA 2.5 + NAA 0	34.44^{b}	0	0	100^{a}	0^{b}	0^{b}
BA 2.5 + NAA 1.5	66.44^{a}	0	0	100^{a}	88.67^{a}	100^{a}
BA 5 + NAA 0	33.22^{b}	0	0	100^{a}	0^{b}	0^{b}

Each number is the mean of 3 experiments with 8 replicates per experiment. Means with the same letters within columns are not significantly different at $p < 0.05$.

Discussion

Among different explants used in this experiment, calli were induced only on mature embryos. Two types of calli, embryogenic and non-embryogenic, were induced on MS medium supplemented with different concentrations of BA and NAA. Calli grown in the medium containing 2.5 mgl^{-1} BA were fast growing, with yellowish green and compact appearance. Those grown in 5 mgl^{-1} BA were however slow growing, with creamy-white and soft. These findings are inconsistent with the role of BA in callus induction in *V. thapsus* (Turker et al., 2001).

Somatic embryogenesis from various explants has been reported in many plant species (Williams and Maheswaran, 1986). It has been reported that growth and morphogenesis are controlled by the types and concentrations of plant growth regulators and the types of explants have also a considerable role in callus induction and somatic embryogenesis (Luo et al., 1999; Fatima et al., 2009). Results from present study showed that the choice of explants are crucial for inducing calli in *V. speciosum* and calli were not induced on young embryos, leaves and petioles. It is supporting the conclusion that the internal state of explant cells is of prime importance in the expression of somatic embryogenesis, with other conditions such as exogenous growth regulators simply enabling expression of this intrinsically determined pattern of development (Zimmerman, 1993; Williams and Maheswaran, 1986). Among the media tested, embryogenic calli were produced only on the medium containing 2.5 mgl^{-1} BA. It is a very rare phenomenon and there are several reports of somatic embryogenesis induced by BA along with 2,4-D or NAA in many plant species (Yasuda et al., 1985; Pasquale et al., 1994).

Result of transferring the embryogenic calli into 3 new media showed that MS medium containing 0.1 mgl^{-1} 2,4-D was more effective than the others in embryogenic calli multiplication. The present report showing the role of 2,4-D in embryogenic callus promotion in the genus *Verbascum*. The optimum concentrations of 2,4-D found for somatic embryo induction or promotion in *V. speciosum,* are not completely consistent with the works on other plant species. However, a high 2,4-D level (up to 2 mgl^{-1}) was reported necessary to enhance embryogenic callus production in many plant species (Nagarajan et al., 1986; Nolan et al., 1989; Luo et al., 1999).

Plants that are conventionally propagated adventitiously may be proliferated rapidly *in vitro* using not only the conventional organs as a source of explants, but also the other tissues not normally associated with vegetative reproduction (Meins, 1986). Shoots along with roots were regenerated directly from embryo explants in hormone free MS medium. Caruso (1971) observed that when stem segments of *V. thapsus* were grown on a simple nutrient medium without hormones, they gave a morphogenetic response and formed shoots. Torregrosa and Bouquet (1996), noted that regeneration could be obtained in the presence of BA alone, but auxin could positively modify the organogenic responses. Our results are inconsistent with those of Skoog and Miller (1957) in general and those of Turker et al., 2001 on *V. thapsus*; in which a low ratio of NAA to BA induces shoot formation, while the reverse condition induces root formation. In addition, MS medium supplemented with 1 mgl^{-1} Kin was more effective than BA, providing strong shoot proliferation (100%) from the explants with a higher number of shoots per explant. A similar result was achieved by stem segments in *V. thapsus* (Turker et al., 2001). The highest frequency of shoot proliferation was obtained, when the shoot buds were transferred from MS medium supplemented with 1.5 mgl^{-1} NAA and 2.5 mgl^{-1} BA. Plant regeneration was also observed in the same condition. *V. speciosum* represented a superior rhizogenesis potential and the calli formed at 1.5 mgl^{-1} NAA without BA or with 2.5 mgl^{-1} NAA, produced adventitious roots vigorously after further subcultures.

Results from present study showed that the choice of explants is crucial for inducing calli in *V. speciosum* and calli were induced only on mature embryos. Embryogenic and non-embryogenic were induced in different concentrations of BA and NAA. It has been reported that types and concentrations of plant hormones and the types of explants have also a considerable role in callus induction and somatic embryogenesis (Luo et al., 1999; Fatima et al., 2009). Embryogenic calli were produced only on the medium containing 2.5 mgl^{-1} BA. This rare phenomenon has been reported by some researchers (Yasuda et al., 1985; Pasquale et al., 1994).

Transferring the embryogenic calli into MS medium containing 0.1 mgl^{-1} 2,4-D was more effective than the other treatments in their multiplication. However, the optimum concentration of 2,4-D in *V. speciosum* is not completely consistent with other species.

Our results were consistent with those of Turker et al., 2001 on *V. thapsus*; in which a low ratio of NAA to BA induces shoots, while the reverse condition induces roots. In addition, MS medium

supplemented with 1 mgl^{-1} Kin was more effective than BA, providing strong shoot proliferation with a higher number of shoots per explant. The highest frequency of shoot proliferation and plant regeneration was obtained, when the shoot buds were transferred from MS medium supplemented with 1.5 mgl^{-1} NAA and 2.5 mgl^{-1} BA.

The *in vitro* culture protocol is established here can provide plant material for future physiological and biochemical studies on *V. speciosum*. These studies will include developing extraction and analytical procedures for the active ingredients of this medicinal plant species.

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References

1- Akdemir Z. S., Tatli I. I., Bedir E. and Khan I. A. (2004) Iridoid and Phenylethanoid Glycosides from *Verbascum lasianthum*. Turkish Journal of Chemistry 28: 227-234.
2- Afifi M. S. A., Ahmad M. M., Pezzuto J. M. and Kinghorn D. (1993) Cytotoxic flavonolignans and flavones from *Verbascum sinaiticum* leaves. Phytochemistry 34: 839.
3- Caruso J. N. (1971) Bud formation in excised stem segments of *Verbascum thapsus* L. American Journal of Botany 58: 429-431.
4- Drandarov K. and Hais I. M. (1997) Separation of EZ isomeric macrocyclic spermine alkakoids of *Verbascum pseudonobile* and *Verbascum phoeniceum* and of their derivatives using thin-layer chromatography. Journal of Chromatography 724: 416-423.
5- Endress R. (1994) Plant Cell Biotechnology. Berlin Springer-Verlag Heidelberg; ISBN 3-540-56947-2; 46-83 pp.
6- Fatima Z., Mujib A., Fatima S., Arshi A. and Umar S. (2009) Callus induction, biomass growth, and plant regeneration in *Digitalis lanata* Ehrh.: Influence of plant growth regulators and carbohydrates. Turkish Journal of Botany 33: 393-405.
7- Hartleb I. and Seifert K. (1994) Sangarosaponin-D a triterpenoid saponin from *Verbacsum songaricum*. Phytochemistry 35: 1009-1011.
8- Huber-Morath A. (1978) *Verbascum* L. In: Davis P. H. ed., Flora of Turkey and the East Aegean Islands. Vol. 6. Edinburgh University Press, 461-600 pp.
9- Judd W. S., Campbell C. S., Kellogg E. A. and Stevens P. F. (2002) Plant Systematics: A phylogenetic approach. Sinauer, Sunderland, 576 p.
10- Karaveliogullari F. A., Duran A. and Hamzaoglu E. (2004) *Verbascum tuna-ekimii* (Scrophulariaceae), a new species from Turkey. Annales Botanici Fennici 41: 227-231.
11- Luo J. P. and Jia J. F. (1999) Callus induction and plant regeneration from hypocotyl explants of the forage legume *Astragalus adsurgens*. Plant Cell Reports 17: 567-570.
12- Meins F. J. R. (1986) Determination and morphogenetic competence in plant tissue culture. In: Yeoman M. M. ed., Plant Cell Culture Technology. Botanical monographs. Vol. 23. Blackwell Scientific Publications, Oxford. 7-25 pp.
13- Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.
14- Nagarajan P., Mckenzie J. S. and Walton P. D. (1986) Embryogenesis and plant regeneration of *Medicago* spp. in tissue culture. Plant Cell Reports 8: 279-281.
15- Nolan K. E., Rose R. J. and Gorst J. R. (1989) Regeneration of *Medicago truncatula* from tissue culture: increased somatic embryogenesis using explants from regenerated plants. Plant Cell Reports 8: 278-281.
16- Pasquale F. D., Carimi F. and Crescimanno F. G. (1994) Somatic embryogenesis from styles of different cultivars of *Citrus limon* (L.) Burm. Australian Journal of Botany 42: 587-594.
17- Sahai A., Shahzad A. and Anis M. (2010) High frequency plant production via shoot organogenesis and somatic embryogenesis from callus in *Tylophora indica*, an endangered plant species. Turkish Journal of Botany 34: 11-20.
18- Sharifnia F. (2007) Notes on the distribution and taxonomy of *Verbascum* in Iran. Iranian Journal of Botany 31: 30-32.
19- Skoog F. and Miller C. O. (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp. Soc. Exp. Biol. 11: 118-130.
20- Torregrosa L. and Bouquet, A. (1996) Adventitious bud formation and shoot development from *in vitro* leaves of *Vitis* × *Muscadinia* hybrids. Plant Cell Tissue and Organ Culture 45: 245-252.
21- Turker A. U., Camper N. D. and Gurel E. (2001) *In vitro* culture of common mullein (*Verbascum thapsus* L.), In vitro Cell and Development Biology- Plant 37: 40-43.
22- Valdes B. (1987) Scrophulariaceae. In: Valdes B., Talveraand S. Fernandez Galiano E. eds., Flora Vascular De Andalucia Occidental. Vol. 2. Barcelona: Ketres, 486-547 pp.
23- Williams E. G. and Maheswaran G. (1986) Somatic embryogenesis: Factors influencing coordinated behavior of cells as an embryogenic group. Annals of Botany 57: 443-462.
24- Yasuda T., Fujii Y. and Yamaguchi T. (1985) Embryogenic callus induction from *Coffea arabica* leaf explant by BA. Plant Cell Physiology 26: 595-597.
25- Zohary M. (1973) Geobotanical Foundations of

Middle East-Jerusalem, Hebrew University, Pp. 307-329.
26- Zimmerman J. I. (1993) Somatic embryogenesis: A model for early development in higher plants. Plant Cell 5: 1411-1423.