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The induction and development of somatic embryos from the *in vitro* cultures of *Catharanthus roseus* (L.) G. Don

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

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Abstract: *Catharanthus roseus* is containing anticancer alkaloids of vincristine and vinblastine and is an important medicinal plant. Several studies have conducted on *in-vitro* culture of this plant. To optimize the somatic embryogenesis, a factorial based on CRD experiment with 10 replications was conducted. Root, hypocotyl and leaf explants grown in-vitro were transferred and cultured on MS media containing different combinations of 2,4-D, NAA and 2,4-D×BAP. The results revealed that in callogenesis, the interaction effects of root and hypocotyl explants×2,4-D and NAA as well as hypocotyl×(1 mg l^{-1} NAA+1 mg l^{-1} BAP) was superior than other treatments (p≤0.01). For calli fresh weight, hypocotyl×NAA and hypocotyl× (1 mg l^{-1} NAA+1 mg l^{-1} BAP) was the treatment of choice (p≤0.01). The calli produced were sub-cultured to attain the pre-embryos and somatic embryos. For the number of pre-embryos and somatic embryos; the interaction of hypocotyl×2,4-D was the most efficient treatment. Seemingly, the production of somatic embryos is accessible in this plant by the logical management of growth regulator combinations. Furthermore, the production and genetic engineering of the somatic embryos could be a promising trend in the subsequent production of high-valued metabolites from this plant.

1. Introduction

Catharanthus roseus (L.) G. Don, generally known as *Madagascar Periwinkle,* is a dicotyledonous plant with 2n=16 belongs to the family *of Apocynaceae*. *Catharanthus roseus* (L.) G. Don is an herbaceous plant that grows to a height of about 80 Cm and is native of Madagascar (Hogan, 2003). More than 130 types of alkaloids have been extracted from the vegetative parts and roots of this plant, which are used to treat several diseases (Aslam *et al.,* 2009). The most important alkaloids extracted from the shoots of this plant are vincristine and vinblastine, with a well-defined anti-cancer properties (Mujib *et al.,* 2012). The amount of these compounds in this plant is about 0.0005% of dry weight of the plant and their extraction is costly and time consuming (Barrales-Cureño *et al*., 2017). These problems have led the scientists to a new approaches of tis-

sue culture studies in *C. roseus* L. (Van Der Heijden *et al.,* 2004). The first study done on tissue culture of *Catharanthus roseus* (L.) G. Don was in 1977. Those, researchers were able to produce callus from the plant (Dhruva *et al.,* 1977). The production of shoots from callus has been successfully done in *Catharanthus roseus* L. (Ramawat *et al.,* 1987). Somatic embryogenesis is a process by which somatic cells differentiate into embryos, called somatic embryos, which are used as one of the practical in vitro techniques for plant micro-propagation (Von Arnold *et al.,* 2002). The first studies on somatic embryogenesis in the *Catharanthus roseus* (L.) G. Don was done in 1994, which were succeeded in producing somatic embryos by the anther culture (Kim *et al.,* 1994). Furthermore, by hypocotyl explant and 1 mgl^{-1} of 2,4-D (2,4-Dichlorophenoxyacetic acid), somatic embryos were obtained (Aslam *et al.,* 2004, 2006). Growth regulators (composition and concentration) and the plant genetic make-up play a role in the success of somatic embryos production. The phenomenon of genotype-dependent plant regeneration also exists in other plant species (Firoozabady and De Boer, 1993).

The physiological conditions, the explant growth stage and embryogenic tissue type affect the production of somatic embryos. The tissue that has the highest metabolism and the least differentiation rate may have the suitable embryogenesis potential (Mikula and Rybczynski, 2001). Auxins, especially 2,4-D, are among the most important plant growth regulators initiate the somatic embryogenesis (Choi *et al.,* 1999; Martin, 2003; Gulzar *et al.,* 2019). In medicinal herb *Spermatozoa hispida* L.; cytokinin, especially BAP (6-Benzylaminopurine), induced frequent somatic embryos (Deepak *et al.,* 2019). With medicinal herb, *Coccinia abyssinica;* 2,4-D and BAP combination helped to produce embryogenic callus, and BAP triggered the production of somatic embryos (Abate *et al.,* 2019). L-glutamine and L-alanine amino acids have had the most positive effect on somatic embryogenesis process (Ji *et al.,* 2011). Various sources of carbon; such as sucrose, fructose, and glucose, as energy sources and osmotic regulators; play an important role in the somatic embryogenesis initiation and frequency (Aslam *et al.,* 2011). MS has been employed as the most common culture medium for most of the plants (Ji *et al.,* 2011). Somatic embryogenesis, leading to the regeneration of intact plants, is an eminent step in the plant transformation. Successful and sustainable transformation requires that a single cell give rise to a whole intact plant. The Ideal transformation takes place through the direct somatic embryogenesisfrom the single cells to reach the intact plants (Aslam *et al.,* 2007).

The aim of the present study was to investigate the different explant types and the diverse plant growth regulators effects to induce somatic embryos and to study of the traits related to the somatic embryogenesis potential in *Catharanthus roseus* (L.) G. Don.

2. Materials and Methods

In vitro seed germination and seedlings production

The present study was conducted in the Research Laboratory, Department of Horticultural Sciences, University of Maragheh, Iran. The seeds were acquired from Pakan Bazr Isfahan Company. The seeds were immersed in water for one day before planting in the dark, and the next day, they were first treated with ethyl alcohol (70%) for one minute and then disinfected with 20% solution of sodium hypochlorite for 10 more minutes. Then, they were washed with sterile distilled water 5 times. The disinfected seeds were cultured in petri dishes containing 20 ml of MS medium (Murashige and Skoog, 1962) without plant growth regulators. In each petri dish; 10 disinfected seeds were cultured at 25-28°C. After emergence, the seedlings were transferred to the photoperiod conditions with 16 hours of light at 25°C and 8 hours of darkness at 20°C. After about 10 days, the 2-4 cm in length seedlings were suitable to continue the experiment.

Preparation of culture media

The culture medium was MS basic medium. Sucrose at a concentration of 30 g l^{-1} was used as a source of carbon. Then, MS culture medium salts were added. pH was set at 5.8. At the last stage, 6 gl^{-1} of agar was added and placed in an autoclave at 121°C for 20 min. Agar-free culture medium (liquid) was employed for the somatic embryos.

Experimental design

A factorial experiment based on completely randomized design (CRD) was planned with 10 replications.

Factor 1: Plant growth regulators, concentration of 1 mgl^{-1} 2,4-D, 2 mgl^{-1} NAA (1-Naphthaleneacetic acid), 1 mgl^{-1} NAA + 1 mgl^{-1} BAP

Factor 2: Explants (Root, hypocotyl and leaf).

Sampling and culture of explants in different treatments

The explants; root, hypocotyl and leaves were excised from the 2-4 cm seedling grown *in vitro*. Hypocotyl explants were cut into a length of about 1 cm and, in order to obtain embryogenic callus; they were cultured in the MS medium supplemented with the same treatments as subcultures. For calli proliferation; 3 weeks after the first date of cultivation, the same plant growth regulator treatments were applied to the subcultures. One month after the first subculture; embryogenic calluses were sub-cultured in MS medium supplemented with concentrations of 0.5, 1, and 1.5 m g l^{-1} BAP. Then, the resulting calli were transferred to MS medium supplemented with 7 g l^{-1} of agar, 30 g l^{-1} of sucrose, 1 mg l^{-1} glutamine and 3 g l^{-1} of polyethylene glycol with 6 replications. At the all stages of cultures and subcultures; the samples were kept in a growth chamber for 16 h light at 25°C and 8 h dark at 20°C.

Data mining and statistical analysis

The callus production percentage, the percentage of necrotic calli and callus weight were noted. After multiple sub-cultures and transfer to the embryogenesis culture medium, the number of pre-embryogenic mass formed were recorded. SAS was used to analyze the variance, and mean comparisons were done with Duncan's multiple range test at 5% probability.

3. Results

Callus production

Hypocotyls of *in vitro* germinated seeds were used as explant on MS medium supplementary with 2,4-D and NAA, which induced white to yellowish callus within 10 days of incubation. The embryogenic callus was white, granular, friable, fast growing within 3 weeks of culture. The number of explants that produced callus and the necrotic calli proportion showed the callogenesis percentage and the percentage of necrotic calli (Fig. 1).

The mean comparison showed that for the callus formation, the interaction between root and 2,4-D; hypocotyl and 2,4-D; root and NAA; hypocotyl and NAA; and hypocotyl and 1 mgl^{-1} NAA + 1 mgl^{-1} BAP were significant at 1% probability level. Figure 2 shows the significant effect of auxin use on the rate of callus production. In this study 2,4-D was very efficient in producing callus and embryos on *Catharanthus roseus*. Embryonic callus was not observed using leaf explants and hypocotyl explants had the highest embryonic callus.

Furthermore, for the callus fresh weight, the interactions of hypocotyl and NAA; and hypocotyl × 1 mg l^{-1} NAA + 1 mgl^{-1} BAP were significantly different from other treatments ($p \leq 0.01$) (Fig. 3).

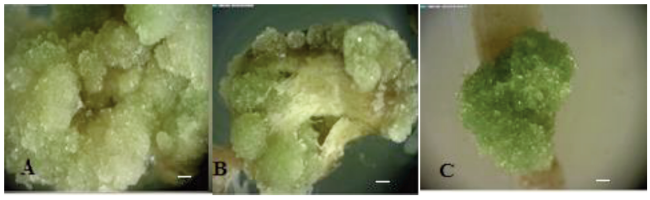

Fig. 1 - The effect of 2,4-D, NAA and BAP on hypocotyl explants of *Catharanthus roseus* and the formation of embryogenic callus (3 week after culture). Scale bars= 1.0 mm. (A) Embryogenic callus from the treatment with 1 mg l^{-1} 2,4-D; (B) Embryogenic callus from the treatment with 2 mg l^{-1} NAA, (C) Embryogenic callus from the treatment with 1 mg l^{1}- NAA + 1 mg l^{-1} BAP.

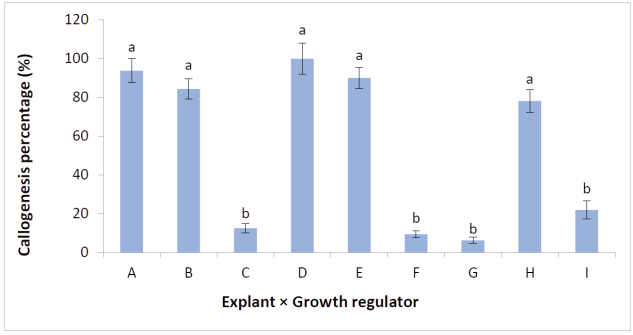

Fig. 2 - The interaction effects of explant×plant growth regulators on the callogenesis percentage of *Catharanthus roseus*. A= 1 mg l^{-1} 2,4-D×root; B= 1 mg l^{-1} 2,4-D×hypocotyl; C= 1 mg l^{-1} 2,4-D×leaf; D= 2 mg l^{-1} NAA×root; E= 2 mg l^{-1} NAA×hypocotyl; F= 2 mg l^{-1} NAA×leaf; G= 1 mg l^{-1} NAA+1 mg l^{-1} BAP×root, H= 1 mg l^{-1} NAA+1 mg l^{-1} BAP×hypocotyl; I= 1 mg l^{-1} NAA+1 mg l^{-1} BAP×leaf).

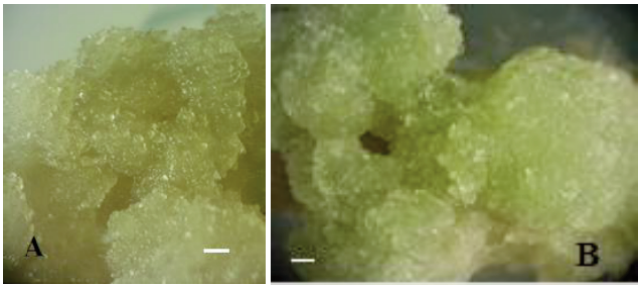

Fig. 3 - The effect of sub-culture of embryogenic calli from *Catharanthus roseus* hypocotyl influenced by different treatments (calli are produced 1 month after subculture). Scale bars= 1.0 mm. (A) Embryogenic calli produced by the treatment of 2 mg l^{-1} NAA; (B) Embryogenic calli produced by the treatment of (1 mgl^{-1} NAA+1 mg l^{-1} BAP).

The calli weight was obtained by weighting 10 samples. The results showed that the growth regulators and explants type and their interaction were significant on the weight of callus production, Treatment composition H and E with 0.85 and 0.81 g, had the most callus weight respectively. The interaction of hypocotyl and NAA; and hypocotyl x NAA + BAP were significant (p≤0.01) on callus fresh weight (Fig. 4).

Pre-embryonic and somatic embryogenic tissues

In this study, two different approaches were employed to increase the formation of embryogenic calluses. Both approaches were aimed at choosing the best combination and concentration of plant growth regulators. Different masses were observed on the embryogenic calli tissues and the pre-embryonic structures were formed evidently. The number of masess of possible pre- and somatic embryos formed were counted. Somatic embryos were isolated by liquied culture and shown with distinct roots and shoots (Fig. 5).

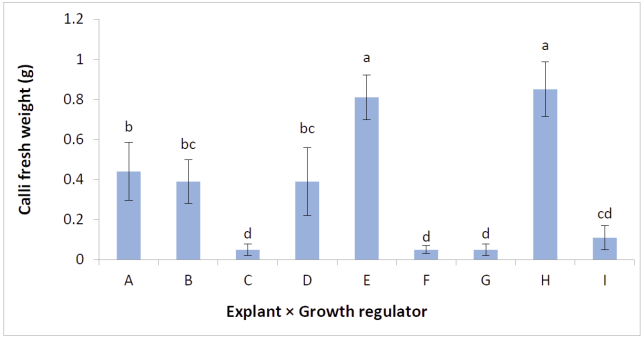

Fig. 4 - The interaction of explant×growth regulators on calli fresh weight at the *in-vitro* cultures of *Catharanthus roseus*. A= 1 mg l^{-1} 2,4-D×root; B= 1 mg l^{-1} 2,4D×hypocotyl; C= 1 mg l^{-1} 2,4-D×leaf; D= 2 mg l^{-1} NAA×root; E= 2 mg l^{-1} NAA×hypocotyl; F= 2 mg l^{-1} NAA×leaf; G= 1 mg l^{-1} NAA+1 mg l^{-1}BAP×root; H= 1 mg l^{-1} NAA+1 mg l^{-1} BAP×hypocotyl; I= 1 mg l^{-1} NAA+1 mg l^{-1} BAP×leaf.

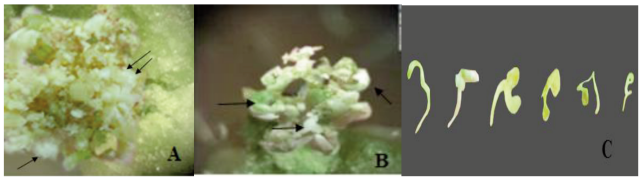

Fig. 5 - (A) Pre-embryo spots (bar= 1 mm); (B) somatic embryos formed on the calli derived from hypocotyl explants of *Catharanthus roseus* (bar= 1 mm); (C) Somatic embryos isolated from liquid cultures including root and shoot ends (bar= 5 mm).

The results showed that the effect of plant growth regulators and explants, and their interactions were significant (p≤0.01) on the number of pre-embryos and somatic embryos produced.

Furthermore, the interactions of hypocotyl × 2,4-D produced the highest number of pre-embryogenic and somatic embryogenic with mean 3.83 and 5.33 respectively in solid culture medium (Fig. 6).

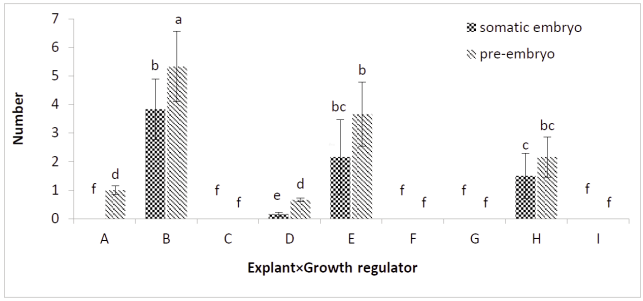

Fig. 6 - The interaction of explant×growth regulators on pre-embryos and somatic embryos number produced in *Catharanthus roseus in-vitro* culture. A= 1 mg l^{-1} 2,4-D × root; B= 1 mg l^{-1} 2,4D × hypocotyl; C= 1 mg l^{-1} 2,4-D × leaf; D= 2 mg l^{-1} NAA × root; E= 2 mg l^{-1} NAA × hypocotyl; F= 2 mg l^{-1} NAA × leaf; G= 1 mg l^{-1} NAA+ 1 mg l^{-1} BAP × root; H= 1 mg l^{-1} NAA + 1 mg l^{-1} BAP × hypocotyl; I= 1 mg l^{-1} NAA + 1 mg l^{-1} BAP × leaf.

4. Discussion and Conclusions

The most frequently used auxin in the studies on somatic embryogenesis is 2,4-D (Bhojwani and Razdan, 1996; Junaid *et al.*, 2006; Jushee *et al.*, 2007). The significant effects of 1 mgl^{-1} 2,4-D has been proven on callus formation and somatic embryogenesis on *Catharanthus roseus* and *Aconitum heterophyllum* (Giri *et al.,* 1993; Aslam *et al.,* 2004). This auxin stimulates cell division and plays an important role in the production of callus (Paramageetham *et al.,* 2004). Auxins alone or in combination with cytokinins are used for somatic embryogenesis induction and initiation (Wojcikowska and Gaj, 2016; Tanida and Shiota, 2019). More often, the use of cytokinins alone induces the production of non-embryogenic callus (Martin, 2004). In dicotyledonous plants, cytokinins are usually added to the culture medium along with auxins for the promotion of callus production (George *et al.,* 2008). In medicinal herb, *Asparagus racemosus* Willd with the embryonic explants cultured on MS- medium and supplemented with 1.5 mgl^{-1} 2,4-D + 0.43 mgl^{-1} kin; 74%

non-embryogenic callus was obtained (Chaudhary and Dantu, 2019). BAP is the major cytokinin used in the studies related to the somatic embryogenesis (Jimenez and Thomas, 2005). Singh *et al.* (2011) obtained 92% of non-embryogenic callus in *Catharanthus roseus* (L.) G. Don in MS culture medium supplemented with 1 mg l^{-1} NAA + 1 mg l^{-1} BAP with hypocotyl explants. Our results are consistent with the findings of Aslam *et al.* (2006) in which hypocotyl explants in MS medium supplemented with 1 mgl^{-1} 2,4-D, resulted in 85% of embryogenic callus. Moreover, they reached 73% of embryogenic callus by hypocotyl explant in the MS medium enriched with 1.5 mgl^{-1} BAP + 1 mgl^{-1} NAA; and 61.75% of embryogenic callus by hypocotyl explants in MS medium supplemented 1 mgl^{-1} NAA + 1 mgl^{-1} BAP as well as 85% of embryogenic callus by hypocotyl explant again in MS medium enriched with 1 mgl^{-1} 2,4-D (Aslam *et al.,* 2007). Studies have shown that 1 mgl^{-1} 2.4-D in *Ocimum basilicum* L., produced about 75% of embryogenic calli (Gopi and Ponmurugan, 2006). Also, the results of another study revealed that by culturing hypocotyl explants in MS medium containing 1 mg l^{-1} NAA +3 mgl^{-1} BA; 80% of non-embryonic callus was observed (Ren *et al.,* 2020).

Auxins and cytokinins form callus tissues by accelerating the division of plant cells (George *et al.,* 2008). In *Withania somnifera* stems cultured in MS medium with 1mgl^{-1} BAP + 1 mgl^{-1} NAA; calli were produced with an approximate weight of 0.22 g (Adhicari and Pant*,* 2013). In another study, internode explants of *Centella asiatica* L. on MS supplemented with auxins and cytokinins produced calli weighting up to 1.5 g (Martin, 2004).

2,4-D causes the rapid cell division and the polarization of cells (Jushee *et al.,* 2007). Our results are almost similar with the findings of Choi *et al.* (1999) On *Eleutherococcus senticosus* (75% of somatic embryos). Moreover, the findings of the present study are in line with the results of Martin (2003) on *Holostema adakodien*, which by using different explants and plant growth regulators; they clearly showed that hypocotyl explants and 2,4-D (1 mg l^{-1}) attained about 50% of somatic embryos. In another study, hypocotyl explants of *Eleutherococcus senticosus* in MS medium produced 89% of somatic embryos (Han and Choi, 2003). Also, our results are the same with Aslam *et al.* (2004) on *Catharanthus roseus* (L.) G. Don, whom described that hypocotyl and 2,4-D (1 m gl^{-1}) were the combination of choice. Also, in another study on *Catharanthus roseus* (L.) G. Don; the most somatic embryos were observed using hypocotyl explants and 2 mgl^{-1} 2,4-D. Auxins have synergistic effects with cytokinins and accelerate the cell division. 2,4-D may also add-up the endogenous levels of IAA in plant tissue, thereby creating dipoles within the cell and forming pre-embryonic structures (Mendez-Hernandez *et al.,* 2019). 2,4-D promotes the accumulation of ROS (oxygen reactive species), and stimulates the ethylene and abscisic acid biosynthesis in plant tissue and ultimately induces stress behavior in plant tissue. As a result, plant cells change or shift to form somatic embryos (Bharatia *et al.,* 2015; Wojcik *et al.,* 2020). 2,4-D application and availability in the culture medium probably results in the expression of cell differentiation genes and the demethylation of DNA. 2,4-D plays an important role in the somatic embryos induction and in the stages of maturation and development of somatic embryos. Whereas, 2,4-D, has an inhibitory role with the biosynthesis of a number of proteins and mRNAs (Krishnan and Siril, 2017). In our study, calli from leaf explants were unable to produce somatic embryos (Gulzar *et al.,* 2019). Otherwise, *Paeonia ostii '*Feng Dan', *Asparagus racemosus* Willd, and *Cnidium officinale* Makino, the combinations of auxins and cytokinins produced reasonable somatic embryos (Adil *et al.,* 2018; Chaudhary and Dantu, 2019; Ren *et al.,* 2020).

Auxins had dominant effect on callus production of *Catharanthus roseus* (L.) G. Don. and the results showed that 2,4-D was much efficient than NAA. More somatic embryos were obtained from hypocotyl explants. Due to the medicinal importance of *Catharanthus roseus*, it is suggested that in the further studies, somatic embryogenesis behavior in this plant should be studied on a larger scale and that the possibility of somatic embryogenesis in a liquid culture medium using suspension culture should be tested in different ways. Therefore, it is possible to study the biosynthesis of secondary metabolites and valuable alkaloids *in vitro* conditions and to optimize the protocols to extract and purify the above mentioned metabolites.

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