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Indirect shoot organogenesis and *in vitro* root formation of *Antirrhinum majus* L. by using of sodium nitroprusside

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Key words: Callus, nitric oxide, plant growth regulator, Snapdragon.

Abstract: The aim of this study was to determine the effect of different concentrations of sodium nitroprusside (SNP) on *in vitro* shoot organogenesis from hypocotyl explant derived from *in vitro* grown seedling as well as root formation of *Antirrhinum majus* L. (Snapdragon). In the first experiment, different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0, 2.26, 4.52, and 6.79 µM) were used for callus formation. The highest callus fresh weight (1.86 g) as well as callogenesis frequency (93.34%) were observed in Murashige and Skoog (MS) medium containing 4.52 µM 2,4-D. In the later experiments, various concentrations (0, 10, 20, 30, 40, and 50 µM) of sodium nitroprusside (SNP) were applied for shoot regeneration from callus that derived from hypocotyl segments. Based on our results, MS medium supplemented with 4.44 µM 6-benzylaminopurine (BAP) plus 0.49 µM 3-indolebutyric acid (IBA) along with 30 µM SNP had the highest shoot organogenesis frequency (93.34%) and shoot number (6.33) from callus. In root induction experiment, different concentrations (0, 20, 40, 60, 80, and 100 µM) of SNP were applied and MS medium containing 60 µM SNP was the best treatment for root induction. The survival rate of plantlets was more than 95% in acclimatization stage. The present study describes an efficient regeneration system for Snapdragon.

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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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1. Introduction

Snapdragon (*Antirrhinum majus* L.) is known as one of the most significant ornamental plants which has worldwide values as cut flowers, herbaceous landscape plants, and flowering potted plants (El-Nashar, 2017). Also, snapdragon has high commercial values with its wide range of color, shape, structure, and size (Weiss *et al.*, 2016). The commercial propagation of snapdragon is via seeds. The seed propagation cannot ensure the whole genetic uniformity so seed-propagated plants may indicate undesired phenotypes, quality, and regeneration potential. Therefore, plants

might be selected randomly without taking necessary care. These features of seeds exert a negative impact on sexual production of this plant (Jaworski *et al*., 2016). Therefore, the development, as well as improvement of *in vitro* culture techniques in this ornamental plant, is of high paramount (Hesami and Daneshvar, 2016). A rapid regeneration pathway for *A. majus* could be useful for commercial propagation of nursery and cut-flower industries as well as breeding programs (Sheyab *et al*., 2010). Also, *in vitro* culture of this plant is necessary for producing high quality/price ratio flower. Moreover, genetic engineering by using biolistic or *Agrobacterium* methods could be known as a viable alternative in traditional breeding methods for developing distinguished snapdragon cultivars in order to satisfy market demands (Davies *et al.,* 2013). On the other hand, the efficiency of gene transformation in snapdragon, obtained via these methods, remains low in this ornamental plant because of the lack of efficiency *in vitro* propagation protocols (Azadi *et al.*, 2016). According to the previous study, Sheyab *et al.* (2010) indicated that high applicability of transformation in *A. majus*is completely depended on the propagation procedures. Therefore, the use of *in vitro* culture for producing Snapdragon could reduce these problems that occur in the commercial production of this plant (Atkinson *et al.*, 1989; Sheyab *et al*., 2010).

Adjusting the culture medium with suitable plant growth regulators (PGRs) in various combinations and concentrations could enhance the propagation potential of various genotypes and explants (Hesami *et al*., 2017 a, b; Jafari *et al*., 2017; Hesami *et al*., 2018 a, b, c; Hesami and Daneshvar, 2018 a, b; Hesami *et al*., 2019 a, b, c). Thus, it is significant to improve the propagation protocols by using suitable PGRs in order to overcome difficulties associated with clonal regeneration and gene transformation strategies to satisfy the increasing demand for *A. majus* (Newbury, 1986; Sheyab *et al*., 2010; Hesami and Daneshvar, 2016).

Nitric oxide is known as a messenger molecule for regulating plant development (Neill *et al*., 2003; Hesami *et al*., 2019 d). This molecule has recently been characterized as one of the phytohormones (Leterrier *et al.*, 2012). Nitric oxide is known as a ubiquitous bioactive molecule that mainly contributed to various plant developmental processes such as fruit ripening, flowering, organ senescence, and germination (Jimenez-Quesada *et al*., 2017). The exterior usage of nitric oxide might improve the tolerance of plants under various stresses such as temperature, heavy metals, ultraviolet radiation, drought, and salinity (Laspina *et al*., 2005; Qiao and Fan, 2008). The activation rate of nitric oxide has been evaluated by the exogenous usage of sodium nitroprusside instead of using NO gas directly because of some technical difficulties (Sarropoulou and Maloupa, 2017). In recent years, nitric oxide is used for developing *in vitro* plant propagation (Rico-Lemus and Rodríguez-Garay, 2014). Kalra and Babbar (2010) indicated that nitric oxide could enhance the regeneration response via increasing the number of meristems and recommended that nitric oxide regulates the gene expression related to differentiation of meristems. Also, Sarropoulou and Maloupa (2017) recommended that nitric oxide exert a powerful impact on cell division and also it could be involved in shoot organogenesis and proliferation. Han *et al*. (2009) and Sarropoulou *et al*. (2014) showed that *in vitro* shoot proliferation as well as root formation of plantlets were promoted significantly by applying SNP to the MS medium in *Malus hupehensis* and cherry rootstocks, respectively. Although there are few studies about the effect of nitric oxide on improving *in vitro* shoot organogenesis (Han *et al*., 2009; Xu *et al*., 2009; Kalra and Babbar, 2010; Tan *et al*., 2013; Sarropoulou *et al*., 2014; Arun *et al*., 2017; Ghadakchiasl *et al*., 2017; Sarropoulou and Maloupa, 2017), there is no research evidence on the effect of this molecule on shoot organogenesis of snapdragon. Thus, the aim of this study was to evaluate the effect of sodium nitroprusside (SNP) on indirect shoot organogenesis as well as root formation that derived from hypocotyl explants of snapdragon in order to reduce the time of *in vitro* shoot propagation.

2. Materials and Methods

The seeds of snapdragon were washed under tap water for 30 min. Further surface sterilization treatments were conducted in a laminar airflow chamber. The seeds were surface sterilized with 70% ethanol for 10 seconds and soaked for 10 min in 10% (v/v) NaOCl. Afterward, the seeds were washed three times in sterilized distilled water. Subsequently, the sterilized seeds were inoculated on one-tenth strength MS medium. After 8-10 days, seeds were germinated, and the hypocotyl segment from *in vitro* seedling was used as a source of explant for the latter experiment.

The MS medium containing 3% (w/v) sucrose, 0.6% (w/v) agar was used as basal medium. The basal

medium was fortified with different PGRs, and pH 5.8 adjusted with 1 N NaOH before autoclaving at 121°C for 20 min. All growth regulators except sodium nitroprusside (SNP) were added before autoclaving. SNP was added after autoclaving by filtering. All cultures were maintained at 25±2°C with 55-60% relative humidity, and 16 h photoperiod (65 µmol m^{-2} s^{-1}) that provided by cool white fluorescent light.

Hypocotyl explants (0.5-1.0 cm) from 1-week-old *in vitro* seedlings (Fig. 1 a) were inoculated on MS medium supplemented with various concentrations (0, 2.26, 4.52, and 6.79 µM) of 2,4-D for callus formation. All of the culture vessels were kept at 25±2°C in the absence of light. Data of callus formation frequency (%) and callus fresh weight (g) were measured after four weeks of culture.

Calli were cultured in the regeneration medium containing 4.44 µM BAP plus 0.49 µM IBA supplemented with different SNP concentrations (0, 10, 20, 30, 40, and 50 µM). The shoots regeneration frequency and the number of shoots per callus were determined after 5 weeks of treatment.

Shoots with 0.5-1.5 cm in length were transferred to MS medium supplemented with 1 mg/l GA_3 (elongation medium) for 4 weeks. Then, the elongated shoots (2-3 cm elongation) were chosen and transferred to the half strength MS medium containing 3% (w/v) sucrose, 0.6% (w/v) agar and different concentrations (0, 20, 40, 60, 80, and 100 µM) of SNP. Rooting percentage (%) and root number including main and secondary roots were evaluated after 30 days.

Plantlets with well-developed root system were removed from the media, washed thoroughly with sterile water and transplanted into potting mixture containing autoclaved perlite and cocopeat mixture (1:1) and covered with transparent plastic to maintain high humidity. The plastic sheets were removed after 4 weeks in order to acclimatize plantlets to greenhouse condition, and the plants were shifted to pots comprising garden soil.

All experiments were performed with a total of 10 replicates per treatment and were repeated 3 sets. The data were analyzed by ANOVA using SAS version 9.3 followed by Duncan's multiple range test (DMRT, $P<0.05$).

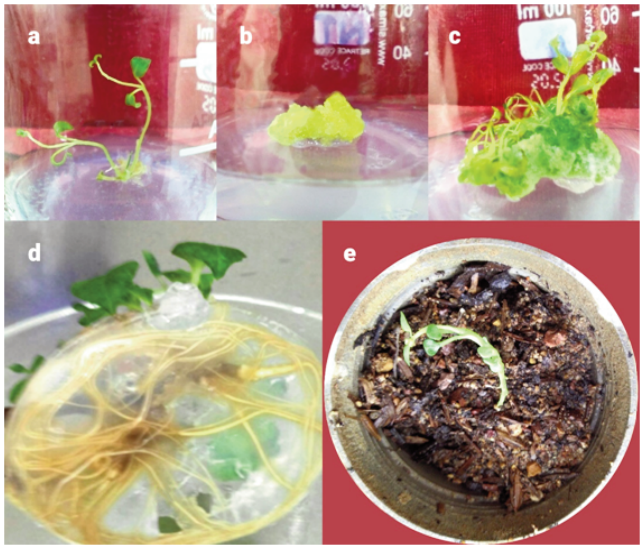

Fig. 1 - *In vitro* shoot regeneration through indirect organogenesis from seedling derived hypocotyl segments of *Antirrhinum majus* L. (a) Seedling from *in vitro* seed germination; (b) Yellow-greenish and friable callus induction on MS + 4.52 µM 2,4-D; (c) Shoot regeneration from callus on MS medium containing 4.44 µM BAP plus 0.49 µM IBA along with 30 µM SNP; (d) *In vitro* root formation on MS + 60 µM SNP; (e) Acclimatized regenerated plants after four weeks.

3. Results and Discussion

The callogenesis experiment was conducted in order to figure out the most suitable and efficient concentration of 2,4-D for callus formation. The result of this study indicated that the maximum percent of callus induction (93.34%) and callus weight (1.86 g) (Fig. 2) were achieved on MS medium containing 4.52 µM 2,4-D (Fig. 1b). In the agreement with our result, Sangwan and Harada (1975) showed that acceptable callus formation of Snapdragon through stem explant was achieved in MS medium containing 4.52 µM 2,4-D. The earlier study proved the positive effect of 2,4-D on the callus formation,

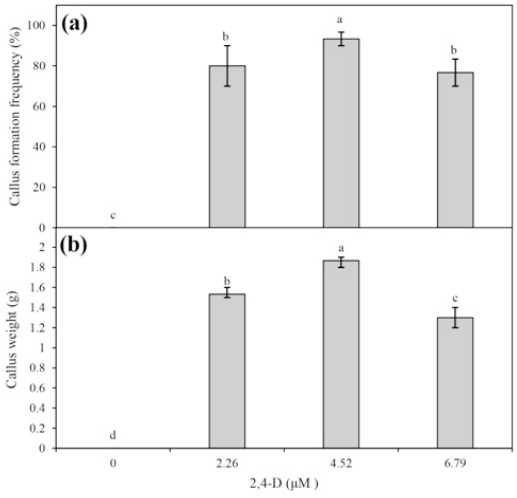

Fig. 2 - Effect of different concentrations of 2,4-Din MS medium on (a) callus formation frequency and (b) callus fresh weight of *A. majus*. Means followed by the same letter are not significantly different at $P<0.05$ as determined by Duncan's multiple range test; Vertical bars: standard error.

and also this study reported that 2,4-D may be involved in endogenous IAA metabolism regulation by inducing some specific proteins and controlling DNA methylation (Pan *et al*., 2010). However, in another study, the callus formation of *A. majus* via hypocotyl explant was obtained on MS medium with different concentrations of NAA plus 10% coconut milk (Atkinson *et al.,* 1989).

By increasing sodium nitroprusside from 10 µM to 30 µM, shoot regeneration was improved (Fig. 3). Also, the maximum frequency of shoot organogenesis (93.34%) and shoots number (6.33) were observed in MS medium supplemented with 30 µM SNP (Fig. 1c, Fig. 3). However, the higher level (more than 30 µM) of sodium nitroprusside might limit the shoots number and shoot organogenesis frequency. Calli can grow in MS medium supplemented with 50 µM sodium nitroprusside. These obtained results recommended that sodium nitroprusside can promote shoot organogenesis in proper doses. Our results indicated that sodium nitroprusside completely promoted shoot organogenesis from hypocotyl segments in MS medium along with 4.44 µM BAP plus 0.49 µM IBA. Thus, BAP and SNP appear to have a synergistic effect on shoot regeneration. The effect of NO on *in vitro* organogenesis is completely associated with cytokinins (Arun *et al*., 2017). It has previously been shown that NO might interact with auxin and cytokinin, linking the regulation of cell division to differentiation during the de-differentiation and re-differentiation of plant cells (Ghadakchiasl *et al.*, 2017; Karalija *et al*., 2017). Tun *et al*. (2001) observed that NO plays a potential role in mediating plant hormone (auxin and cytokinin) signal transduction during growth and development. Carimi *et al*. (2005) found that BA stimulates the release and accumulation of NO in plant suspension cell cultures. Therefore, in the present study, SNP may have functioned as an intermediary for adventitious shoot differentiation and regeneration, as suggested by Han *et al*. (2009) in *Malus hupehensis*.

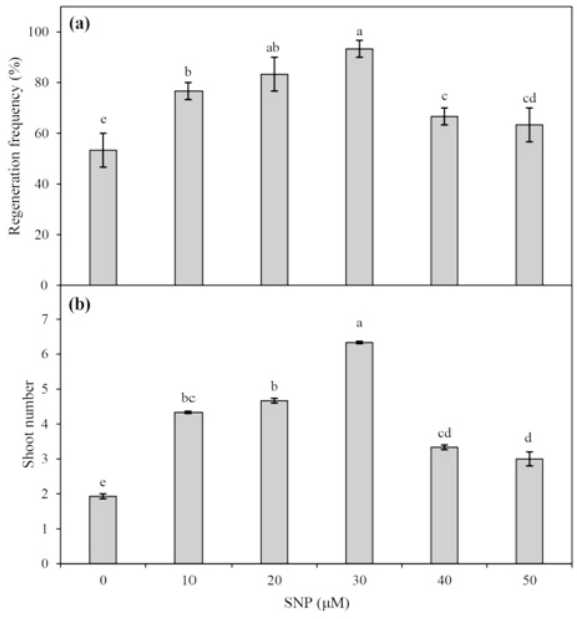

Fig. 3 - Effect of different concentrations of SNP in MS medium containing 4.44 µM BAP plus 0.49 µM IBA on (a) regeneration frequency and (b) shoot number of *A. majus*. Means followed by the same letter are not significantly different at P<0.05 as determined by Duncan's multiple range test; Vertical bars: standard error.

Our results showed that MS medium supplemented with 1 mg/l GA_3 caused shoot elongation. NO (precursor of SNP) has been reported to influence several plant developmental events in which gibberellins (GAs) play crucial roles such as seed germination, hypocotyl elongation, acquisition of photomorphogenic traits, and primary root growth (Beligni and Lamattina, 2000). However, the actual interaction between NO and GAs has been described for only a limited number of these physiological events. In fact, most of our current knowledge of the mechanisms underlying the interplay between GAs and NO is restricted to the regulation of seed germination (Neill *et al*., 2003) and the inhibition of hypocotyl elongation during seedling de-etiolation (Lozano-Juste and León, 2011). NO has been described as acting upstream of GAs (Bethke *et al*., 2007), regulating both biosynthesis and perception/transduction of GAs (Lozano-Juste and León, 2011).

There was no root formation in the MS medium without sodium nitroprusside while adding SNP promoted root formation significantly. By increasing the concentration of SNP from 0 to 60 µM, the root formation frequency (100%) and roots number (8.33) (Fig. 4) were increased significantly (Fig. 1d). However, the roots number was decreased when the SNP level was over 60 µM. Root formation is known as the meristematic development of tissues after removing the primary root system (Dash *et al*., 2017; Jafari *et al*., 2017). It was indicated that nitric oxide was involved in the response of auxins during root induction in cucumber (Pagnussat *et al*., 2003) and another report demonstrated that a NO-mediated cGMP dependent pathway was involved in this process (Pagnussat *et al*., 2003). In order to form the root meristem, auxins promoted parenchyma cells dedifferentiation and entrance to cell division (Klerk *et al*., 1995; Fujita and Syono, 1996). Also, Gouvea *et*

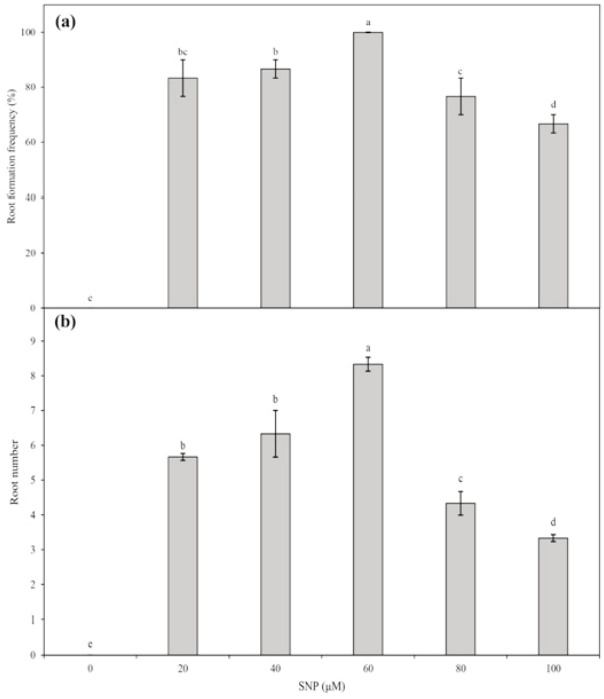

Fig. 4 - Effect of different concentrations of SNP in MS medium on (a) root formation frequency and (b) root number of *A. majus*. Means followed by the same letter are not significantly different at P<0.05 as determined by Duncan's multiple range test; Vertical bars: standard error.

al. (1997) suggested that the role of nitric oxide in signal transduction pathways for root elongation is similar to the role of auxins in this step. Therefore, it became clear that nitric oxide might have an interaction with auxins in regulating cell division to differentiation in ''de-differentiation'' and ''re-differentiation'' steps of plant cells (Ötvös *et al*., 2005). The positive effect of nitric oxide on improving root induction is reported in various species (Huang and She, 2003; Correa-Aragunde *et al.,* 2004; Han *et al*., 2009). Sarropoulou *et al.* (2014) recommended that nitric oxide could (a) produce an antioxidant condition that protects auxins from deteriorations as well as oxidation, (b) speed up cell expansion in order to improve rooting in plants, (c) serve as a downstream messenger in the IAA signaling pathway, (d) regulate enzyme activities or cell-cycle genes that are associated with auxin signal transduction, and (e) reduce the lignification of cell wall. By using exogenous sodium nitroprusside, the root induction in mung bean was promoted significantly (Huang and She, 2003). Furthermore, sodium nitroprusside can induce root hair induction in lettuce (Lombardo *et al*., 2006), and development of lateral roots in tomato (Correa-Aragunde *et al*., 2004).

Plantlets that had well-developed roots were transferred successfully into small pots consisting of perlite and cocopeat mixture (1:1). Our results showed that the rooted plants had 95% survival rate in the acclimatization stage. Afterwards, within 20 days after transferring plantlets to the greenhouse, the normal growth of plantlets was resumed (Fig. 1e). Similar to our results, Hesami and Daneshvar (2016) indicated that by acclimatization of the snapdragon plantlets in the perlite and cocopeat mixture (1:1), 90% survival rate was obtained.

In conclusion, we have developed a method for indirect shoot organogenesis from hypocotyl explants of *A. majus*. It is of note that SNP, a donor of NO, has a direct effect on *in vitro* shoot differentiation and rooting of the snapdragon explants. SNP may interact with auxin and cytokinin, linking the regulation of cell division to cell differentiation during the dedifferentiation and redifferentiation of plant cells. The improvement of ornamental plant by conventional methods (hybridization, inbreeding and mass selection) is time and labor consuming, depends on the existing gene pool(s) and violently influenced by environmental conditions. On the other hand, callus culture can be utilized as a powerful tool for genetic cell transformation via somaclonal variation and promoting mutagenesis and genetic engineering that can be either more rapid than traditional breeding and leading to new genes and genotypes. The indirect plant regeneration system developed for *A. majus* provided a step towards the application of such methodology, for this ornamental plant. Moreover, this protocol is rapid with induction of callus to acclimatizing of plantlets to greenhouse completed within 21 weeks.

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