

## ***In Vitro* Culture of Carnation (*Dianthus caryophyllus* L.) Focusing on the Problem of Vitrification**

**Mahdiyeh Kharrazi<sup>1\*</sup>, Hossein Nemati<sup>1</sup>, Ali Tehranifar<sup>1</sup>, Abdolreza Bagheri<sup>2</sup> and Ahmad Sharifi<sup>3</sup>**

<sup>1</sup> Department of Horticulture, College of Agriculture, Ferdowsi University of Mashhad, Mashhad, IRAN

<sup>2</sup> Department of Biotechnology and Plant Breeding, College of Agriculture, Ferdowsi University of Mashhad, Mashhad, IRAN

<sup>3</sup> Iranian Academic Culture for Education, Culture and Research-Branch of Mashhad, Mashhad, IRAN

### **ABSTRACT**

This study was conducted to evaluate the effect of plant growth regulators on *in vitro* shoot multiplication, vitrification and rooting of two carnation cultivars (Eskimo Mogr and Innove Orange Bogr). Isolated axillary buds were cultured on MS medium supplemented with different levels of Benzyl amino purine (BAP) or kinetin (Kin) in combination with 0.2 mg/l NAA and shoot multiplication and vitrification rate were tested. Then different concentrations of Naphthalene acetic acid (NAA) or Indole butyric acid (IBA) on root induction of regenerated shoots were surveyed. Shoot regeneration and vitrification were influenced by the cultivar, kind of cytokinin and its concentration. Eskimo Mogr produced more shoots in comparison with Innove Orange Bogr (6.17 and 3.83 shoots/explant, respectively). BAP-containing media had more shoot multiplication and vitrification than Kin and by increasing the concentration of cytokinin, shoot multiplication and vitrification increased. There was a negative correlation between plant height and internode length with vitrification (-77% and -85%, respectively). By considering the amount of multiplication and rate of vitrification for obtaining the highest number of normal shoots, MS medium containing lower concentration of BAP (1mg/l for Eskimo Mogr and 2 mg/l for Innove Orange Bogr) and 0.2 mg/l NAA is recommended. Rooting of regenerated shoots was dependent on the cultivar. The highest root percentage for Eskimo Mogr (83%) was achieved on the medium supplemented with 0.5 mg/l NAA, while for Innove Orange Bogr (98%) it was obtained on the medium containing 1.5 mg/l IBA.

**Key Words:** *Dianthus caryophyllus* L., micropropagation, shoot multiplication, vitrification, *in vitro* rooting.

### **INTRODUCTION**

Carnation (*Dianthus caryophyllus* L.) is one of the world's most popular cut flower. The importance of this ornamental flower is due to its beauty, diversity of colors, excellent keeping quality, and wide range of different forms (Ali et al. 2008, Kanwar and Kumar 2009). Plant tissue culture technique has been used in this plant for virus elimination, commercial micropropagation, gene transformation and induction of somaclonal variation (Brar et al. 1995, Altvorst et al. 1992). Adventitious shoot regeneration in carnation was affected by culture environment, plant growth regulators and kind of explant (Kanwar and Kumar 2009, Casanova et al. 2008, Pareek et al. 2004, Onamu et al. 2003). The commonly used explants were axillary buds and shoot tips (Brar et al. 1995, Miller et al. 1991, Salehi 2006). Regenerated shoots from axillary buds and shoot tips are true to type because in their regeneration process there is no callus phase (Brar et al. 1995). The results of previous studies showed that the highest multiplication rate of carnation was achieved in the range of 0.5 to 3 mg/l cytokinins and depended on the cultivar. Different types of cytokinin such as BAP, Kin, TDZ and 2ip has been used (Ali et al. 2008, Brar et al. 1995, Kovac 1995, Mujib and Pal 1995, Salehi 2006). On the other hand, shoot regeneration and multiplication of carnation affected by the type of cytokinin and its concentration (Ali et al. 2008, Brar et al. 1995).

Vitrification, a physiological and morphological disorder in regenerated plants, is one of the most drastic problems during micropropagation of carnation (Mii et al. 1990, Ziv 1991). This abnormality affects the production at commercial level (Yadav et al. 2003). In vitrified plantlets, leaves have large vacuolated mesophyll cells, fewer stomata and less photosynthetic capacity. Their stem and leaves are often rigid, thick and breakable (Sharma and Mohan 2006, Kevers et al. 2004, Leshem 1983). During acclimatization, they have shown difficulties and the ability of vitrified plants to grow normally reduced (Yadav et al. 2003). Vitrification, which is a hindrance to tissue culture, is not yet fully understood (Kevers et al. 2004).

The results of previous studies showed that different factors affect the micropropagation, vitrification and rooting of carnation cultivars and one of the most important factors is the type of growth regulators and their concentration (Hazarika and Bora 2010, Paques 1991, Leshem 1988). So the objective of this study was to evaluate the effect of different types of cytokinin and auxin and their concentrations on micropropagation, vitrification and rooting of carnation axillary bud explants under *in vitro* condition.

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\* Corresponding author: ma\_kharrazi@yahoo.com

## MATERIALS AND METHODS

In this study stem segments of two carnation cultivars, Eskimo Mogr and Innove Orange Bogr, were selected in their active growth stage. After defoliation, shoot explants about 5-6 cm length were washed thoroughly with tap water for 30 min and surface sterilization was done by immersing them in 2% sodium hypochlorite solution containing Tween 20 (2 drops/100 ml solution) for 15 min. Then explants were rinsed 3 times with sterile distilled water. The axillary buds with about 3-5 mm length were removed and then cultured on the basic MS medium containing BAP or Kin (0, 1, 2, 3 and 4 mg/l) in combination with 0.2 mg/l NAA, 30 g<sup>-1</sup> sucrose, 8 g<sup>-1</sup> agar. For rooting of regenerated shoots the effect of NAA or IBA (0.5, 1.5, 2.5 and 3.5 mg/l) on MS medium was evaluated. The pH of the medium was adjusted to 5.7-5.8 prior to autoclaving (15 min at 121 °C and 1.5 kg cm<sup>-2</sup> pressure). The cultures were incubated in a growth chamber at 25±2°C with a 16-h photoperiod (2500-3000 Lux) provided by cool-white fluorescent lamps. Subcultures were done every 4 weeks. Finally, rooted plants were transferred to pots containing pasteurized mixture of sand and perlite (1:1, v/v) for acclimatization. The pots were covered with polyethylene bags initially for 2 weeks to maintain humidity. Acclimatized plants were transferred to greenhouse condition.

Treatments were verified based on ANOVA using MSTAT-C program and the means were compared using Duncan's multiple range test (DMRT) at 1% level.

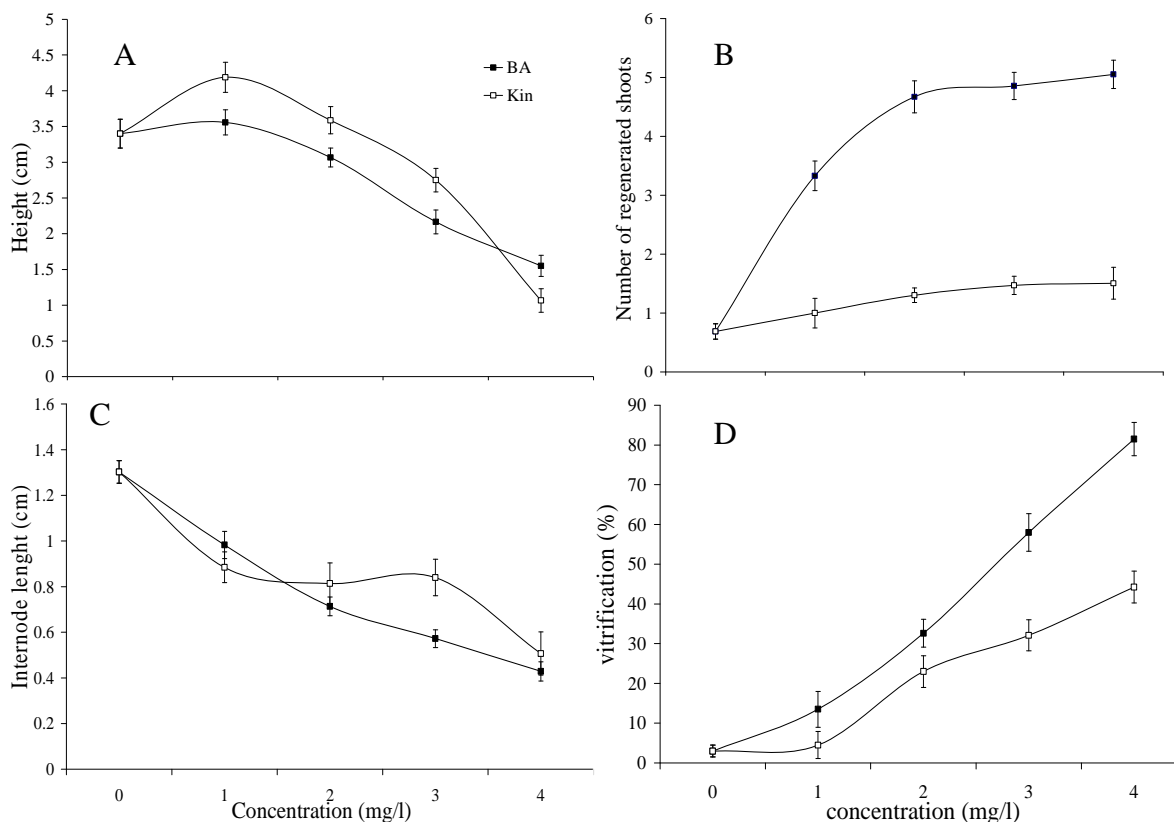
## RESULTS AND DISCUSSION

### *Proliferation*

The results of this experiment showed that there was a significant difference between two cultivars in shoot regeneration. In addition, plant growth regulators significantly influenced the proliferation and vitrification of regenerated shoots. In all treatments, Eskimo Mogr produced more shoot than Innove Orange Bogr (2.94 and 1.97, respectively). Also two carnation cultivars had the same reaction to the different types of cytokinin. Medium containing BAP was more effective than Kin on shoot regeneration (Fig. 1-B). The highest number of shoots (5 shoots/explant) was formed on medium supplemented with 4 mg/l BAP compared to 0.69 shoot on basal medium (Fig. 1-B). But shoot regeneration in different levels of Kin was nearly constant (1.18 shoots/explant) (Fig. 1-B). In most concentrations of cytokinins, Kin led to produce longer shoots in compare with BAP and as the level of cytokinins increased, the height of shoots decreased (Fig. 1-A). As regards BAP induces the loss of apical dominance and accelerates the growth of lateral buds, it seems that increasing the concentration of this growth regulator induces the growth of axillary buds.

The results of previous studies have shown that application of cytokinin stimulated sprouting and development of higher number of axillary buds and apical dominance release was suppressed with the increase of cytokinin concentration (Kapchina-Toteva and Yakimova 1997). In addition, shoot height and internode length tended to decrease with increasing cytokinin concentration (Brar et al. 1995). These results are in close agreement with our findings.

Ali et al. (2008) reported that the media supplemented with BAP produced more shoots than Kin. They also reported that using Kin in the medium caused the regeneration of longer shoots, in comparison with BAP. Brar et al. (1995) found that increasing the concentration of cytokinin led to decreasing the height of regenerated shoots and by increasing the level of cytokinin, the rate of regeneration increased. While, Majib and Pal (1995) reported that among the various concentration of BAP, the lowest amount of it (0.5 mg/l) caused the highest number of shoots per explant. Our results are similar to Ali et al. (2008) and Brar et al. (1995) reports but in contrast to the finding of Mujib and Pal (1995). Since they reported that among the various concentration of BAP, the lowest amount of it (0.5 mg/l) caused the highest number of shoots per explant. This contradiction may be due to different cultural condition or genotype.



**Figure 1.** The effect of different concentrations of BAP and Kin on height (A), number of regenerated shoots (B), internode length (C) and vitrification (D) of carnation.

### Vitrification

Vitrification, a commonly problem in carnation tissue culture caused morphological changes of regenerated shoots during *in vitro* micropropagation. The results of our experiment indicate that vitrified shoots were short and thick with luminous and breakable leaves. Friable callus developed at the base of the stem, from which many roots grew in all direction. This phenomenon was affected by genotype, kind of plant growth regulators and their concentrations. Eskimo Mogr had more vitrification percentage than Innove Orange Bogr (34% and 23% respectively). The percentage of vitrified regenerated shoots in medium containing BAP was more than Kin in all concentrations (40% and 26% respectively) (Fig. 1-D). In addition, with increasing the concentration of these growth regulators, vitrification increased. There was a negative correlation between vitrification percentage and height of regenerated shoots and their internode length ( $R = -77\%$  and  $-85\%$ , respectively). It seems that the internode length can be an appropriate indicator for determining the vitrification rate. The results of this experiment showed that regenerated shoots treated with Kin, were longer and had more internode length and less vitrification, in comparison with BAP.

Cytokinins have been shown to induce hyperhydricity in many species, usually in a concentration-dependent manner and when other conditions in the culture system are not optimized (Kataeva et al. 1991, Olmos et al. 1997, Ochatt et al. 2002, Ivanova and Van Staden 2008). Overcoming vitrification is an integral part in establishing a satisfactory micropropagation method for carnation. Sharma and Mohan (2006) reported that cytokinin type is one of the most important factors that affected the rate of vitrification, So that using Kin on the medium declined the rate of vitrification, while the application of BAP increased the rate of this phenomenon. They also reported that the highest shoot multiplication and vitrification was occurred on MS medium supplemented with 2 mg/l BAP. They noted that by decreasing the concentration of BAP from 2 to 0 mg/l during subsequent subcultures, the rate of vitrification decreased.

Based on the results of this study, it can be concluded that BAP stimulates shoot proliferation in carnation, but at the same time it increases the rate of vitrification (especially in higher concentrations). Consequently it is suggested that for obtaining normal plantlets with minimum vitrification rate, lower concentrations of BAP (1mg/l for Eskimo Mogr and 2 mg/l for Innove Orange Bogr) is more suitable (Table 1).

**Table 1.** Effect of different concentrations of cytokinin in combination with 0.2 mg/l NAA on number and height of regenerated shoots and vitrification of two carnation cultivars.

PGRs (mg/l)	Eskimo Mogr			Innove Orange Bogr		
	Number of shoots	height (cm)	Vitrification (%)	Number of shoots	height (cm)	Vitrification (%)
0 BAP	0.75 c	3.67 a	4 e	0.62 c	3.12 b	2 d
1 BAP	4.16 b	3.50 a	18 de	2.5 b	3.6 b	9 cd
2 BAP	5.50 a	3.3 a	37.25 c	3.83 a	2.83 b	28 bc
3 BAP	5.85 a	2.5 b	68.5 b	3.86 a	1.83 c	47.5 b
4 BAP	6.16 a	1.9 b	84.2 a	3.93 a	1.2 c	78.5 a
0 Kin	0.75 c	3.67 a	4 e	0.62 c	3.12 b	2 d
1 Kin	1.16 c	3.4 a	9 e	0.83 c	4.97 a	0 d
2 Kin	1.45 c	2.5 b	28 cd	1.15 c	4.67 a	18 cd
3 Kin	1.76 c	2.5 b	37.2 c	1.17 c	3 b	27 bc
4 Kin	1.83 c	0.7 c	58 b	1.18 c	1.43 c	30.5 bc

Means with similar letters in each column are not significantly different by Duncan's multiple range test ( $p < 0.01$ ).

#### **Rooting of regenerated shoots**

Results showed that there was a significant difference between two cultivars of carnation in rooting percentage. So that the highest root percentage for Eskimo Mogr (83%) were achieved on the medium supplemented with 0.5 mg/l NAA, while for Innove Orange Bogr (98%) it was obtained on the medium containing 1.5 mg/l IBA. These findings confirmed the different reactions of various cultivars of carnation to different kinds of auxins. In addition, plant growth regulators significantly influenced the height and number of produced roots. Generally, the highest root number and root length was occurred at the range of 1.5-2.5 mg/l of NAA or IBA but the best rooting percentage was achieved at the range of 0.5-1.5 mg/l of these two growth regulators. By increasing the concentration of auxin up to 0.5 mg/l, rooting percentage increased. But generally higher concentrations of auxin led to decreasing the rooting percentage (Table 2).

Auxin plays a major role in root induction through its effect on the first cell division which forms root initials (Farooq et al. 2008). Salehi (2008) found that there is a great deal of variation among cultivars of carnation in their requirements for plant growth regulators *in vitro* and rooting of carnation cultivars is highly genotype dependent. He reported that recommending the general medium for rooting of different carnation cultivars, is not possible. Results of his study showed that plantlet with higher root numbers have the lower root lengths. But our results did not show this negative relation. Ali et al. (2008) found that among the various concentrations of NAA and IBA, the best rooting response were obtained on MS medium containing 1 mg/l NAA. Also, there are several reports on suitability of auxin- free medium for rooting of carnation cultivars (Mii et al. 1990, Ilahi et al. 1995). Notwithstanding these reports, we observed that auxin- free medium did not result in a good rooting percentage, may be due to genotype differences. These results are in accordance with the finding of Cuzzuol et al. (1996) and Jagannatha et al. (2001).

**Table 2.** Effect of different concentrations of auxin on rooting percentage, root length and root number of two carnation cultivars.

PGRs (mg/l)	Eskimo Mogr			Innove Orange Bogr		
	Rooting percentage (%)	Root length (cm)	Root number	Rooting percentage (%)	Root length (cm)	Root number
0 NAA	12.2 d	0.5 g	0.5 f	51.8 d	1.6 ab	5.3 c
0.5 NAA	83.2 a	1.33 de	4.3 d	65.8 bc	1.58 ab	7.54 b
1.5 NAA	70.4 b	1.56 cd	5.44 bc	60.2 c	1.6 ab	3.28 de
2.5 NAA	64.4 b	2.9 a	7.28 a	51.2 d	1.32 bc	3.06 e
3.5 NAA	15.4 d	1.12 def	4.8 cd	24.4 f	0.5 d	0.5 f
0 IBA	12.2 d	0.5 g	0.5 f	51.8 d	1.6 ab	5.3 c
0.5 IBA	72 b	1.9 bc	2.42 e	70.6 b	1.9 a	9.3 a
1.5 IBA	65.8 b	2.1 b	5.9 b	98 a	1.4 b	4.34 cd
2.5 IBA	65.6 b	0.92 efg	4.8 cd	65.8 bc	1.36 bc	4.7 c
3.5 IBA	51.8 c	0.78 fg	4.6 cd	33.2 e	1.04 c	4.46 c

Means with similar letters in each column are not significantly different by Duncan's multiple range test ( $p < 0.01$ ).

## CONCLUSIONS

From the present study it was concluded that several factors (cultivar, kind and concentration of growth regulators) can influence the micropropagation, vitrification and rooting of carnation. There was a significant difference in the average number of regenerated shoots between the cultivars. Eskimo Mogr produced more regenerated shoots than Innove Orange Bogr. The use of Kin resulted in a significantly lower shoot multiplication compared to BAP. Increasing the concentration of cytokinin increased the number of regenerated shoots and also vitrification rate. Application of BAP in the medium compared with Kin stimulates the rate of this phenomenon. The greatest shoot formation was obtained on media with 4 mg/l BAP, however in this medium vitrification was very high. Since BAP has better effect on shoot regeneration, it is suggested that lower concentration of this growth regulator can be used for obtaining the desirable shoot regeneration with less vitrification rate. Rooting of regenerated shoots was depended on genotype and the best rooting response took place on 0.5 to 1.5 mg/l of each auxins. The rooted plantlets were successfully acclimatized and transferred to greenhouse (with a 90% success), where they displayed normal growth.

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