EFFICIENT PLANT REGENERATION VIA INDIRECT ORGANOGENESIS IN CARNATION (*DIANTHUS CARYOPHYLLUS SEMPERFLORENS FLORE PLENO*) CULTIVARS

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ABSTRACT

Callus induction and plant regeneration are important steps of in vitro plant breeding of ornamental plants. In this study, the effects of different combinations of plant growth regulators (PGRs), promoters, and minerals on callus induction and plant regeneration in different carnation cultivars were studied in a completely randomized design with three replications. For callus induction, 16 different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BA), 1-naphthaleneacetic acid (NAA), and casein hydrolysate (CH) were studied using in vitro leaf explants. The Murashige and Skoog (MS) medium supplemented with 0.2 mg·dm⁻³ of 2,4-D and 200 mg·dm⁻³ of CH showed the highest frequency of callus induction. Among the cultivars, ‘Noblesse’ showed the highest rate of callus induction (91.67%). Regarding regeneration, BA, NAA, silver nitrate (AgNO₃), and adenine hemisulfate (As) were used in ten different combinations. The ‘Cameron’, ‘Tabasco’, and ‘Noblesse’ cultivars with 95.24% regeneration percentage showed the highest rate of plant regeneration. Generally, in most cultivars, the highest regeneration rate and shoot number per explant were found in the MS medium supplemented with 3 mg·dm⁻³ of BA, 0.6 mg·dm⁻³ of NAA, 5 mg·dm⁻³ of AgNO₃, and 40 mg·dm⁻³ of As. According to the results, the highest regeneration frequency was obtained when 40 mg·dm⁻³ of As was added to the medium. Finally, the flow cytometry analysis indicated that there were no significant differences between in vitro regenerated and control plants in terms of DNA ratios.

Key words: silver nitrate, adenine hemisulfate, callus induction, shoot regeneration

INTRODUCTION

Carnation (*Dianthus caryophyllus* L.) is a member of the large Caryophyllaceae family, with 80 genera and about 2000 species, which are either annual or perennial and grow mostly in the northern hemisphere (Maurya et al. 2020). The standard carnation

species (2n = 30) with a large flower size is the most important of these, used as a cut flower around the world. Due to its excellent storage quality, a wide range of forms and colors, the ability to withstand long-distance transportation, and its remarkable ability to rehydrate after continuous shipping, this plant has special value in the flower industry (Iantcheva 2016).

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There are several obstacles for carnation hybridization breeding, including lack of viable and sufficient pollen, cross-incompatibility, chromosomal aberrations, lack of viable gametes, post-zygotic barriers at an immature stage, and embryo without endosperm (Dyaberi et al. 2015). Therefore, in vitro breeding methods are widely used to breed this species (Azadi et al. 2016). Direct or indirect plant regeneration is a major prerequisite for genetic transformation and in vitro mutation breeding techniques (Casas et al. 2010; Ntui et al. 2010). Various plant growth regulators (PGRs) have been used in earlier studies, and in most cases, a high concentration of cytokinin, including TDZ was necessary for shoot regeneration in different carnation cultivars (Thakur & Kanwar 2018; Thu et al. 2020; Maurya et al. 2021). In the in vitro regeneration other additions can play a useful role. Addition of silver nitrate (AgNO_3) to the regeneration media was successful in direct plant regeneration from cotyledon explants in *Cosmos bipinnatus* (Jaberi et al. 2018). Also casein hydrolysate (CH) and adenine hemisulfate (As) were effective in the regeneration of different species (Khaleida & Al-Forkan 2006; Gatica Arias et al. 2010; Arora et al. 2011; Pandey & Tamta 2014; Cardoso 2019; Al Ramadan et al. 2021; Lakshmi et al. 2021; Maurya et al. 2021).

In vitro culture techniques are useful in overcoming hybridization barriers in carnation, in in vitro mutagenesis and genetic transformation. In this study, an efficient protocol for high-frequency callus induction from leaf explants and plant regeneration via indirect shoot organogenesis was established using different PGRs, AgNO_3 , As, and CH, in nine standard carnation cultivars. The objective of this study is to develop a plant regeneration protocol for different cultivars of carnation via callus induction, using in vitro leaf explants.

MATERIALS AND METHODS

Plant materials

Nine standard carnation cultivars – ‘Cameron’, ‘Eskimo’, ‘Grand Slam’, ‘Liberty’, ‘Mariposa’, ‘Noblesse’, ‘Tabasco’, ‘Tabor’, and ‘White Liberty’ – were studied.

The in vitro leaf explants obtained from axillary buds were excised into 0.5–0.7 cm pieces, followed by incubation with the axial side down on the Murashige and Skoog (1962) (MS) basal medium. In all experiments, culture conditions included a temperature of $25 \pm 2^\circ\text{C}$ under a PPFD of $80 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ using fluorescent light with a 16-h photoperiod.

Callus induction

The leaf segments were incubated on the MS media containing $30 \text{ g}\cdot\text{dm}^{-3}$ of sucrose, solidified with 0.7% (w/v) Plant agar (Duchefa Biochemie) at pH 5.8, before autoclaving and supplemented with different concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) (0, 0.2, 0.5, and $1 \text{ mg}\cdot\text{dm}^{-3}$), 1-naphthaleneacetic acid (NAA) (0 and $0.5 \text{ mg}\cdot\text{dm}^{-3}$), 6-benzylaminopurine (BA) (0, 0.2, and $0.5 \text{ mg}\cdot\text{dm}^{-3}$), and CH (0 and $200 \text{ mg}\cdot\text{dm}^{-3}$) in 16 different combinations (Table 1). For callus induction leaf explants were cultured in 80-mm Petri dishes containing 25 ml of fresh MS medium.

Indirect organogenesis

For shoot regeneration, three-week-old calluses were transferred to the MS media supplemented with BA (0, 2, 3, and $5 \text{ mg}\cdot\text{dm}^{-3}$), NAA (0, 0.2, and $0.6 \text{ mg}\cdot\text{dm}^{-3}$), As (0, 20, 40, and $80 \text{ mg}\cdot\text{dm}^{-3}$), and AgNO_3 (0 and $5 \text{ mg}\cdot\text{dm}^{-3}$) in 10 different combinations, in culture jars ($10 \times 6 \text{ cm}$). AgNO_3 was filter-sterilized through a $0.24\text{-}\mu\text{m}$ Millipore membrane and added to the autoclaved culture media. The other compounds were added prior to the autoclaving at 121°C for 15 min.

Overcoming hyperhydricity and shoot proliferation

To eliminate hyperhydricity of regenerated shoots caused by high concentrations of cytokines, hyperhydric shoots were transferred to a modified MS recovery medium containing $0.275 \text{ g}\cdot\text{L}^{-1}$ of NH_4NO_3 with $0.5 \text{ mg}\cdot\text{dm}^{-3}$ gibberellic acid (GA_3), $0.3 \text{ mg}\cdot\text{dm}^{-3}$ BA, and $0.1 \text{ mg}\cdot\text{dm}^{-3}$ NAA based on a pilot experiment (data not shown) for about two weeks. GA_3 was filter-sterilized through a $0.24\text{-}\mu\text{m}$ filter and added to the media after autoclaving.

Shoot elongation, root formation, and acclimatization

The regenerated shoots were cut off from the base and transferred for elongation to an agar-solidified

MS elongation medium supplemented with $1 \text{ mg} \cdot \text{dm}^{-3}$ of kinetin (KIN) and $0.1 \text{ mg} \cdot \text{dm}^{-3}$ of NAA for three weeks. For root induction, the shoots with 4–7 leaves of 2–3 cm long were transferred to the $\frac{1}{2}$ MS medium (rooting medium) supplemented with $0.26 \text{ mg} \cdot \text{dm}^{-3}$ of indole-3-butyric acid (IBA). After 14 days, root formation was observed (Figure 1G–I). The rooted plantlets with 5–8 leaves and a height of 3–4 cm were removed from the jars, and the agar was washed away gently from the roots with tap water. The plantlets were transplanted to plastic trays ($50 \times 40 \times 10 \text{ cm}$) containing perlite, coco peat, and peat moss (1:1:1, v/v). They were then kept in a greenhouse under controlled conditions (a temperature of $25 \pm 2^\circ \text{C}$ under a PPFD of $100\text{--}110 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and a relative humidity of 90%) for acclimatization.

Flow cytometry analysis

The leaves of in vitro-regenerated plants and donor plants were finely chopped in 500 μl of ice-cold nuclei extraction buffer (PVP: 10 mg, DNA-extraction buffer: 1 cc, RNase: 15 μl , and PI: 30 μl). Then, 2 ml of nuclei staining solution (CyStain® PI Absolute P) was added to the crude mixture. To eliminate cell debris, the suspension was filtered with a 50- μm filter, and the nuclei were collected into pre-chilled tubes. The ploidy level of the samples was analyzed by a flow cytometer (BD FACS Calibur, Biosciences, USA) using a 488-nm argon laser to excite the PI fluorochrome and an FL-2 detector with a 585/42 bandpass filter. Samples were run on low pressure, and 104 nuclei were counted within the double gate (Nalousi et al. 2019).

Data collection and statistical analyses

Each treatment contained 12 explants cultured in 80-mm Petri dishes with three replications. Different callus induction and plant regeneration parameters were collected, and the significant differences in means were statistically analyzed by one-way analysis of variance (one-way ANOVA). For non-normal distribution data, logarithmic transformation was performed prior to further analysis. Values represent means $\pm$ SD and different letters show a significant difference ($p < 0.05$) based on Duncan's multiple range test (DMRT).

RESULTS

Callus induction

Significant differences were observed in callus formation depending on different combinations or concentrations of PGRs and genotype (Tables 1 & 2). The highest callus induction frequency was observed in the 'Noblesse' cultivar with 91.67% and the 'White Liberty' cultivar with 90.33%. The results indicated that in treatments with a high callus growth rate, callus induction frequency significantly decreased. In this regard, 'Noblesse' and 'White Liberty' cultivars with the highest callus frequency percentage showed a slow growth rate (Table 2). And *vice versa*, in cultivars with a high callus growth rate (earlier callus induction), such as 'Cameron' and 'Eskimo', a lower callus induction frequency was observed. The MS medium supplemented with $0.2 \text{ mg} \cdot \text{dm}^{-3}$ of 2,4-D and $200 \text{ mg} \cdot \text{dm}^{-3}$ of CH showed the highest quality of organogenic calli (the calli with the highest rate of plant regeneration with a green-yellow color and a semi-compact characteristic) among the cultivars (Figure 1A, B).

Plant regeneration

After four weeks, some green spots were observed on the surface of the calli (Figure 1C). After eight weeks, shoot regeneration was observed. Addition of As to the media significantly increased the percentage of shoot induction and the shoot number in all tested cultivars. While, in the MS medium supplemented with $3 \text{ mg} \cdot \text{dm}^{-3}$ of BA, $0.6 \text{ mg} \cdot \text{dm}^{-3}$ of NAA, and $5 \text{ mg} \cdot \text{dm}^{-3}$ of AgNO_3 without As, the mean shoot regeneration rate was 53.96% among the cultivars. Adding $40 \text{ mg} \cdot \text{dm}^{-3}$ of As significantly increased the regeneration rate to 78.83% (Table 3). The highest rate of regeneration was observed in 'Noblesse', 'Cameron', and 'Tabasco' cultivars with an average regeneration rate of 95.24%. The 'Cameron' cultivar with 28.3 shoots per explant showed the highest number of shoots in each explant. In high concentrations of BA ($> 3 \text{ mg} \cdot \text{dm}^{-3}$) and As ($> 40 \text{ mg} \cdot \text{dm}^{-3}$), the rate of regeneration and the number of shoots decreased. In the MS medium supplemented with $3 \text{ mg} \cdot \text{dm}^{-3}$ of BA, $0.6 \text{ mg} \cdot \text{dm}^{-3}$ of NAA, $5 \text{ mg} \cdot \text{dm}^{-3}$ of AgNO_3 , and $40 \text{ mg} \cdot \text{dm}^{-3}$ of As, the highest rate of regeneration was observed in all the cultivars (Table 4).

Recovery of hyperhydricity, shoot elongation, rooting, and acclimatization

In the majority of regenerated shoots, hyperhydricity was observed (Figure 1E). Such shoots were recovered from hyperhydricity on the recovery medium after two to three weeks (Figure 1F). Subsequently, the recovered plantlets were transferred to the MS elongation medium on which the shoots of all cultivars increased by approximately 2–3 cm after three weeks (Figure 1G).

All shoots were rooted after two weeks when transferred to the rooting medium (Figure 1H). More than 80% of the plantlets survived transfer to greenhouse condition and acclimatized successfully (Figure 1I–K).

Genetic stability assessment

The genetic stability assessment using the flow cytometry analysis did not detect significant differences between in vitro-regenerated and control plants in terms of DNA ratios (Figure 2).

Figure 1. Different stages of organogenesis from leaf explants in carnation cultivars: A, B – explant type and callus induction after 21 days; C – green spots on the surface of the callus 25 days after sub-culture on the regeneration medium; D – efficient shoot induction on the MS medium supplemented with $3 \text{ mg} \cdot \text{dm}^{-3}$ of BA, $0.6 \text{ mg} \cdot \text{dm}^{-3}$ of NAA, $5 \text{ mg} \cdot \text{dm}^{-3}$ of AgNO_3 , and $40 \text{ mg} \cdot \text{dm}^{-3}$ of As two months after incubation; E – hyperhydric shoots regenerated in most explants; F – recovery from hyperhydricity on the modified MS ($0.275 \text{ mg} \cdot \text{dm}^{-3}$ of NH_4NO_3) containing $0.5 \text{ mg} \cdot \text{dm}^{-3}$ of GA_3 , $0.3 \text{ mg} \cdot \text{dm}^{-3}$ of BA, and $0.1 \text{ mg} \cdot \text{dm}^{-3}$ of NAA after 2–3 weeks; G – shoot elongation on the MS medium with $1 \text{ mg} \cdot \text{dm}^{-3}$ of KIN and $0.1 \text{ mg} \cdot \text{dm}^{-3}$ of NAA; H – rooting on the $\frac{1}{2}$ MS macro- and full micronutrients and $1.5 \text{ mg} \cdot \text{dm}^{-3}$ of IBA; I – the acclimatization of plantlets in the greenhouse with controlled climate system and high relative humidity; J, K – acclimatized plantlets transferred to the soil in the greenhouse (scale bars = 1 cm)

Figure 2. Ploidy level of the regenerated (right) and mother plants (left) of carnation cultivars through flow cytometry analysis. DNA content histogram in 'Tabor', 'Mariposa', and 'Cameron' cultivars showed no significant differences. The nuclei were isolated from the leaves of the mother and regenerated plants

Table 1. Different PGR treatments used for callus induction from leaf segments of carnation

PGRs (mg·dm ⁻³)	Treatment number															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2,4-D	0.5	0.5	0.5	0.5	0.2	0.2	1	1	0.5	0.5	0.5	0.5	0.5	0.5	0.2	0.2
BA	0.2	0.2	0.5	0.5	0.2	0.2	0.2	0.2	0.2	0.2	0.5	0.5	0	0	0	0
NAA	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0	0	0	0	0	0	0	0
CH	0	200	0	200	0	200	0	200	0	200	0	200	0	200	0	200

Table 2. Callus induction response to treatments among different carnation cultivars based on Duncan's multiple range grouping

Callus induction in different carnation cultivars											
Dependent variable	Visual score	SS	‘Mariposa’	‘Eskimo’	‘Cameron’	‘Tabasco’	‘Tabor’	‘Noblesse’	‘Liberty’	‘White Liberty’	‘Grand Slam’
CF 0–100%	excellent	≥ 86						91.67%		90.33%	
	good	51–85	72.33			62.25%					
	medium	25–50					38.92%				37.72%
	weak	≤ 25		18.67%	22.73%				19.67%		
DCI 2–30 day	excellent	≤ 9		6			5				
	good	10–19				14			14		
	medium	20–29	25		21			21			21
	weak	≥ 30								33	
CGR score 1–5	excellent	5		5			5				
	good	4				4			4		
	medium	3			3					3	3
	weak	≤ 2	2					1			
CC score 1–5	green	5	5	5	5	5	5		5		
	green-yellow	4	4	4	4	4	4				
	white	3								3	3
	brown	≤ 2						1			

CF – callus frequency, DCI – days to callus induction (showing the number of days required for visible callus induction), CGR – callus growth rate scored from 1 for lowest to 5 for highest, CC – callus color scored from 1 for brown to 5 for green, SS – statistical score

Table 3. Effects of different PGR treatments on regeneration frequency for plant regeneration in different carnation cultivars

Regeneration frequency (%) for carnation cultivars													
PGR (mg·dm ⁻³)				‘Mariposa’	‘Eskimo’	‘Cameron’	‘Tabasco’	‘Tabor’	‘Noblesse’	‘Liberty’	‘White Liberty’	‘Grand Slam’	Mean for all cultivars
BANAAAs AgNO ₃													
3	0.2	0	0	4.76±0.74d	23.81±0.74e	23.81±0.74e	28.57±1.3e	19.05±0.74d	23.81±0.74d	19.05±0.74d	23.81±0.74e	4.76±0.74d	19.05±0.95f
3	0.6	0	0	19.05±0.74b	66.67±0.74b	71.43±1.3b	66.67±0.74b	66.67±0.74b	42.86±2.24c	66.67±0.74b	66.67±0.74b	19.05±0.74b	53.96±2.38b
2	0.2	0	0	4.76±0.74d	14.29±1.3g	4.76±0.74h	14.29±0.14f	9.53±0.74e	14.29±1.3e	9.53±0.74e	4.76±0.74h	9.53±0.74c	9.53±0.46h
2	0.6	0	0	0e	38.1±0.74d	9.53±1.5f	38.1±0.74d	0f	23.81±1.98d	0f	9.53±1.5g	0e	13.23±1.79g
2	0.2	20	5	9.53±1.4c	38.1±0.74d	33.34±0.74d	38.1±0.74d	9.53±1.44e	33.34±0.74c	9.53±1.5e	23.81±1.98e	23.81±1.98b	24.34±1.36e
3	0.2	20	5	9.53±0.74c	33.34±1.5d	76.19±0.74b	38.1±1.98d	33.34±1.5c	80.95±0.74b	76.19±0.74b	38.1±1.98d	23.81±1.5b	45.51±2.86b
2	0.6	40	5	4.76±0.74d	57.14±1.27c	61.91±1.5c	61.91±1.5c	42.86±0.79c	61.91±1.5c	33.34±1.5c	42.86±1.3c	9.53±0.74d	41.80±2.46c
3	0.6	40	5	38.1±0.74a	90.47±0.74a	95.24±0.74a	95.24±0.74a	80.95±0.74a	95.24±0.74a	90.47±0.74a	90.47±0.74a	33.34±0.74a	78.83±2.76a
5	0.6	40	5	4.76±0.74d	23.81±0.74e	66.67±0.74c	61.91±0.74c	28.57±2.24d	14.29±1.3e	28.57±2.24d	19.05±1.98f	9.53±0.74c	28.57±2.42d
3	0.6	80	5	0e	19.05±0.74f	19.05±0.74e	19.05±0.74f	33.34±1.5c	0f	28.57±1.3d	19.05±0.74f	4.76±0.74d	15.87±1.32g

Different letters in a column indicate significant differences according to Duncan's multiple range test ($p < 0.05$); values represent mean $\pm$ SE

Table 4. Effects of different treatments on shoot number per explant for plant regeneration in different carnation cultivars

Shoot number per explant for carnation cultivars													
PGR (mg·dm ⁻³)				‘Mariposa’	‘Eskimo’	‘Cameron’	‘Tabasco’	‘Tabor’	‘Noblesse’	‘Liberty’	‘White Liberty’	‘Grand Slam’	Mean for all cultivars
BA NAAAs AgNO ₃													
3	0.2	0	0	0.43±0.07c	1.81±0.08d	3.85±0.2f	1.81±0.78f	2.81±0.12e	1.81±0.08d	2.24±0.7c	1.81±0.077c	0.43±0.07d	1.89±0.12e
3	0.6	0	0	1±0.02a	1.15±0.022c	8.53±0.12e	8.5±0.11d	7.76±0.12d	1.91±0.13e	1.91±0.13d	5.86±0.18b	1±0.02c	4.18±0.38d
2	0.2	0	0	0.29±0.05d	0.67±0.055e	0.29±0.044g	1.05±0.1g	1.05±.1f	0.76±0.06e	0.67±0.5e	0.29±0.04d	0.57±0.04d	0.63±0.03f
2	0.6	0	0	0e	1.81±0.077d	3.71±0.59g	4.76±0.56g	0g	1.81±0.08d	0f	1.05±0.1c	0 e	1.46±0.20e
2	0.2	20	5	0.76±0.12c	2.29±0.0c	20.67±0.91d	3.14±0.066f	0.76±0.12f	5±0.33c	0.76±0.12e	1.53±0.12c	1.95±0.17c	4.22±0.85d
3	0.2	20	5	0.76±0.06b	7.09±0.17b	28.3±0.29a	21.24±1.29c	21.24±1.29c	6.47±0.21b	7.81±0.21b	7.09±0.18b	2.28±0.22b	11.36±1.08b
2	0.6	40	5	2.71±0.34a	5.76±0.37c	24.19±0.38b	27.81±0.66a	24.19±0.38a	5.76±0.38c	11.57±0.31b	21.81±0.38a	2.71±0.34a	14.06±1.15b
3	0.6	40	5	1±0.033a	22.33±0.11a	23.76±0.17b	22±0.1b	24.14±0.12a	19±0.36a	20.19±0.11a	22.33±0.38a	0.67±0.22b	17.27±1.05a
5	0.6	40	5	0.19±0.033d	6.4±0.1b	7.81±0.21e	16.19±0.51c	4.76±0.41e	2.9±0.36c	5±0.55d	4.76±0.41c	0.62±0.06c	5.4±0.53c
3	0.6	80	5	0e	0.76±0.033e	1.05±0.01g	5.09±0.01e	1.57±0.04e	0 f	2.38±0.01c	1.28±0.011c	0.38±0.06c	1.39±0.18e

Note: See Table 1

DISCUSSION

Because of carnation hybridization breeding barriers (Dyaberi et al. 2015), an efficient protocol for plant organogenesis is important for in vitro mutation breeding and genetic engineering purposes. Direct or indirect shoot regeneration of range of carnation genotypes using different explants has been reported. Taking into account that different carnation cultivars showed different responses to plant regeneration treatments, the optimization of efficient plant regeneration protocol would be valuable (Zia et al. 2020). In the present study, we developed an efficient indirect organogenesis protocol for shoot regeneration for different standard carnation cultivars (Iantcheva 2016). The type and the concentration of PGRs are extremely important in callus induction, and they will subsequently affect the plant regeneration ability (Muhamad et al. 2018). The previous studies showed that a concentration higher than $0.5 \text{ mg} \cdot \text{dm}^{-3}$ 2,4-D resulted to embryogenic calli induction in carnation (Choudhary & Chin 1995; Karami et al. 2008). In this study, a high rate of organogenesis was observed in the calli induced with a low concentration of 2,4-D. Thakur et al. (2002) also reported efficient organogenic callus induction in carnations using $0.5 \text{ mg} \cdot \text{dm}^{-3}$ of 2,4-D and $0.5 \text{ mg} \cdot \text{dm}^{-3}$ of NAA. Moreover, for organogenic callus induction from the leaf explant of carnations, the MS medium containing $0.5 \text{ mg} \cdot \text{dm}^{-3}$ of 2,4-D and $1 \text{ mg} \cdot \text{dm}^{-3}$ of BAP was used. In our experiment the medium supplemented with a low concentration 2,4-D ($0.2 \text{ mg} \cdot \text{dm}^{-3}$) and $200 \text{ mg} \cdot \text{dm}^{-3}$ of CH showed the highest quality of organogenic calli too.

The MS medium with BAP or without PGRs caused the formation of hyperhydric shoots (Jain et al. 2001). Similarly, in our study, although high concentrations of BAP showed a high regeneration rate, this resulted in the formation of hyperhydric shoots.

The source of the explant is very important for efficient callus induction and, subsequently, for mode of regeneration (Kanwar & Kumar 2009). For example, carnation petal explants were successfully used for callus induction and somatic embryogenesis (Karami et al. 2008). In most cases leaf explants were used for plant regeneration

in carnations (Esmail et al. 2013) as the most suitable for plant regeneration in different carnation cultivars (Abu-Qaoud 2013; Esmail et al. 2013). Among various types of cytokines and auxins, BA and 2,4-D showed the highest effect on plant regeneration (Yantcheva et al. 1998; Jain et al. 2001; Kantia & Kothari 2002; Kanwar & Kumar 2009). Our results showed that a high concentration of auxins (2,4-D or NAA) increased the callus growth rate; however, it had a negative effect on the plant regeneration ability. Using promoters such as silver nitrate, adenine hemisulfate, and casein hydrolysate significantly increased the callus growth rate and the initiation of direct and indirect plant regeneration in different species (Yantcheva et al. 1998; Dubois et al. 2000; Akasaka-Kennedy et al. 2005; Jaber et al. 2018). In the present study, the above promoters were used as cell division and organogenesis enhancers.

For indirect organogenesis, the possibility of somaclonal variation is an important challenge (Nalouisi et al. 2019). Flow cytometry has been confirmed as a rapid, simple, and reproducible technique for the assessment of DNA content and ploidy variation occurring in indirect plant regeneration (Escobedo-Gracia-Medrano et al. 2018). Therefore, in the present study, to evaluate the genetic stability of regenerated plants, flow cytometry was used for genome size estimation compared with donor plants. The results showed that there was a high similarity in terms of ploidy levels between the regenerated and donor plants. Previously, flow cytometry has been used to detect somaclonal variation in *Arracacia xanthorrhiza* plants regenerated via indirect morphogenesis, and no genomic changes were found (Vitamvas et al. 2019).

CONCLUSION

Here, an efficient protocol for high-frequency organogenic callus induction from leaf explants and plant regeneration via indirect shoot organogenesis was established using different BA, 2,4-D, NAA, AgNO_3 , As, and CH, in nine standard carnation cultivars. A highest frequency of organogenic callus induction was obtained in medium containing $0.2 \text{ mg} \cdot \text{dm}^{-3}$ of 2,4-D and $200 \text{ mg} \cdot \text{dm}^{-3}$ of CH.

In the most of studied cultivars, the highest regeneration rate and shoot number per explant were obtained in the MS medium supplemented with 3 mg·dm⁻³ of BA, 0.6 mg·dm⁻³ of NAA, 5 mg·dm⁻³ of AgNO₃, and 40 mg·dm⁻³ of As. Silver nitrate and adenine hemisulfate showed a significant effect as regeneration promoters. The resulted carnation regenerants proved to be stable against ploidy level, which make the obtained protocol suitable for in vitro breeding programs.

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