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High Frequency Plant Regeneration from Leaf Derived Callus of *Dianthus caryophyllus* L.

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Abstract

An efficient procedure was developed for *in vitro* callus induction, proliferation and regeneration of carnation cultivar (*Dianthus caryophyllus* L.) using leaf, nodal and inter-nodal explants on Murashige and Skoog's medium (MS) supplemented with exogenous plant growth regulators. For morphogenic callus induction and proliferation from various explants, MS medium supplemented with 3.0 mg/l 2,4-D was highly efficient with 100% callus induction frequency from inter-nodal explants. Leaf explants showed quicker response than nodal and inter-modal explants, for callus initiation within 6 days of inoculation. Best grown callus was obtained from leaf explant. The leaf-derived callus was maintained up to several weeks, which indicated that 8-week incubation period was the most suitable for obtaining well proliferated, morphogenic callus. Temperature variation also affected the growth of *in vitro* induced morphogenic callus from various explants. Results have shown that 27˚C proved to be the best temperature for morphogenic callus induction and proliferation from leaf and inter-nodal explants. Among the auxin-cytokinin combination, MS medium containing 1.0 mg/l N(6)-benzylaminopurin (BAP) and 2.0 mg/l NAA showed the highest efficiency of callus initiation and proliferation from leaf, nodal and inter-nodal explants. Light conditions proved better for callogenesis and proliferation from leaf, nodal and inter-nodal explants. Regeneration response from well grown morphogenic callus was prominent on MS medium supplemented with 3.0 mg/l BAP alone and 1.0 mg/l NAA with 3.0 mg/l BAP.

Keywords

***Dianthus caryophyllus* L., Regeneration Potential, Plant Growth Regulators, Morphogenic Callus Induction**

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1. Introduction

Carnation, a member of the family *Caryophyllous*, has 88 genera and 1750 species, which were cultivated over 2000 years ago in Asia and Europe. There are over 300 species, mostly perennials having showy petals of the colors of the rainbow except blue [1]. World's major exporters of carnation are Europe, Latin America and Israel. Asian countries like Japan, India and Pakistan are also concentrating upon the consumption of carnation. Considering the benefits of this crop and to fulfill the world's demand for carnation, these countries have started to propagate it and its varieties are maintained year after year by cuttings or by other vegetative propagules. Thus plants remain same phenotypically and genotypically. Since 1950s researchers have been trying to get rid of internal infections of carnation using Plant Tissue Culture Technique, which refers to growing plant cells, tissues and organ in an artificially prepared nutrient medium static or liquid, under aseptic conditions [2]. Callogenesis has been used as a tool to study plant physiology, cell biology and genetics. It has also been exploited in agricultural practices, forestry and industry in order to achieve mass-propagation of disease free plants. Moreover, plants with immense medicinal value are becoming endangered due to over-exploitation. Therefore, rapid multiplication of important drug yielding plant is becoming imperative [3]. Plants regenerated via callogenesis exhibit heritable mutations which influence the plant morphology, flower color and shape. It is novel source of genetic variation which contradicts the concept of clonal uniformatiy in micropropagation. Plant variants obtained thus are called somaclones. Carnation is also observed to be influenced by somaclonal variations. Although somaclonal variation is undesirable in the context of micropropagation, it can be used for genetic improvement. As a result of these variations different colors have been observed in carnation flowers [4]. Although efficient systems have been reported for the regeneration of plants from leaf base [5], stem explants [6] and for the induction of direct somatic embryogenesis from leaf, petal as well as anther explants by Pareek and Kothari [7], Karami *et al.* [8], and Xiaopeng *et al.* [9], but still more efficient protocols are needed to increase the number of plants in a shorter period of time. With realization of foreseen advantages and unprecedented applications this technique has received great attention all over the world including Pakistan and India. Keeping in view all the above facts, the present investigation was undertaken to optimize the culture conditions for efficient callogenesis, proliferation and regeneration of *Dianthus caryophyllous* L. The main aim was to determine the optimum concentration of plant growth regulators, best source of explants and well suited micro environment conditions for callus induction, proliferation and regeneration of carnation cultivar (*Dianthus caryophyllous* L.)

2. Materials and Methods

2.1. *In Vitro* Growth Conditions

Nodal and inter-nodal explants of 0.5 - 0.7 cm size were excised and used as explants, while soft leaf explants of 0.5 - 0.7 cm^2 size were excised and used as explants obtained from carnation cultivar (*Dianthus caryophyllous* L.). Explants were obtained from pot grown plants which were washed thoroughly with tap water and house hold detergent to remove all the traces of dust particles for 20 minutes. The explants were surface sterlised and inoculated according to the method described by Ali *et al.* [2]. MS medium [10] supplemented with different concentrations of auxin and cytokinin along with 3% sucrose was used and pH of the medium was adjusted to 5.71 ± 0.5 and 10% of Agar-agar was used for solidification of media which was autoclaved at 121˚C for 15 minutes at 15 lbs/in^2 pressure. For callus induction and proliferation, MS media containing different concentration and combination of auxin and cytokinin was used. Ten explants were cultured in each test tube and were sub-cultured for proliferation. Sub-culturing was carried out after every 4-week interval unless otherwise indicated. The temperature of 27˚C ± 1˚C was maintained in the growth chamber and photoperiod was 16 hours with 8 hours dark period in every 24 hours cycle. 21˚C - 28˚C temperature was maintained in individual smaller growth chamber to check the effect of temperature on callus proliferation. The cultures were maintained under light intensity of 2500 - 3000 lux.

Callogenesis: Callus was induced from three different explants, leaf, node and inter-nodal explants in MS medium supplemented with different concentrations of 2,4-D alone and BAP in combination with NAA.

2.2. Effect of Dark and Light Conditions

For callus induction, leaf explants were cultured in MS medium supplemented with growth regulators and incubated under light (35 $\mu mole \cdot m^{-2} \cdot s^{-1}$ and dark conditions (24 h dark) at 27˚C ± 1˚C.

2.3. Callus Induction Frequencies (CIF)

Callus induction frequencies were calculated as the percent explants inducing callus by using following equation and was converted to mean CIF, as described [11].

Callus induction frequency (%) = number of calli producing explants/total number of explants in the culture × 100.

2.4. Growth Assessment Tests

Total fresh weight of proliferating calli and multiplied shoots were assessed after specific interval. Total fresh weight was determined and growth value (GV) was calculated according to the following equation as described [12].

Fresh weight (%) = (final weight – initial weight/initial weight) × 100.

Growth value (%) = (final fresh weight – initial fresh weight/initial fresh weight) × 100.

Total dry weight of calli was measured after dehydration treatment at 70˚C ± 1˚C for two days.

2.5. Induction of Somatic Embryos and Plantlets by Regeneration

To standardize the medium for the regeneration frequencies of somatic embryos, the well proliferated embryogenic calli from leaf explants were transferred to regeneration media comprising MS medium supplemented with different concentration of BAP and NAA.

2.6. Plantlet Establishment for Multiple Shooting

After initiating germination of somatic embryos, the plantlets were isolated and transferred onto the MS medium for shoot multiplication. These shoots were sub-cultured on MS medium supplemented with NAA for root induction.

2.7. Callus Regeneration Frequencies (CRF)

Callus regeneration frequencies were calculated as the percent calli inducing shoots, using following equation and was converted to mean CRF.

Callus regeneration frequency (%) = (number of calli inducing shoots/total number of calli in the culture) × 100.

3. Results and Discussion

3.1. Effect of Auxin on Callus Induction Response

Mass scale production of carnation from shoot tip culture (Apical Meristem) was described by many scientists [13] [14]. Numerous studies have also been made to regenerate carnation *in vitro* using different explants through callus phase [15]-[17]. In present study callus induction was investigated using MS medium supplemented with different concentrations of 2,4-D ranging from 1 - 5.0 mg/l. leaf explants showed 100 % callus induction response within 6 days of inoculation on MS medium containing 3.0 mg/l 2,4-D (**Table 1**). Callus induction started from the cut edges of the leaf explants and covered the whole tissue (**Figure 1**). Calli obtained from leaf explants were friable and granular, well-proliferated, greenish yellow and morphogenic. Increased or decreased concentration of exogenous auxin, not only reduced the rate of callus initiation but also suppressed the proliferation efficiency. The higher concentration of auxin caused necrosis in calli which appeared in the form of dark brownish patches on yellow callus (**Figure 1**, **Table 1**). Callus induction response of nodal explants was 90% within 9 days of inoculation on MS medium containing 3.0 mg/l 2,4-D and calli were friable and granular, well-proliferated, yellowish white and morphogenic (**Figure 1**, **Table 2**). 90% callogenic response within 10 days of inoculation was obtained by inter-nodal explants of carnation on MS medium supplemented with 3.0 mg/l 2,4-D (**Table 3**). Calli obtained from inter-nodal explants were also friable and granular, well-proliferated, yellowish white and morphogenic.

3.2. Effect of Auxin-Cytokinin Interaction on Callus Induction Response

Many researchers reported the best callus induction response of carnation on MS medium supplemented with

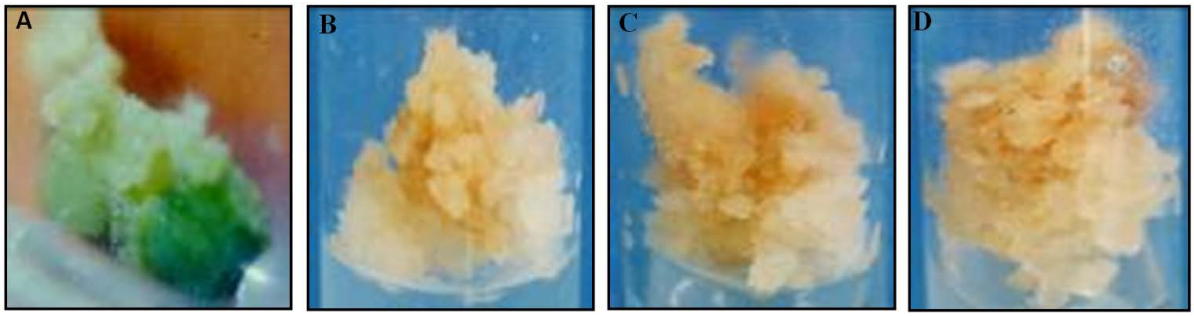

Figure 1. *In vitro* callus induction and proliferation in carnation. (A) Morphogenic callus induction derived from cut edges of leaf explant after 1 week of culture on MS medium containing 3.0 mg/l 2,4-D; (B) Shown is 3-week-old culture of proliferated callus induced from leaf explants on MS medium containing 3.0 mg/l 2,4-D; (C) Higher concentration of auxin (5.0 mg/l 2,4-D) caused necrosis in callus in the form of brownish patches; (D) 2-week-old friable, granular, yellowish-white and morphogenic callus derived from nodal explants on MS medium containing 3.0 mg/l 2,4-D.

Table 1. Effect of various concentrations of auxin (2,4-D) on callus induction frequencies (%) in carnation using leaf explants.

Media composition (mg/l)	Days for callus induction	Mean No. of cultures showing callus induction	Types of callus	CIF (%)
1.0 mg/l 2,4-D	18	2 ± 0.247	Whitish yellow, granular, soft, less proliferated	20
2.0 mg/l 2,4-D	10	4 ± 0.420	Whitish yellow, granular, friable, morphogenic	40
3.0 mg/l 2,4-D	6	10 ± 0.414	Greenish yellow, granular, morphogenic, soft	100
4.0 mg/l 2,4-D	8	5 ± 0.414	Yellowish brown, globular, morphogenic, rough	50
5.0 mg/l 2,4-D	12	2 ± 0.609	Brown, non-morphogenic, less proliferated	20

Abbreviation: ±: standard error of mean; CIF: callus induction frequency.

Table 2. Effect of auxin (2,4-D) on callus induction frequencies (%) in carnation using nodal explants.

Media composition (mg/l)	Days for callus induction	Mean No. of cultures showing callus induction	Types of callus	CIF (%)
1.0 mg/l 2,4-D	22	3 ± 0.346	Whitish yellow, friable, soft, less proliferated	30
2.0 mg/l 2,4-D	12	5 ± 0.171	Whitish yellow, soft, granular, less proliferated	50
3.0 mg/l 2,4-D	9	9 ± 0.39	Whitish yellow, friable, well proliferated	90
4.0 mg/l 2,4-D	10	8 ± 0.579	Whitish yellow, granular, well proliferated	80
5.0 mg/l 2,4-D	14	4 ± 0.5	Brownish yellow, compact, non-morphogenic	40

Abbreviation: ±: standard error of mean; CIF: callus induction frequency.

Table 3. Effect of auxin (2,4-D) on callus induction frequencies (%) in carnation using inter-nodal explants.

Media composition (mg/l)	Days for callus induction	Mean No. of cultures showing callus induction	Types of callus	CIF (%)
1.0 mg/l 2,4-D	24	3 ± 0.269	Whitish yellow, granular, less proliferated	30
2.0 mg/l 2,4-D	15	4 ± 0.5	Whitish yellow, granular, less proliferated	40
3.0 mg/l 2,4-D	10	9 ± 0.171	Whitish yellow, granular, less proliferated	90
4.0 mg/l 2,4-D	11	7 ± 0.577	Yellow, globular, well proliferated	70
5.0 mg/l 2,4-D	15	3 ± 0.25	Brownish yellow, compact, less proliferated	30

Abbreviation: ±: standard error of mean; CIF: callus induction frequency.

auxin alone or auxin along with cytokinins. Esmaiel *et al.* [5], reported the best callus induction response from leaf base explants MS medium supplemented with 0.5 mg/l of NAA + 0.5 mg/l of BAP and medium supplemented with 0.01 mg/l of NAA + 1.0 mg/l of BAP. To observe the effects of different higher concentration of exogenous auxin in combination with cytokinin, MS medium supplemented with 1 - 5.0 mg/l NAA and 1.0 mg/l BAP was used. Leaf explants showed 20% callogenic response on MS medium supplemented with 2.0 mg/l NAA and 1.0 mg/l BAP within 16 days of inoculation (**Table 4**). Calli were brownish yellow, compact, less proliferated and non morphogenic. Nodal explants showed 20% callogenic response on MS medium supplemented with 2.0 mg/l and 3.0 mg/l NAA in combination with 1.0 mg/l BAP within 21 and 24 days of inoculation respectively. Calli were brownish yellow, compact, less proliferated and non morphogenic (**Table 5**). Inter-nodal explants showed 20% and 10% callogenic response in MS medium supplemented with 2.0 mg/l and 3.0 mg/l NAA in combination with 1.0 mg/l BAP within 18 and 23 days of inoculation respectively (**Table 6**).

Calli were brownish yellow, compact, less proliferated and non morphogenic. Higher and lower concentration of cytokinin along with BAP showed a reduction in callus induction efficiency (**Tables 4-6**).

Table 4. Effect of NAA with BAP on callus induction frequencies (%) in carnation using leaf explants.

Media composition (mg/l)	Callus induction	Mean No. of cultures showing callus induction	Types of callus	CIF (%)
1.0 mg/l BAP + 1.0 mg/l NAA	NI	---	---	---
1.0 mg/l BAP + 2.0 mg/l NAA	16	2 ± 0.609	Whitish yellow, compact, prolifrated	20
1.0 mg/l BAP + 3.0 mg/l NAA	20	1 ± 0.216	Brownish yellow, less proliferated	10
1.0 mg/l BAP + 4.0 mg/l NAA	20	1 ± 0.856	Brownish yellow, less proliferated	10
1.0 mg/l BAP + 5.0 mg/l NAA	26	2 ± 0.609	Brownish yellow, less proliferated	10

Abbreviation: ±: standard error of mean; NI: no induction; CIF: callus induction frequency.

Table 5. Effect of NAA with BAP on callus induction frequencies (%) in carnation using nodal explants.

Media composition (mg/l)	Days for callus induction	Mean No. of cultures showing callus induction	Types of callus	CIF (%)
1.0 mg/l BAP + 1.0 mg/l NAA	NI	---	---	---
1.0 mg/l BAP + 2.0 mg/l NAA	21	2 ± 0.5	Brownish yellow, friable, soft	20
1.0 mg/l BAP + 3.0 mg/l NAA	24	2 ± 0.25	Brownish yellow, friable, less proliferated	20
1.0 mg/l BAP + 4.0 mg/l NAA	NI	---	---	---
1.0 mg/l BAP + 5.0 mg/l NAA	NI	---	---	---

Abbreviation: ±: standard error of mean; NI: no induction; CIF: callus induction frequency.

Table 6. Effect of NAA with BAP on callus induction frequencies (%) in carnation using inter-nodal explants.

Media composition (mg/l)	Days for callus induction	Mean No. of cultures showing callus induction	Types of callus	CIF (%)
1.0 mg/l BAP + 1.0 mg/l NAA	NI	---	---	---
1.0 mg/l BAP + 2.0 mg/l NAA	18	2 ± 0.5	Yellow, friable, less proliferated, non-morphogenic	20
1.0 mg/l BAP + 3.0 mg/l NAA	23	1 ± 0.25	Brownish yellow, friable, less proliferatednon-morphogenic	10
1.0 mg/l BAP + 4.0 mg/l NAA	NI	---	---	---
1.0 mg/l BAP + 5.0 mg/l NAA	NI	---	---	---

Abbreviation: ±: standard error of mean; NI: no induction; CIF: callus induction frequency.

3.3. Effect of Environmental Factors on Callus Proliferation Response

To observe the effects of environmental factors on callus proliferation response, we incubated *in vitro* grown well-induced calli by leaf, nodal and inter-nodal explants on MS media supplemented with 3.0 mg/l 2,4-D under the temperature of 21˚C, 23˚C, 25˚C, 27˚C, and 30˚C for the period upto 3, 5, 8 and 10 weeks. The observation has shown that 8 weeks proved to be the excellent incubation period to obtain highly proliferated, *in vitro* grown whitish yellow, friable, soft granular and morphogenic callus. After 8-week of incubation the rate of proliferation was lost as callus started to lose the vigor and appeared brownish yellow (**Figure 2**, **Table 7**). Fresh weight of these calli proliferated at various temperatures was measured which indicated the highest fresh weight gain in callus mass proliferated at 27˚C temperature. Dry weight of these calli obtained after dehydration at 70˚C for 2 days indicated the highest dry mass of calli proliferated at 27˚C of incubation temperature. Our experiment proved that 27˚C is most optimal incubation temperature for attaining fully proliferated callus (**Figure 3**). Under

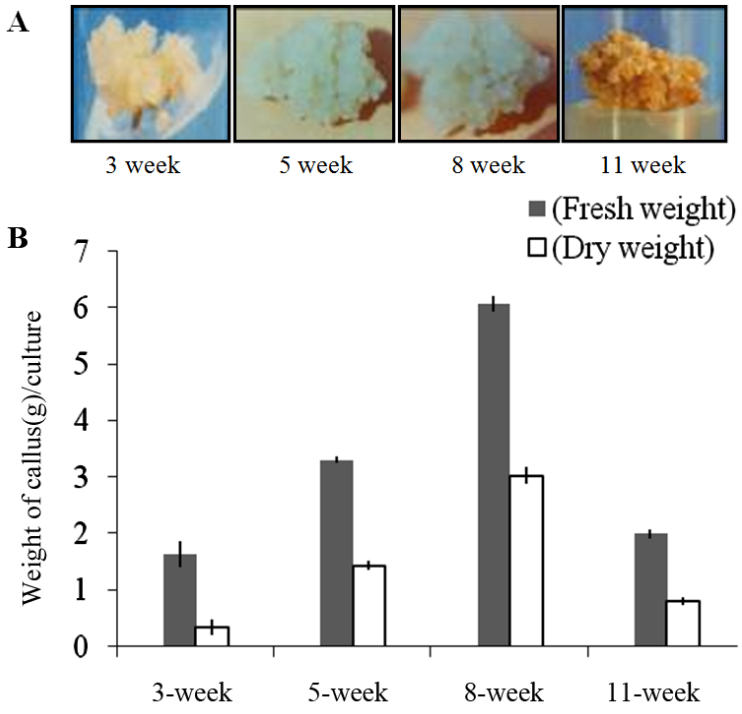

Figure 2. Effect of incubation period on callus proliferation responesin carnation. (A) *In vitro* callus induction and proliferation from leaf explants on MS medium containing 3.0 mg/l 2,4-D. Note that well proliferated morphogenic callus was obtained at 8 week of inoculation; (B) Fresh and dry weight measured after 3, 5, 8 and 10 weeks of inoculation. Error bars represent means ± SE ($n \geq 5$).

Table 7. Effect of incubation period on callus growth and proliferation response in carnation using leaf explants.

Media: MS + 3.0 mg/l 2,4-D		
Incubation time (weeks)	Type of callus	Proliferation response
3	Whitish yellow, soft, friable, granular, less proliferated	++
5	Whitish yellow, soft, friable, granular, less proliferated and morphogenic	+++
8	Whitish yellow, soft, friable, granular, well proliferated, morphogenic	++++
10	Brownish yellow, globular, non-morphogenic, decreased rate of proliferation	+

Key: --- = no induction; + = slow response of proliferation; ++ = moderate; +++ = good; ++++ = excellent.

dark condition callus induction frequency was very low in leaf, nodal and inter-nodal explants. All calli were brownish yellow with strong necrotic signs. Leaf explants proved to be the best with 100% callus induction efficiency within 6-days of callus initiation when exposed to white light. Morphologically these calli were embryogenic, greenish yellow, compact and smooth (**Table 8**).

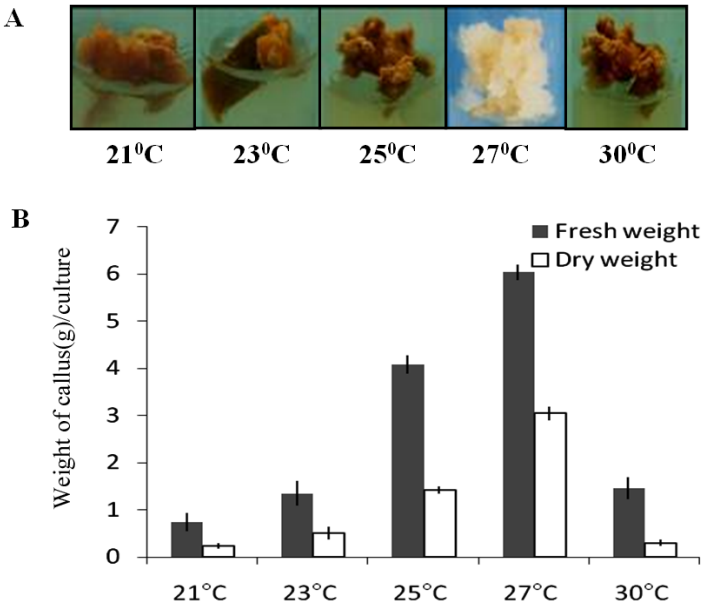

Figure 3. Effect of temperature variation on callus proliferation response. (A) *In vitro* callus induction and proliferation from leaf explants on MS medium containing 3.0 mg/l 2,4-D. After 8-week of inoculation well proliferated morphogenic callus was obtained at 27˚C incubation. (B) Fresh and dry weight of calli measured after incubating at 21, 23, 25, 27 and 30˚C for 8 weeks. Error bars are means ± SE (n ≥ 5).

Table 8. Effect of light and dark conditions on callogenesis response in *Dianthus caryophyllus* L.

Leaf explant				
Treatment	Media	CIF (%)	Days for callus induction	Callus morphology
Light	MS + 2,4-D 3.0 mg/l	100	6	Greenish yellow, morphogenic, proliferated
	MS + 2,4-D 1.0 mg/l + BAP 3.0 mg/l	80	9	Whitish yellow, morphogenic, proliferated
Dark	MS + 2,4-D 3.0 mg/l	30	16	Brownish yellow, necrotic, non proliferated
	MS + 2,4-D 1.0 mg/l + BAP 3.0 mg/l	20	18	Brownish yellow, necrotic, non proliferated
Nodal explant				
Light	MS + 2,4-D 3.0 mg/l	30	9	Whitish yellow, morphogenic, proliferated
	MS + 2,4-D 1.0 mg/l + BAP 3.0 mg/l	20	15	Brownish yellow, necrotic, non proliferated
Dark	MS + 2,4-D 3.0 mg/l	10	20	Brownish yellow, necrotic, non proliferated
	MS + 2,4-D 1.0 mg/l + BAP 3.0 mg/l	10	24	Brownish yellow, necrotic, non proliferated
Inter-nodal explant				
Light	MS + 2,4-D 3.0 mg/l	20	10	Whitish yellow, morphogenic, proliferated
	MS + 2,4-D 1.0 mg/l + BAP 3.0 mg/l	10	16	Brownish yellow, necrotic, non proliferated
Dark	MS + 2,4-D 3.0 mg/l	10	24	Brownish yellow, necrotic, non proliferated
	MS + 2,4-D 1.0 mg/l + BAP 3.0 mg/l	10	25	Brownish yellow, necrotic, non proliferated

Abbreviation: CIF: callus induction frequency.

3.4. Regeneration and Shoot Multiplication from Morphogenic Callus

Callus differentiation on MS medium supplemented with 0.2 mg/l NAA and 1.0 mg/l BAP have been reported by Esmaiel *et al.*, [5] while regeneration protocols for *in vitro* propagation of carnation via somatic embryogenesis have also been reported [2] [18]. However, Shibat and Mii [19], reported difficulty in regenerating plants from callus due to their highly dedifferentiated nature. Shoot regeneration in carnation is influenced by genotype, explant source and the balance of plant growth regulators [20]. Our research showed that best grown calli were obtained from leaf explant and transferred to regeneration medium to study regeneration potential. Somatic embryos from morphogenic calli gave rise to shoots with the frequency of 100 % on MS medium supplemented with 3.0 mg/l BAP alone and 1.0 mg/l NAA in combination with 3.0 mg/l BAP. Initially originated shoots were sub-cultured for shoot multiplication onto 3.0 mg/l BAP supplemented medium to assess growth value of *in vitro* multiplied shoots (**Table 9**). Healthy shoots were further separated and transferred onto the medium supplemented with root induction growth regulator 1.0 mg/l NAA alone and 1.0 mg/l NAA in combination with 3.0 mg/l BAP which showed 70% root regeneration efficiency in both. Fully grown plantlets were sub-cultured for maintenance in growth chamber in controlled conditions (**Figure 4**, **Table 10**). Mangal *et al.* [21], and Onamu *et al.* [22], used MS medium supplemented with combination of NAA and Kinetin for shoot induction from meristem. Liquid MS medium was found to be more effective for efficient shoot multiplication than MS medium solidified with agar or phytagel [2]. Kanwar and Kumar [23], reported the rooting response from *in vitro* multiplied shoots regenerated from callus, on half strength MS medium supplemented with 2.0 mg/l indole butyric acid (IBA) and 0.2% activated charcoal. In current study, well developed multiple shoots when attained considerable heights (~3 cm) were shifted to MS medium containing growth regulator 1.0 mg/l NAA alone and 1.0 mg/l NAA in combination with 3.0 mg/l BAP which showed best *in vitro* rooting response. The callus mediated regeneration not only improves genetic variability of otherwise limited germplasm of carnation but may be utilized in carnation improvement program via *in vitro* cell selection, somaclonal variations, genetic transformation and for various biotic and abiotic stresses in this crop. In the present study tissue culture methodology was used in three steps to optimize and standardized the *in vitro* growth conditions for efficient carnation cultivation *i.e.*, callus induction, callus proliferation and callus regeneration to induce organogenesis directly from somatic embryogenesis. Additionally, variable responses are observed depending on the kind of exogenous-hormone, which are largely related to the source of explants and micro-environment such as incubation period, temperature and light conditions.

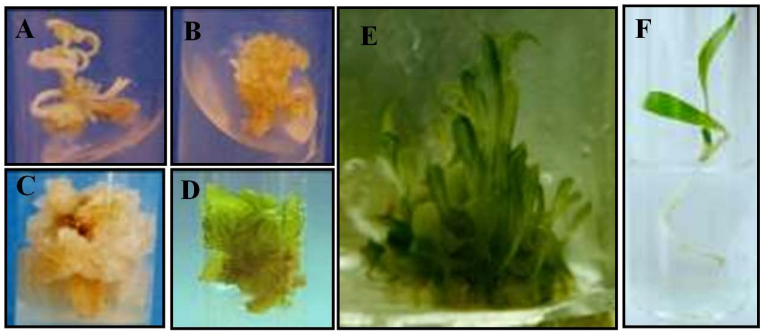

Figure 4. *In vitro* plant regeneration in carnation. (A) Callus induction and proliferation from leaf explant on MS medium containing 3.0 mg/l 2,4-D. (B) Callus regeneration from leaf derived calli on MS medium containing 3.0 mg/l BAP. (C)-(E) Shoot multiplication from *in vitro* induced leaf derived regenerated callus on MS medium containing 3.0 mg/l BAP. (F) Rooting of *in vitro* raised shoots on liquid MS medium supplemented with 3.0 mg/l BAP and 1.0 mg/l NAA.

Table 9. Growth value assessment test in MS medium supplemented with 3.0 mg/l BAP.

MS Media	Shoot induction (%)	No. of shoots/culture	Average length of shoots (cm)	Growth value
3.0 mg/l BAP	100	15	3.5 cm	79.56

Table 10. Study of regeneration frequencies (%) of *Dianthus caryophyllus* L. in MS medium supplemented with NAA and BAP.

MS media	Leaf explant		MS + NAA (mg/l)	Inter-nodal explant	
	$CRF_{(S)}$ (%)	$CRF_{(R)}$ (%)		$CRF_{(S)}$ (%)	$CRF_{(R)}$ (%)
Basal medium (control)	0	0	Basal medium (control)	0	0
1.0 mg/l BAP	10	20	1.0 mg/l NAA	10	30
2.0 mg/l BAP	50	40	2.0 mg/l NAA	20	50
3.0 mg/l BAP	100	70	3.0 mg/l NAA	70	100
4.0 mg/l BAP	60	50	4.0 mg/l NAA	30	40
5.0 mg/l BAP	10	10	5.0 mg/l NAA	10	20
1.0 mg/l BAP + 1.0 mg/l NAA	10	10	1.0 mg/l BAP + 1.0 mg/l NAA	30	20
2.0 mg/l BAP + 1.0 mg/l NAA	50	40	2.0 mg/l BAP + 1.0 mg/l NAA	50	40
3.0 mg/l BAP + 1.0 mg/l NAA	100	70	3.0 mg/l BAP + 1.0 mg/l NAA	80	90
4.0 mg/l BAP + 1.0 mg/l NAA	70	40	4.0 mg/l BAP + 1.0 mg/l NAA	50	40
5.0 mg/l BAP + 1.0 mg/l NAA	20	10	5.0 mg/l BAP + 1.0 mg/l NAA	10	20

Abbreviation: $CRF_{(S)}$: callus regeneration frequencies of shoots; $CRF_{(R)}$: callus regeneration frequencies of roots.

4. Conclusion

From the present study it was concluded that although the callus can be produced under several growth conditions using leaf, nodal and inter-modal explants but the differentiation of callus induced from nodal and inter-nodal explants was difficult. Thus the efficient protocol is developed to elevate the efficiency of callogenesis using leaf explants source, by optimizing hormonal concentrations suitable for high frequency of callus induction and regeneration via somatic embryogenesis that can facilitate mass propagation of carnation predominantly.

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