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AN EFFICIENT PROTOCOL FOR *IN VITRO* PROPAGATION OF CARNATION (*DIANTHUS CARYOPHYLLUS*)

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Abstract

The present research work involves shoot formation, their multiplication and rooting in carnation *Dianthus caryophyllous*. For shoot formation both apical and nodal meristems were used. MS medium containing BAP alone or in combination with kinetin was tested. Best shoot formation response was obtained after 6 days of inoculation from apical meristem and after 7 days of inoculation from nodal meristem on MS medium supplemented with 4.0 mg/l BAP. Apical meristem showed more pronounced effect for shoot formation than nodal meristem. Well-developed shoots were shifted for their multiplication. Maximum number of multiple shoots were obtained on MS medium containing 1.0 mg/l BAP. These multiple shoots increased in their number when were given subsequent incubation period. Addition of Kinetin to BAP failed to show good shoot multiplication response. Shoots after attaining the size of 5.0 cm were shifted for rooting. Best rooting response was obtained on MS medium containing 1.0 mg/l NAA. Well rooted plants were shifted into glass house for hardening and acclimatization and were shifted to natural climatic conditions.

Introduction

Carnation *(Dianthus caryophyllus* L.) is most famous for its use as a cut flower in the florist trade, but also perform well in the garden as a bedding plant. Carnation a member of the family Caryophyllous has 88 genera and 1750 species. Carnations were cultivated over 2000 years ago. Modern varieties were developed first in France in 1840. The name carnation is derived from the latin tern *"Carnatio"* meaning fleshness. Caryophyllous means pink refers to the colour of blooms of the original species.

It is the perennial plant having cut growth habit. Its crop succeeds in sunny environment and salt laden air. It dislikes acidic soil but tolerate pH range from 6-8. This plant is one of the world's most important cut flowers due to perpetual flowering (Mii *et al*., 1990) and single and multi-color cultivars. Carnation flowers from July to August and seed ripens from August to September. Flowers are attractive to moths and butterflies (*Lepidoptera*) and are pollinated by them (Gender, 1994).

The carnation flower is a wonderful accent to bouquets and carnation home floral arrangements. It has been used as an unconventional food plant. It is used in salad garnishing, in fruit salads and for flavoring fruits etc. It can be used as substitute for rose petals in making syrup. An essential oil is also obtained from its flowers, which is used in perfumery where 500 Kg of flower produce 100 gm of oil. The flower heads are dried and used in cosmetic and sachats. Its flowers are considered to be alexiteric, antispasmodic, cardiotonic, diaphoretic and nervine (Chopra *et al*., 1986).

Consumption of carnation in world floriculture market in 1985 was about 12.5 billion dollars. In 1990, the total consumption rose to about 25 billion dollars. In 1995, the total consumption increased up to 31 billion dollars. Continuous development in production, imports and economic variables into account, has raised the consumption of carnation in world's market, up to 35 billion dollars in 2000.

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World's major exporters of carnation are Europe, Latin Americas and Israel. Latin America exports carnation to the market of Europe and USA like Mexico, Costa Rica, Columbia, Peru, Chili, Argentina and Dominican Republic. 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 vegetatively, thus its varieties are maintained year after year by cutting or other vegetative propagules. In this way the plants remain same phenotypically and genotypically. But unfortunate events can happen causing clone to decline. They may become internally infected through chance infection by pathogen like fungi, bacteria and viruses which decrease their yield significantly.

Since 1950,s researchers have found technique to rid the carnation from internal infection, called 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. By using this technique, better quality and disease resistant plants can be obtained. This technique is based upon the concept of totipotency which means single cell can give rise to whole plant by cell division.

The potential advantages of this technique involves the evaluation of large number of genotypes by using very small space in the laboratory and reduction of time between two successive generations by controlling the environmental and nutritional conditions along with reduction of differences in morphology and stages of development. In recent years this technique has gained greater momentum on commercial application in the field of plant biotechnology and floriculture.

The most successful and most widely used discipline of plant tissue culture technique is micrpopropagation which refers to the propagation of plants by using meristem tip culture which is the transfer of apical buds and surrounding leaf primordia to sterile culture conditions (Ali *et al.*, 2004; Mangal *et al*., 2002; Ioannov, 1990; Villabobos, 1986). With the realization of foreseen advantages and unprecedented applications this technique has received great attention all over the world including Pakistan and India. The present investigation was undertaken to optimize the culture conditions for micropropagation of Carnations (*Dianthus caryophyllous* L.).

Material and Methods

Apical and nodal meristems of 1.0 mm size were used as explants. Explants were obtained from pot grown plants. They were washed thoroughly with tap water and then with house hold detergent to remove all the traces of dust particles. The explants were then immersed in 7.5% aqueous solution of Sodium hypochlorite for 15 minutes and were thoroughly rinsed. Sodium hypochlorite solution was decanted and apical shoots were rinsed three times with autoclaved distilled water to remove all the traces of sterilent. Inoculation was carried out in laminar air flow cabinet. It was cleaned by scrubbing with 70% ethanol solution and was irradiated with UV irradiations for 25 minutes before use.

MS medium (Murashiage & Skoog's, 1962) supplemented with different concentrations of auxin and cytokinin along with 3% sucrose was used. pH of the medium was adjusted to 5.71±0.5. Agar was used for solidification of media. The media was autoclaved at 121 °C for 15 minutes at 15 lbs/In^2 pressure.

For shoot induction and proliferation, MS media containing different concentration and combinations of auxins and cytokinins was used. For rooting MS media was supplemented with different concentration of NAA or IBA. Ten explants were cultured in each test tube. All the cultures were maintained under light intensity of 2500- 3000 lux

having temperature of 27±1.0°C and photoperiod was 16 hour with 8 hours dark period in every 24 hour cycle. Sub-culturing was carried out after every 4 week interval.

Results

Shoot formation: For shoot formation MS medium was used either in basal form or it was supplemented with different concentrations of BAP ranging from 1-5mg/l. Best shoot formation response was obtained in MS medium containing 4.0 mg/l BAP. At this concentration all the explants showed shoot formation response within 7 days of inoculation both from apical and nodal meristem (Table 1a; Figs. 1 and 2). By increase or decrease in the concentration of BAP not only rate of shoot formation was decreased but time taken for shoot induction was also increased.

When combination of 1.0 mg/l BAP with different concentrations of Kinetin was used it was observed that by the addition of Kinetin the rate of shoot formation was decreased. When 0.5 mg/l Kinetin was used with 1.0 mg/l BAP 80% shoot formation was obtained after 11 days of inoculation from apical meristem and 70% after 20 days of inoculation from nodal explant. All other concentrations of Kinetin with same concentration of BAP failed to give satisfactory results for shoot formation in both kinds of explants.

Multiple shoot formation: For multiplication of shoots, MS medium was supplemented with BAP alone or in combination with Kinetin. It was observed that when BAP was used alone at 1.0 mg/l concentration, total 25.2 shoots were obtained in all the cultures. By increase in the concentration of BAP the rate of shoot multiplication was decreased and at 5.0 mg/l BAP only 10.4 shoots per culture were formed (Table 2a; Fig. 7).

No good response of shoot multiplication was obtained when different concentrations of Kinetin were added with 1.0 mg/l BAP. Rate of shoot multiplication was decreased by increase in the concentration of Kinetin (Table 2b).

Rooting and hardening: Well developed *In vitro* plants after attaining the size of 5.0 cm were shifted for rooting. For *In vitro* rooting MS medium was supplemented with NAA ranging from 1.0 mg/l to 5.0 mg/l. It was observed that best response for rooting was obtained when 1.0 mg/l of NAA was used (Fig. 8). By increasing the concentration of NAA, root induction response was decreased and at 2.5 mg/l of NAA very poor results were obtained (Table 3a).

Well rooted plants after two weeks of culturing were hardened in glasshouse. Best response for hardening was obtained in mixture containing sand + peat + soil (1:1:1) at 95% humidity level under natural light conditions.

Discussion

In vitro techniques offer new possibilities in commercial clonal propagation of plants as well as in high valued secondary products (Short, 1991). The present study was also undertaken to propagate commercially important cultivar of carnation. For shoot formation both apical and nodal meristems were used. Mass scale production of carnation from shoot tip culture (Apical Meristem) were also described by many scientists (Dantas *et al*., 2001; Manoj *et al*., 2003; Saher, 2004).

Table 1a. Effect of different concentrations of BAP on shoot formation.

S. No.	Media	Composition	No. of explants cultured	Days for shoot formation		Mean number of cultures showing shoot formation	
				Apical	Nodal	Apical	Nodal
1.	MS1	MS Basal	10	19.6 ± 0.669^{a}	24.6 ± 0.726^{a}	2.2 ± 0.334^{d}	2.0 ± 0.4^{d}
2.	MS2	BAP 1.0 mg/l	10	14.0 ± 0.489^{b}	16.4 ± 0.456^{b}	3.0 ± 0.4^{d}	2.0 ± 0.282^{d}
3.	MS3	BAP 2.0 mg/l	10	10.2 ± 0.334^{c}	16.0 ± 0.4^{b}	5.0 ± 0.489^{c}	3.0 ± 0.282^{d}
4.	MS4	BAP 3.0 mg/l	10	9.4 ± 0.357^{c}	9.8 ± 0.657^{c}	8.4 ± 0.456^{b}	6.4 ± 0.456^{c}
5.	MS5	BAP 4.0 mg/l	10	6.2 ± 0.521^{d}	7.0 ± 0.489^{d}	10.2 ± 0.438^{a}	10.6 ± 0.669^{a}
6.	MS6	BAP 5.0 mg/l	10	7.2 ± 0.438^{d}	9.4 ± 0.456^{c}	8.2 ± 0.521^{b}	8.0 ± 0.282^{b}

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new multiple range test.

Table 1b. Effect of different concentration of BAP and kinetin on shoot formation.

S.No.	Media	Composition	No. of explants cultured	Days for shoot formation		Cultures showing shoot formation	
				Apical	Nodal	Apical	Nodal
1.	MS1	BAP 1.0 mg/l+ Kin 0.5 mg/l	10	11.2 ± 0.521^{d}	20.0 ± 0.632^{c}	8.2 ± 0.334^{a}	7.4 ± 0.219^{a}
2.	MS2	BAP 2.0 mg/l+ Kin 1.0 mg/l	10	15.2 ± 0.334^{c}	26.2 ± 0.769^{b}	5.4 ± 0.357^{b}	5.4 ± 0.456^{b}
3.	MS3	BAP 3.0 mg/l+ Kin 1.5 mg/l	10	15.0 ± 0.632^{c}	26.0 ± 0.692^{b}	4.0 ± 0.4^{c}	4.4 ± 0.219^{c}
4.	MS4	BAP 4.0 mg/l+ Kin 2.0 mg/l	10	20.2 ± 0.334^{b}	30.0 ± 1.356^{a}	2.2 ± 0.178^{d}	2.0 ± 0.282^{e}
5.	MS5	BAP 5.0 mg/l+ Kin 2.5 mg/l	10	22.2 ± 0.438^{a}	30.2 ± 1.035^{a}	3.4 ± 0.456^{c}	3.4 ± 0.219^{d}

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new multiple range test.

Table 2a. Effect of different concentrations of BAP on *In vitro* shoot multiplication.

S. No.	Media	Composition	Number of test tubes cultured	Number of multiple shoot formed	Days for shoot multiplication	Average shoot length (cm)
1.	MS1	MS Basal	10	7.4 ± 0.219^{e}	24.2 ± 0.769^{b}	3.4 ± 0.456^{b}
2.	MS2	BAP 1.0 mg/l	10	25.2 ± 0.521^{a}	16.2 ± 0.334^{e}	5.2 ± 0.593^{a}
3.	MS3	BAP 2.0 mg/l	10	19.4 ± 0.536^{b}	18.4 ± 0.456^{d}	4.4 ± 0.357^{ab}
4.	MS4	BAP 3.0 mg/l	10	12.2 ± 0.521^{c}	21.6 ± 0.920^{c}	4.4 ± 0.219^{ab}
5.	MS5	BAP 4.0 mg/l	10	12.0 ± 0.565^{c}	$23.0 \pm 0.565b^{c}$	3.2 ± 0.178^{b}
6.	MS6	BAP 5.0 mg/l	10	10.4 ± 0.357^{d}	27.2 ± 0.521^{a}	3.4 ± 0.219^{b}

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new multiple range test.

Table 2b. Effect of different concentrations of BAP and kinetin on *In vitro* shoot multiplication.

S. No.	Media	*Composition*	Number of test tubes cultured	Number of multiple shoot formed	Days for shoot multiplication	Shoot length (cm)
1.	MS1	BAP 1.0 mg/l + Kin 0.5 mg/l	10	9.4 ± 0.456^{a}	24.0 ± 0.632^{a}	5.4 ± 0.219^{a}
2.	MS2	BAP 1.0 mg/l + Kin 1.0 mg/l	10	7.6 ± 0.456^{b}	16.2 ± 0.593^{d}	4.0 ± 0.282^{b}
3.	MS3	BAP 1.0 mg/l + Kin 1.5 mg/l	10	4.4 ± 0.219^{c}	18.2 ± 0.334^{c}	4.2 ± 0.438^{b}
4.	MS4	BAP 1.0 mg/l + Kin 2.0 mg/l	10	2.2 ± 0.178^{d}	21.0 ± 0.8^{b}	3.4 ± 0.219^{b}
5.	MS5	BAP 1.0 mg/l + Kin 2.5 mg/l	10	2^{e}	23.4 ± 0.456^{a}	3.8 ± 0.334^{b}

Means followed by different letters in the same column differ significantly at P = 0.05 according to Duncan's new multiple range test.

Table 3a. Effect of different concentrations of NAA on *In vitro* rooting.

Media	Composition (mg/l)	No. of cultured inoculated	Mean number of cultures showing root induction	Days for root induction	Root induction response
MS1	NAA 0.5	10	4.0 ± 0.219^{c}	12.2 ± 0.715^{a}	+
MS2	NAA 1.0	10	9.6 ± 0.357^{a}	9.2 ± 0.334^{b}	++++
MS3	NAA 1.5	10	9.0 ± 0.282^{ab}	9.0 ± 0.282^{b}	+++
MS4	NAA 2.0	10	8.0 ± 0.4^{b}	9.8 ± 0.334^{b}	++
MS5	NAA 2.5	10	2.0 ± 0.282^{d}	12.6 ± 0.456^{a}	+

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new multiple range test.
+ = Poor, ++ = Fair, ± = Standard error of mean, +++ = Good, ++++ = Excellent

Table 3b. Effect of different concentrations of IBA on *In vitro* rooting.

Media	Composition (mg/l)	No. of cultured inoculated	Mean number of cultures showing root induction	Days for root induction	Root induction response
MS1	IBA 0.5	10	2.2 ± 0.334^{b}	14.2 ± 0.334^{a}	+
MS2	IBA 1.0	10	2.2 ± 0.178^{b}	12.0 ± 0.632^{b}	+
MS3	IBA 1.5	10	3.0 ± 0.282^{b}	12.2 ± 0.334^{b}	+
MS4	IBA 2.0	10	5.2 ± 0.334^{a}	10.4 ± 0.456^{c}	+++
MS5	IBA 2.5	10	6.0 ± 0.282^{a}	10.0 ± 0.282^{c}	+++

Means followed by different letters in the same column differ significantly at P = 0.05 according to Duncan's new multiple range test.
+ = Poor, ++ = Fair, ± = Standard error of mean, +++ = Good, ++++ = Excellent

Table 3c. Effect of different concentrations of NAA and IBA on *In vitro* rooting.

Media	Composition (mg/l)	No. of cultured inoculated	Mean number of cultures showing root induction	Days for root induction	Root induction response
MS1	NAA 0.5 + IBA 0.5	10	5.2 ± 0.178^{c}	12.8 ± 0.334^{a}	++
MS2	NAA 0.5 + IBA 1.0	10	6.0 ± 0.282^{c}	10.2 ± 0.334^{b}	+++
MS3	NAA 0.5 + IBA 1.5	10	8.0 ± 0.489^{b}	10.0 ± 0.489^{b}	+++
MS4	NAA 1.0 + IBA 2.0	10	10.6 ± 0.456^{a}	8.0 ± 0.296^{c}	++++

Means followed by different letters in the same column differ significantly at P = 0.05 according to Duncan's new multiple range test.
+ = Poor, ++ = Fair, ± = Standard error of mean, +++ = Good, ++++ = Excellent

Fig. 1. *In vitro* shoot induction from apical meristem on MS medium containing 4.0 mg/l BAP after 6 days of inoculation.

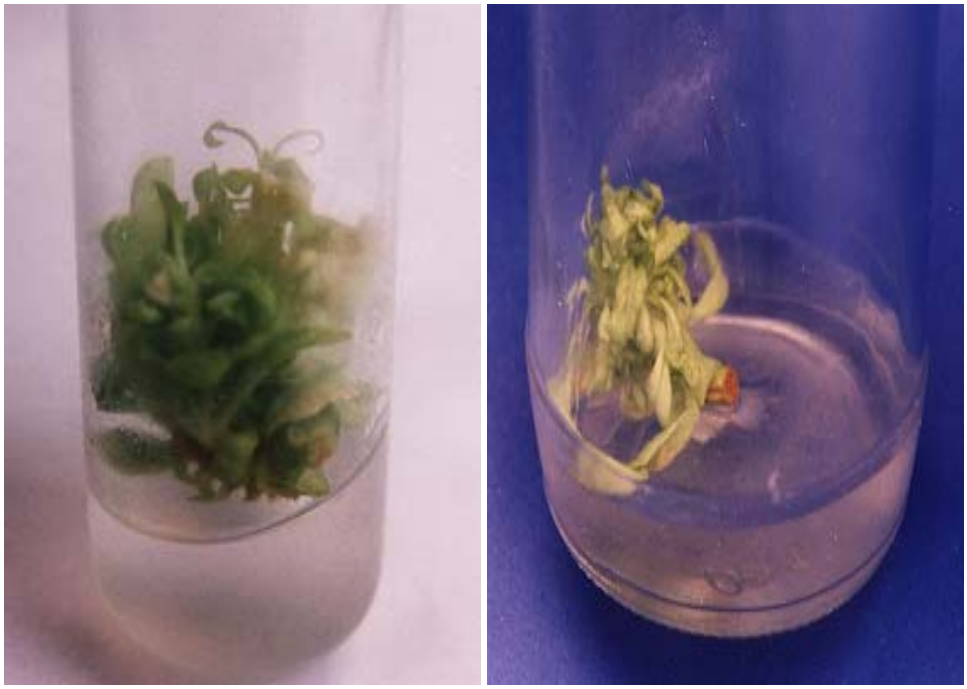
Fig. 2. *In vitro* shoot induction from nodal meristem on MS medium containing 4 .0mg/l BAP after 7days of inoculation.

Fig. 3. *In vitro* grown shoots of *Dianthus caryophyllousfrom* from apical meristem on MS medium containing 4.0 mg/l BAP after 25 days of inoculation.

Fig. 4. *In vitro* grown shoots of *Dianthus caryophyllousfrom* from apical meristem on MS medium containing 4.0 mg/l BAP after 25 days of inoculation.

a **b** **c** **d** **e**

Fig. 5. Different stages of shoot induction and growth from shoot apical meristem.



Figs. 6a. and 6b. Multiple shoot formation from *In vitro* grown shoots of *Dianthus caryophyllous* on MS medium containing 2.0 mg/l BAP from apical and nodal explant.

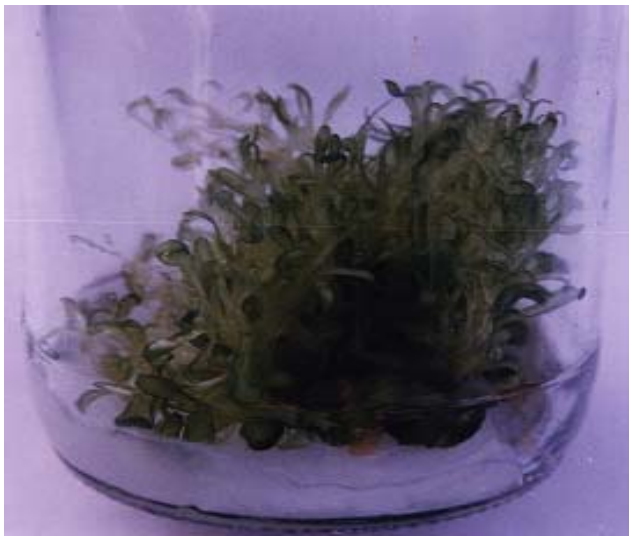


Fig. 7. Well developed multiple shoots of *Dianthus caryophyllous* from *In vitro* grown shoots on MS medium containing 2.0 mg/l BAP after eight week of culturing.

Fig. 8. *In vitro* rooting of *Dianthus caryophyllous* on MS medium containing 1.0 mg/l NAA from well developed *in vitro* shoots.

In the present study two different media were standardized i.e., shoot formation medium and shoot proliferation medium. The results of present study reveals that best shoot formation response was obtained on MS medium supplemented with 4.0 mg/l BAP (Table 1a; Figs. 1, 2, 3 and 4). By increase or decrease in the concentration of BAP shoot formation response was decreased.

Lubomski & Jerry (1989) also reported the best shoot formation response of carnation on MS medium supplemented with BAP. However, Siddiqui (1993) reported best shoot induction response in MS medium containing 5.0 mg/l Kinetin while Mangel *et al*., (2002) and Onamu *et al*., 2003 used MS medium supplemented with combination of NAA and Kinetin for shoot induction from meristem, but in present study it was observed that addition of kinetin failed to stimulate shoot induction response. On the other hand there was a decline in shoot induction with increase in the concentration of Kinetin (Table 1b).

In the present study it was also observed that apical meristem responded earlier as compared to nodal meristem (Tables 1a & b). This potential effect of explants is also discussed by Bressan *et al*., (1982).

Different concentrations of BAP were used for multiplication of induced shoots. It was observed that by decrease in the concentration of BAP rate of shoot multiplication was increased and maximum 25 shoots were obtained when MS medium was supplemented with 1.0 mg/l BAP (Fig. 7). Kovac (1995) also reported highest shoot multiplication in carnation in MS medium containing 1.0 mg/l BAP. Although 2.0 mg/l BAP also showed good shoot multiplication response but time taken for shoot multiplication was more and number of shoots formed were comparatively less than 1.0 mg/l BAP alone. However Van (1992) and Yanrcheve *et al.*, 1998 reported highest numbers of shoots per explant on MS medium containing 0.9 mg/l BA and 0.3 mg/l NAA.

In the present investigation liquid MS medium was found to be more effective for efficient shoot multiplication than MS medium solidified with agar or phytagel. Better *In vitro* shoot multiplication response in liquid medium has also been reported by Fisher *et al*., (1993) Majada *et al*., (1997) in carnation and by Ali *et al*., (2004) in turmeric.

Well developed multiple shoots when attain considerable height were shifted to MS medium containing different concentrations of NAA and IBA either alone or in combination with each other for *In vitro* rooting. Good rooting response was obtained in MS medium containing 1.0 mg/l NAA within 9 days of inoculation (Fig. 8).

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