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Studies on standardization of *in vitro* culture establishment and shoot multiplication of carnation (*Dianthus caryophyllus* L.)

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Abstract
The present investigation was carried out at the Plant Tissue Culture Laboratory and Experimental Farm of Department of Floriculture and Landscape Architecture, Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan (HP). The experiments were laid out in a Completely Randomized Design (factorial) consisting of two cultivars i.e. 'Parendillo' and 'Yellow Star'. Out of the two carnation cultivars under study, 'Parendillo' showed superiority over 'Yellow Star' for all the characters observed during *in vitro* propagation. Shoot tips were rated better explants than nodal sections for mass multiplication in carnation cultivars. A protocol was developed for production of high quality virus indexed carnation plants *in vitro*. For culture establishment, MS medium supplemented with 2.5 mgl^{-1} BA showed maximum per cent response. High quality shoots could be produced from shoot tip raised shoots in multiplication medium containing 2 mgl^{-1} BA, 0.1 mgl^{-1} NAA and 1 mgl^{-1} GA_3. The *in vitro* multiplied shoots were serologically indexed using DAS-ELISA for important carnation viruses viz. Carnation Latent Virus (CLV), Chrysanthemum Virus B (CVB) and Chrysanthemum Aspermy Virus (CAV). All the shoots showing multiplication under *in vitro* conditions were tested free of viruses.

Keywords: *In vitro*, explants, MS medium, micropropagation

Introduction
Carnation (*Dianthus caryophyllus* L.) is one of the most important commercial cut flowers of the world grown under protected conditions. With the development of innovative production technologies, farmers have adopted the trend of growing low volume high value crops for sustainable income. As a result greenhouse cultivation of flower crops has been picking up in the country. Among different greenhouse cut flowers grown and traded in the market, carnation has occupied a place of importance. It is one of the major floriculture crops in many countries of the world with high ornamental and commercial value (Burchi *et al*., 1996) [2].
Many important viruses have been reported to infect the crop. The important viruses infecting carnation include Carnation Latent Virus (CLV), Carnation Etched Ring Virus (CERV), Chrysanthemum Virus B (CVB), Chrysanthemum Aspermy Virus (CAV) etc.It is important to establish virus free mother block for raising a successful crop of carnation because, if the infection enters the mother block it would be propagated alongwith the rooted cuttings. With the advancement made in biotechnology, tissue culture for rapid multiplication of disease free plants is becoming popular. Remarkable progress *in vitro* regeneration of carnation was made, involving organogenesis from various explants sources (Frey and Janick 1991; Van Altvorst *et al*., 1992; Messeguer *et al.*, 1993; Zuker *et al.*, 1995; Dharma, 2003; Ali *et al.*, 2008 and, Kanwar and Kumar, 2009) [5, 20, 14, 22, 4, 1, 9]. The technology of virus indexing is also another advancement made in vegetative propagation of carnation (Ruiten, 1988 and Kofranek, 1992) [18, 11]. To reduce diseases, commercial propagators have developed a process called disease indexing, which allows the production of disease-free cuttings of carnation. Virus indexing of mother block is therefore important for raising healthy propagules. The application of tissue culture for regeneration and commercial propagation of whole plants is an established technique. The *in vitro* regenerated plants are used for the establishment of mother block which further acts as a source of cuttings for mass multiplication of carnation. Therefore, the present investigation will be carried out with following objectives:-

1. Refinement of protocol for *in vitro* propagation of carnation.
2. Production of virus indexed plants of carnation.

Materials and Methods

The present investigations, "Studies on standardization of *in vitro*culture establishment and shoot multiplication of Carnation (*Dianthus caryophyllus* L.)" were carried out in the Plant Tissue Culture Laboratory and Experimental Farm of Department of Floriculture and Landscape Architecture, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (HP).Two commercial standard cultivars of carnation viz. 'Parendillo' and 'Yellow Star' were selected for conducting different *in vitro* experiments. The mother block of selected cultivars was established under polyhouse conditions at the experimental farm of the department. Two explants namely, shoot tips and nodal sections were used for the establishment of cultures. Stem cuttings were taken from healthy donor plants and the leaves covering the stem were removed gently. Shoot tips and nodes were excised from these stem cuttings. All the explants were treated in the 0.1% teepol solution for 10 minutes and washed with tap water. These were then treated with Dithane M-45 (0.2%) and Bavistin (0.1%) for 20 minutes and were washed under running tap water for 15-20 minutes. All the explants were cut to a size of 5-6 mm. Under laminar flow cabinet, nodes and shoot-tips were treated with different surface sterilants (Mercuric Chloride, Sodium Hypochlorite and Calcium Hypochlorite) for varying durations. After surface sterilization, explants were washed thrice with distilled water before inoculation. All the cultures were kept in the culture trollies of culture room maintained at a temperature of 25±2 °C was maintained under artificial light (16 hours light and 8 hours dark period daily) having intensity of 1.5 Klux at plant level. The *in vitro* cultures during establishment, multiplication and rooting were incubated in culture room.

Experiment 1: Standardization of culture establishment medium:

The sterilized explants (apical and nodal) were inoculated in the culture tubes containing MS medium supplemented with different concentration of Benzyl Adenine (0.5, 1, 1.5, 2, 2.5, 3 mgl^{-1}) for the initial establishment of cultures. Observations like per cent response after 4 weeks and number of shoots/explant were recorded.

Experiment 2: Standardization of culture medium for shoot multiplication of carnation

The best establishment medium was used for establishment of cultures in both the cultivars. For shoot multiplication, healthy shoots of approximately 3.5-4 cm were selected for multiplication. Such shoots were individually cultured on basal MS medium supplemented with different concentration of Benzyl Adenine (BA) i.e. 1, 1.5, 2, 2.5, 3 mgl^{-1} BA combined with NAA (0.1 mgl^{-1}) and GA_3 (1 mgl^{-1}) for multiplication. The observations like number of shoots, length of shoots/shoot (cm), number of leaves/shoot and quality of shoots were recorded after 4 weeks of culturing.

Virus Indexing

The *in vitro* multiplied shoots were indexed for important viruses before rooting. For this, DAS-ELISA techniques was followed. ELISA is a simple, effective and powerful tool for identification of the viruses. The present studies were carried out by following double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) techniques. Commercially available immuno reagents (M/s BIOREBA AG, Switzerland) by following the protocol of suppliers of ELISA kits with little modification were used.

Results and Discussion

In vitro culture establishment

Results indicate that the cultivars differed significantly from each other for *in vitro* culture establishment with cv. 'Parendillo' showing superiority over 'Yellow Star'. Better per cent response (Table 1a and 1b) combined with more number of shoots/explant (Table 2a and 2b) were found in 'Parendillo' as compared to 'Yellow Star'. The inter varietal differences among cultivars could be attributed to genetic makeup of cultivars. Mubarack *et al*., 1991 [16] and Kallak *et al*., 1997 [8] also confirmed that *in vitro* propagation in carnation is genotype dependent.

Among explants, nodal sections proved better for producing more number of shoots/explants in establishment medium (Table 2a) in comparison to shoot tip explants. In a similar study, Mujib *et al.* (1993) also obtained the highest number of adventitious shoots from nodal explants in comparison to shoot tip explants in carnation.

Among BA concentrations in this medium, a gradual increase in per cent response of cultures was, however, observed with increasing BA concentration in the medium upto 2.5 mgl^{-1}. The optimum value, however, varied among cultivars (Table 1b). On the other hand, maximum number of shoots during establishment were obtained when MS medium was supplemented with 3 mgl^{-1} BA (Table 2a). The role of cytokinins for cell division and shoot proliferation is well established. Dharma (2003) [4] through his studies observed that maximum number of shoots were observed on MS full salts supplemented with 5 µM BAP in carnation cv. 'Tempo' and 'Diplomat'. Ilahi *et al*. (1995) [7] also induced rapid propagation from nodal segments of carnation on MS medium supplemented with 1, 2 or 3 mgl^{-1} BA. They also concluded that more number of shoots/explant were produced from the nodal section than shoot tip explants.

Table 1(a): Effect of cultivars, Benzyl Adenine (BA) in MS medium and explant source on per cent response of cultures during culture establishment (4 weeks after culture)

Benzyl Adenine (mgl^{-1})	Cultivars of carnation		Mean	Explants	
	Parendillo	Yellow Star		Shoot tip	Nodal
0	51.25 (45.74)*	54.99 (47.48)	52.71 (46.60)	56.25 (48.68)	49.16 (44.54)
0.5	66.00 (54.45)	66.99 (59.97)	70.29 (57.21)	67.92 (55.69)	72.66 (58.73)
1.0	84.17 (66.79)	54.17 (65.21)	83.12 (66.00)	83.75 (66.53)	82.50 (65.47)
1.5	85.00 (67.56)	74.58 (70.09)	86.39 (68.83)	86.25 (68.66)	86.54 (69.00)
2.0	95.00 (82.07)	82.08 (66.61)	89.27 (74.34)	89.38 (72.53)	89.17 (76.15)
2.5	96.25	78.75	87.50	87.50	87.50

	(83.23)	(62.76)	(73.00)	(70.97)	(75.03)
3.0	87.50 (70.88)	75.83 (60.77)	81.67 (65.83)	82.50 (65.58)	80.83 (66.07)
Mean	80.74 (67.25)	76.68 (61.84)	-	79.08 (64.09)	78.34 (65.00)
Explants			$CD_{0.05}$ for:		
Shoot tip	78.57 (64.35)	79.58 (63.83)	Cultivars		2.07
			Explants		NS
Nodal	82.90 (70.14)	73.77 (59.86)	Benzyl Adenine		3.88
			Cultivars x Benzyl Adenine		5.49
			Explant xBenzyl Adenine		NS
			Cultivars x Explant		2.93

Table 1(b): Interaction effect between cultivars, Benzyl Adenine (BA) in MS medium and explant source on per cent response of cultures during culture establishment (4 weeks after culture)

Benzyl Adenine (mgl^{-1})	Cultivars of carnation			
	Parendillo		Yellow Star	
	Shoot tip	Nodal	Shoot tip	Nodal
0	47.50 (43.56)*	55.00 (47.92)	65.00 (53.80)	43.33 (41.16)
0.5	65.00 (53.78)	66.99 (55.13)	70.83 (57.60)	78.33 (62.34)
1.0	85.00 (67.50)	83.33 (66.07)	82.50 (65.55)	81.66 (64.87)
1.5	85.00 (67.50)	85.00 (67.62)	87.50 (69.82)	88.08 (70.37)
2.0	90.00 (74.14)	100.00 (90.00)	88.75 (70.91)	78.33 (62.30)
2.5	92.50 (76.47)	100.00 (90.00)	82.50 (65.47)	75.00 (60.06)
3.0	85.00 (67.50)	90.00 (74.26)	80.00 (63.66)	71.66 (57.89)

*values in parenthesis are arc sine transformed values
$CD_{0.05}$ for: Cultivarsx Benzyl Adenine x Explant : 7.76

Table 2(a): Effect of Benzyl Adenine (BA) in MS medium and explant source on number of shoots/ explant during culture establishment (4 weeks after culture)

Benzyl Adenine (mgl^{-1})	Cultivars of carnation		Mean	Explants	
	Parendillo	Yellow Star		Shoot tip	Nodal
0	1.21	0.10	1.10	1.05	1.16
0.5	1.37	1.93	1.28	1.14	1.42
1.0	1.81	1.55	1.68	1.61	1.74
1.5	1.99	1.71	1.85	1.79	1.91
2.0	2.89	2.41	2.65	2.51	2.79
2.5	2.97	2.43	2.70	2.68	2.73
3.0	2.88	2.65	2.77	2.69	2.84
Mean	2.16	1.85	-	1.92	2.08
Explants			$CD_{0.05}$for:		
Shoot tip	2.05	1.80	Cultivars		0.11
Nodal	2.27	1.90	Explants		0.11
			Benzyl Adenine		0.21
			Cultivars x Benzyl Adenine		NS
			Explants x Benzyl Adenine		0.59
			Cultivars x Explant		0.28

Table 2(b): Interaction effect between cultivars, Benzyl Adenine (BA) in MS medium and explant source on number of shoots/explants during culture establishment (4 weeks after culture)

Benzyl Adenine (mgl^{-1})	Cultivars of carnation			
	Parendillo		Yellow Star	
	Shoot tip	Nodal	Shoot tip	Nodal
0	1.10	1.32	1.00	0.10
0.5	1.21	1.53	1.07	1.31
1.0	1.73	1.89	1.50	1.60
1.5	2.03	1.96	1.55	1.87
2.0	2.55	3.23	2.48	2.35
2.5	2.90	3.05	2.45	2.42
3.0	2.85	2.91	2.53	2.77

$CD_{0.05}$ for: Cultivars x Benzyl Adenine x Explant : NS

***In vitro* shoot multiplication:**

Genotypic differences among cultivars became more obvious in multiplication also. Both the cvs 'Parendillo' and 'Yellow Star' behaved differently for *in vitro* shoot multiplication. More number of shoots/shoot, longer shoots and more number of leaves/shoot were produced by 'Parendillo' as compared to 'Yellow Star' (Table 3a and 3b). Among explants also shoot tip raised cultures showed better multiplication over nodal sections. These results are in conformity with the findings of Ali *et al.* (2008) [1] who obtained more proliferation in the shoot tip explants of carnation.

In the present study, maximum number of shoots/shoot were obtained in the shoot tip regenerated shoots of cultivar 'Parendillo' (Table 3b) when cultured on MS medium supplemented with 3 mgl^{-1} BA, 0.1 mgl^{-1} NAA and 1 mgl^{-1} GA_3. Influence of auxin and cytokinin combination on plantlet regeneration *in vitro* is a well-established fact (Skoog and Miller, 1957) [19] and was confirmed by several researchers (Pennazio, 1975 and Hempel, 1979) [17, 6]. Kharrazi *et al.* (2011) [10] also noticed highest number of shoots on medium supplemented with 4.4 µM BAP + 1.47 µM NAA.

Table 3(a): Effect of BA, NAA and GA_3 in MS medium and explant source on number of shoots/shoot in multiplication medium (4 weeks after culture)

Growth regulators (mgl^{-1})			Cultivars of carnation		Mean	Explants	
BA	**NAA**	**GA_3**	**Parendillo**	**Yellow Star**		**Shoot tip**	**Nodal**
2.5	-	-	2.82	2.90	2.66	3.02	2.30
1.0	0.1	-	2.87	4.03	2.90	3.31	2.49
1.5	0.1	-	4.26	4.19	4.03	4.51	3.56
2.0	0.1	-	4.18	4.81	4.19	4.24	4.14
2.5	0.1	-	4.97	5.09	4.81	4.79	4.83
3.0	0.1	-	5.12	4.53	5.09	5.01	5.17
2.0	0.1	1.0	4.61	4.91	4.53	4.67	4.39
2.5	0.1	1.0	5.09	5.17	4.91	4.75	5.09
3.0	0.1	1.0	5.31	5.04	5.17	5.09	5.25
Mean			4.36	4.15	-	4.38	4.13

Explants			CD $_{0.05}$ for:	
Shoot tip	4.38	4.37	Cultivars	0.20
Nodal	4.34	3.93	Explants	0.20
			Growth regulators	0.42
			Cultivars x Growth regulators	NS
			Explant x Growth regulators	0.59
			Cultivars x Explant	0.28

Table 3 (b): Interaction effect between cultivars, Benzyl Adenine (BA) in MS medium and explant source on number of shoots/explants during culture establishment (4 weeks after culture)

Benzyl Adenine (mgl^{-1})	Cultivars of carnation			
	Parendillo		Yellow Star	
	Shoot tip	**Nodal**	**Shoot tip**	**Nodal**
0	1.10	1.32	1.00	0.10
0.5	1.21	1.53	1.07	1.31
1.0	1.73	1.89	1.50	1.60
1.5	2.03	1.96	1.55	1.87
2.0	2.55	3.23	2.48	2.35
2.5	2.90	3.05	2.45	2.42
3.0	2.85	2.91	2.53	2.77

$CD_{0.05}$ for: Cultivars x Benzyl Adenine x Explant : NS

Higher shoot length was observed in 'Parendillo' than 'Yellow Star'. Maximum length of shoots was observed when the shoot tips raised shoots of cv. 'Parendillo' were cultured when MS medium was supplemented with 2 mgl^{-1} BA, 0.1 mgl^{-1} and 1 mgl^{-1} GA_3. The results are supported by the work carried out by Mujib *et al*. (1993) who used shoot tips and node cuttings as explants for *in vitro* regeneration of shoots in carnation cv. 'William Sim'. The greatest plant height on the main shoot from a shoot tip (8.81 cm and 12.87 cm respectively) was obtained with 0.5 mgl^{-1} BAP and 0.2 mgl^{-1} NAA.

In our studies, addition of GA_3 to MS medium containing BA and NAA has a profuse effect on shoot elongation with maximum shoot length found with 3 mgl^{-1} BA, 0.1 mgl^{-1} NAA and 1 mgl^{-1} GA_3 (Table 4). The effect of GA_3 on shoot elongation has been clearly reported by Can and Koc (1992) [3] in carnation. Maitra *et al.* (2011) [12] carried out a study in which explants cultured on MS medium supplemented with 1 mgl^{-1} NAA and 2.5 mgl^{-1} kinetin produced longest shoots (6.60 cm).

Table 4: Effect of BA, NAA and GA_3in MS medium and explant source on average length of shoots (cm) in multiplication medium(4 weeks after culture)

Growth regulators (mgl^{-1})			Cultivars of carnation		Mean	Explants	
BA	NAA	GA_3	Parendillo	Yellow Star		Shoot tip	Nodal
2.5	-	-	2.75	2.54	2.64	2.58	2.71
1.0	0.1	-	2.78	2.55	2.67	2.44	2.89
1.5	0.1	-	2.85	2.87	2.86	2.84	2.88
2.0	0.1	-	3.15	3.14	3.14	2.94	3.35
2.5	0.1	-	2.88	2.80	2.84	2.92	2.75
3.0	0.1	-	2.70	2.53	2.61	2.61	2.62
2.0	0.1	1.0	6.70	5.75	6.22	6.44	6.01
2.5	0.1	1.0	6.33	5.80	6.05	6.13	5.98
3.0	0.1	1.0	6.49	6.02	6.25	6.27	6.24
Mean			4.07	3.78	-	3.91	3.94
Explants					CD $_{0.05}$ for:		
Shoot tip			3.96	3.86	Cultivars		0.08
Nodal			4.18	3.70	Explants		NS
					Growth regulators		0.17
					Cultivars x Growth regulators		0.24
					Explant x Growth regulators		0.24
					Cultivars x Explant		0.11

The parameters like number of shoots/shoot, length of shoots and visual quality contributed to overall quality of shoots (Table 5a, 5b). Better quality shoots obtained in cv. 'Parendillo could be attributed to its genetic superiority over 'Yellow Star'. Shoot tip explant regenerated shoots produced high quality shoots over nodal explants. Among growth regulator combinations, best quality shoots were obtained when MS medium was supplemented with 2 mgl^{-1}BA, 0.1 mgl^{-1}NAA and 1 mgl^{-1} GA_3 irrespective of cultivar and explant source. The superiority of contributing characters in this medium resulted in maximum score and production of highest quality shoots. The synergistic effects of cytokinins with auxins and gibberellins is responsible for the production of high quality shoots in this medium.

Table 5(a): Effect of BA, NAA and GA_3 in MS medium and explant source on quality of shoots in multiplication medium (score out of 15) (4 weeks after culture)

Growth regulators (mgl^{-1})			Cultivars of carnation		Mean	Explants	
BA	NAA	GA_3	Parendillo	Yellow Star		Shoot tip	Nodal
2.5	-	-	8.25	7.88	8.06	8.38	7.75
1.0	0.1	-	6.63	6.25	6.44	6.88	6.00
1.5	0.1	-	8.00	7.25	7.63	8.25	7.00
2.0	0.1	-	10.50	9.75	10.13	10.38	9.88
2.5	0.1	-	10.50	10.25	10.38	10.38	10.38
3.0	0.1	-	8.75	8.38	8.56	8.50	8.66
2.0	0.1	1.0	14.13	14.16	14.14	14.28	14.00
2.5	0.1	1.0	12.63	12.38	12.50	12.50	12.50
3.0	0.1	1.0	12.88	12.31	12.59	12.56	12.63
Mean			10.25	9.84	-	10.23	9.86
Explants					CD $_{0.05}$ for:		
Shoot tip			10.36	10.10	Cultivars		0.08
Nodal			10.25	9.58	Explants		0.08
					Growth regulators		0.16
					Cultivars x Growth regulators		0.23
					Explant x Growth regulators		0.23
					Cultivars x Explant		0.11

Table 5(b): Interaction effect of cultivars, BA, NAA and GA_3 in MS medium and explant source on quality of shoots in multiplication medium (score out of 15) (4 weeks after culture)

Growth regulators (mgl^{-1})			Cultivars of carnation			
			Parendillo		Yellow Star	
BA	NAA	GA_3	Shoot tip	Nodal	Shoot tip	Nodal
2.5	-	-	8.25	8.25	8.50	7.25
1.0	0.1	-	7.25	6.00	8.50	7.50
1.5	0.1	-	8.50	7.50	8.00	6.50
2.0	0.1	-	10.75	10.25	10.00	9.50
2.5	0.1	-	10.50	10.50	10.25	10.25
3.0	0.1	-	8.50	9.00	8.50	8.25
2.0	0.1	1.0	14.25	14.00	14.31	14.00
2.5	0.1	1.0	12.50	12.75	12.50	12.25
3.0	0.1	1.0	12.75	13.00	12.38	12.25

$CD_{0.05}$for:Cultivars x Growth regulators x Explant : 0.32

Virus Indexing

The *in vitro* multiplied shoots were serologically indexed for presence of important viruses viz. Carnation Latent Virus (CLV), Chrysanthemum Virus B (CVB) and Chrysanthemum Aspermy Virus (CAV). It is evident from the data (Table 6) that shoots regenerated from shoot tips and nodal explants were assayed negative i.e. free from viruses. *In vitro* culture techniques have already established their role in producing disease/ virus free plants (Waithaka, 1992) [21]. In another study, successful elimination of Carnation Latent Virus (CLV) from carnation cv. 'Scania' through meristem tip culture has been reported by Mangal *et al*. (2001) [13].

Table 6: DAS-ELISA based virus indexing of shoots regenerated from shoot tip and nodal explants in carnation cultivars against important viruses:

Virus / Positive control		Explants							
		Shoot tips				Nodal			
		Parendillo		Yellow Star		Parendillo		Yellow Star	
		Samples 1	Samples 2	Samples 1	Samples 2	Samples 1	Samples 2	Samples 1	Samples 2
Carnation Latent Virus	1.631(+++)	0.301(-)	0.328(-)	0.268(-)	0.271(-)	0.306(-)	0.340(-)	0.273(-)	0.281(-)
Chrysanthemum Virus B	1.628(+++)	0.266(-)	0.362(-)	0.215(-)	0.249(-)	0.276(-)	0.369(-)	0.214(-)	0.257(-)
Chrysanthemum Aspermy Virus	1.617(+++)	0.308(-)	0.390(-)	0.301(-)	0.388(-)	0.402(-)	0.392(-)	0.310(-)	0.398(-)
Negative control Buffer control		0.272 0.231							

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