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Research Paper

Standardization of efficient protocol for callus induction and shoot regeneration in border carnation (*Dianthus caryophyllus* L.)

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ABSTRACT

An experiment was conducted to standardize the protocol for *in vitro* callusing of border carnation (*Dianthus caryophyllus* L.) var. 'Can Can'. Different explants tried were leaf segments and nodal segments. For callus induction, 12 different combinations of 2,4-D, BAP and NAA were used.Among both types of explants, the best callus formation was observed in MS medium supplemented with BAP 1.00 + 2,4-D 2.00 mg l^{-1}, showing the highest development of callus and callus weight per explant (2.15 g $explant^{-1}$).For shoot regeneration,BAP,NAA.IAA and Kinetin were used in eight different combinations.Cultures on media supplemented with NAA showed a higher percentage of calli producing shoots and a greater shoot number compared to those containing IAA in combination in both the explants. The highest regeneration rate and shoot number per explant were found in the MS medium supplemented withkinetin 2.00 + NAA 0.50 mg l^{-1}.

Key words: *Callusing, development, media, shoot regeneration*

Carnation (*Dianthus caryophyllus* L.) is a member of Caryophyllaceae family, which includes 80 genera and around 2000 species, primarily annual or perennial plants found predominantly in the northern hemisphere (Maurya *et al*. 2020).Carnations are extensively utilized as cut flowers, bedding plants, potted plantsand are ranked as the third-most important cut flowers after roses and chrysanthemums in the global floriculture industry owing to their diverse flower color, resilience during long-distanct transportation, and remarkable ability to rehydrate effectively after prolonged shipping. The global floricultural industry has seen a growing demand for new carnation cultivars with unique traits over the past few decades (Kumar *et al*. 2016).Therefore, in vitro breeding methods are widely employed to breed this species (Azadi *et al.* 2016).Earlier studies have utilized various plant growth regulators (PGRs), with a high cytokinin concentration often required for shoot regeneration in different carnation cultivars (Thakur & Kanwar 2018; Thu *et al*. 2020; Maurya *et al.* 2021).

Plant callus refers to a proliferating mass of disorganized plant parenchyma cells. In a living plant, callus cells are those that cover a plant wound. Callus can undergo direct organogenesis and/or embryogenesis, where the cells develop into a new plant, a process known as callus culture. Callus formation is stimulated from plant tissue explants after they undergo surface sterilization and are placed onto tissue culture medium in vitro. This culture medium is supplemented with plant growth regulators like auxins, cytokinins, and gibberellins to initiate callus formation or somatic embryogenesis. The specific ratio of auxin to cytokinin in the plant tissue culture medium leads to the formation of a proliferating and dividing mass of callus cells (Qadri *et al.* 2018). Callus cultures are often broadly categorized as compact or friable. Friable calluses are easily fragmented and can be used to establish cell suspension cultures. Callus can undergo direct organogenesis and/or embryogenesis, where the cells develop into a new plant, a process known as callus culture. Thakur *et al*. (2002) produced friable and

pale yellow callus from internodal segments of two perpetual carnation cultivars, 'Cabaret' and 'Feyenoord', on MS medium containing 0.5 ppm each of 2,4-D and NAA. The callus induced on medium supplemented with 2,4-D alone or in combination with NAA failed to regenerate shoots when directly transferred to the regeneration medium. Shoot initiation only occurred after the calli were cultured on MS basal medium containing 0.3% activated charcoal. The callus became green on this medium, and several shoot primordia appeared after one month of culture. Border Carnations are popular perpetually flowering carnation types that are used in gardens and in containers, owing to their complex ancestry and heterozygous nature.The first step towards somatic embryogenesis is usually the induction of callus from explants. To overcome this problem, a quick micropropagation system is exigent needed, which has not yet been explored in our region so far. Therefore, it is important to devise an efficient protocol of callus proliferation to initiate *in vitro* manipulation of carnation for both production and the introduction of new varieties in border carnations.

Callus induction is often the preferred route for plant multiplication in tissue culture due to its ability to generate a large number of plants from a small piece of tissue. It is the first step towards somatic embryogenesis. This mass propagation is advantageous for maintaining genetic uniformity, also allowing for the introduction of variation in flower characteristics. Additionally, Callus induction allows for rapid propagation, especially useful for species with slow growth rates or challenging propagation methods. Border Carnations are popular perpetually flowering carnation types that are used in gardens and in containers. Owing to their complex ancestry and heterozygous nature, they do not set seeds. To overcome this problem, a quick micropropagation system is exigently needed, which has not yet been explored in our region so far. Therefore, it is important to devise an efficient protocol of callus proliferation to initiate *in vitro* manipulation of border carnation for both production and the introduction of new varieties.

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MATERIALS AND METHODS

Culture media and culture condition: The nutrient medium used to develop a propagation protocol for border carnation consisted of MS (Murashige and Skoog, 1962) medium supplemented with macro elements, microelements, and vitamins. Myo- inositol, Sucrose (usually 30 gl^{-1}), and Plant Growth Regulators (PGRs) were added to the medium as needed for each stage. The pH of the medium was adjusted to 5.7using 1N NaOH or 1N HCl dropwise with a micropipette, ensuring that the electrode did not touch the walls of the beaker and that the electrode bulb remained fully immersed in the solution. The medium was gently heated in a microwave oven, and then the required amount of agar (usually 7g/l) was added, stirred well with a scalpel or stirrer, and heated again in the microwave oven until boiling to completely dissolve the agar. After cooling for a few minutes, the medium was dispensed into culture vessels, which were plugged with non-absorbent cotton plugs. Test tubes and flasks containing the prepared culture medium were autoclaved at 121 °C and 15 psi for 15–20 minutes. The cultures were then incubated under controlled conditions in a culture room at a temperature of 24 ± 1 °C with a 16-hour light and 8-hour dark cycle.

Explant and hormonal regimes for callus induction: In the standardization experiments for callus induction, leaf and nodal segment explants were utilized. A total of 12 PGR combinations in MS media supplemented with0.50 and 1.00 mg l^{-1} BAP in combination with 1.00, 1.50 and 2.00 mg l^{-1} 2,4-D or 1.00,1.50 and 2.00 mgl^{-1} NAA were utilized. Each treatment consisted of 12 explants, with one explant per test tube, and was replicated three times. Callus formation data were recorded based on the following parameters.

Callus development (%): Callus development refers to the development of visible callus that can be observed by the naked eye and subsequently subcultured to promote proliferation or multiplication. Data collection was conducted after a 4-week culture period.

Callus weight (g explant^{-1}): At the conclusion of a six-week culture period, the weight of the callus was measured. Each test tube or flask containing fresh media was weighed individually. The callus from actively growing cultures was then carefully separated from the explants and transferred to pre-weighed test tubes or flasks containing fresh media. The test tubes or flasks were re-weighed, and the difference in weight before and after adding the callus was recorded as the callus weight per explant.

Callus type: The type of callus in each case was visually evaluated, and observations were recorded accordingly.

Callus regeneration : Various strategies were employed for achieving the regeneration of shoots from calluses derived from two different morphological structures like leaf and nodal segments. Callus pieces of uniform size and age originating from different morphological structures were placed on regeneration media supplemented with BAP 1.00 and 2.00 mg l^{-1}in combination with 0.50 mgl^{-1} IAA or 0.50 mg l^{-1} NAA, 1.00 and 2.00 mg l^{-1} Kinetin in combination with 0.50 mg l^{-1} IAA or 0.50 mg l^{-1} NAA. Data for regeneration studies was recorded under following parameters.

Regeneration (%): Regeneration means development of callus into shoots. Data was recorded after 8 weeks of culture.

Number of shoots callus^{-1}: Data was recorded after 8 weeks of culture.

Statistical analysis: The data collected for various parameters in the current study was subjected to statistical analysis using analysis of variance (ANOVA) for a Completely Randomized Design (CRD) with three replications (Gomez and Gomez 1983). In order to adhere to the model assumptions required for analysis of variance, the percentage data underwent square root transformation, following the recommendation of Steel and Torrie (1980).

RESULTS AND DISCUSSION

Callus induction: In the current study, twelve growth regulator combinations were used, comprising auxins (2,4-D and NAA at concentrations of 1.00, 1.50, and 2.00 mg l^{-1}) in combination with cytokinin (BAP at concentrations of 0.50 or 1.00 mg l^{-1}), for standardizing various callusing parameters of leaf and nodal segment explants of border carnation (Fig.1 and 2).In case of leaf explants, all the combinations of growth regulators induced callus formation. Perusal of data (Table 1) reveals that there were significant differences in means of callusing parameters viz., callus development per cent and callus weight explant^{-1} under the influence of various growth regulator combinations.

Table 1: Influence of growth regulators on callusing in leaf explant of border carnation (*Dianthus caryophyllus* L.)

MS + PGRs	Concentration (mg l^{-1})	*Callus development (%)	**Callus weight (g explant^{-1})	Callus type
BAP + 2,4-D	0.50 + 1.00	72.22^{g} (8.56)	1.58^{e}	Compact, creamy white
BAP + 2,4-D	0.50 + 1.50	74.30^{e} (8.68)	1.59^{e}	Compact, creamy white
BAP + 2,4-D	0.50 + 2.00	77.78^{d} (8.89)	1.71^{d}	Compact, creamy white
BAP + NAA	0.50 + 1.00	69.44^{i} (8.39)	1.30^{h}	Soft yellowish green
BAP + NAA	0.50 + 1.50	67.53^{j} (8.28)	1.32^{gh}	Soft yellowish green
BAP + NAA	0.50 + 2.00	64.05^{l} (8.07)	1.07^{j}	Soft yellowish green
BAP + 2,4-D	1.00 + 1.00	78.47^{c} (8.92)	1.81^{c}	Compact creamy white
BAP + 2,4-D	1.00 + 1.50	80.76^{b} (9.04)	2.00^{b}	Compact, creamy white
BAP + 2,4-D	1.00 + 2.00	91.00^{a} (9.59)	2.15^{a}	Compact, creamy white
BAP + NAA	1.00 + 1.00	73.19^{f} (8.61)	1.43^{f}	Soft yellowish green
BAP + NAA	1.00 + 1.50	70.00^{h} (8.43)	1.37^{g}	Soft yellowish green
BAP + NAA	1.00 + 2.00	72.11^{g} (8.55)	1.16^{i}	Soft yellowish green

*Data recorded after 4 weeks of culture ; ** Callus weight recorded after 6 weeks of culture
Figures in the parenthesis are square root transformed value of the percentage data.
Different letters in a column indicate significant differences according to Duncan's multiple range test ($p < 0.05$); values represent mean ± SE

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The highest percentage of callus development was observed in leaf explants incubated with higher concentrations of 2,4-D(1.50 mg l^{-1} and 2.00 mg l^{-1}) in combination with BAP (0.50mg l^{-1} or 1.00 mg l^{-1}) whereas, significantly lowest percentage of callus development was observed in NAA treatments at higher concentrations in combination with BAP at 0.50mg l^{-1} or 1.00 mg l^{-1}. The results are in conformity with Kanwar and Kumar (2009), who reported highest callus induction in leaf explant of carnation cv. 'Indios' achieved on MS media supplemented with 2.00 mg l^{-1} 2, 4-D in combination with 1.00 mg l^{-1} BA and lowest on media containing NAA or kinetin.

In this study, callus formation occurred on all media containing 2,4-D, while media containing NAA and kinetin showed poor growth and lower callus induction. This suggests that 2,4-D is a crucial factor for initiating callus in carnations.Qadri*et al*. (2018) obtained callus induction with MS medium supplemented with BAP 1.00 mg l^{-1}+ 2,4-D 2.00 mg l^{-1} from leaf and nodal segment explants of two carnation (*Dianthus caryophyllus* L.) cultivars, namely 'Scania' and 'Indios'. Perusal of data regarding callus weight explant $^{-1}$ revealed maximum callus weight on media containing 2, 4-D 2.00 mg l^{-1} in combination with BAP 1.00 mg l^{-1}. Other 2, 4-D treatments also resulted in higher callus weight in comparison to NAA based treatments.

Table 2: Influence of growth regulators on callusing in nodal segment explant of border carnation (*Dianth us caryophyllus* L.)

MS + PGRs	Concentration (mg l^{-1})	* Callus development (%)	** Callus weight (g explant^{-1})	Callus type
BAP + 2,4-D	0.50 + 1.00	67.22^{e} (8.26)	1.54^{ef}	Compact creamy white
BAP + 2,4-D	0.50 + 1.50	74.17^{d} (8.67)	1.56^{e}	Compact creamy white
BAP + 2,4-D	0.50 + 2.00	77.63^{c} (8.87)	1.67^{d}	Compact creamy white
BAP + NAA	0.50 + 1.00	50.33^{h} (7.17)	1.31^{h}	Soft yellowish green
BAP + NAA	0.50 + 1.50	47.67^{i} (6.98)	1.15^{i}	Soft yellowish green
BAP + NAA	0.50 + 2.00	44.28^{j} (6.73)	1.01^{j}	Soft yellowish green
BAP + 2,4-D	1.00 + 1.00	77.86^{c} (8.88)	2.05^{c}	Compact creamy white
BAP + 2,4-D	1.00 + 1.50	82.00^{b} (9.11)	2.16^{b}	Compact creamy white
BAP + 2,4-D	1.00 + 2.00	88.19^{a} (9.45)	2.42^{a}	Compact creamy white
BAP + NAA	1.00 + 1.00	58.88^{f} (7.74)	1.55^{ef}	Soft yellowish green
BAP + NAA	1.00 + 150	55.49^{g} (7.52)	1.50^{f}	Soft yellowish green
BAP + NAA	1.00 + 2.00	53.75^{g} (7.40)	1.41^{g}	Soft yellowish green

*Data recorded after 4 weeks of culture ; ** Callus weight recorded after 6 weeks of culture
Figures in the parenthesis are square root transformed value of the percentage data.
Different letters in a column indicate significant differences according to Duncan's multiple range test ($p < 0.05$); values represent mean ± SE

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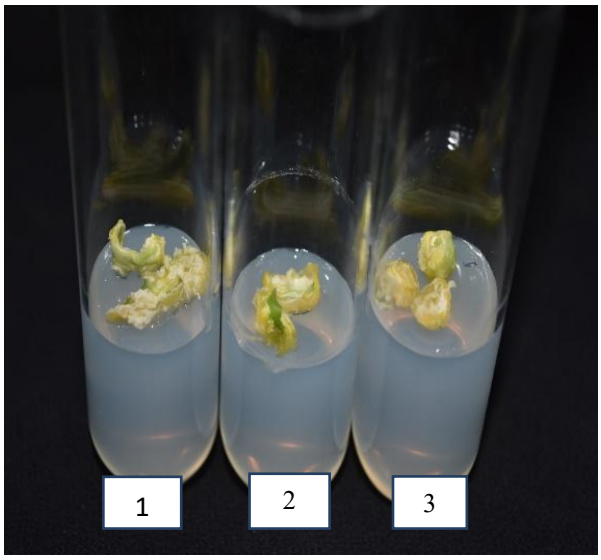


Fig. 1. Callusing in leaf explant

1) BAP + 2,4-D = 1.00 + 1.00 mg l^{-1}
2) BAP + 2,4-D = 1.00 + 1.50 mg l^{-1}
3) BAP + 2,4-D = 1.00 + 2.00 mg l^{-1}

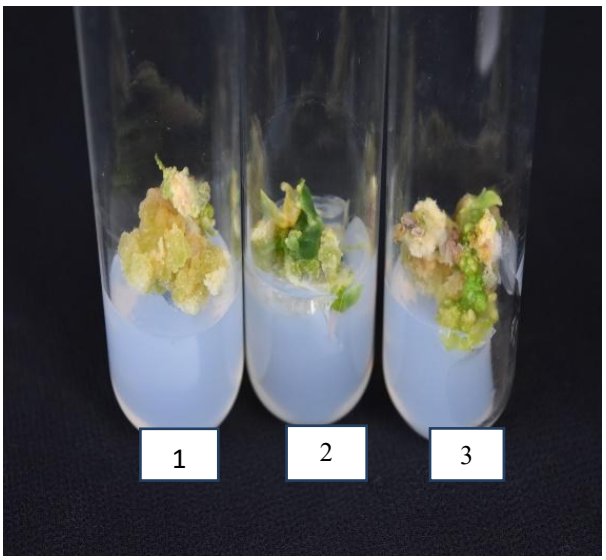


Fig.2 Callusing in nodal explant

1) BAP + 2,4-D = 1.00 + 1.00 mg l^{-1}
2) BAP + 2,4-D = 1.00 + 1.50 mg l^{-1}
3) BAP + 2,4-D = 1.00 + 2.00 mg l^{-1}

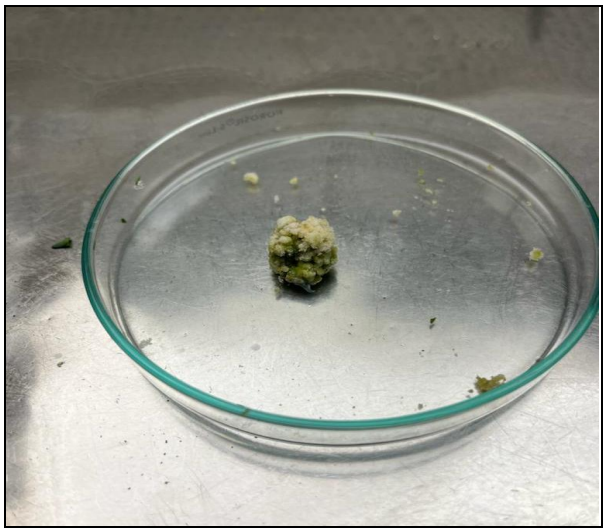


Fig. 3: Callus type-I (Compact creamy white)

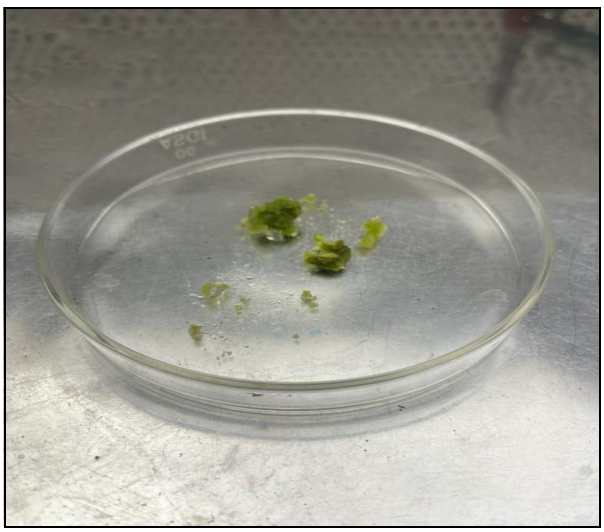


Fig. 4 Callus type- II (Soft yellowish green)

Nodal segment explants exhibited a similar trend as recorded in leaf explants (Table 2). Nodal sections incubated on MS media fortified with 2, 4-D (1.00, 1.50 or 2.00 mg l^{-1}) in combination with BAP 1.00 mg l^{-1}showed significantly earlier callus formation. The percentage of callus development was significantly higher on MS media enriched with 2, 4-D (1.00, 1.50 or 2.00 mg l^{-1}) in combination with BAP 1.00 mg l^{-1}, while as NAA based treatments at 1.00, 1.50 and 2.00 mg l^{-1} in combination with BAP 0.50 mg l^{-1} exhibited lowest percentage of callusing. Kiss and Mandy (2003) similarly reported callus induction on MS media supplemented with 2, 4-D and BA at 0.50 + 2.00, 1.00 + 1.50, 1.50 + 1.00mg l^{-1} and 2.00 + 0.50 mg l^{-1} from explants of carnation cv. 'Rimini'. They found callus formation was more stimulated in the medium containing 1.50 mg 2, 4-D + 1.00 mg BA per litre compared to the medium containing 2, 4-D 0.50 + BA 2.00 mg l^{-1}. On further perusal of data regarding callus weight explant^{-1}, it was observed that higher concentrations of 2, 4-D in combination with BAP at 1.00 mg l^{-1} produced significantly greater callus weight than other treatments including NAA based growth regulator combinations. Callus type in 2, 4-D based treatment combinations was compact creamy white and the

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treatments containing growth regulator NAA in combination produced soft yellowish green (Fig. 3 and 4) type of callus.

Table 3: Influence of growth regulator combinations on shoot regeneration in leaf derived callus of border carnation (*Dianthus caryophyllus* L.)

MS + PGRS	Concentration (mg l^{-1})	Callus regeneration (%)	Shoot number $callus^{-1}$
BAP + IAA	1.00 + 0.50	0.00^{h} (1.00)	0.00^{h}
BAP + IAA	2.00 + 0.50	5.30^{f} (1.74)	2.01^{f}
Kinetin + IAA	1.00 + 2.00	4.17^{g} (1.52)	1.31^{g}
Kinetin + IAA	2.00 + 0.50	6.90^{e} (2.00)	3.01^{e}
BAP + NAA	1.00 + 0.50	15.19^{c} (2.52)	5.33^{d}
BAP + NAA	2.00 + 0.50	17.84^{b} (3.56)	11.67^{b}
Kinetin + NAA	1.00 + 0.50	13.31^{d} (3.15)	8.93^{c}
Kinetin + NAA	2.00 + 0.50	20.29^{a} (4.13)	16.01^{a}

Data recorded after 8 weeks of culture
Figures in the parenthesis are square root transformed value of the percentage data
Different letters in a column indicate significant differences according to Duncan's multiple range test ($p < 0.05$); values represent mean ± SE

Table 4: Influence of growth regulator combinations on shoot regeneration in nodal segment derived callus of border carnation (*Dianthus caryophyllus* L.)

MS + PGRS	Concentration (mg l^{-1})	Callus regeneration (%)	Shoot number $callus^{-1}$
BAP + IAA	1.00 + 0.50	3.41^{h}(2.10)	0.80^{h}
BAP + IAA	2.00 + 0.50	5.13^{f}(2.48)	1.79^{f}
Kinetin + IAA	1.00 + 2.00	4.01^{g}(2.24)	1.53^{g}
Kinetin + IAA	2.00 + 0.50	5.90^{e}(2.63)	2.60^{e}
BAP + NAA	1.00 + 0.50	12.91^{d}(3.73)	4.50^{d}
BAP + NAA	2.00 + 0.50	18.33^{b}(4.40)	9.80^{b}
Kinetin + NAA	1.00 + 0.50	16.22^{c}(4.15)	7.07^{c}
Kinetin + NAA	2.00 + 0.50	23.33^{a}(4.92)	13.01^{a}

Data recorded after 8 weeks of culture
Figures in the parenthesis are square root transformed value of the percentage data
Different letters in a column indicate significant differences according to Duncan's multiple range test ($p < 0.05$); values represent mean ± SE

Regeneration and organogenesis: In present investigation, different experiments were conducted to study the influence of various growth regulators and explants on regeneration potential of callus in border carnation. The growth regulator combinations consisting auxins (IAA and NAA at 0.50 mg l^{-1} each) in combination with cytokinin (BAP and kinetin both at 1.00 and 2.00 mg l^{-1}) were tested in regeneration of leaf and nodal segment derived callus.Our findings align with various studies that have documented a synergistic impact of BAP or Kinetin when combined with NAA for the proliferation of multiple shoots from shoot tips and nodal segments in *Dianthus caryophyllus* L. (Danial *et al*., 2009; Kharrazi *et al*., 2011). In current study, data on regeneration of leaf derived callus observed a poor response when only 5.30 to 20.29 per cent calli differentiated shoots under the influence various growth regulator treatments. While as treatment BAP 1.00 + IAA 0.50 mg l^{-1} failed to differentiate shoots from leaf callus. Kanwar and Kumar (2009) reported, although the callus was produced with

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most of the growth regulators, the differentiation of callus (i.e., leaf and internodal derived callus) in carnation cv. 'Indios' was difficult, as only a few treatments responded.Regarding shoot yield, Comparatively among IAA containing cultures, shoot yield was significantly less as against NAA supplemented treatments. Leaf derived callus incubated on media combination kinetin 2.00 + IAA 0.50 mg l^{-1} produced maximum shoots $callus^{-1}$ (3.01).Whereas, calli failed to develop shoots incubated on media containing BAP 1.00 + IAA 0.50 mg l^{-1}.

Regarding the influence of growth regulator treatments on regeneration of shoots from nodal segment derived callus, a similar trend was followed as observed in leaf derived callus differentiating shoots. Significantly, more per cent of calli producing shoots and shoot number was recorded in cultures on media supplemented with NAA than those containing IAA in combination (Table 3and 4). Growth regulator combination kinetin 2.00 mg l^{-1} + NAA 0.50 mg l^{-1} was superior to all other treatments in differentiating high percentage of calli producing shoots and shoot number per culture (Fig. 5) These results align with those of Thakur *et al*. (2002) who achieved the highest average number of shoots in carnation cultivars 'Cabaret' and 'Feyenoord' by culturing calli derived from internodes on MS medium containing 2.00 ppm kinetin and 0.50 ppm NAA. Significantly maximum average number of shoots 13.01 was produced in calli on media supplemented with kinetin 2.00 mg l^{-1}+ NAA 0.50mg l^{-1}.

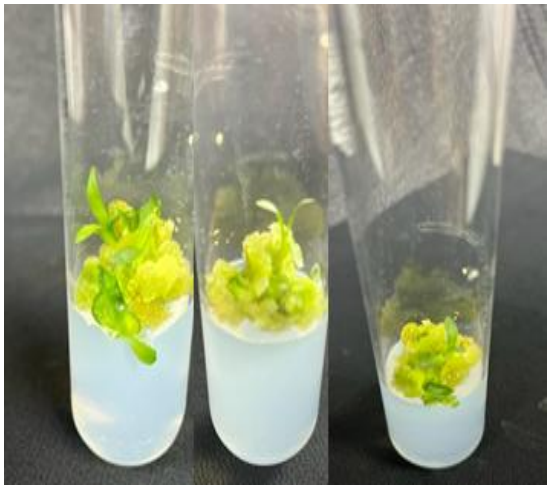


Fig 5. Shoot regeneration from callus on MS media supplemented with Kinetin 2.00 mg l^{-1} + NAA 0.50 mg l^{-1}

Callus regeneration (%) directly influenced number of shoots /culture in both leaf derived or nodal segment derived callus (Fig. 6 and 7) With the increase in callus regeneration per cent number of shoots/ culture increased.

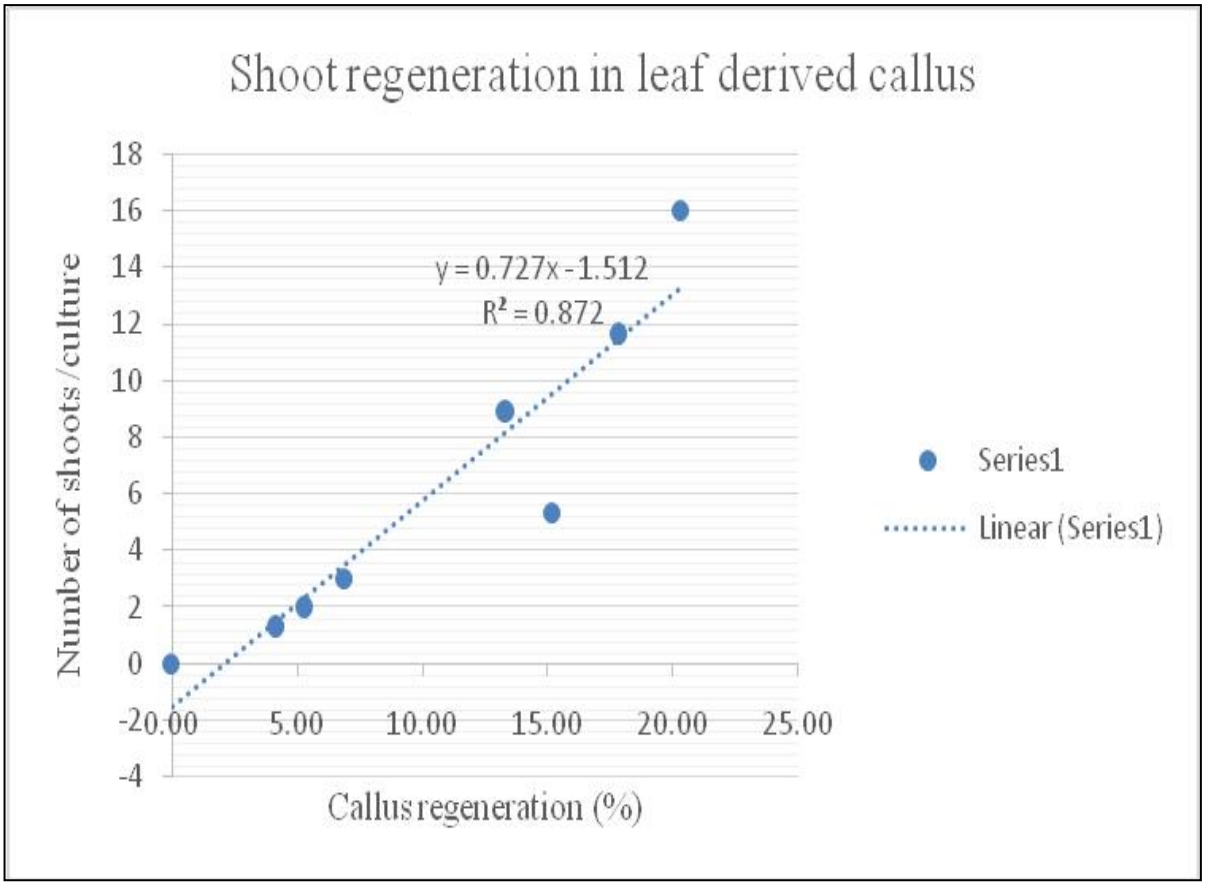


Fig. 6: Effect of callus regeneration per cent on number of shoots/culture in leaf derived callus

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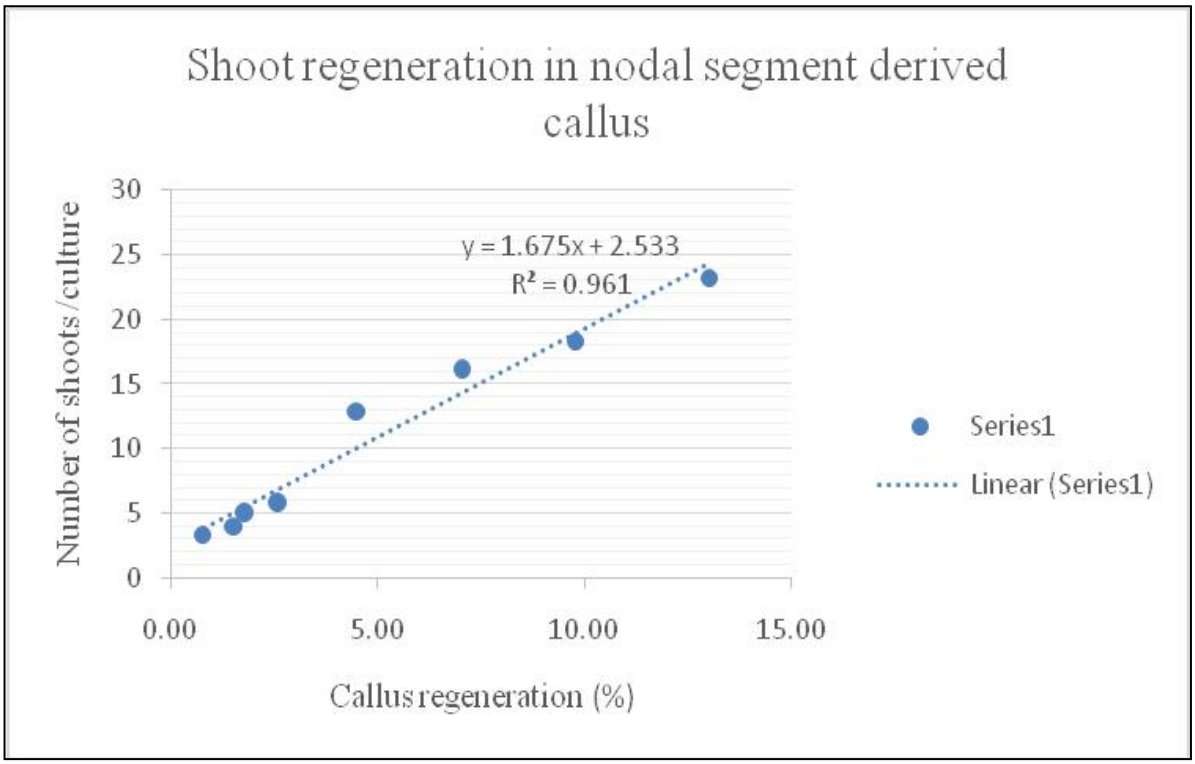


Fig. 7: Effect of callus regeneration per cent on number of shoots/culture in nodal segment derived callus

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CONCLUSION

The study concludes that MS medium supplemented with BAP at 1.00 mg l^{-1} and 2,4-D at 2.00 mg l^{-1} yielded the best results for callusing of both leaf and nodal segment explants, exhibiting the highest callus induction rate, and the greatest callus weight per explant.Highest callus development of 91.00 and 88.19 per cent and significantly maximum mean callus weight 2.15 and 2.42 g explant-1 was recorded in media fortified with BAP 1.00 + 2, 4-D 2.00 mg l^{-1} in leaf and nodal segment explant of border carnation. The highest regeneration rate and shoot number per explant were obtained in MS media supplemented with Kinetin 2.00 + NAA 0.50 mg l^{-1}.

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