

# **An Effective Protocol for Carnation (*Dianthus caryophyllus* L.) cv. 'Geolei' explants Sterilization for successful callusing and shoot regeneration**

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## **Abstract**

Carnation is a popular floricultural crop grown widely for its attractive cut flowers. Micro-propagation can be used to create large-scale carnation output. For growth and development, plants require some necessary nutrients as well as growth regulators. Due to the importance of carnation, the present work is carried out using leaf and nodal segments to examine the potential of several plant growth regulators for *in vitro* callus formation and adventitious shoot regeneration. Explants were sterilized properly with **bavistin, sodium hypochlorite and mercuric chloride**. The minor contaminated cultures were created by consecutively treating the explants with 0.25% bavistin, 0.50% sodium hypochlorite, and 0.1% mercuric chloride for ten, fifteen, and two minutes.

MS media with 2.5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) in combination with 0.75 mg/l naphthalene acetic acid (NAA) resulted in the maximum callus induction (90.47%) from leaf explants. Maximum shoots (76.47%) were produced in MS media supplemented with 2.0 mg/l Thidiazuron (TDZ) + 0.25 mg/l NAA. NAA at 1.25 mg/l was most efficient for maximum root induction (83.32%). In the present study, an effective protocol of carnation explants sterilization was optimized for successful callusing and shoot regeneration.

**Key Words:** Bavistin, Callus induction; 2,4-Dichlorophenoxy acetic, Naphthalene acetic acid, Mercuric chloride, Regeneration, Sodium hypochlorite, Thidiazuron.

**Abbreviations:** MS: Murashige and Skoog

## **1. INTRODUCTION**

Carnation is a member of the *Caryophyllaceae* family, derived from the Latin phrase "Carnation," which means "fleshness"[1]. The term 'Dianthus' comes from the Greek words 'dios' which means 'God' or 'divine,' and 'anthos,' which means 'flower,' and is thus known as 'Divine Flower.' The name 'caryophyllus' comes from the Greek words 'caryan' which means 'nut,' and 'phylon,' which means 'leaf.' Due to the clove-like aroma of carnation, Linnaeus selected the name 'caryophyllus' after the genus name of clove [2]. *Dianthus* species are adapted to cold alpine areas of Europe and Asia. However, they are also found along the Mediterranean coast. *D. caryophyllus* is thought to have commenced in the Mediterranean regions of Greece and Italy (including Sicily and Sardinia). However, its precise origin is difficult to determine due to its lengthy period in cultivation [3]. *Dianthus* species can be cultivated through seeds in the spring and cuttings in late summer [4]. Seeds, stem cuttings, and layering are the most common methods of propagation for carnations [5]. However, these approaches are not suitable for commercial development due to the possibility of genetic variation and slow multiplication rates. As a result, tissue culture techniques have been effectively used for quick plant replication, breeding cycle reduction, and the generation of superior cultivars [6,7]. However, propagation can be done at any time of year in a glasshouse setting. For large-scale replication of disease-free plants, *in vitro* propagation procedures have also been standardized. Carnation's heterozygous makeup makes it difficult to develop using traditional breeding procedures [8]. As a result, combining plant tissue culture technology and genetic transformation approaches to improve it could be a more promising strategy. It is generally recognized that to conduct effective genetic transformation research the plant must regenerate successfully through *in vitro* methods. The response of plants to *in vitro* regeneration conditions is critical for genetic manipulation strategy. Establishing effective *in vitro* regeneration methods is also necessary condition for establishing genetic transformation methods for introducing novel features or studying gene expression regulation *in vitro*. This report describes the optimization of efficient sterilization methods using suitable explants (leaf and nodal explants) for successful callus induction and direct shoot regeneration in carnation.

## **2. MATERIALS AND METHODS**

### **2.1 Plant Material Collection**

The current study was conducted in the Department of Agricultural Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology in Meerut, (U.P., India). The plant material was collected from the Department of Floriculture and Landscaping, Dr. YSP University of Horticulture & Forestry, Nauni, Solan, (H.P., India), in rooted cuttings of carnation (*Dianthus caryophyllus* L. cv. 'Geolei'). Leaf and nodal segments were used as explants.

### **2.2 Explants surface sterilization**

The leaf and nodal segments were washed thoroughly under running tap water for 15-20 minutes. Subsequent steps were conducted under laminar airflow (LAF). The leaf explants were then surface sterilized with different concentrations of bavistin (w/v) (A systemic fungicide, Carbendazim) for 10 minutes and after that the same explants were treated with sodium hypochlorite (NaOCl) (v/v) solution for fifteen minutes. These combinations were also tried with 0.1% mercuric chloride ( $\text{HgCl}_2$ ) to check the surface sterilization efficacy on nodal explants for two minutes. After every step, the explants were washed with autoclaved water four to five times. Effects of different concentration were recorded as uncontaminated cultures after four weeks of inoculation.

### **2.3 Culture Conditions and Callusing**

Surface sterilized leaf segments were inoculated on solid Murashige and Skoog [9] medium containing sucrose (3% w/v), agar (0.8 %w/v) and various concentrations of growth regulators for *in vitro* culture establishment. Before autoclaving at 121 °C and 1.06 kg/cm<sup>2</sup> for 20 minutes, the pH of the culture media was adjusted to 5.8±0.2 [7]. All cultures were incubated at 25±1 °C for 16 hours light and 8 hours dark photoperiods under cool white fluorescent tubes (Philips, India). Except for rooting, all trials were performed using full Murashige and Skoog-salt strength [10]. **Murashige and Skoog** media was supplemented with different concentrations of 2,4-D and NAA for callus induction. The cultures were kept in darkness for seven days to induce callus before being transferred to a 16-hour photoperiod for three weeks. Following four weeks of inoculation, observations were recorded about the percentage of explants producing callus [callus induction frequency (CIF)], callus weight, induction response, as well as the type of callus. Each treatment had six repetitions (Petri dishes), with four to five (1-1.5cm) leaf explants in each Petri dish [13], with slight modifications.

### **2.4 *In vitro* direct shoot regeneration**

Surface disinfected nodal segments were cultured onto solid MS media supplemented with varied quantities of 6-Benzylaminopurine (BAP) and different combinations of TDZ and NAA for *in vitro* culture establishment. The cultures were incubated for four weeks. Observations were recorded for percent shoot induction, average shoot length (cm), and shoot induction response.

### **2.5 *In vitro* rooting of plantlets**

Regenerated adventitious shoots from the nodal segment were tweaked off and inoculated for rooting, because the section of the explants in touch with the media gets contaminated in most cases, but the shoots remain unaffected. Half strength MS-media with 0.8% agar supplemented with various concentrations of NAA was used as rooting media. Observations were recorded on root induction (%) and average root length. After that, the rooted plants were withdrawn from the culture tubes, appropriately cleaned to remove agar gel that stuck to the roots, and transferred into small plastic cups filled with sterilized soil and compost (1:1) for hardening [26]. For acclimatization, the plantlets were housed in a green house facility.

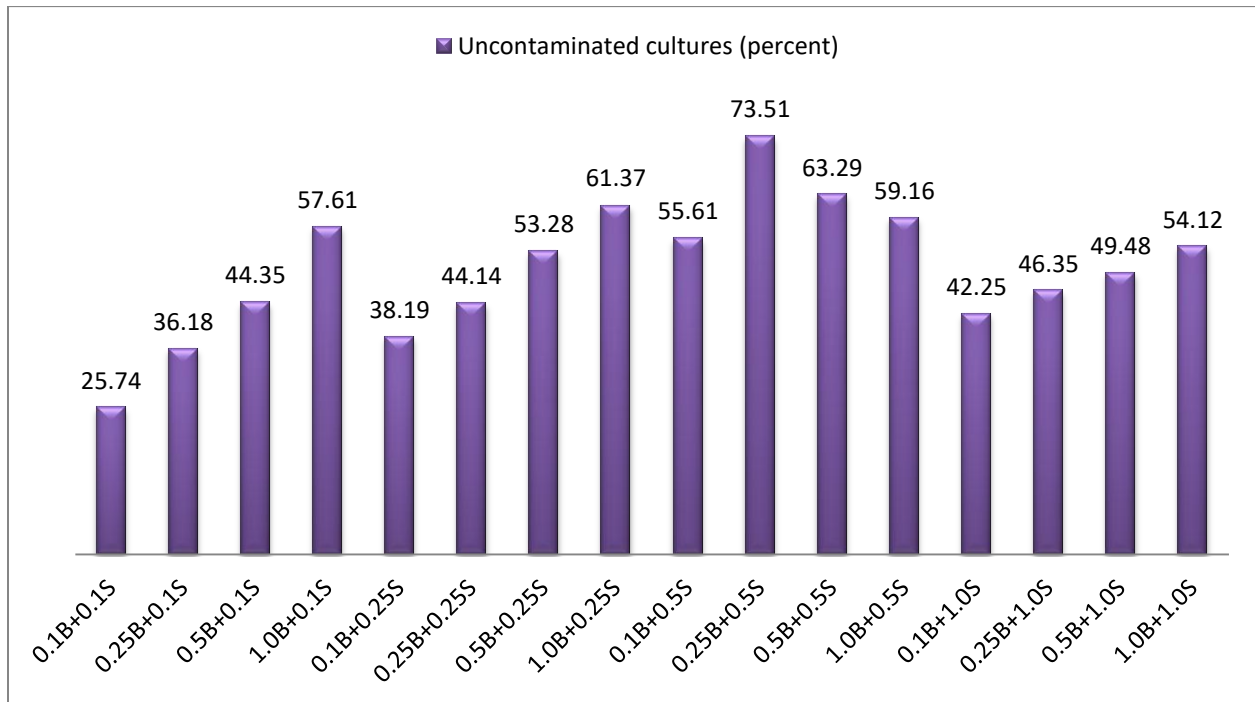
## 2.5 Statistical Analysis

All the experiments were set up in complete randomized design (CRD) with a minimum of three replications per treatment. All the experiments were studied through statistical software OPSTAT ([http:// 14.139.232.166/opstat/index.asp](http://14.139.232.166/opstat/index.asp)) [28].

## 3. RESULTS

### 3.1 Explants surface sterilization

The effects of different doses of bavistin and sodium hypochlorite applied for surface sterilization of leaf explants were observed for 10 and 15 minutes, respectively. After four weeks, the concentration combination of 0.25% (w/v) bavistin and 0.50% (v/v) sodium hypochlorite proved to be the most effective, but it caused minor callus contamination (Figure1). A 0.25 % (w/v) bavistin and 0.50 % (v/v) sodium hypochlorite with 0.1%  $\text{HgCl}_2$  produced the least contamination for nodal segments.



**Figure1:** The effect of different concentrations of bavistin (B) and sodium hypochlorite (S) solution treated for 10 and 15 minutes, respectively, on surface sterilization of explants after four weeks of incubation of cultures of carnation cv. Geolei

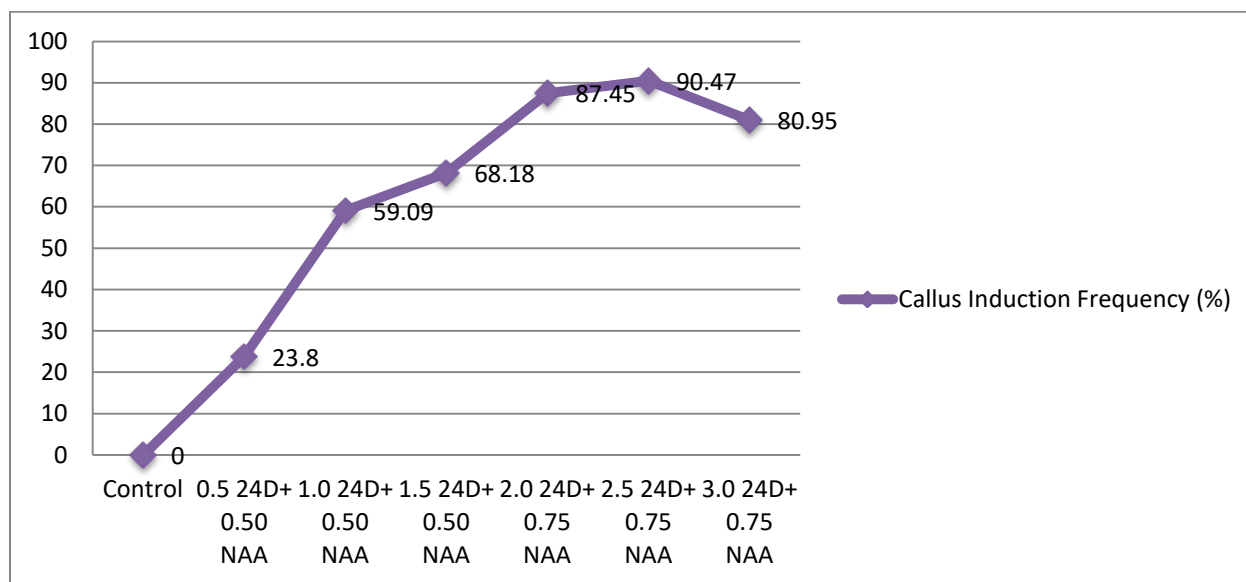
### 3.2 Callusing

A total of six culture media combinations with control were tested (Table 1). In the present study, all different culture media compositions induced callusing through leaf segments differently. On culture media supplemented with 2.5 mg/l 2,4-D and 0.75 mg/l NAA showed the maximum callus induction frequency(CIF)(90.47 %)(Figure: 3b), followed by 88.95 % CIF on culture media supplemented with 2.0 mg/l 2,4-D + 0.75 mg/l NAA. Callus induction (CIF) was minimum (23.80%) in media when supplemented with 0.5 mg/l 2,4-D + 0.50 mg/l NAA. On the other hand, no response was seen in media with no growth regulator (Figure 2).

**Table 1:** Effect of growth regulators added in MS medium on callus induction from leaf explants after four weeks old culture of carnation cv. Geolei

SNo.	Growth Regulator (mg/l)	Callus weight/ explants (gm)	Callus induction Response	Type of Callus
T0	Control	0.45	--	NR
T1	0.5 24D+ 0.50 NAA	0.65	+	Less proliferated, soft
T2	1.0 24D+ 0.50 NAA	1.06	++	Greenish white, friable
T3	1.5 24D+ 0.50 NAA	1.29	+++	Yellowish-white, friable
T4	2.0 24D+ 0.75 NAA	2.15	++++	Greenish white, well proliferated
T5	2.5 24D+ 0.75 NAA	2.43	++++	Greenish, well proliferated
T6	3.0 24D+ 0.75 NAA	1.93	+++	Brownish whiteproliferated

Symbol; --: no response (NR), +: poor response, ++: moderate, +++: good, ++++: excellent



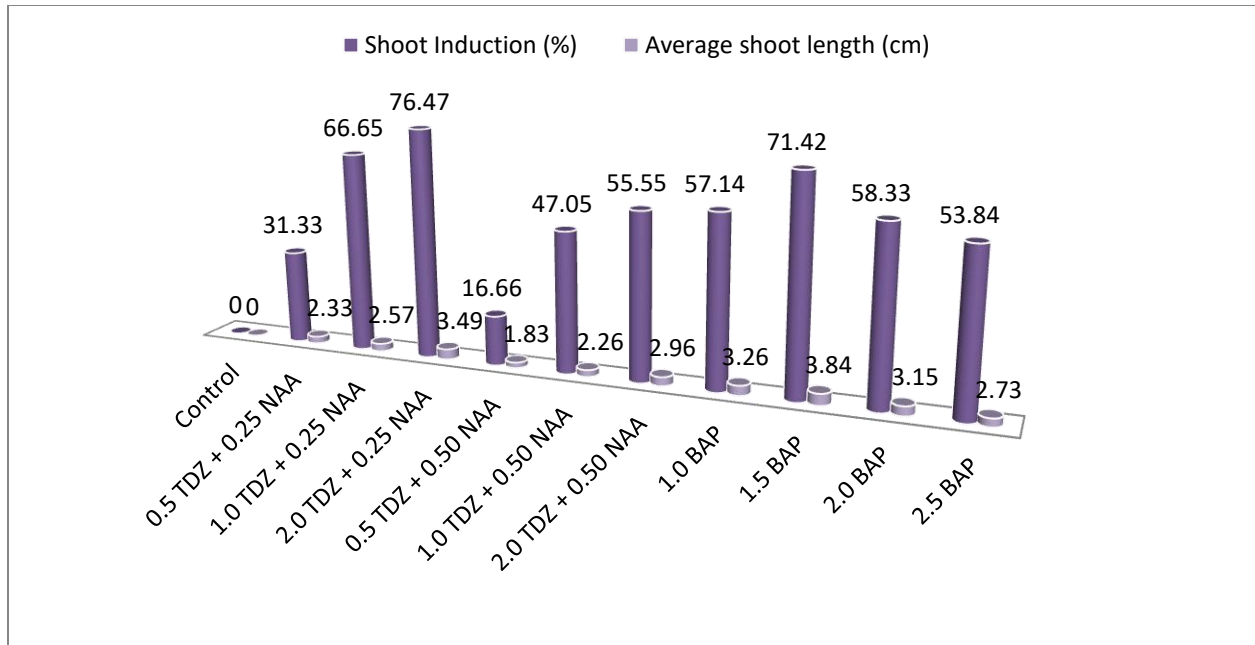
**Figure2:**Effect of plant growth regulators (2,4-D and NAA) supplemented in MS medium on callus induction frequency from leaf explants after four weeks old culture of carnation.



**Figure 3 (a):** Leaf segments inoculated for callus induction; (b) proliferated callus after four weeks old culture; (c) callus showing no response towards shooting of carnation cv. Geolei

### 3.3 Adventitious shoot induction

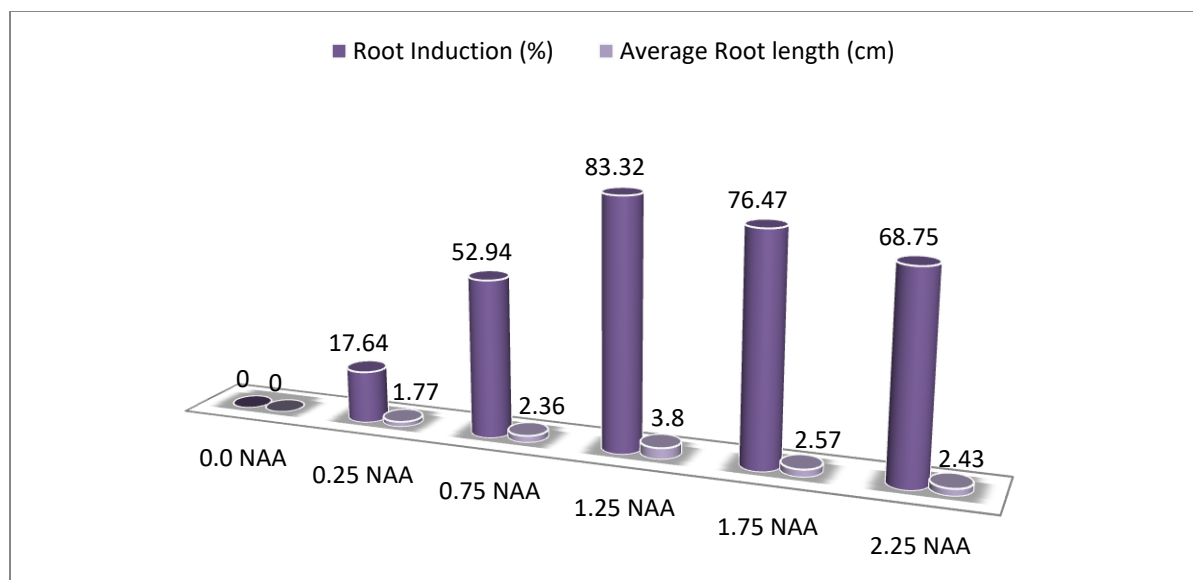
The four-week-old callus taken from leaf explants was transferred to shoot induction media. Still, no differentiation towards shooting was seen (Figure 3c). As a result, we have decided to pursue regeneration employing nodal segments through adventitious shoot induction. Nodal segment explants were inoculated on MS media supplemented with differing proportions of BAP alone and TDZ + NAA. In seven to ten days, the explants began eliciting adventitious shoots. However, only one to two shoots were produced on each explant (Figure:3 a,b). After four weeks of culture, the last observations for data collecting were taken (Figure3). Maximum shoots (76.47%) and (71.42%) were produced by nodal segments maintained on MS medium supplemented with 2.0 mg/l TDZ + 0.25 mg/l NAA and 1.5 mg/l BAP, respectively. Using 1.5 mg/l BAP in MS media, the average shoot length was recorded 3.84 cm, followed by 3.49 cm with concentration combination of 2.0 mg/l TDZ + 0.25 mg/l NAA.



**Figure 4:** Effect of plant growth regulators supplemented in MS medium on adventitious shoot induction and average shoot length from nodal segment explants after four weeks of the culture of carnation cv. Geolei

### 3.4 Rooting and Acclimatization

In the present study, half-strength MS media was used for rooting which was supplemented with 0.25 to 2.25 mg/l NAA including control and media with 1.25 mg/l NAA resulted in the highest response in terms of maximal root induction (83.32 %) and most extended root length (3.8 cm) (Figure 4, 3c). The rooted shoots were withdrawn from the culture vessels after four weeks of culture (Figure 3d), cleaned carefully to remove the culture media, and dipped in 0.01% carbendazim (bavistin) solution for 10-15 minutes before being transplanted to small plastic cups containing sterilized soil and vermicompost (1:1). To maintain relative humidity, the cups were covered with polythene bags. The combination was then saturated with 1/10th MS medium to maintain the moisture and nutrition, allowing the plant to grow well [13].



**Figure 5:** Effect of plant growth regulators added in MS medium on root induction and average root length after four weeks of the culture of carnation cv. Geolei

#### 4. DISCUSSION

In the present study we tried to check the effectiveness of sterilizing agents Sodium Hypochlorite (NaOCl) with bavistin and  $\text{HgCl}_2$  in combination and alone and their sterilizing efficacy were tested on Leaf and Nodal segment explants. Two different time combinations were used to make the explants (leaf and nodal segment) contamination-free. Our finding showed that the leaf and nodal segments are more susceptible to fungal contamination, thus increasing the bavistin concentration found more effective; however, increasing the sodium hypochlorite concentration makes the plants less sensitive in the culture. The reason for this is that the NaOCl has a high oxidation capability, which is extremely powerful against bacteria, fungi, and viruses present on the surface of the explants and infest the deep tissues of the explants. The reaction between amino acids and NaOCl yields the aldehyde,  $\text{NH}_4\text{Cl}$ , and  $\text{CO}_2$ . As a result, direct contact of the tissue with NaOCl during sterilizing process, depending on the concentration, application time, and temperature, may damage the tissue's health [11].

Several authors have also reported the effectiveness of bavistin and sodium hypochlorite [13, 19, 20, 23, 25]. Plant materials were sterilized by treating them for five minutes with an aqueous solution of 0.1 %  $\text{HgCl}_2$  and two drops of Tween 20 under aseptic conditions, followed by five rinses with autoclaved distilled water to remove any residual of  $\text{HgCl}_2$  [23]. Leaf explants were surface-sterilized for bavistin (w/v) and 5–20 minutes with 0.5 % sodium hypochlorite (v/v) [13]. Leaf explants were surface-sterilized by soaking them in 70% ethanol for a minute was useful. In 0.1 %  $\text{HgCl}_2$  containing one drop of Tween 20, for 12 minutes, then washed six times with sterile distilled water [25]. Our study showed clearly that the leaf and nodal segment

explants sterilized with the various concentration of NaOCl, bavistin and HgCl<sub>2</sub> were effective and reduced contamination in the culture.

In our study the best callusing (90.47%) was observed when media was supplemented with 2.5 mg/l 2,4-D + 0.75 mg/l NAA after effective sterilization of explants. Our study supported by many researchers such as Quadri [14] supplemented MS media with BAP with 2,4-D in combination for callus induction, resulting in 91.66 percent callus induction from leaf and nodal segment explants. Thakur and Kanwar [13] found highest callus induction when MS medium supplemented with 2,4-D and NAA. Jorapur [8] obtained cent percent percent callusing with MS supplemented with 2,4-D and NAA. When MS medium supplemented with NAA and kinetin, Mehta [17] obtained 92 percent callus induction.

Growth regulators play a critical function in the proliferation of shoots and roots. Present study was an effort to get good callus and shoot regeneration with less contamination. Hyperhydricity is a serious physiological condition that develops during carnation *in vitro* cultures and causes excessive hydration and decreased mechanical strength, leading to culture failure. Culture media containing 0.15 mM FeSO<sub>4</sub>·7H<sub>2</sub>O and 2.25 mM MgSO<sub>4</sub>·7H<sub>2</sub>O [12], were used to overcome this situation. The regenerated plantlets showed substantially less hyperhydricity in the carnation.

After callus induction shoot regeneration were achieved using nodal explants on the MS medium supplemented with TDZ, NAA and BAP in combination. This combination gave the maximum shoot regeneration with good shoot length. We also observed that BAP alone at 1.5 mg/l showed the good shoot regeneration but this did not generate good shoot length. It is evident from the present study that TDZ in combination with NAA produced better results for shoot regeneration while BAP alone also proved better but not in terms of shoot length. Therefore combinations of PGRs may prove a better option for shoot regeneration because they have the synergistic effects on the growth of the cell and induce the cell division faster. The 6-Benzylaminopurine, often known as benzyl adenine, BAP, or BA, is a synthetic cytokinin that stimulates the growth and development of plants.

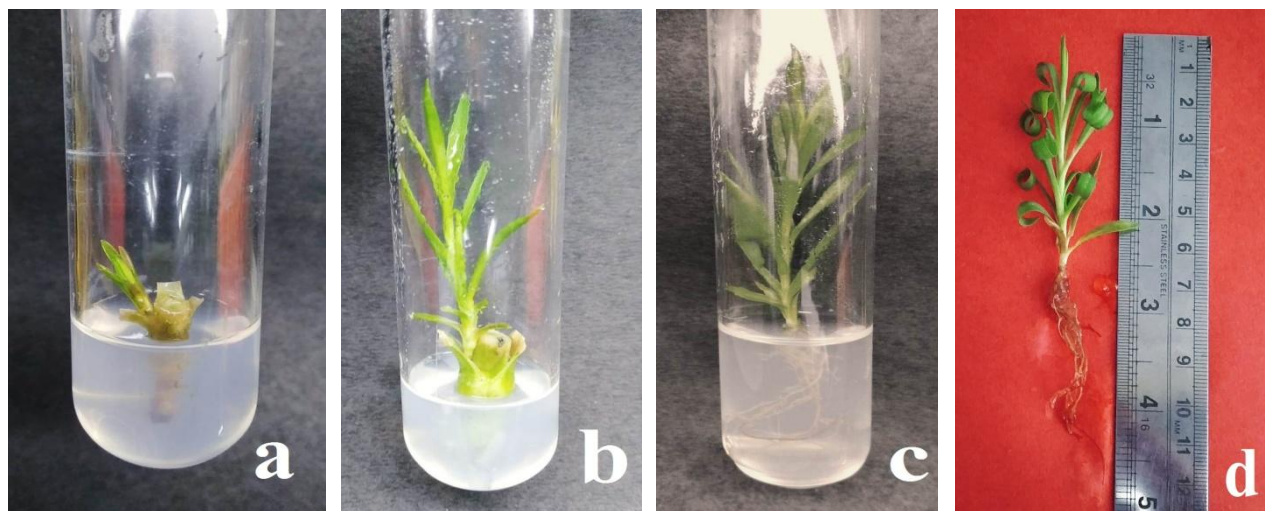
Our study was supported the work of several researchers such as Khatun [20] who reported producing the highest number of shoots (25±0.4) with modified MS media which was supplemented with 1.0 mg/l TDZ and 1.0 mg/l TIBA, the explants demonstrated adventitious shoot regeneration (12.33 %). In comparison the maximum frequency of shoot regeneration (18.33 percent) was observed with 1.0 mg/l TDZ and 0.5 mg/l TIBA [21]. When inoculated on MS media supplemented with 2.0 mg/l BA, nodal sections produced more shoots-, than the apical segment [22]. Average shoot regeneration (80.56 percent), average number of shoots (6.01), and average shoot length (1.93 cm) were found to be highest in MS medium supplemented with 1.5 mg/l TDZ, 0.25 mg/l Kinetin, and 0.25 mg/l NAA [13]. In another study, MS medium was used with 10% coconut water + 1.0mg/l BAP, the maximum percentage of shoot induction was recorded [23]. Jorapur[8] reported that the number of shoots dropped when

BAP concentration was increased from 2 mg/l to 3 mg/l. Thakur [2] used 2.0 mg/l BAP to achieve maximum shoots (77.77 %) with a shoot length of 2.44 cm. On the same media containing 2.0 mg/l BA, Al-Mizory [24] demonstrated *in vitro* shoot regeneration from nodal segment.

For healthy growth and successful commercial production, regenerated shoots must form the root. Because of its effect on fast cell division, auxin plays a critical function in root induction. In the present study, half-strength MS media with 1.25 mg/l NAA proved better and resulted in the highest response in terms of maximal root induction and extended and healthy roots were obtained. Regenerated microshoots were survived well with soil and vermicompost (1:1) mixture under glasshouse conditions. Several researchers have reported that auxins alone or in combination for rooting in *Dianthus caryophyllus* L. In half MS medium enhanced with combination of IBA and NAA, rapid roots induction and a maximum number of roots per culture were attained [18]. Esmail [25] noticed various responses of ten carnation cultivars on MS medium without adding any growth regulator. When cultivated individually on half-strength MS medium with 0.5 mg/l IBA + 0.5 mg/l NAA, 78.2 % of regenerated shoots rooted [26]. For 15 days, Casas [27] placed the proliferating shoots on a rooting media of half-strength MS medium with 0.7 % agar + 5.4  $\mu$ M NAA. The shoots were also rooted well in half-strength MS medium supplemented with 2 mg/l indoles butyric acid (IBA) and 0.2 % activated charcoal [15].

## **Conclusion**

The finding of the present investigation has developed an effective surface sterilization protocol. A high frequency *in vitro* callus induction procedure for carnation cv. Geolei was developed in this study. The possible reasons for callusing may be that they are not differentiating towards shooting might be the culture conditions, certain lab practices which went overlooked, or endogenous growth regulators that might have been generated in the culture, which produced an inhibitory impact. A suitable concentration combination of TDZ, NAA and BAP for direct adventitious shooting using nodal segment produced the best results. For rooting, NAA gave the optimum response. The present research indicated that the use of explants sterilized with bavistin, sodium hypochlorite in combination with mercuric chloride is most effective.



**Figure 6:**(a)Adventitious shoot induction and regeneration from nodal segment; (b) shooting after fourweeks in shooting media; (c)elongated roots after four weeks in rooting media; (d) fully developed plantlet.

### Competing Interests

There are no competing interests stated by the authors.

### Author's Contribution

The present research has been equally contributed by all the authors.MKY, AT, MK, RSS and LKG designed the study, and US performed the experiments and recorded the data. US, SS and VDB did the statistical analysis. US has prepared the draft and MKY has checked the manuscript. All authors read and approved the present manuscript.

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