Original Research Article

An effective protocol for sterilization of Carnation (*Dianthus caryophyllus* L.) cv. 'Geolei' explants for successful callusing and shoot regeneration

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 various sterilants and their various concentration and time combination were optimized before inoculation in the culture media. The least contaminated cultures were created by consecutively treating the explants with 0.25 percent bavistin, 0.50 percent sodium hypochlorite, and 0.1 percent mercuric chloride for ten, fifteen, and two minutes, respectively.

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

Abbrevaiations: MS: Murashige and Skoog

1. INTRODUCTION

Carnation is a member of the *Caryophyllaceae* family. Carnation is 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 'phyllon,' 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, but 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), but its precise origin is difficult to determine due to its lengthy period in cultivation [3]. In general, *Dianthus* species can be cultivated through seed in the spring and cuttings in the late summer [4]. Seeds, stem cuttings, and layering are the most common methods of propagation for carnations [5]. These approaches, however, are not suitable for commercial development due to 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 and genetic transformation approaches to improve it could be a more promising strategy. It is generally recognized that in order to conduct effective genetic transformation research, the plant must be able to regenerate 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 a necessary step in establishing genetic transformation methods for introducing novel features or studying gene expression regulation and plant physiology *in vitro*. Optimization of efficient sterilization methods using suitable explants (leaf and nodal explants), callus induction and direct shoot regeneration in carnation is described in this report.

2. MATERIALS AND METHODS

2.1 Plant Material

The current study was conducted in Department of Agricultural Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology in Meerut, (U.P., India). The plant material was collectedd from the Department of Floriculture and Landscaping, Dr. YSP University of Horticulture & Forestry, Nauni, Solan, (H.P., India), in the form of 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 air flow (LAF). The explants were then surface sterilized with different concentrations of bavistin (w/v) (systemic fungicide, Carbendazim) for 10 minutes and with sodium hypochlorite (NaOCl) (v/v) solution for fifteen minutes. 0.1 percent mercuric chloride ($HgCl_2$) was used only for two minutes with nodal segments only. After every step, the explants were washed with autoclaved water for four to five times. Effects of different concentration were recorded as uncontaminated cultures after four weeks of inoculation (Figure 1).

2.3 Culture Conditions and Callusing

Surface sterilized leaf segments were inoculated on solid Murashige and Skoog [9] medium containing sucrose (3 percent w/v), agar (0.8 percent w/v) and various concentrations of growth regulators for *in vitro* culture establishment. Prior to autoclaving at 121 ºC and 1.06 kg/cm^2 for 20 minutes, the pH of the culture media was adjusted to 5.8±0.2. All cultures were incubated at 25±1 ºC 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 MS-salt strength [10]. MS 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 dish.

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 mixed combinations of TDZ and NAA for *in vitro* culture establishment. The cultures were incubated for four weeks and observations were recorded for percent shoot induction, average shoot length (cm) and shoot induction response. The developed adventitious shoots were sub-cultured after two weeks.

2.5 *In vitro* rooting of plantlets

After fourth week of culture, 2.5-3.0 cm long *in vitro* grown shoots were transferred to half strength MS media with different concentrations of NAA for root induction. Observations were recorded on root length and development. Thereafter, the rooted plants were withdrawn from the culture tubes, cleaned properly to remove agar gel that stuck to the roots, and transferred in into small plastic cups filled with sterilized soil and compost (1:1) for hardening. For acclimatization, the plantlets were housed in a green house facility.

3. RESULTS

3.1 Explants surface sterilization

The effects of different doses of bavistin and sodium hypochlorite on surface sterilization of leaf explants were studied for 10 and 15 minutes, respectively. After four weeks, the combination of 0.25 percent (w/v) bavistin and 0.50 percent (v/v) sodium hypochlorite proved the most effective and caused least callus contamination (Figure 1). For nodal segments also, 0.25 percent (w/v) bavistin and 0.50 percent (v/v) sodium hypochlorite with HgCl2 for two minutes produced the least contamination. 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. 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 also infested the deep tissues of the expalnts. The reaction between amino acids and NaOCl yields the aldehyde, NH_4Cl, and CO_2. As a result, direct contact of the tissue with NaOCl during the sterilizing process, depending on the concentration, application time, and temperature, and may be damaging to the tissue's health [11].

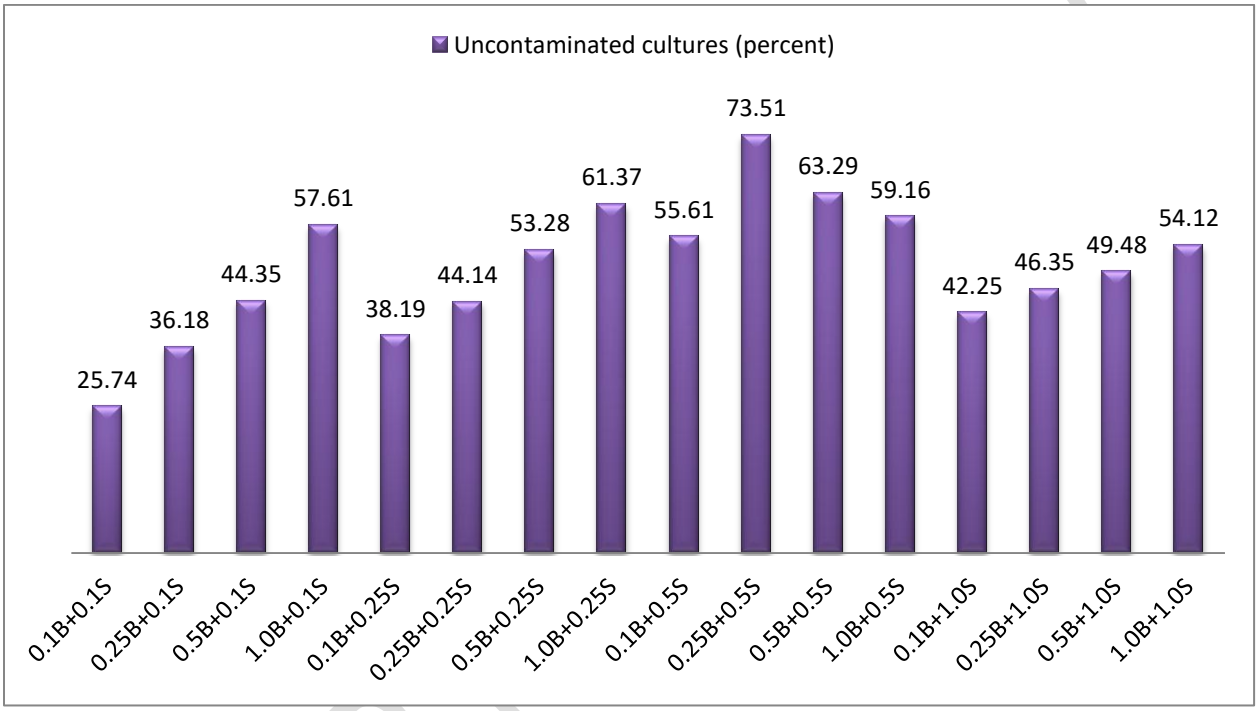

Figure 1: The effect of different concentrations of bavistin (B) and sodium hypochlorite (S) solution treated for 10 and 15 minutes, respectively, on surface sterilization of explants after four weeks of incubation of cultures of carnation cv. Geolei

3.2 Callusing

A total of six culture media combinations with a 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 + 0.75 mg/l NAA, the highest callus induction frequency(CIF) (90.47 percent) was recorded (Figure: 3b), followed by 88.95 percent CIF on culture media supplemented with 2.0 mg/l 2,4-D + 0.75 mg/l NAA. CIF was lowest (23.80 percent) in media supplemented with 0.5 mg/l 2,4-D + 0.50 mg/l NAA, whereas 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 (grams)	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 white, proliferated

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

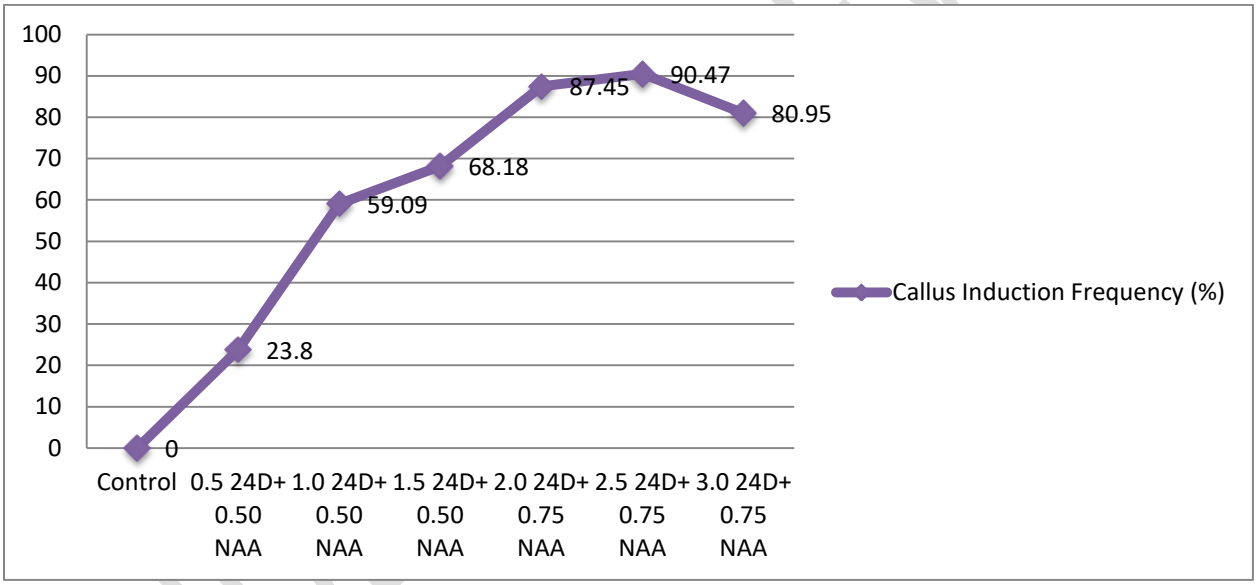

Figure 2: Effect of plant growth regulators added in MS medium on callus induction frequency from leaf explants after four weeks old culture of carnation.

Figure 3 (a): Leaf segments inoculated for callus induction; (b) proliferated callus after 4 weeks old culture of carnation cv. Geolei; (c) callus showing no response towards shooting

3.3 Adventitious shoot induction

The four-week-old callus taken from leaf explants was transferred to shoot induction media, but 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, although only one to two shoots were produced on each explants (Figure:3 a,b). After four weeks of culture, the last observations for data collecting were taken (Figure 3). Maximum shoots (76.47 percent) and (71.42 percent) 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. With 1.5 mg/l BAP, the average shoot length was 3.84 cm, followed by 3.49 cm with 2.0 mg/l TDZ + 0.25 mg/l NAA. Hyperhydricity is a serious physiological condition that develops during carnation *in vitro* cultures and causes excessive hydration and decreased mechanical strength which mostly leads to cultures failure. To overcome this situation, we used culture media containing 0.15 mM $FeSO_4.7H_2O$ and 2.25 mM $MgSO_47H_2O$ [12], and the regenerated plantlets showed substantially a less hyperhydricity in the carnation.

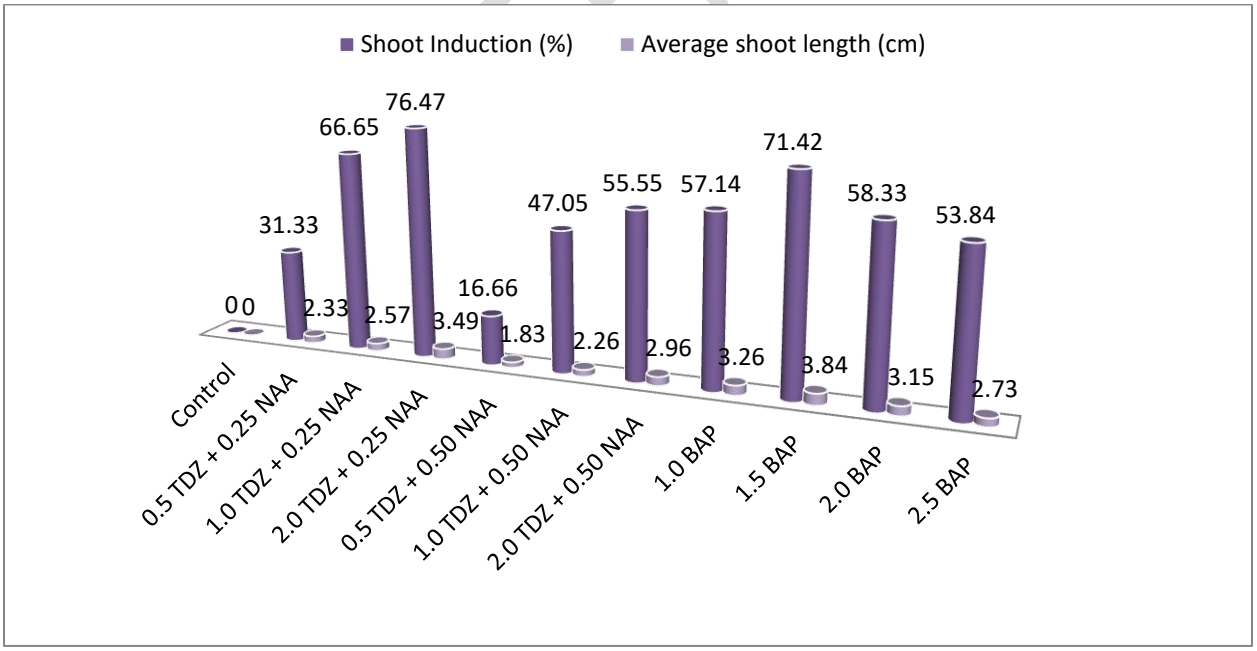

Figure 4: Effect of plant growth regulators supplemented in MS medium on adventitious shoot induction and average shoot length from nodal segment explants after four weeks of culture of carnation cv. Geolei

3.4 Rooting and Acclimatization

For healthy growth, regenerated shoots must form the root. Because of its effect on fast cell division, auxin plays a critical function in root induction. Supplementing half strength MS media with 1.25 mg/l NAA resulted in the highest response in terms of maximal root induction (83.32 percent) and longest 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 percent cabendazim (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].

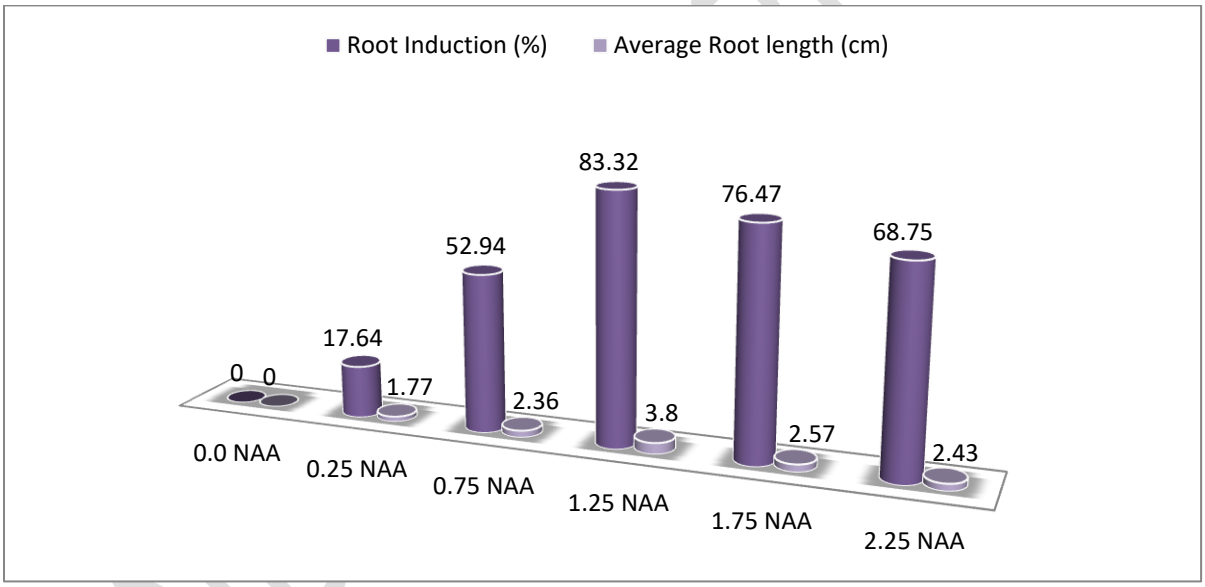

Figure 5: Effect of plant growth regulators added in MS medium on root induction and average root length after four weeks of culture of carnation cv. Geolei

4. DISCUSSION

In the present study we tried to check the effectiveness of sterilizing agents with different time combinations to make the explants (leaf and nodal segment) contamination free. 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 percent $HgCl_2$ and two drops of Tween 20 under aseptic conditions,

followed by five rinses with autoclaved distilled water to remove any residual of $HgCl_2$ [23]. Leaf explants were surface sterilized for 1–10 minutes with 0.2 percent bavistin (w/v) and 5–20 minutes with 0.5 percent sodium hypochlorite (v/v) [13]. Leaf explants were surface sterilized by soaking them in 70% ethanol for a minute, then in 0.1 percent HgCl2 containing one drop of Tween 20, for 12 minutes, then washed six times with sterile distilled water [25]. The explants sterilized with the various concentration of sterilants for different time were effective and reduced contamination in our study.

After sterilization explants were inoculated on 2,4-D in combination of NAA at various doses for producing callus in carnation leaf segments. Quadri [14] supplemented MS media with 1.00 mg/l BAP with 2.00 mg/l 2,4-D for callus induction, resulting in 91.66 percent callus induction from leaf and nodal segment explants. Thakur and Kanwar [13] found that MS medium supplemented with 2.0 mg/l 2,4-D and 0.5 mg/l NAA resulted in the highest callus induction of 94.44 percent. Jorapur [8] obtained 100 percent callusing with MS + 2,4-D 1.5 mg/l + NAA 0.5 mg/l. 74 percent with 2.0 mg/l 24D and 75.17 percent with 2,4-D + BA (2.0 + 1.0) according to Kanwar and Kumar [15]. According to Kumar [16], the combination of 1.0 mg/l 2,4-D + 2.2 mg/l TDZ + 0.12 mg/l zeatin had the CIF of 76.80 percent. 2.0 mg/l 2,4-D + 0.5 mg/l NAA, on the other hand, produced considerable (40 percent) callus. When MS medium supplemented with 2.0 mg/l NAA and 2.0 mg/l kinetin, Mehta [17] obtained the 92 percent of callus induction.

Growth regulators play a critical function in the proliferation of shoots and roots. 6-Benzylaminopurine, often known as benzyl adenine, BAP, or BA, is a synthetic cytokinin that stimulates growth and development of plants. In the half strength MS medium, BAP, TDZ, and silver nitrate (AgNO3) in concentrations of 1.5, 0.25, and 0.5 mg/L, respectively, responded with early micro-shoots formation, and maximum regeneration was observed; while BAP (2.5 mg/L), TDZ (1.0 mg/L), and $AgNO_3$ (1.5 mg/L) produced maximum length of micro-shoots [18]. Kharrazi [19] found that BAP (4.0 mg/l) produced better results. When nodal segment explants were cultivated on MS + 1.0mg/l BAP, Khatun [20] reported that they produced 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 percent), while 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 an another study, MS medium was used with 10 percent coconut water + 1.0mg/l BAP, the maximum percentage of shoot induction was recorded [23]. Jorapur [8] reported that when BAP concentration was increased from 2 mg/l to 3 mg/l, the quantity of shoots dropped. Thakur [2] used 2.0 mg/l BAP to achieve maximum shoots (77.77

percent) 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.

Regenerated adventitious shoots from the nodal segment were tweaked off and inoculated for rooting on half strength MS media with 0.8 percent agar supplemented with various concentrations of NAA after four weeks of shooting. The section of the explants in touch with the media gets contaminated in most cases, but the shoot is unaffected. Several researchers have reported that auxins alone or in combination for rooting in *Dianthus caryophyllus* L. In half MS medium enhanced with 1.5 mg/L IBA and 0.5 mg/L NAA, rapid induction of roots and maximum number of roots per culture were attained [18]. According to Thakur [2], half strength MS medium mixed with 0.04 percent activated charcoal produced the maximum rooting percentage (62.49), 4.50 roots per shoot, and 1.61 cm root length. Maximum rooting (98.19 percent) was achieved using half strength MS medium supplemented with 1.5 mg/l IBA and 0.02 percent activated charcoal, with 9.60 average numbers of roots per microshoot and 4.24 cm root length [13]. Khatun [20] tested varied concentrations of IBA, IAA, and NAA in half-strength MS medium. With 1.0 mg/l NAA, the best results were obtained (80 percent of the shoots rooted effectively after 8-9 days of culture). Esmaiel [25] noticed various responses (0.0 to 72.8 percent mean root formation) 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 percent of regenerated shoots rooted [26]. For 15 days, Casas [27] placed the proliferating shoots on a rooting media made up of half strength MS medium with 0.7 percent agar + 5.4 µM NAA. The shoots were also rooted well in half strength MS medium supplemented with 2 mg/l indole butyric acid (IBA) and 0.2 percent activated charcoal [15].

Conclusion

The finding of present investigation has developed an effective surface sterilization methodology. A high frequency *in vitro* callus induction procedure in carnation Cv. Geolei that is effective and dependable is developed. The possible reasons of callus not differentiating towards shooting might be the culture conditions, certain lab practices which went overlooked or endogenous growth regulators might have been generated in the culture, which produced an inhibitory impact. The concentration combination 2.0 mg/l TDZ + 0.25 mg/l NAA and 1.5 mg/l BAP for direct adventitious shooting with nodal segment produced the best results. For rooting, 1.25 mg/l NAA gave the best response. The present research indicated that use of explants sterilized with bavistin, sodium hypochlorite is most effective.

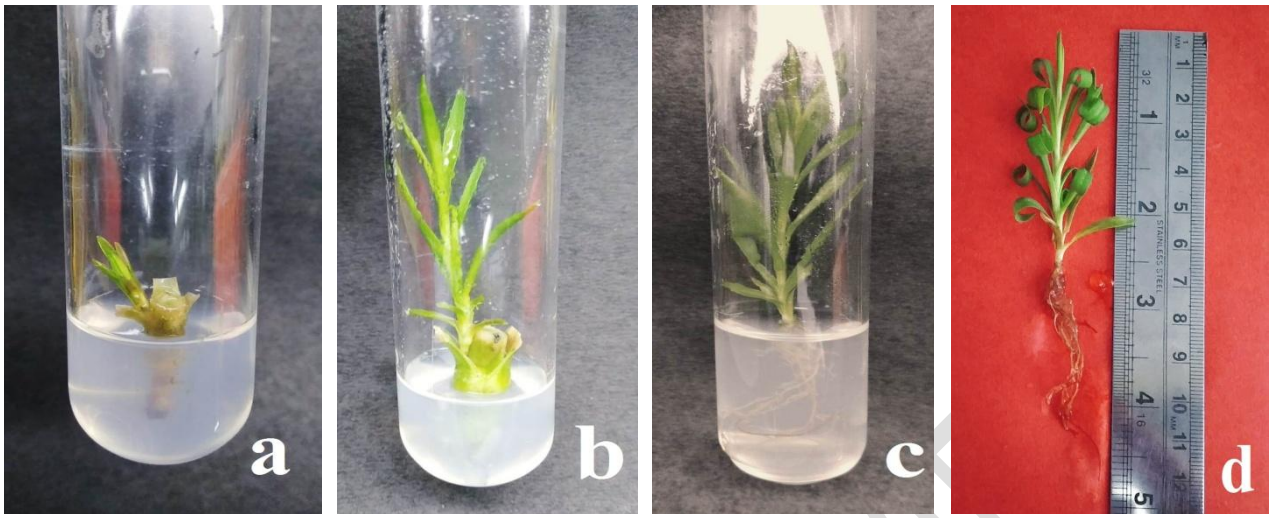

Figure 6: (a) Adventitious shoot induction and regeneration from nodal segment; (b) shooting after 4 weeks in shooting media; (c) elongated roots after 4 weeks in rooting media; (d) fully developed plantlet.

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