**Effect of 2, 4-D and kinetin on *in vitro* callogenesis of carnation**

**Abstract**

The study was carried out in 2019-22, at the Plant Tissue Culture Laboratory, Veer Chandra Singh Garhwali, Uttarakhand University of Horticulture and Forestry, Bharsar, Pauri Garhwal, Uttarakhand-246123. The study was conducted to optimize the protocol for callus induction in carnation as it is a basic step of micropropagation and commercial production of carnation can be boosted through plant tissue culture techniques. In this experiment, MS media was fortified with 2, 4-D and Kinetin at different concentrations for callogenesis where leaf, apical bud and node were used as explants. Additionally, light and dark condition for 24 h with 25±2ºC temperature and 60% relative humidity were also provided in culture room to observe the effect of culture conditions on callus induction. The results showed that the maximum callus induced in treatment T4 in node explant under light condition and in treatment T5 less number of days taken in leaf explant under light condition. We found that, the varied concentrations of plant growth regulators impacts the growth and quality of callus induced from different explants. By optimizing the suitable dose of plant growth regulators, explant and culture conditions will help in prospects and research directions in carnation micropropagation studies.

**Keywords:** Carnation, callus, micropropagation, 2, 4-D, Kinetin, etc.

**Introduction**

Carnation (*Dianthus caryophyllus* L.) is one of the major cut flower crops belonging to the family Caryophyllaceae. It is endemic to Southern Europe, namely the Mediterranean coasts. The genus name ‘Dianthus’ derived from the Greek words ‘*dios*’, which means divine and ‘*anthos*’, which means flower and was designated to the group by the Greek plant biologist Theophrastrus (300 B.C.) and is also known as the ‘divine flower’(Bhatt, 1989). Apart from being a cut flower, it is also used for bedding, potting plants, borders, edging and in rock gardens. The importance of this ornamental flower is due to its beauty, diversity of colours, excellent keeping quality and wide range of different forms (Ali *et al*., 2008; Kanwar and Kumar, 2010). Besides aesthetic value, carnation flowers are considered to be cardio tonic, diaphoretic and alexiteric. The whole plant is used as a vermifuge in China while in France it is used for perfume extraction (Poucher, 1950).

Both sexual and asexual methods are used for its propagation. For the development of new varieties, it is propagated by seeds. However, the terminal cuttings are commonly used for commercial propagation. For commercial cut flower production, carnations are grown in greenhouses by maintaining an optimum growing environment. The quality planting materials for carnations are imported from European countries, particularly the Netherlands at very high prices (Qadri *et al.,* 2018) and production through conventional methods requires a huge amount of planting materials. The requirement for skilled manpower has always been increasing due to the production of a lesser amount of saplings per unit of time. Tissue culture techniques, on the other hand, can be a vital tool for generating an enormous number of plantlet clones within a short period of time for large-scale production (Pareek *et al.,* 2004; Kanwar and Kumar, 2010). Hence, micropropagation is the commercial method for propagating carnations on a large scale. Although few protocols are available for *in vitro* regeneration and micropropagation of carnation, the process of callusing, shoot regeneration and root establishment is affected by the genotype used, culture environment, plant growth regulators and kind of explant. Therefore, it is another important study to generate a protocol for *in vitro* callus induction. Because it is the first and most important stage in the *in vitro* production of a crop by tissue culture techniques. To promote callus formation, plant growth regulators including auxins, cytokinins, and gibberellins are added to the culture media. Plant tissue culture media with specific auxin to cytokinin ratios promotes disorderly growth and division of callus cells. Callus cultures are often characterized as either compact or friable. Friable calluses break readily and can be utilized to create cell suspension cultures. Callus culture involves the direct organogenesis or embryogenesis of cells, resulting in the formation of a whole new plant and the tissue culture techniques have been developed to produce *in vitro* culture to obtain disease-free planting material (Bhatt, 1989). The experiment was aimed to optimize callus induction protocol from different explants (*viz.,* leaf, apical bud and node) of carnation (*Dianthus caryophyllus* L.) var. Pink Dona in Murashige and Skoog (1962) media with different concentrations of plant growth regulators (2, 4-D and Kinetin).

**Materials and Methods**

The newly emerging young and immature, leaves, apical buds and upper nodal segments from healthy and disease free plant material were collected from the experimental farm of Department of Floriculture and Landscaping of College of Horticulture, VCSG, Uttarakhand University of Horticulture and Forestry, Bharsar, Uttarakhand. The plant material was collected in clean polythene bags and washed thoroughly in running water with labolin for 15-20 minutes immediately. Then, treated with 0.1 % HgCl2 solution for 30 seconds for surface sterilization and rinsed thoroughly with double distilled water 3-4 times under aseptic conditions in Laminar Airflow Cabinet. The edges of the explants were cut with a scalpel. The size of explants was kept 10 mm for all explants and were placed on filter paper in petri dishes under Laminar Airflow Chamber. All the instruments were first steam sterilized in an autoclave at 15 psi (121°C) for 20 minutes and then flame sterilized before each inoculation. The culture media used in this experiment was Murashige and Skoog (1962) media supplemented with freshly prepared 2, 4-D and Kinetin solutions. The concentration of 2, 4-D and kinetin were 0.5, 1.0, 1.5 and 2.0 mgL-1. The pH of the culture media was adjusted to 5.8 prior to autoclaving at 15 psi (121ºC) and for 40 minutes. All the cultures were incubated at 25±2ºC under 4000 lux light intensity for 24h light/dark photoperiods, provided by cool white fluorescent tubes (Philips, India). All the above mentioned in vitro practices were carried out at Tissue Culture Laboratory, Department of Basic and Social Sciences, VCSG, Uttarakhand University of Horticulture and Forestry, Bharsar, Uttarakhand. The incubated cultures were observed regularly. Number of callus induced and days taken for callus induction were recorded. Three replications with 6 explants in each replication (24 explants) were maintained for each treatment and statistically analyzed using factorial completely randomized design (Gomez and Gomez, 1984) and analysis of variance by 3 way factorial anova technique. The comparative Duncan’s multiple range (P=0.05) test was used to determine the difference between treatments.

**Results and discussion**

The data in table 1 showed that in treatment T4 maximum number of callus were induced by node explant in light condition and by leaf in dark condition followed by T7 in dark condition in leaf explant. There was no induction of callus recorded treatment T2 and T16 in leaf explant. The data in table 2 showed that in treatment T5 less number of days (14) taken for callus induction followed by T3, T11 and T15 in leaf explant. Whereas maximum days in treatment T4 in node under dark condition. In previous studies, leaf explants excised from the *in vitro* growing plantlets were cultured on solid MS media supplied with 2, 4-D (0.0, 0.5, 1.0 and 2.0 mgL-1) and Kn (0.0, 0.5, 1.0 and 2.0 mgL-1). Callus initiation was observed within two weeks of culturing (Mohammed *et al*. 2017). Similarly, Castillo *et al*. (1998) reported that auxin 2, 4-D alone or in combination with cytokinins has been widely used to enhance callus induction and maintenance. This elongation of the cell was due to the response of 2.4-D, but the cell cannot divide rapidly because there was no addition of kinetin. The combination of the growth regulators, 2.4-D, and kinetin, in all explant resulted in a larger callus size compared to only 2.4-D. In 2.4-D without kinetin, callus growth response in all explant with a small callus needed extended time to grow bigger. This suggests that the ant nest plant callus cannot grow optimally with a single auxin administration without the addition of a growth regulator from the cytokinin group. Morini *et al.* (2000) also revealed that the callus formation of quince leaves was found only in the abaxial surface part. The appearance of the callus on the injured part might be because of the excitement of the tissue on the explant to cover the wound. Explant response on the treatment medium is started with explant swelling or elongation. The size of the explant becomes bigger from the beginning and the callus begins to form on part of the injured explant. The combination of auxin (2.4-D) and cytokinin (kinetin) as growth regulators on the MS medium can induce callus formation, both in single and combination treatments. The callus began to appear on the edge of the explants and on the wounded parts and continued to grow until the end of the observation at 10 weeks after planting. In addition, Stobbe *et al*. (2002) revealed that the wound-induced calli regenerate new organs or new tissues, suggesting that they are highly pluripotent. Kallak *et al.* (2020) )found that callus induction, shoot tips and nodal or internodal stem segments of carnation cultivars (Coral, Jaguar, Salome and Sannah) were grown on MS basal medium with 2, 4-D and kinetin. Muhammed *et al.* (2014) established a protocol for callus induction from leaves of carnation. MS media fortified with 2.0 mgL-1 2, 4-D and 0.5 mgL-1 Kn was found to be the appropriate medium for producing callus cultures. In contrast, in the present investigation at the similar concentration, leaf explant showed poor response for callus induction. This is might be due to higher concentration of 2, 4-D is inhibitory for the induction of callus in leaf explant. Maitra *et al*. (2012) also reported that auxin (2, 4-D) and cytokinin (Kinetin) are responsible for the callus production of carnation from terminal young leaf explants. Basal MS medium along with different concentrations and combinations of auxin and cytokinin produced nodular, greenish yellow to greenish profuse callus within 8 to 10 days of inoculation. Kami *et al*. (2010) also reported that light sensors mediate numerous adaptive response and developmental transitions. Light intensity at sub optimal condition is desirable for callus induction. Higher light intensity leads to browning of callus. Callus browning is caused by polyphenolic compounds present when the explants were wounded and enzymatic browning reaction of phenolic compounds, oxidized by polyphenol oxidase, peroxidase or exposure for air. The oxidation process of phenolic compounds is enhanced by light intensity (Taranto *et al*., 2017). Therefore, light intensity has a pronounced effect on the colour of callus induced. During the experiment the culture tubes and conical flasks were placed in the culture room maintained at a temperature of 25±2ºC and 60% humidity under light (florescent light for 24 hours) and dark (for 24 hours) condition. In tissue culture, factors including auxins, cytokinins, light and temperature influence callus induction and at suboptimal concentrations and combinations can cause callus browning and necrosis (Afshari e*t al*., 2011; Dou *et al*., 2017 and Ikeuchi *et al*., 2013). Light is an external factor that influences callus formation. The colour change that exists in the callus was because of pigment, nutrients and environmental factors, such as light (Evans *et al*., 2003). George and Sherrington (1984) stated that white light could induce callus formation and organogenesis in the plant tissue. A callus that has yellowish green and green colour was formed with the addition of kinetin. The green colour was because of chlorophyll, resulting from the 2, 4-D interaction with kinetin, mainly because kinetin (cytokinin) has a function in the formation of chlorophyll in the callus and due to environmental factors such as exposure to light. Light is one of the important environmental factors that controls plant growth, development, morphogenesis, metabolism and chlorophyll content in plant cell, tissue and organ cultures (Dou *et al.*, 2017). It can also influence the efficacy of plant growth regulators (PGRs) as well as adjustment of endogenous hormone levels. Recent studies have resolved the light regulation of gibberellin and auxin metabolism (Martinez and Gil 2001; Halliday *et al.*, 2009; Stavang *et al.*, 2007). An enhanced callus proliferation may lay in the fact that natural auxin levels increase under lower light intensity or darkness (Chory *et al.*, 1994).

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Treatment** | **Concentrations** | **Growing condition** | | | | | |
| **Light** | | | **Dark** | | |
| **MS media + 2, 4-D + kinetin mgL-1** | **Leaf**  **(E1)** | **Apical bud (E2)** | **Node (E3)** | **Leaf**  **(E1)** | **Apical bud (E2)** | **Node (E3)** |
| T1 | 0.5 : 0.5 | 0.417hijkl | 0.417hijkl | 0.75efghijkl | 1.083defghijkl | 0.667fghijkl | 1.5cdefgh |
| T2 | 0.5 : 1.0 | 0l | 1.083defghijkl | 1.417cdefghi | 2.333bc | 0.417hijkl | 0.417hijkl |
| T3 | 0.5 : 1.5 | 1.75cdef | 1.167defghijk | 0.917defghijkl | 1.333cdefghij | 1.083defghijkl | 0.583ghijkl |
| T4 | 0.5 : 2.0 | 0.417hijkl | 1.333cdefghij | 3.583a | 3.583a | 0.75efghijkl | 0.333ijkl |
| T5 | 1.0 : 0.5 | 1defghijkl | 0.75efghijkl | 1.5cdefgh | 1.167defghijk | 1.917cd | 0.25jkl |
| T6 | 1.0 : 1.0 | 0.583ghijkl | 1.333cdefghij | 1.25cdefghij | 2.333bc | 1.5cdefgh | 0.5ghijkl |
| T7 | 1.0 : 1.5 | 1.417cdefghi | 0.917defghijkl | 0.667fghijkl | 3ab | 1defghijkl | 0.25jkl |
| T8 | 1.0 : 2.0 | 0.25jkl | 0.833defghijkl | 1.167defghijk | 1.417cdefghi | 1.833cde | 1defghijkl |
| T9 | 1.5 : 0.5 | 0.25jkl | 1.167defghijk | 1.417cdefghi | 1.333cdefghij | 1.167defghijk | 1.417cdefghi |
| T10 | 1.5 : 1.0 | 0.75efghijkl | 1.417cdefghi | 1.25cdefghij | 0.917defghijkl | 1.833cde | 0.583ghijkl |
| T11 | 1.5 : 1.5 | 0.833defghijkl | 0.583ghijkl | 1defghijkl | 0.917defghijkl | 1defghijkl | 0.667fghijkl |
| T12 | 1.5 : 2.0 | 0.667fghijkl | 1.417cdefghi | 1.167defghijk | 0.917defghijkl | 1.417cdefghi | 0.667fghijkl |
| T13 | 2.0 : 0.5 | 1.167defghijk | 0.333ijkl | 1.083defghijkl | 1.5cdefgh | 0.75efghijkl | 1defghijkl |
| T14 | 2.0 : 1.0 | 1.167defghijk | 1.167defghijk | 1.25cdefghij | 1.583cdefg | 0.917defghijkl | 0.917defghijkl |
| T15 | 2.0 : 1.5 | 0.25jkl | 0.417hijkl | 1.417cdefghi | 1.083defghijkl | 1.833cde | 0.917defghijkl |
| T16 | 2.0 : 2.0 | 0.5ghijkl | 0.417hijkl | 0.667fghijkl | 1.333cdefghij | 0.083kl | 0l |

**Table 1: Number of callus induced in leaf, apical bud and node under light and dark condition**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Treatment** | **Concentrations** | **Growing condition** | | | | | |
| **Light** | | | **Dark** | | |
| **MS media + 2, 4-D + kinetin mgL-1** | **Leaf**  **(E1)** | **Apical bud (E2)** | **Node (E3)** | **Leaf**  **(E1)** | **Apical bud (E2)** | **Node (E3)** |
| T1 | 0.5 : 0.5 | 19bcdefghijklm | 22bcdefghijklm | 21bcdefghijklm | 22bcdefghijklm | 22bcdefghijklm | 20.333bcdefghijklm |
| T2 | 0.5 : 1.0 | 19.333bcdefghijklm | 21bcdefghijklm | 22bcdefghijklm | 15.667hijklm | 21bcdefghijklm | 23.333bcdefghij |
| T3 | 0.5 : 1.5 | 14.667klm | 24.333bcdefg | 24.667bcdefg | 18efghijklm | 24.333bcdefg | 21.667bcdefghijklm |
| T4 | 0.5 : 2.0 | 18.333defghijklm | 19bcdefghijklm | 19.333bcdefghijklm | 22bcdefghijklm | 19bcdefghijklm | 33.667a |
| T5 | 1.0 : 0.5 | 14m | 23.333bcdefghij | 18.667cdefghijklm | 21bcdefghijklm | 23.333bcdefghij | 18.667cdefghijklm |
| T6 | 1.0 : 1.0 | 18.333defghijklm | 22.333bcdefghijklm | 18.333defghijklm | 22.333bcdefghijklm | 22.333bcdefghijklm | 27abc |
| T7 | 1.0 : 1.5 | 19.667bcdefghijklm | 21.333bcdefghijklm | 17.667efghijklm | 20.333bcdefghijklm | 21.333bcdefghijklm | 25bcdef |
| T8 | 1.0 : 2.0 | 19bcdefghijklm | 23.667bcdefghi | 17.667efghijklm | 18efghijklm | 23.667bcdefghi | 24.333bcdefg |
| T9 | 1.5 : 0.5 | 15jklm | 22.667bcdefghijkl | 19bcdefghijklm | 16.333ghijklm | 22.667bcdefghijkl | 24.667bcdefg |
| T10 | 1.5 : 1.0 | 20bcdefghijklm | 25.333bcde | 20.333bcdefghijklm | 16.667fghijklm | 25.333bcde | 23bcdefghijk |
| T11 | 1.5 : 1.5 | 14.667klm | 23.333bcdefghij | 23.333bcdefghij | 18efghijklm | 23.667bcdefghi | 25.333bcde |
| T12 | 1.5 : 2.0 | 15.333ijklm | 18.667cdefghijklm | 21.667bcdefghijklm | 15.667hijklm | 18.667cdefghijklm | 22.667bcdefghijkl |
| T13 | 2.0 : 0.5 | 15.667hijklm | 21.667bcdefghijklm | 33.667a | 22bcdefghijklm | 27abc | 24bcdefgh |
| T14 | 2.0 : 1.0 | 18efghijklm | 22bcdefghijklm | 18.667cdefghijklm | 21.333bcdefghijklm | 21.667bcdefghijklm | 25.667abcde |
| T15 | 2.0 : 1.5 | 14.333lm | 21.333bcdefghijklm | 27abc | 18.667cdefghijklm | 23bcdefghijk | 27.333ab |
| T16 | 2.0 : 2.0 | 15.333ijklm | 25.667abcde | 25bcdef | 18efghijklm | 26abcde | 26.667abcd |

**Table 2: Number of days taken to callus induction in leaf, apical bud and node under light and dark condition**

**Conclusion**

From the study we can conclude that the best explant for callus induction was leaf and node. In light condition the callus induced in minimum days as compared to dark condition. The auxin and cytokinin both play vital role in callus induction and slightly high amount of kinetin comparative to 2, 4-d was favourable for callogenesis. This study can further be used as a protocol for the development of new plantlets *via* plant tissue culture techniques.

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**References**

Afshari, R., Angoshtari, R. and Kalantari, S. (2011). Effects of light and different plant growth regulators on induction of callus growth in rapeseed (*Brassica napus* L.) genotypes. *Plant Omics* 4:60-67.

Ali, A., Afrasiab, H., Naz, S., Rauf, M. & Iqhbal, J. (2008). An efficient protocol for *in vitro* propagation of carnation (*Dianthus caryophyllus* L.). *Pakistan Journal of Botany*, 40(1): 111-121.

Bhatt, R. N. (1989). Carnation. *In*: *Commercial Flowers.* (Ed.). Bose, T. K. and Yadav, L. P. Kolkata: Naya Prokash, pp. 355-416.

Castillo, AM, Egana, B, Sanz, JM and Cistue L. (1998). Somatic embryogenesis and plant regeneration from barley cultivars grown in Spain. *Plant Cell Reports* 17: 902-906.

Chory, J., Reinecke, D., Sim, S., Washburn, T. and Brenner, M. (1994). A role for cytokinins in desolation in Arabidopsis (mutants have an altered response to cytokinins). *Plant Physiology* 104:339-347.

Dou, H., Niu, G., Gu, M. and Masabni, JG. (2017). Effects of light quality on growth and phytonutrient accumulation of herbs under controlled environments. *Horticulturae* 3:36.

Evans, D. E. Colenman, J. O. D. and Kearns, A. (2003). *Plant Cell Culture*. BIOS Scientific Publishers, London.

George, EF. Sherrington, PD. (1984). Plant propagation by tissue culture: Handbook and directory of common laboratories. Exegetic, Eversley, UK. 709.

George, EF., Hall, MA. and De, Klerk, GJ. (2008). The components of plant tissue culture media I: macro and micro nutrients. *Plant Propagation by Tissue Culture* 65-113.

Gomez, K. A. and Gomez, A. A. (1984). Statistical procedures for agricultural research. 2nd ed. John Wiley and Sons Inc. New York. USA.

Halliday, KJ., Martínez-García, JF. and Josse, EM. (2009). Integration of light and auxin signaling. *Cold Spring Harbor Perspectives Biology* 1: a001586-a001586.

Ikeuchi, M., Sugimoto, K. and Iwase, A. (2013). Plant callus: mechanisms of induction and repression. *Plant Cell* 25: 3159-3173.

Kallak, H. (1997). Effect of genotype explant source and growth regulators on Organogenesis in carnation callus. *Plant Cell, Tissue and Organ Culture* 5(2): 127-135.

Kami, C. Lorrain, S. Hornitschek, P. and Fankhauser, C. (2010). Light-regulated plant growth and development. *Current Topics Developmental Biology* 91: 29-66.

Kanwar, J. K. & Kumar, S. (2010). Influence of growth regulators and explants on shoot regeneration in carnation. *Horticulture Science*, 36: 140-146.

Maitra, S., Ghosh, Roychowdhary and Satya. (2012). Effect of cytokinins and auxins on *in vitro* regeneration of Carnation (*Dianthus caryophyllus* L.) through Callusing. *International Journal of Bio-resource and Stress Management* 3(1): 31-34.

Martinez, García, JL. and Gil, J. (2001). Light regulation of gibberellin biosynthesis and mode of action. *Journal of Plant Growth Regulators* 20: 354-368.

Mohammed, A. M., Moeman, S. Hanafy. and Usama, I. A. (2017). Effect of methyl jasmonate and mannitol application on growth and eugenol content in callus cultures of carnation. *Plant Biotechnology Department, Genetic Engineering and Biotechnology Division* 27(2): 227-240.

Morini, S., Claudio, D. O., Bellocchi, G., and Fisichella, M. (2000). Effect of 2, 4-D and light quality on callus production and differentiation from *in vitro* cultured quince leave. *Plant Cell, Tissue and Organ Culture* 63(1): 47-55.

Muhammad, A., Rauf, S., Din, U. A., Rauf, M. and Afrasiab, H.(2014).High frequency plant regeneration from leaf derived callus of (*Dianthus caryophyllus* L.). *American Journal of Plant Sciences* 5(15): 24-54.

Murashige, T. (1974). Plant propagation through tissue cultures. *Annual Review of Plant Physiology* 25: 135-166.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-479.

Pareek, A., Kantia, A. & Kothari, S.L. (2004). *In-vitro* cloning of ornamental species of *Dianthus*. *Indian Journal of Biotechnology*, 3: 263-266.

Poucher, W.A. (1950).Perfumes. *Cosmetics and Soaps.* Chapman and Hall Ltd. London.

Qadri, Z. A., Neelofar, N. H. Masoodi, D. Ambreena & Wani, A. Muneeb. (2018). *In vitro* callusing of carnation (*Dianthus caryophyllus* L.) cv. Scancia and Indios. *Current Journal of Applied Science and Technology*,27(1): 1-10.

Stavang, JA., Moe, R., Olsen, JE. and Junttila, O. (2007). Differential temperature regulation of GA metabolism in light and darkness in pea. *Journal of Experimental Botany* 58: 3061-3069.

Stobbe, H., Schmitt, U., Eckstein, D. and Dujesiefken, D. (2002). Developmental stages and fine structure of surface callus formed after debarking of living lime trees (*Tillia* sp.). *Annals of Botany* 89(6): 773-782.

Taranto, F., Pasqualone, A., Mangini, G., Tripodi, P. and Miazzi, M. (2017). Polyphenol oxidases in crops: biochemical, physiological and genetic aspects. *International Journal of Molecular Sciences* 18(2): 337.