43. *In vitro* callogenesis, shoot multiplication and flowering in *Dianthus chinensis* L. cv. Pink

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Abstract: Present study revealed *in vitro* callogenesis, shoot multiplication and flowering in *D. chinensis* L. cv. Pink, a cultivated ornamental plant. Both callus proliferation and shoot regeneration induced from nodal explants cultured on MS medium supplemented with 2,4-D1mg/l and BAP1mg/l of which callus was green in colour, non-embryogenic and non-friable. The callogeneic root regeneration observed on MS medium fortified with 2,4-D1mg/l+BAP0.5mg/l+NAA1mg/l and BAP1mg/l+Kin0.5mg/l. The cultures supplemented with gibberellic acid showed lesser number of multiple shoots compared to other cultures. Among the treatments, 2mg/l gibberellic acid supplemented cultures exhibited higher shoot multiplication. Nodal explants grown on ½ MS with BAP1mg/l+Kin0.5mg/l and BAP0.5mg/l showed better rate of shoot regeneration and flower induction within 14 days.

Key words: Callogenesis, shoot multiplication, *in vitro* flowering, growth hormones, gibberellic acid.

Introduction: *Dianthus chinensis* L. cv. Pink belongs to the family Caryophyllaceae which includes 88 genera and 1750 species. It is one among the most important commercial cut flower of the world and enjoys second preference after rose in many countries including India probably due to its excellent keeping quality, wide range of colours, ability to with stand large distance transportation and remarkable ability to rehydrate after continuous shipping (Staby *et al*., 1978). The plant is native to northern China, Korea, Mongolia and Southeastern Russia. It has a pleasant spicy, clove like taste and ideal for decorating or adding to cakes (Lim, 2014).

Dianthus contains a variety of chemical compounds namely anthocyanins, saponins, eugenol, phenylethyl alcohol, benzyl benzoate, methyl salicylate, phosphoric acid, vitamin A, alkaloids and flavones. The whole plant is a bitter tonic herb that stimulates the digestive and urinary systems. It shows anthelmintic, febrifuge, antibacterial, antiphlogistic, antioxidant, anticancer, diaphoretic, diuretic, emmenagogue and haemostatic properties. The species used internally in

the treatment of acute urinary tract infections, urinary stones, constipation and failure to menstruate.

Due to the medicinal and ornamental significance, carnation should be multiply and conserve the germplasm. Since 1950, the researchers have been trying to produce *in vitro* carnation using plant tissue culture technique. The micropropagation refers to growing plant cells, tissues and organ in an artificially prepared nutrient medium under aseptic conditions. Callogenesis is a better method to regenerate numerous plantlets without more expense. It is novel source of genetic variation which contradicts the concept of clonal uniformatiy in micropropagation. To overcome the somaclonal variations, *in vitro* shoot multiplication from a single explant is more relevant. *In vitro* plant regeneration is based on the supplementation of proper concentration and combinations of different plant growth hormones in basic MS medium.

The flowering process is one of the critical events in the life of a plant. This process involves the switch from vegetative stage to reproductive stage of growth and is believed to be regulated by both internal and external factors. A flowering system *in vitro* is considered to be a convenient tool to study specific aspects of flowering, floral initiation, floral organ development and floral senescence. The application of cytokinins, sucrose concentrations, photoperiods and subculture time to promote flowering *in vitro* is well documented in many plant species (Wang *et al*., 2002; Vu *et al*., 2006). This study is part of a programme to investigate the *in vitro* conservation protocol of *D. chinensis* L. cv. Pink and describes callogenesis, shoot multiplication and *in vitro* flowering system. Hence the protocol may open up new gates in the conservation and sustainable exploitation of this very important ornamental plant.

Materials and methods: All *in vitro* culture works were carried out from the tissue culture laboratory of KAHM Unity Women's College, Manjeri, Kerala, India.

Plant material: Healthy plants of *D. chinensis* cv. Pink were collected from ornamental plant nursery, Calicut, Kerala. The young internodes were used as explant for *in vitro* studies.

Surface sterilization of explant: The explant collected and washed with running tap water for 15 minutes to remove the soil particles. The explants were treated with 0.5% Bavistin for 1 hour and 1% Tween 20 for 15 minutes followed by washing with sterile distilled water for 4-5 times. The explants were taken into Laminar Air Flow (LAF) chamber for further processes of surface

sterilization. Within the UV sterilized LAF, the explants treated with 0.1 % $HgCl_2$ for 3-5 minutes followed by washing with sterile distilled water for 4-5 times. Then, 70% ethanol wash for 3 min was also done to reduce the microbial contamination.

Callogenesis from internodes: Inside the LAF, the cut ends of surface sterilized internodes removed using a sterile knife. Explants inoculated on to the callus inducing medium (MS, MS+2,4-D1mg/l+BAP1mg/l, MS+2,4-D0.5mg/l+BAP0.5mg/l, MS+2,4D1mg/l+BAP0.5mg/l+NAA1mg/l and MS+2,4D0.5mg/l+BAP1mg/l+NAA0.5mg/l) using a sterile forceps under an aseptic condition. The culture flasks closed carefully and sealed using cling film. Cultures kept in the growth room at 25±2 ^{0}C with a photoperiod of 8 h daylight and light intensity of 1500 $\mu Em^{-2}S^{-1}$.

Shoot multiplication: For shoot multiplication the internodes cultured on different media compositions including MS+BAP1mg/l+NAA0.5mg/l, MS+BAP1mg/l+Kin0.5mg/l, MS+BAP1mg/l+Kin0.5mg/l+GA0.5mg/l, MS+BAP1mg/l+Kin0.5mg/l+GA1.0mg/l, MS+BAP1mg/l+Kin0.5mg/l+GA1.5mg/l and MS+BAP1mg/l+Kin0.5mg/l+GA2.0mg/l. The cultures kept under same photoperiod and light intensity.

***In vitro* flower induction**: The half strength MS medium used for *in vitro* flower induction. The internodes cultured on media like ½MS, ½MS+BAP1mg/l+Kin0.5mg/l, ½MS+BAP0.5mg/l+Kin1mg/l, ½MS+BAP0.5mg/l and ½MS+BAP1.0mg/l. All cultures incubated under above cultural conditions until the induction of *in vitro* flowers. The observed data statistically analyzed using One-way ANOVA.

Results: Best callus proliferation, callus induction frequency (CIF) and shoot regeneration were showed on MS+2,4-D1mg/l+BAP1mg/l. Callus was green in colour, non-embryogenic and non-friable. The roots induced from the calli which cultured on MS+2,4-D1mg/l+BAP0.5mg/l+NAA1mg/l.

Table 1. Callogenesis and shoot regeneration in *D. chinensis* cv. Pink on different media compositions within 21 days.

Sl. No.	Media composition	CIF (%)	Number of shoots	Number of roots
1	MS	0	0	Nil
2	MS+2,4-D1mg/l+BAP1mg/l	75±2.1	4±0.98	Nil
3	MS+2,4-D0.5mg/l+BAP0.5mg/l	40±1.4	0	Nil
4	MS+2,4D1mg/l+BAP0.5mg/l+NAA1mg/l	70±2.8	2±0.71	Numerous
5	MS+2,4D0.5mg/l+BAP1mg/l+NAA0.5mg/l	60±1.3	0	Nil

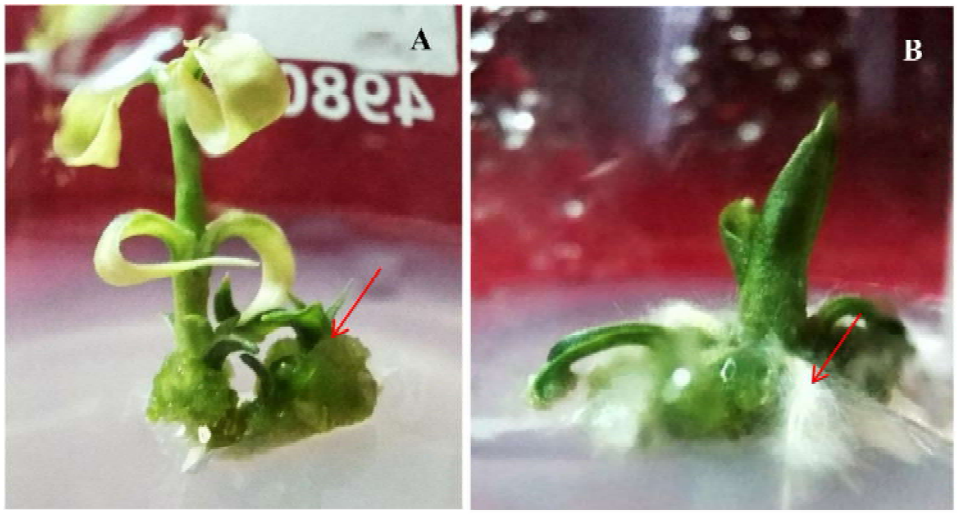

Figure 1. Callogenesis and shoot regeneration in *D. chinensis* cv. Pink on different culture media. A. MS+2,4-D1mg/l+BAP1mg/l and B. MS+2,4-D1mg/l+BAP0.5mg/l+NAA1mg/l.

In the present study, the better number of multiple shoots was observed on MS medium supplemented with BAP1mg/l+NAA0.5mg/l and BAP1mg/l+Kin0.5mg/l+GA2mg/l within 21 days, while the shoot length was highest in MS medium supplemented with BAP1mg/l+Kin0.5mg/l. The multiple shoots grown on MS medium fortified with BAP1mg/l+Kin0.5mg/l also showed large number of elongated roots. Nodal explant cultured on hormone free MS medium showed lesser number of multiple shoots with reduced height. In the study, the callus induction detected in culture grown on MS medium with BAP1mg/l+NAA0.5mg/l. The cultures treated with GA showed lesser number of shoots with reduced length. Among the GA concentrations, 2mg/l GA supplied cultures exhibited higher number of shoots and better shoot length.

Table 2. Shoot multiplication in *D. chinensis* cv. Pink from nodal explants on different media compositions within 21 days.

Sl. No.	Media compositions	Number of shoots	Shoot length (cm)	Number of flowers	Callus induction
1	MS	5	6.6	Nil	Nil
2	MS+BAP1mg/l+NAA0.5mg/l	11	8.9	Nil	Present
3	MS+BAP1mg/l+Kin0.5mg/l	9	15.4	Nil	Nil
4	MS+BAP1mg/l+Kin0.5mg/l+GA0.5mg/l	5	5.2	Nil	Nil
5	MS+BAP1mg/l+Kin0.5mg/l+GA1.0mg/l	7	7.6	Nil	Nil
6	MS+BAP1mg/l+Kin0.5mg/l+GA1.5mg/l	9	6.1	Nil	Nil
7	MS+BAP1mg/l+Kin0.5mg/l+GA2.0mg/l	10	8.7	Nil	Nil

Nodal explants grown on ½ MS+BAP1mg/l+Kin0.5mg/l showed shoot regeneration and flower induction within 14 days. While in *in vitro* internode culture on ½ MS+BAP0.5mg/l, two shoots with two flower buds were noticed within 14 days. On the 21st day, the explant cultured on ½ MS+BAP1.0mg/l showed induction of 7 shoots with 1 flower and 1 flower bud.

Table 3. Flower and shoot induction in *in vitro* culture of *D. chinensis* cv. Pink on different media compositions within 14 days.

Sl. No.	Media compositions	Number of flowers	Number of flower buds	Number of shoots
1	½ MS	0	Nil	4
2	½ MS+BAP1mg/l+Kin0.5mg/l	1	Nil	1
3	½ MS+BAP0.5mg/l+Kin1mg/l	1	1	2
4	½ MS+BAP0.5mg/l	0	2	3
5	½ MS+BAP1.0mg/l	1	1	7

Discussion: *D. chinensis* cv. Pink is one of the most popular ornamental plants worldwide and also among the most studied ones, mainly in cut flower postharvest physiology. The protocols for *in vitro* propagation of this species including nodal segment culture, adventitious shoot induction and flower induction are described in this study. Internode segments were excised from field grown healthy plants and there after cultured on MS medium supplied with different concentrations and combinations of growth regulators for callus induction, root proliferation, shoot regeneration and flower induction.

Figure 2. Shoot multiplication in *D. chinensis* cv. Pink from nodal explants on different media compositions within 21 days. **A**. Hormone free MS medium. **B**. MS+BAP1mg/l+NAA0.5mg/l and **C**. MS+BAP1mg/l+Kin0.5mg/l.

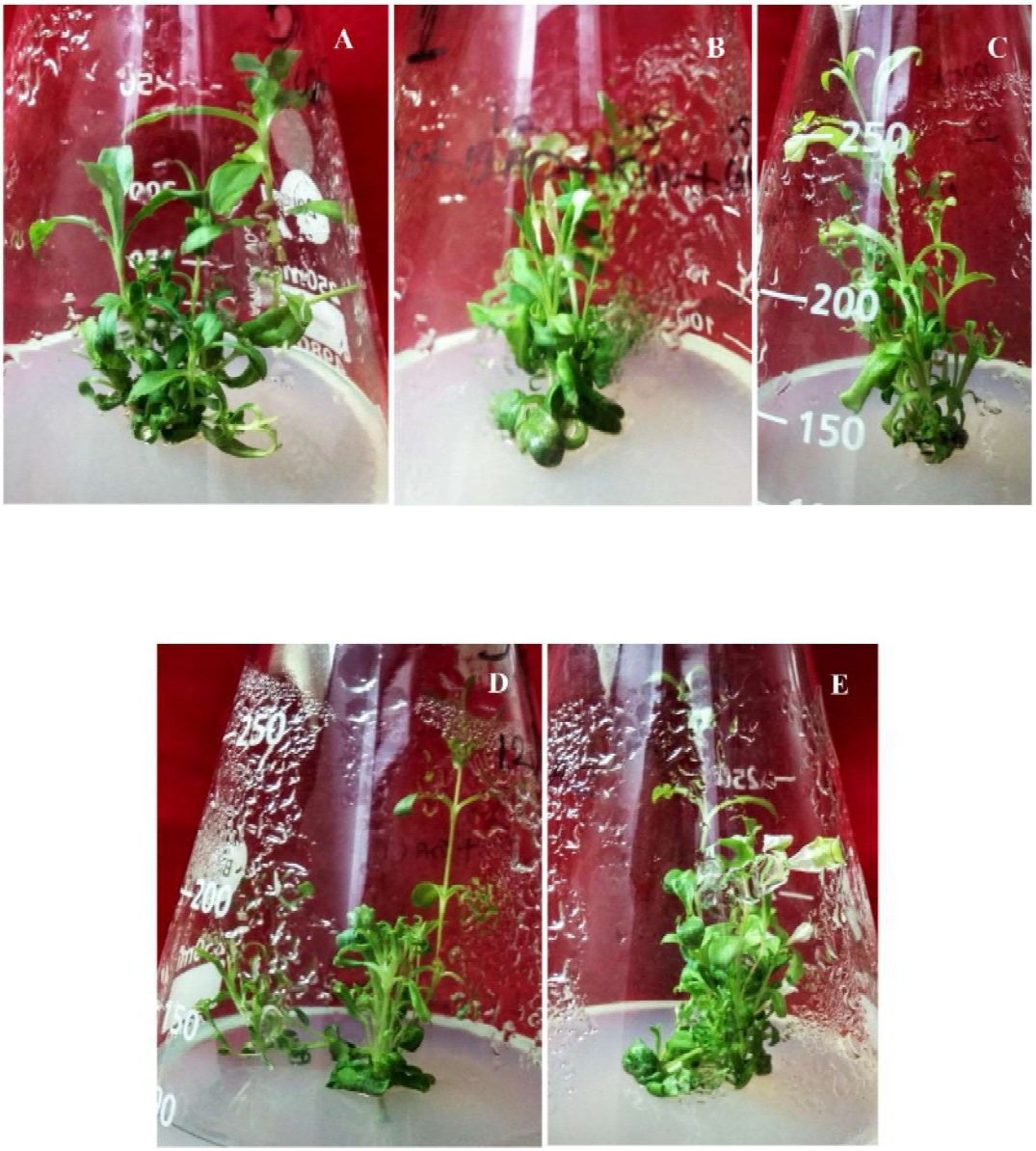

Figure 3. Shoot multiplication in *D. chinensis* cv. Pink from nodal explants on different media compositions (**A**. MS+BAP1mg/l+Kin0.5mg/l. **B**. MS+BAP1mg/l+Kin0.5mg/l+GA0.5mg/l. **C**. MS+BAP1mg/l+Kin0.5mg/l+GA1mg/l. **D**. MS+BAP1mg/l+Kin0.5mg/l+GA1.5mg/l. **E**. MS+BAP1mg/l+Kin0.5mg/l+GA2mg/l.) within 21 days.

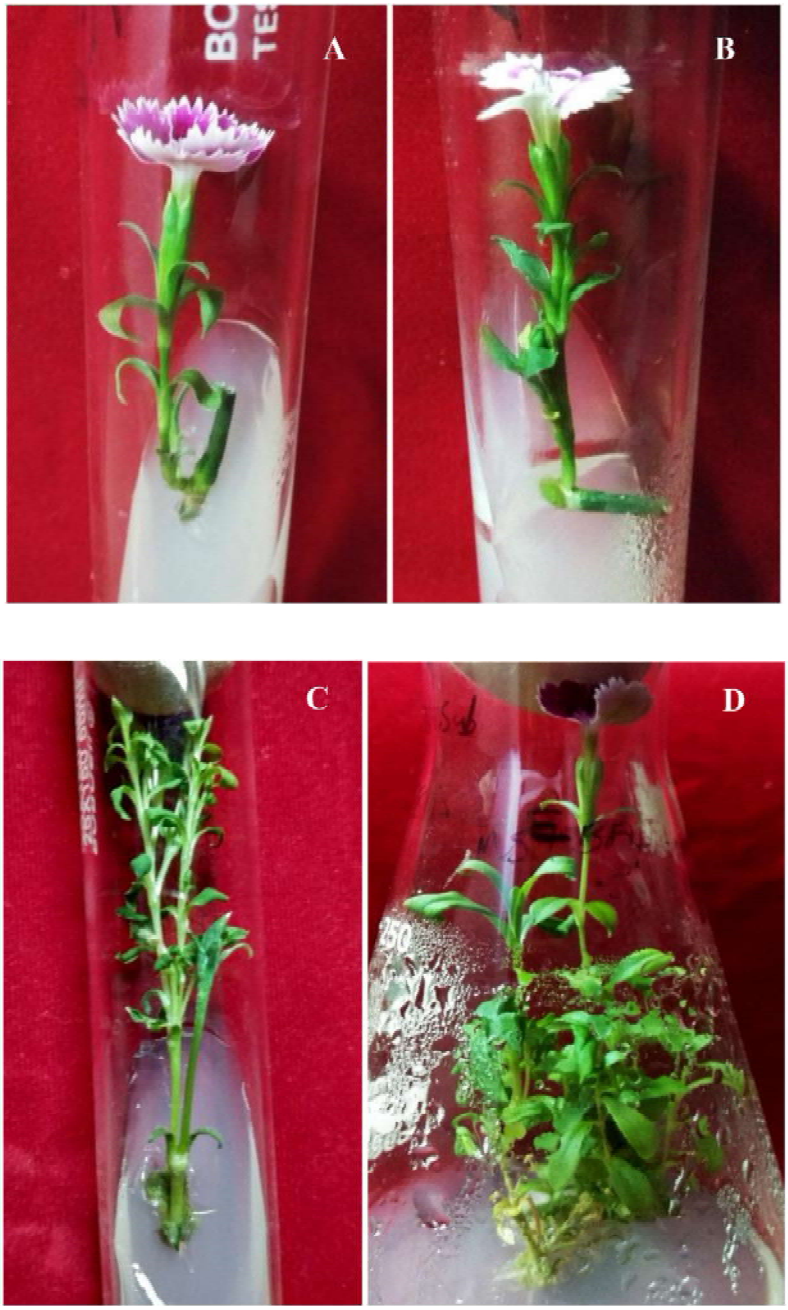

Figure 4. Flower induction in *in vitro* culture of *D. chinensis* cv. Pink on different media compositions within 14 days. **A.** ½MS+BAP1mg/l+Kin0.5mg/l, **B**. ½ MS+BAP0.5mg/l+Kin1mg/l, **C**. ½MS+BAP0.5mg/l and D. ½MS+BAP1.0mg/l.

In the present investigation, the best callus proliferation, CIF and indirect shoot regeneration were observed on MS+2,4-D1mg/l+BAP1mg/l. While MS medium supplied with 2mg/l2,4-D and 0.5mg/lNAA induced better calli in *D. caryophyllus* L. (Thakur & Kanwar, 2017). Similarly callus induction from anthers of *D. chinensis* was observed on MS medium supplied with 1mg/l2,4-D and 0.1mg/lNAA (Nontaswatsri *et al*., 2007). Presence of green coloured, non-embryogenic and non-friable callus was observed in the study. However, the roots induced from the calli cultured on MS+2,4-D1mg/l+BAP0.5mg/l+NAA1mg/l within 21 days. In the work of Shi *et al*. (2013), the hairy roots were developed from calli cultured on MS+BA1.0-3.0mg/l+NAA0.1-0.2mg/l within 15 days.

Axillary shoot proliferation has much potential as supporting technology for micropropagation of ornamental plants. *In vitro* shoot formation from nodal segments is useful in producing type plants and in maintaining genetic stability of the plants. In most of the *in vitro* ornamental plant culture, the cytokinins showed better role in multiple shoot induction. The effectiveness of cytokinin especially BAP in promoting axillary shoot proliferation is well documented in many *Rosa indica* (Hameed *et al*., 2007; Ali *et al*., 2008). In the present study, a successfull protocol for *in vitro* shoot multiplication of *D. chinensis* cv. Pink was standardized using different combinations of BAP, NAA, Kin and GA. The better number of multiple shoots observed on MS medium supplemented with BAP1mg/l+NAA0.5mg/l and BAP1mg/l+Kin0.5mg/l+GA2mg/l within 21 days, while the shoot length was highest in MS medium fortified with BAP1mg/l+Kin0.5mg/l. Similar to this experiment, Kin, 2,4-D, NAA and BAP induced increased number of multiple shoots in *Celosia cristata* L. (Warhade & Badere, 2015). The combination of BAP1mg/l and NAA0.5mg/l in MS medium induced both calli and shootlets in *Dianthus*. In *Arabidopsis*, the endogenous GA reduced regeneration of shoot buds (Ezura & Herberd, 1995). While better shoot multiplication was exhibited in *Lotus corniculatus* L. grown on MS medium supplemented with GA3 (Nikolic *et al*., 2010). Absence of multiple shoots on hormone free MS medium indicates the necessity of plant growth hormones in organogenesis.

Floriculture is a sunrise industry and owing to steady increase in demand of cut flowers. Transition from vegetative state to reproductive development in plants is of great interest to botanist. Flowering is considered to be a complex process regulated by a combination of environmental and genetic factors. *In vitro* flowering has been reported in a number of plant species including *Oscimum basilicum*, *Panax ginseng*, *Withania somnifera* etc (Chang & Hsing,

1980; Sudhakaran & Sivasankari, 2002; Saritha & Naidu, 2007). In many plants, *in vitro* flowering was normally achieved by the application of exogenous hormones to the culture medium. In *D. chinensis*, the propagation is mainly by stem cuttings and seeds. In the present study different concentrations and combinations of BAP and kinetin were used to induce *in vitro* flowering. Half strength MS medium was successfull in the flower induction study. Nodal explants grown on ½MS+BAP1mg/l+Kin0.5mg/l showed shoot regeneration and flower induction within 14 days. However, the explant cultured on ½MS+BAP1.0mg/l induced 7 shoots with 1 flower and 1 flower bud within 21 days. Flowers were large and dark pink in colour. In contrast most efficient flower induction was observed on MS and B5 media supplemented with 0.5mg/lTDZ + 0.1mg/lNAA or 0.5mg/lTDZ + 0.1mg/lNAA in many plants (Wang *et al*., 2002).

Most of the ornamental plants raised through seeds are highly heterozygous and show tremendous variations in growth, habit and yield. While vegetative propagation gives rise to plantlets similar to parent plants. The use of *in vitro* propagation can be overcome these difficulties and can manipulate the phenotypic variation. Recent progress in plant tissue culture techniques has opened new possibilities for improvement of ornamental pot plants (Sharma & Agrawal, 2012).

Conclusion: In the study, a better protocol for *in vitro* callus induction, shoot multiplication and flower induction from inter nodal segments of *D. chinensis* cv. Pink was standardized. MS medium fortified with 2,4-D1mg/l and BAP1mg/l induced higher rate of callus proliferation and shoot regeneration while root induction showed in MS medium supplemented with 2,4-D1mg/l+BAP0.5mg/l+NAA1mg/l and BAP1mg/l+Kin0.5mg/l. *In vitro* shoot multiplication from internodes observed on MS medium with BAP1mg/l+NAA0.5mg/l and BAP1mg/l+Kin0.5mg/l+GA2mg/l within 21 days. The effect of GA on shoot multiplication was significant in the study in which the medium, MS+BAP1.0mg/l+Kin0.5mg/l, supplemented with 2mg/lGA showed best shoot multiplication. The *in vitro* flowering was observed on ½ MS medium supplemented with BAP1mg/l+Kin0.5mg/l, BAP0.5mg/l+Kin1.0mg/l and BAP1.0mg/l within 14 days. The *in vitro* study should be more useful to ameliorate the production of this enchanting cut flower in floriculture field.

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