EFFECT OF SILVER NITRATE AND NICKEL CHLORIDE ON SHOOT MORPHOGENESIS IN *DIANTHUS*

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ABBREVIATIONS

BAP	**:**	**6-Benzyl Aminopurine**
NAA	**:**	**α-Naphthalene Acetic Acid**
cm	**:**	**Centimeter**
2, 4-D	**:**	**2, 4- dichlorophenoxyacetic acid**
DW	**:**	**Distilled water**
Fig.	**:**	**Figure**
gm.	**:**	**gram**
mg/l	**:**	**milligram per liter**
ml	**:**	**milliliter**
mm	**:**	**millimeter**
MS	**:**	**Murashige and Skoog (1962) medium**
S. D.	**:**	**Standard Deviation**
UV	**:**	**Ultraviolet**
W	**:**	**Watt**
w/v	**:**	**Weight by volume**

µmol : Micromole

INTRODUCTION

Dianthus is one of the world's most popular, economic and important cut flowers due to its excellent keeping quality, wide range of forms, ability to withstand long distance transportation and remarkable ability to rehydrate after continuous shipping, perpetual flowering and presence of new single- and multi-colour cultivars and is native of the Mediterranean region.

It belongs to family Caryophyllaceae. The Caryophyllaceae, commonly called the pink family or carnation family, is a family of flowering plants. It is a large family, with 88 genera and some 2,000 species. This cosmopolitan family consists of mostly herbaceous plants and is best represented in temperate climates, with a few species growing on tropical mountains. Some of the more commonly known members include pinks and carnations (*Dianthus*), and Firepink and Campions (*Lychnis* and *Silene*). Many species are grown as ornamental plants, and some species are widespread weeds. Most species grow in the Mediterranean and bordering regions of Europe and Asia.

CLASSIFICATION

Kingdom	:	Vegetabile
Sub-Kingdom	:	Angiospermae
Division	:	Dicotyledonae
Class	:	Polypetalae
Order	:	Caryophyllinae
Family	:	Caryophyllaceae
Genus	:	*Dianthus*

The genus *Dianthus* consists of more than 300 species including the ornamentals such as Carnations (*D. caryophyllus* L.), Pinks (*D. chinensis*) and Sweet Williams (*D. barbatus*). *D. caryophyllus* is a top-selling ornamental crop worldwide. *D. chinensis* is short-lived, perennial garden plants, native to China and northern parts of India, blooming in several colours. Certain varieties of pinks have a strong clove scent, which has made them popular in perfumes for more than 2,000 years. It has a wide range of medicinal uses as well. *D. barbatus* (biennial carnation) is native to the mountains of southern Europe from the Pyrenees east to the Carpathians and the Balkans, with a variety disjunct in north-eastern China, Korea, and south-easternmost Russia. It is traditional used in landscaping and cut flowers. *Dianthus* is also used for culinary purpose. Crystallized petals are used for decorating cakes, while fresh petals

can be used in salads, pies, and sandwiches. Petal bases are removed before using it for culinary purposes because of the better taste.

Dianthus is an herbaceous perennial plant growing upto 80 cm tall. The leaves are glaucous greyish green to blue-green, slender and up to 15 cm long. The flowers are produced singly or in group of five together in a cyme, and sweetly scented. The leaves are linear, lance-shaped and blue-grey or grey-green in colour, with a waxy texture. The fruit is non-fleshy. It is usually a capsule, less frequently a small nut. The original natural flower colour is bright pinkish-purple, but cultivars of other colours, including red, white, yellow and green have been developed through breeding and planted in well-drained soil in a sunny location.

Dianthus cultivation is associated with various problems .The seeds show the high level of heterogeneity in the seed population which acts as a constraint to propagation of agronomic traits and for commercial seed production. Carnation breeders constantly seek new varieties with improved horticultural traits such as disease and pest resistance and long vase-life. The traditional breeding programs for selection of superior clones are tedious and utilizing huge amount of seed results in wastage of the seeds significantly. *In vitro* technology has often been used as a promising avenue to plant improvement for research and practical applications. Plant tissue culture, is of great value, particularly as an alternative to conventional breeding and propagation procedures.

PLANT TISSUE CULTURE MEDIUM

Growth and morphogenesis of plant tissue under *in vitro* conditions are largely governed by the composition of the culture medium. Various culture media formulation proposed consist of several basal components which include mineral nutrients, vitamins, amino acids, growth regulators, sugars, agar and water in various concentrations. Mineral nutrients are the building blocks for the synthesis of organic molecules and also act as catalysts in enzyme reactions. The ions of the dissolved salts also play important role as counter-ion in the transport of ionized molecules by the plant, in the osmotic regulation and in maintaining the electrochemical potential of the plant. The mineral salts in media can be broadly classified into two groups: microelements and macroelements. Microelements including Fe, Cu, Zn, Co, Mo, B, Cl and I are

required in relatively low concentration but their deficiency can have catastrophic effects. Mg, Ca, S, P, K and N are considered as macroelements since they are required in high concentration. Vitamins, amino acids, growth regulators and sugars act as organic supplements in the medium. Agar and Phytagel are added as gelling agents. All these components fulfill the one or more requirements of plant under *in vitro* conditions.

The formulation of the mineral components of the media has been an empirical process since the beginning of tissue culture. Knop (1865) proposed a salt solution which formed the basis for culture medium. Kotte (1922) cultured small root tips of pea and maize which could not survive indefinitely on medium proposed by him. White (1934), Gautheret (1939) , Hildebrandt *et al* (1946) did remarkable work and provided the necessary impetus on developing plant cell and tissue culture medium.

A landmark in media formulation was put forward by Murashige and Skoog (1962) by the development of MS medium. They were able to achieve several fold increase in yield of tobacco callus cultures by addition of leaf extract to the modified White's medium. The concentration of all ingredients in MS medium was increased as compared to the White's medium. Scanning of the plant tissue culture literature leads to the conclusion that MS is the most frequently used medium. Many different media are basically MS with minor changes.

The MS basal medium proposed by Murashige and Skoog (1962) was originally developed for the culture of tobacco pith callus. It may not be optimum for the culture of other plant species, as different plant species have different nutrient requirement during successive stages of growth and development. Nutrient at particular level has the potential to partially substitute the requirement of plant growth regulators in the medium (Preece, 1995). An understanding of optimal nutrient concentration could lead to increased growth and could evoke morphogenesis *in vitro* more efficiently.

Several heavy metals also play important role in the regeneration of plant as microelements. Sliver as silver nitrate and cobalt as cobalt chloride act as ethylene inhibitor. Nickel as nickel chloride proved to be effective in stimulating morphogenesis, shoot regeneration and callus induction. Sliver nitrate ($AgNO_3$) has proved to be a very potent inhibitor of ethylene action and widely used in plant tissue culture. $AgNO_3$ has been employed in plant tissue culture studies for

inhibiting ethylene action because of its water solubility and lack of phytotoxicity at effective concentration (Beyer, 1976).

Considering these facts, the present work has been taken up with the following objectives:

- Standardization of reproducible regeneration protocol for *Dianthus*.
- To study the effects of heavy metals (sliver and nickel) on shoots morphogenesis.
- Acclimatization and hardening of regeneration plantlets.

REVIEWOF LITERATURE

Carnation is one of the most important cut flower crops grown worldwide on a commercial scale, and ranks the top among cut flowers (Duhoky *et al*, 2009). High commercial value and consumer demand for new varieties act as the driving force for carnation breeding (Moyal-Ben Zvi and Vainstein, 2007). This review present a consolidated account of micropropagation and effect of heavy metals on shoot morphogenesis.

Plant tissue culture is the science of growing plant cells, tissues or organs isolated from the mother plant on artificial media. One of the most exciting and important aspects of *in vitro* cell and tissue culture is the capability to regenerate and propagate plants from cultured cells and tissues. A wide range of plants has now been regenerated through this tissue culture technique

has been found particularly useful for propagation of plant species whose production is less as compared to its rate of consumption, so that demand for the same could be meet.

The development of a reliable and efficient *in vitro* regeneration system for a particular species becomes essential to facilitate genetic transformation and a future aim of genetic modification (Hansen and Wright, 1999; Tawfik and Noga, 2001 & 2002). Although it is now possible to dispense with micropropagation for genetic transformation, such methods are currently limited to a small number of plant species. However, micropropagation which tends to introduce certain random genetic changes resulting from somaclonal variation is still sought after. Somaclonal variation tends to negatively affect transgene expression (Birch, 1997; Matzke and Matzke, 1998) minimize the organogenetic potential of the plant and is often accompanied by hyperhydricity (Cassells and Curry, 2001).

Moreover, a suboptimal in vitro environment may exacerbate the occurrence of somaclonal variants and consequently, hamper further microplant development, such as rooting and acclimatization (Cassells and Curry, 2001).Subsequently, omission of a callus stage as a precursor for organogenesis is the preferred strategy for the avoidance of somaclonal variation (Skirvin *et al.,* 1994; Cassells and Curry, 2001; Susek *et al*., 2002).

Explant selection is a very important factor affecting the morphogenic potential of plant system. Adventitious shoot regeneration in carnation has been reported from various explants such as shoot tips (Baker and Philips, 1962; Earle and Langhans, 1975; Jethwani *et al*., 1994) hypocotyls (Petru and Landa, 1974), leaf (Van Altvorst *et al*., 1992, Kantia and Kothari, 2002), stem segments (Van Altvorst and Koehorst, 1995), axillary buds (Van Altvorst and Koehorst, 1995; Ghosh, 1987), petals (Leshem *et al*., 1988; Nugent *et al*., 1991; Van Altvorst *et al.*, 1992; Messeguer *et al*., 1993; Fisher et al., 1993; Sankhla *et al*., 1994; Nakano *et al*., 1994), anthers (Villalobos, 1981), cotyledons (Nontaswatsri and Fukai, 2005) and ovules (Demmink *et al*., 1987).

Messeguer *et al.,* (1992) reportated adventitious shoot regeneration in carnation (*Dianthus caryophyllus L.*) using leaves, basal segments of flower and petals from greenhouse grown *Dianthus* as explant .Explants were cultured on MS medium containing various concentrations of 6-benzylaminopurine (BA) and α-naphthalene acetic acid (NAA). Petals and floral segments exhibited a high morphogenetic potential when cultured on various growth regulator. Basal

segments were the only part of the leaf that generated shoots when cultured on medium supplemented with 0.01 mg/ l NAA and l mg/ l BA. Dark conditions and an agar concentration of 5.5 g/ l significantly improved the percentage of regenerating leaves and the number of shoots per leaf explant. These shoots were normal, with a vegetative shape and could easily be transferred to greenhouse conditions.

Van Altvorst *et al.,* (1992) reported adventitious shoot formation from in vitro leaf explants of *Dianthus caryophyllus*. Leaves were cultured on MS medium with 0.3 mg/l BA and 0.3 mg/l NAA. After 2 weeks, adventitious shoots developed at the bases of the explants. Both BA and NAA in the range 0.1–0.9 mg/l affected the average numbers of shoots per regenerating explant, but not the regeneration percentage. The highest number of adventitious shoots was obtained on medium containing 0.9 mg/l BA and 0.3 mg/l NAA. Adventitious shoot regeneration was compared among leaf, stem and petal explants of *D.caryophyllus L. cv. Scania* on MS medium containing different concentrations of 6-BA and NAA by Nakano *et al.,* (1993).

High frequency regeneration was obtained only from petal explants on the media containing 5 to 10 M BA with or without 5 M NAA. Among the cytokinins tested, N-2-chloro-4-pyridyl-N'-phenylurea and N-1, 2, 3-thiadiazol-5-yl-N'-N'-phenylurea were more effective than BA, kinetin, N6-2-isopentenyl adenine and zeatin on regeneration from petal explants.Miller *et al.,* (1991) reported shoot regeneration in Carnation (*D. caryophyllus*) from axillary bud explants (excised from leaf axils of the young shoots), leaf and stem. The explants cultured on a MS medium supplemented with 15µM BA and 0-5 µM NAA and solidified with Gelrite.The best response in terms of shoot induction was obtained from the axillary bud explants. Shoots were transfer onto a medium solidified with agar to minimize visible signs of vitrification. Miller *et al.,* (1992) studied the shoot morphogenesis from fragmented flower buds, petals explants were cultured on MS medium supplemented with 4-8 µM NAA and 4-8 µM BAP. The yield of shoots from a single flower bud was high, ranging between 70 and 275, for the11 different cultivars tested.

Salehi (2005) reported the shoot regeneration of carnation (*D. caryophyllus* L.) using shoot tip as explant when cultured on MS fortified with 3 mg/l kinetin and 0.5 mg/l NAA or 1 mg/l BA and 1 mg/l NAA. Pareek *et al.,* (2009) reported the micropropogation of *D. caryophyllus, D. barbatus* and *D. chinensis* using shoot tip and nodes as explant. They were cultured on MS

medium supplemented with NAA and BAP. The best response in terms of shoot multiplication was obtained on MS medium supplemented with NAA (0.5mg/l) and BAP (1mg/l).

Danial *et al.,* (2009) reported *in vitro* propagation of carnation (*D. caryophllus L)* by using shoot apical meristems as explant cultured on MS medium supplement with BAP and kinetin (0.0-2.0 mg/l).The results revealed that maximum multiplication of shoot tips occurred in the presence of 1.0 mg/l of either BAP or Kinetin. Duhoky *et al.,* (2008) reported that *in vitro* micropropagation of carnation, Shoot tip and single node explants were obtained from actively growing healthy shoots of two cultivars of carnation *D. caryophyllus* L.: early growth cultivar (E-cultivar) and late growth (L-cultivar) cultured on MS media supplemented with different concentrations of cytokinins and auxins. The highest response percentage was (71.84%) noticed by culturing the explants of E-cultivar. The best results in initiation stage were occurred when the explants of E-cultivar cultured on MS medium supplemented with a high concentration of BA and low concentration of IAA. The highest multiplication rate (2.563 shoots/explant) were obtained by culturing the explants of L-cultivar on MS medium supplemented with 2.0 mg/ l BA+ 0.1 mg/ l IAA, but the highest growth rate were occurred in shoots of L cultivar which was cultured on MS medium contained low concentration of BA or without it, and low concentration of IAA. All the plantlets were rooted easily in multiplication media.

Silver ions in the form of nitrate, such as $AgNO_3$, play a major role in influencing somatic embryogenesis, shoot formation and efficient root formation which are the prerequisites for successful genetic transformation (Bais *et al.,* 1999; Bais *et al.,* 2000a; Bais *et al.,* 2000b; Bais *et al.,* 2001a; Bais *et al.,* 2001b; Bais *et al.,* 2001c). Silver ions are also employed in the form of silver thiosulphate in several tissue culture studies (Eapen and George, 1997).Ethylene is recognized as a ubiquitous plant hormone (Lieberman, 1979; Yang, 1985), which influences growth and development of plants (Abeles, 1973; Yang and Hoffman, 1984; Mattoo and Suttle, 1991). *In vitro* studies have indicated that ethylene can affect callus growth, shoot regeneration and somatic embryogenesis *in vitro* (Purnhauser *et al.,* 1987; Songstad *et al.,* 1988; Roustan *et al.,* 1989; Roustan *et al.,* 1990; Biddington, 1992; Pua and Chi, 1993). Thus, by regulating the production or action of ethylene, the growth and development of some tissue cultures can be

controlled to a certain extent (Beyer, 1976c; Davies, 1987; Purnhauser *et al.,* 1987; Songstad *et al.,* 1988; Chi and Pua, 1989; Bais *et al.,* 2000a; Giridhar *et al.,* 2003).

AgNO3 has been known to inhibit ethylene action (Beyer, 1976a) and cobaltous ions are known to inhibit ethylene synthesis (Lau and Yang, 1976). Increased shoot and root induction using high $AgNO_3$ concentrations were reported for *Dianthus* (Gutiérrez-Miceli *et al*., 2010), *Triticum* and *Nicotiana* (Purnhauser *et al*., 1987) and *Brassica napus* (De Block *et al*., 1989). Gutiérrez-Miceli *et al.,* (2010) reported effect of $AgNO_3$ in stimulating morphogenesis in callus redifferentiation in *D. caryophyllus* by using meristems as explant. Callus clusters were cultured on MS medium containing kinetin (0, 33, and 66 μM), NAA (0, 7.95, and 15.9 μM) and $AgNO_3$ (0, 23.54 and 47.08 μM) for shoot and root induction.

A maximum of 78% calluses with shoots was obtained on medium supplemented with NAA (15.9 μM) and $AgNO_3$ (47.08 μM). The influence of silver nitrate ($AgNO_3$) and cobalt chloride ($CoCl_2$) on shoot multiplication and *in vitro* flowering in *Capsicum frutescens* was investigated by Sharma *et al*., (2008). Both $AgNO_3$ and $CoCl_2$ at a concentration of 30 μM resulted in the maximum tissue response in terms of shoot length and number of shoots after 45 days culturing on MS medium. The morphogenetic response of *Brassica campestris* genotype R500 was reported by Palmer (1992) medium containing NAA, BAP and $AgNO_3$ significantly enhanced both percentage shoot regeneration and number of shoots per cotyledon explant.

The impact of Ni toxicity on the physiology of plants depends on the type of plant species, growth stage, cultivation conditions, Ni concentration and exposure time (Krupa *et al.,* 1993; Xylander and Braune 1994; Marschner 1995; Kabata-Pendias and Pendias 2001; Assuncao et al. 2003) in the soil. The toxic effects of higher concentration of Ni are observed at multiple levels, these include inhibition of mitotic activities (Rao and Sresty 2000), reduction in plant growth (Molas 2002), plant water relation and photosynthesis (Chen *et al.,* 2009), inhibition of enzymatic activities as well as nitrogen metabolism (Gajewska *et al.,* 2009), interference with the uptake of other essential metal ions (Chen *et al.,* 2009), induction of oxidative stress (Chen *et al.,* 2009). All of these alter physiological processes culminating ultimately in reduced fruit yield and quality (Gajewska *et al.,* 2006). Germination of seeds and seedling growth of *Brassica juncea* were significantly reduced by Ni (25, 50, 100 mg dm-3) treatment (Sharma et al. 2008).

Moreover, the roots of Nicotiana *tabacum* became dark brown within 7–10 days of exposure to Ni (0.43 mM) and the growth of plants was severely inhibited (Boominathan and Doran 2002) by Ni and other heavy metals results from general metabolic disorders and immediate inhibition of cell division. However, it is not clear whether Ni enters cell nuclei at high concentrations and if it does, how important is immediate interference of Ni with DNA and nuclear proteins. (Yusuf *et al.,* 2011)

Hyperhydricity which is of common occurrence in plant tissue culture is much more severe in Caryophyllaceae (Mii *et al.,* 1990). In *Dianthus caryophyllus* it has been reported that the hyperhydric shoots showed reduced apical dominance, hypertrophy and defective cell walls (Werker and Leshem, 1987). Hyperhydricity during micropropagation of carnation was reduced to 0% by media modification. Increased concentration of iron and/or magnesium reduced hyperhydricity with 0.7-0.8% agar and increased shoot multiplication (Yadav *et al*., 2003**).**Vitrified shoots regenerated from carnation petals *D. caryophyllus* were recovered by culturing them in a medium containing 3.0 g/l bactopeptone reported by Sato *et al*., (1993).

Jain *et al.,* (2001) reported regeneration of de novo shoots from leaf derived callus of carnation using induction medium contained 2, 4-D and BAP. Shoot buds were formed when the callus was further subculture on 2, 4-D- and BAP-containing medium, or MS medium without any growth regulators. The shoots so formed were hyperhydric, bushy in appearance with reduced stem length and watery leaves. The normal conformation of shoots was restored by culturing the hyperhydric shoots onto medium supplemented with GA3 and bactopeptone. The recovered shoots were rooted on MS medium added with NAA (1 mg/l) or IBA (2 mg/l).

MATERIALS AND METHODS

PLANT MATERIAL

Dianthus, an important horticultural plant has been taken up for the present study. The seeds of *Dianthus* (L.) were procured from Namdhari Seed Agency, Bengaluru (India).

SEED VIABILITY TEST

Dormancy of seeds is a major factor affecting its germination. Hence the seeds obtained were subjected to a seed viability test to check the percentage of viable seed in the provided seed-lot. 10 seeds were placed on a wet filter paper which was placed over a thin wet layer of cotton in a Petri plate. This set up was kept in a dark chamber for 48 hours. The seed viability percentage was calculated by the formula:

Seed Viability Percentage = **No. of seeds germinated / Total no. of seeds kept for germination × 100**

EXPLANTS TAKEN FROM ASEPTICALLY GROWN SEEDLINGS

Various explants from aseptically germinated seeds were taken for the present study.

Explants to be taken from aseptically grown seedlings:

1. Hypocotyl (0.5-0.7 cm)
2. Cotyledon (whole expanded lamina along with petiole 1-2 mm)
3. Cotyledonary node with shoot tip and part of hypocotyls (1-2 mm) after separating both the cotyledons

Explants to be taken from aseptically grown mature plants:

1. Leaf
2. Nodes
3. Internodes
4. Shoot tips

EQUIPMENTS AND APPARATUS

The following equipments and apparatus were used during the study.

1. Glassware (Borosil)

i)	Erlenmeyer conical flasks 1000 ml capacity	:	100-250 ml capacity
ii)	Test tubes	:	25 × 150 mm
iii)	Beakers	:	100-1000 ml capacity
iv)	Measuring Cylinders	:	10-1000ml capacity
v)	Pipettes	:	0.1-10 ml capacity
vi)	Petri plates	:	100 × 50 mm diameter
vii)	Reagent bottles	:	100-150 ml capacity

2. Electronic balance : Shimadzu, 0.001-310 g weighing capacity
3. Autoclave : Life, India
4. Laminar Air Flow cabinet : Deepak Meditech Pvt. Ltd., India
5. pH meter : Elico, India
6. Refrigerator : Samsung
7. Miscellaneous : Forceps(different sizes), scalpels, spatula, spirit lamp and aluminium foil.

STERILIZATION AND PREPARATION OF EXPLANTS

The seeds were washed with 20% (v/v) Extran followed by four rinses in distilled water. Seeds were surface sterilized in 0.1% (w/v) mercuric chloride ($HgCl_2$) aqueous solution for 3 minutes with continuous stirring and washed thoroughly with three changes of sterile distilled water to remove any traces of $HgCl_2$ in the Laminar Air Flow cabinet. The seeds were then germinated on half strength MS media with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar, pH adjusted to 5.8. Explants as stated above were excised from respective donor plants. These explants were inoculated on MS medium supplemented with varying concentrations of cytokinins added singly or in combination with different auxins to standardize the protocol for *Dianthus* micropropagation.

BASAL MEDIUM PREPARATION

Basal medium used in this study was MS (Murashige and Skoog, 1962) medium. The constituents and composition of this medium is given in Table 1. Stock solutions of various organic and inorganic nutrients were prepared at various concentrations by dissolving their weighed amounts in distilled water. Each component was added according to the list of ingredients. Each ingredient was completely dissolved before adding the next.

The stock solutions of growth regulators were also prepared. For preparing the stock solutions of the growth regulators; auxin was first dissolved in few drops of absolute alcohol and the final volume was made up by adding distilled water (20 mg growth substance was dissolved in 100 ml distilled water).These stock solutions were stored in different reagent bottles and placed in the refrigerator. They were used within a week of their preparation.

The medium was prepared in 1000 ml measuring cylinder containing distilled water and all the mineral nutrients and sugar (dissolved in small volume of distilled water) was added. The final volume was made up by adding distilled water. The medium was poured in different beakers and growth regulators added and solidified with agar (0.8%). Similarly medium were prepared which contain induction medium with the different concentration of $AgNO_3$ and $NiCl_2$ were added exogenously into separate beakers .The pH of the medium was adjusted to 5.8 by adding 0.1N HCl and 1N NaOH. The medium was finally dispensed into Erlenmeyer 100 ml flasks or culture tubes. Each flask contained approximately 30 ml of medium and the culture tubes contained 20 ml medium. They were marked for their medium composition. The mouth of the culture vessels was plugged with non-absorbent cotton and autoclaved at 121^0C and 1.06-kg/cm^2 (105 kPa) pressure for 20 minutes.

ASEPTIC MANIPULATION

All precautions were taken for aseptic operation. All the culture vessels, measuring cylinders and bottles etc. were cleaned and rinsed with distilled water prior to use. The Petri plates and accessories were wrapped and autoclaved along with the medium. The measured volume of distilled water required for seed sterilization was also autoclaved in plugged conical flasks.

Table 1: Composition of MS (Murashige and Skoog, 1962) basal medium

Sucrose : 3% (w/v)

S. No.	Nutrient	Weight of nutrient in stock solution (mg)	Total volume of stock solution (ml)	Concentration of nutrient in stock solution as compared to the final medium	Volume of stock solution in 1 l of final medium (ml)	Final concentration in medium (mg/ l)	Final concentration in medium (mM)
A	NH_4NO_3	33000	400	50 X	20	1650	20.6
B	KNO_3	38000	400	50 X	20	1900	18.8
C	KH_2PO_4	6800	200	200 X	5	170	1.25
	KI	33.2				0.83	5×10^{-3}
	H_3BO_3	248				6.2	0.1
D	$Na_2MoO_{4.2}H_2O$	50	200	1000 X	1	0.25	1.03×10^{-3}
	$CoCl_2.6H_20$	5				0.025	0.11×10^{-3}
E	$CaCl_2.2H_2O$	17600	200	200 X	5	440	2.99
F	$MgSO_4.7H_2O$	14800	200	200 X	5	370	1.5
	$MnSO_4.H_20$	676				16.9	0.1
	$ZnSO_4.7H_2O$	344				8.6	29.9×10^{-3}
G	$CuSO_4.5H_2O$	5	200	1000 X	1	0.025	0.1×10^{-3}
H	$Fe_2(SO_4)_3.7H_20$	1114	200	200 X	5	27.8	0.1
	Na_2EDTA	1490				37.3	0.1
I	Thiamine HCl	10	100	1000 X	1	0.1	0.3×10^{-3}
	Pyridoxine HCl	50				0.5	2.43×10^{-3}
	Nicotinic Acid	50				0.5	4.06×10^{-3}
	Glycine	200				2	26.64×10^{-3}
J	Myo-inositol	2000	200	100 X	10	100	0.56

Agar : 0.8-1% (w/v)

pH : 5.

The transfer chamber and culture room were also kept clean. *In vitro* transfer of explant was carried out in the Laminar Air Flow cabinet. The cabinet platform was swabbed with absolute alcohol along with the autoclaved flasks containing the medium and distilled water. Autoclaved and wrapped Petri plates, scalpels and forceps were placed in the cabinet. Spirit lamp, spirit column and cotton were also arranged inside the cabinet. The cabinet was then irradiated by UV rays for 35-40 minutes by UV tubes. After irradiation, the cabinet door was opened and in a continuous airflow from the Laminar HEPA filters the transfer process was carried out. Hands were properly wiped with spirit and the accessories were heated in the spirit lamp flame until red-hot. Special care was taken to avoid the tissue being touched by hot forceps/scalpels as it reduces viability of the tissue. All the glassware and instruments wiped with spirit were kept away from the burning spirit lamp. The rim of the Petri plates was also heated properly in the flame before inoculation.

INDUCTION OF SHOOT BUDS

Desired explants were cultured on MS medium supplement of various levels of plant growth regulators.

SUB-CULTURING PROCEDURE

The primary cultures were transferred to fresh medium after 4-5 weeks of culture incubation .The shoot bud induced from the explant were sectored into groups each having 3-4 of them. These clusters were then transferred on elongation medium cytokinins with various concentration of auxin.

ROOTING OF EXPLANTS

The elongated shoots were transferred on medium containing various concentrations of auxins for proper root system.

EFFECT OF HEAVY METALS

The explants (shoot tip and node) were excised from 20-25 days old aseptically grown seedling. The explants inoculated on MS medium supplemented with various concentrations of BAP and in combination with NAA. Effect of heavy metals ($AgNO_3$ and $NiCl_2$) was examined on shoot morphogenesis. The explants were cultured into two different types of media:

i) The induction medium (0.5mg/l BAP and 0.5mg/l NAA)

ii) Induction medium with various concentration of heavy metals ($AgNO_3$ and $NiCl_2$)

INCUBATION

The culture flask was incubated in a growth chamber. The growth chamber was equipped with air-conditioner and temperature controller to maintain the temperature of the culture room at 26±1°C. A photoperiod of 16 hours light and 8 hours dark was maintained with the help of a timer and the light intensity was facilitated with fluorescent tubes.

OBSERVATION

The cultures were observed weekly for the formation of shoot or shoot initials. The origin of the shoot and the general state of the node and shoot tip explants was recorded. The numbers of shoot formed on each the node and shoot tip was counted. Additional records on the appearance of the shoot (colour and size) were taken.

STATISTICAL ANALYSIS

The observations recorded for the various experiments were subjected to following statistical analysis:

Average: The average (mean) was calculated by dividing the sum values of observations for a particular treatment by the total number of observations for that treatment:

$$\textbf{AVERAGE} = \sum \mathbf{n} / \mathbf{N}$$

Where

$\sum n$ = summation of values of observations for a treatment

N = total number of observations for that treatment

Standard Deviation: This is a measure of dispersion which was calculated by squaring the deviation of each observation from the mean, adding the squares, dividing by the number of observation and extracting the square root according to:

$$\sigma = \pm\sqrt{(\sum d^2 / N)}$$

Where

σ = standard deviation

$\sum d^2$ = summation of squares of deviation of each observation from the mean

N = number of observations

OBSERVATIONS AND RESULTS

SEED VIABILITY TEST

The viability percentage of *D. caryophyllus, D. barbatus* and *D. chinensis* was found to be 90%, 70% and 0% respectively (Fig. 2a). Hence seeds of *D. caryophyllus* were considered for further studies.

SEED GERMINATION

Seeds of *D.caryophyllus* were procured from Namdhari Seed Agency, Bengaluru. After proper sterilization, the seeds were inoculated aseptically on half strength MS media (Fig.2b). The first visible germination of seeds was observed after 4 days of inoculation. Seedling explants were taken from 12 days old seedlings and mature plant explants were taken from 25 days old seedlings.

EXPLANT CULTURE

Explants taken from aseptically grown seedlings

Effect of various concentrations of cytokinins (BAP) alone or in combination with auxins (NAA) was studied in hypocotyl, cotyledon and cotyledonary node.

EFFECTS OF CYTOKININ

Hypocotyl

Swelling was induced in hypocotyl when cultured on MS medium supplemented with BAP (0.2 -5 mg/l) after 2 week of the culture. The explant turned Yellowish on MS medium supplemented with BAP (0.2mg/l).Even higher levels of BAP in the medium could not evoke shoot regeneration. Hence they were discarded and were not used for further studies.

Cotyledon

Cotyledons were inoculated with adaxial surface in contact with medium. The explants were cultured on various concentration of BAP (0.2, 0.5, 1, 2, 3 and 5 mg/l). After incubation of 4 weeks cotyledon explants did not show any morphogenic response on medium supplemented with various concentration of BAP. Hence they were discarded and were not used as explant for further studies.

Cotyledonary node

Shoot regeneration was not achieved from Cotyledonary nodal explants when cultured on medium with various concentration of BAP. Hence they were not taken for further studies.

Explants taken from aseptically grown mature plants

Effect of various concentrations of cytokinins (BAP) alone or in combination with auxins (NAA) was studied in leaf, nodes and shoot tips.

EFFECTS OF CYTOKININS

Leaf

Leaf explants were inoculated with adaxial surface in contact with medium. Swelling of the explants was observed after 1week of culture on MS medium supplemented with various concentrations of BAP (0.2-5 mg/l).But the explants turned yellowish after incubation of 4 weeks and did not show any sign of shoot regeneration.

Nodes

Nodal explants were inoculated on MS medium supplemented with various concentration of BAP (0.2-5 mg/l). Formation of 2-3 shoots accompanied with little browing at the base of the explants was observed on BAP (0.2 mg/l).On increasing the level of BAP upto 0.5mg/l, increasing in the number of shoot buds was observed. A maximum of 4-5 shoot buds regenerated from the explant. On further raising the levels of BAP (1-5mg/l), shoots were formed but they were highly vitrified. The nodal explants showed better response than other explants in terms of quality and number of shoot bud induced per explant. Maximum number of shoots was regenerated on MS medium supplemented with 0.5 mg/l BAP (Fig. 3).

Shoot tip

Shoot tip explants were cultured on MS medium fortified with various concentration of BAP. Shoots with brownish margins was observed on medium supplemented with (0.2 mg/l) BAP. The explants were cultured on MS medium supplemented with BAP (0.5mg/l) formation of 3-4 shoot buds with slight vitrification (Fig4). On raising the levels of BAP (1-3mg/l), highly vitrified abnormal shoots were formed. On further increasing the concentration of BAP to 5mg/l abnormal shoots with enlarged leaves were formed.

EFFECTS OF CYTOKININ AND AUXIN (Table 2)

Nodes

Nodal explants were culture on the medium supplemented with BAP (0.2, 0.5, 1mg/l) in combination of various concentration of NAA (0.1, 0.2, 0.5 and1mg/l). Lower level of BAP (0.2mg/l) in combination with NAA (0.5mg/l) formed 6-7 vitrified shoot buds after 4 week of

incubation. BAP (0.5mg/l) in combination with NAA (0.5mg/l) induced a maximum of 11-12 shoot buds formation per explant. On further raising the concentration of BAP (1mg/l) in combination of NAA (0.2mg/l), highly vitrified multiple shoots were formed. No profound effect of BAP (1mg/l) in combination of NAA (0.5-1mg/l) was observed. Best response was observed on medium supplemented with of BAP (0.5mg/l) in combination with NAA (0.5mg/l) on which 11-12 shoots were formed (Fig 5b).

Shoot tip

Shoot tip explants were cultured on the MS medium supplemented with BAP (0.2, 0.5, 1mg/l) and various concentration of NAA (0.1, 0.2, 0.5, 1mg/l). Higher level of NAA (0.5 mg/l) in combination with BAP regenerated shoots, but on lower levels of NAA (0.1- 0.2), highly vitrified, shoots were formed. Best response was observed on MS supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA (Fig. 5e)

SUBCULTURE

The primary shoots obtained from the culture of explants on MS medium augmented with cytokinins and auxins were further subcultured in a clump of 1-3 shoots on proliferation medium which consisted medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA resulted in sprouting of new shoot buds from the axil. Subculturing of the shoots was done after every 3-4 weeks.

Table 1. Formation of shoot buds from mature seedling explant of *Dianthus caryophyllus* cultured on MS supplemented with BAP

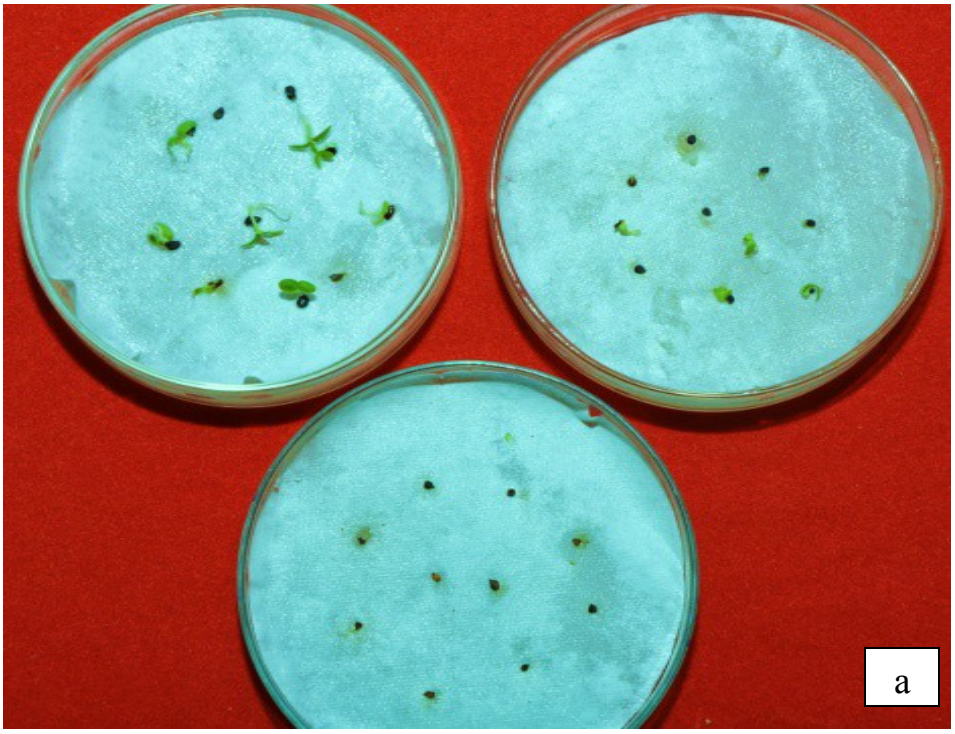
BAP(mg/l)	Mean no.of shoot ±S.D.	
	Node	Shoot tip
0.2	2.8±0.6	3.2±0.9
0.5	4.9±1.5	3.9±1.1
1	4.3±0.9	3.5±0.8
2	3.8±1.2	2.7±1.2
3	3.5±0.9	2.5±1.1
5	2.9±1.1	2.3±0.9

Figure 2

Seed germination in aseptic conditions to test for its viability and in aseptic conditions to initiate explant culture *in vitro* conditions

(a) Seed Viability Test

(b) Seed Germination


a


b

Figure 3

Morphogenic response of nodal explants of *Dianthus caryophyllus* cultured on MS medium supplemented with

(a) 0.2 mg/l BAP

(b) 0.5 mg/l BAP

(c) 1 mg/l BAP

(d) 2 mg/l BAP

(e) 3 mg/l BAP

(f) 5 mg/l BAP

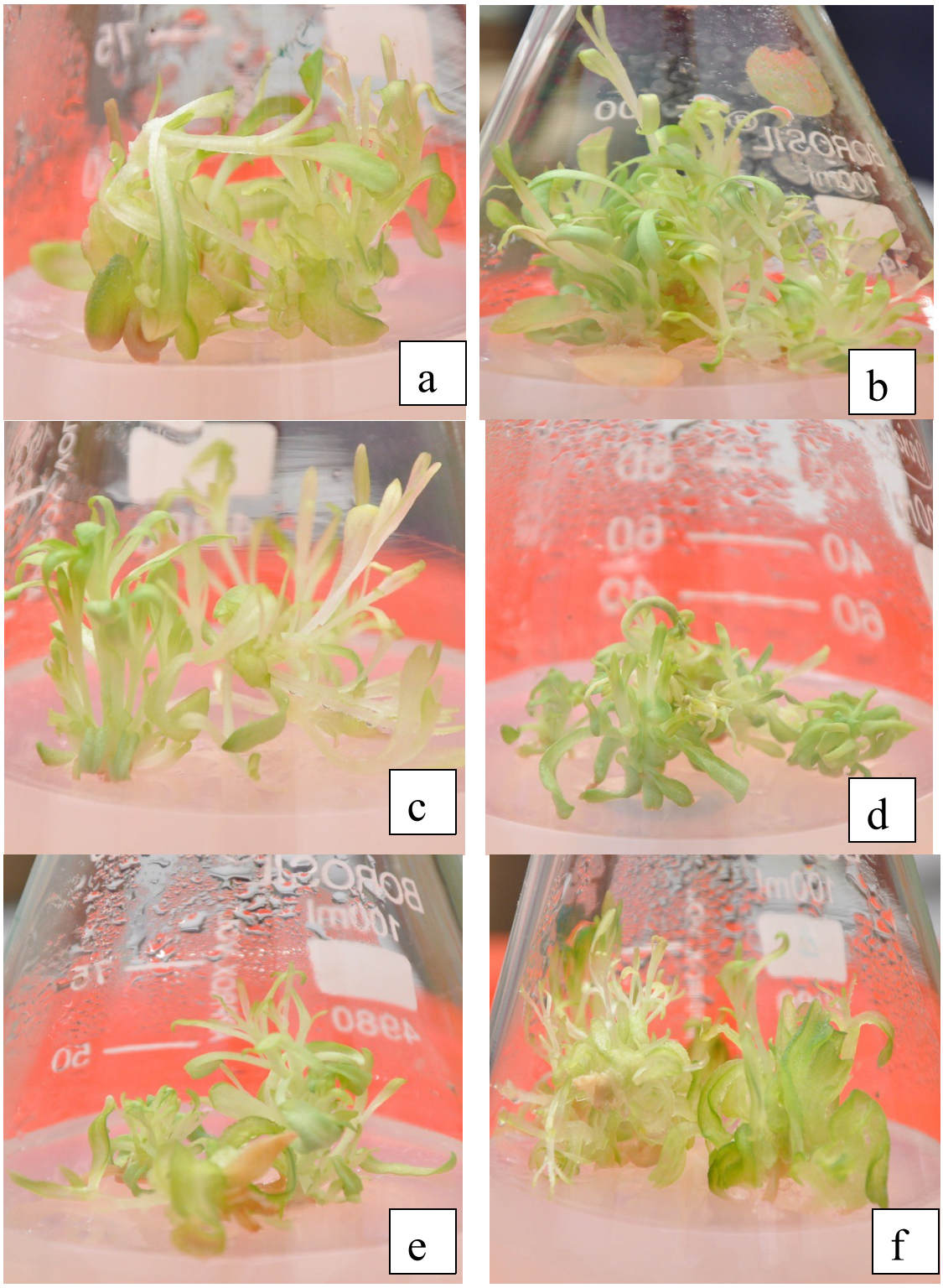

a
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Figure 4

Morphogenic response of shoot tip explants of *Dianthus caryophyllus* cultured on MS medium supplemented with

(a) 0.2 mg/l BAP

(b) 0.5 mg/l BAP

(c) 1 mg/l BAP

(d) 2 mg/l BAP

(e) 3 mg/l BAP

(f) 5 mg/l BAP

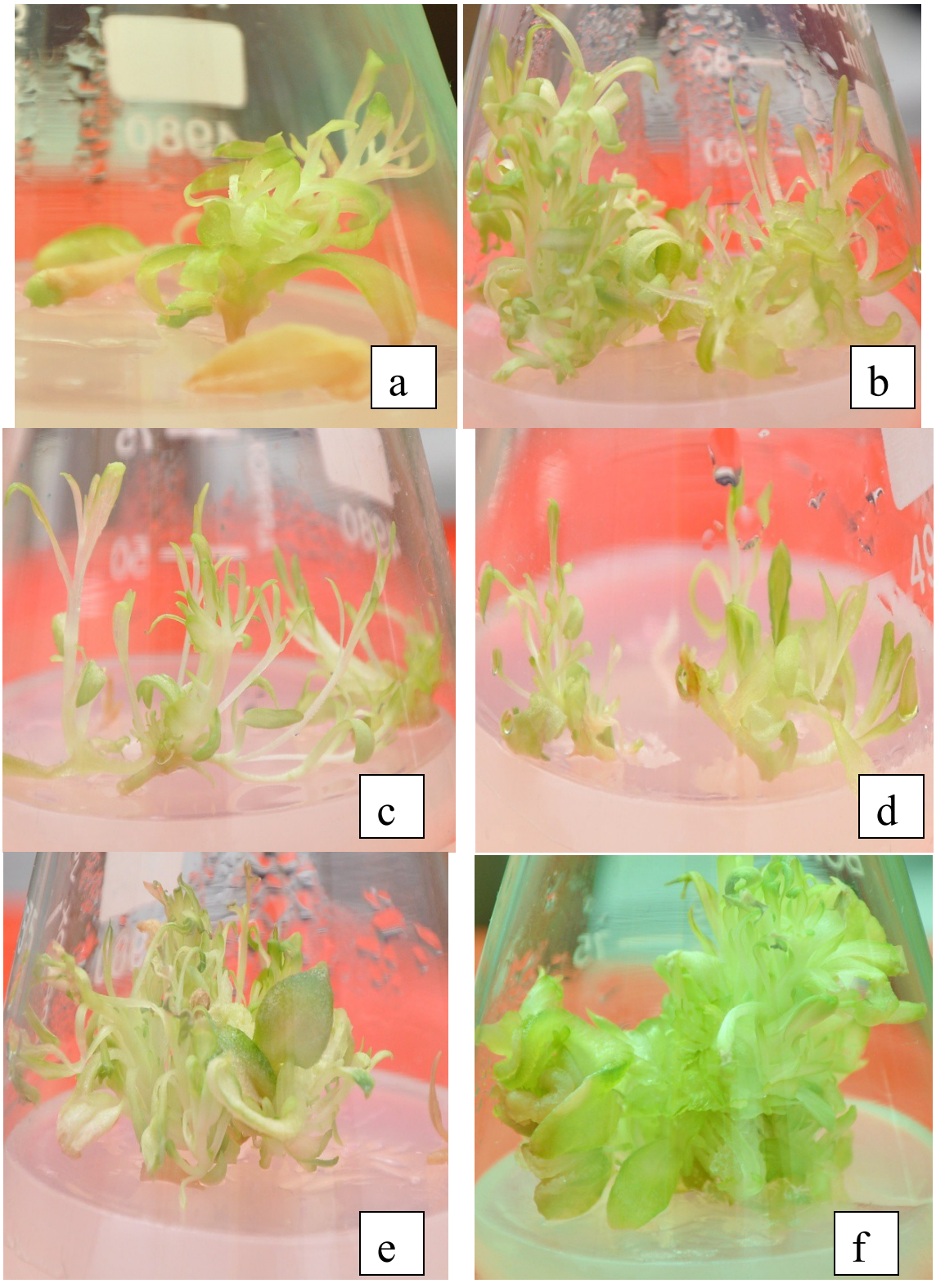
a
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Table 2. Formation of shoot buds from mature seedling explant of *Dianthus caryophyllus* cultured on MS supplemented with BAP in combination with NAA

PGR (mg/l)		Mean no. of shoot ±S.D.	
BAP	NAA	Node	Shoot tip
0.2	0	2.9± 0.6	3.3±0.8
	0.1	6.3±0.8	5.5±0.9
	0.2	7.2±0.7	6.9±1.1
0.5	0.1	6.5±0.9	5.3±0.9
	0.2	8.5±0.7	7.2±0.8
	0.5	11.5±0.6	9.2±0.7
1	0.1	4.8±0.8	3.9±0.8
	0.2	3.5±0.7	3.3±0.9
	0.5	3.2±0.8	2.9±0.7

Figure 5

(a-c) Morphogenic response of nodal explants on MS medium supplemented with BAP in combination with NAA

(a) MS+ BAP (0.2 mg/l) +NAA (0.2 mg/l)

(b) MS+ BAP (0.5 mg/l) +NAA (0.5 mg/l)

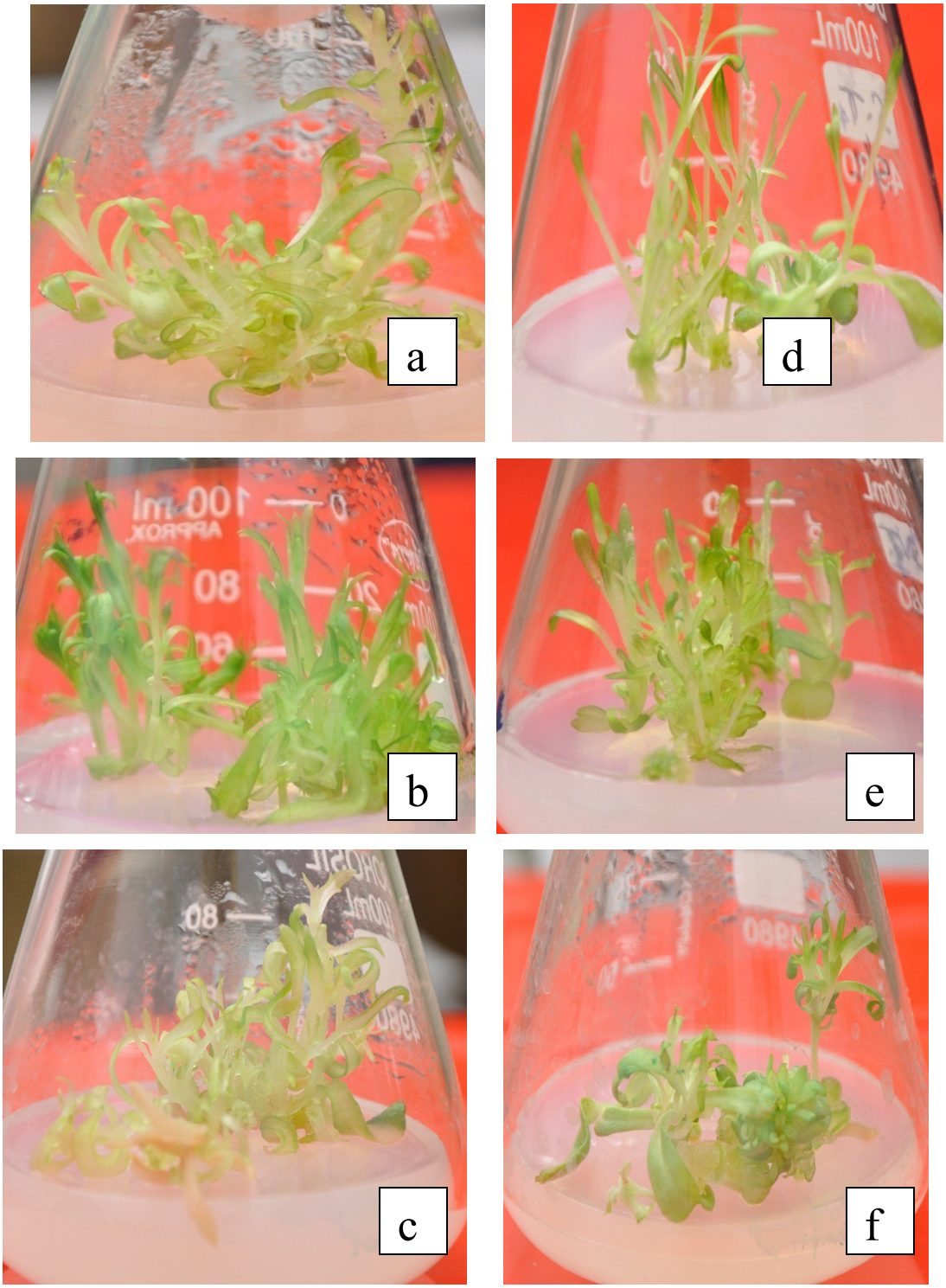
(c) MS+ BAP (1 mg/l) +NAA (0.5 mg/l)

(d-f) Morphogenic response of shoot tip explants on MS medium supplemented with BAP in combination with NAA

(d) MS+ BAP (0.2 mg/l) +NAA (0.2 mg/l)

(e) MS+ BAP (0.5 mg/l) +NAA (0.5 mg/l)

(f) MS+ BAP (1 mg/l) +NAA (0.5 mg/l)



EFFECT OF HEAVY METALS ($AgNO_3$ &$NiCl_2$)

The effect of heavy metals (which are not constituent of MS media) was also investigated on the induction of shoot bud from nodes and shoot tips of *Dianthus* and the subsequent proliferation of primary shoots to form multiple shoots per explant.

B.1 Silver as Silver Nitrate ($AgNO_3$) (Table 3)

Node and shoot tip explants were cultured on MS medium supplemented with BAP (0.5 mg/l), NAA (0.5 mg/l) and different concentrations of $AgNO_3$ (0, 0.1, 0.5, 1 and 1.5 mg/l). Incorporation of $AgNO_3$ in to the medium induced positive response. The explants showed swelling during the first week of culture. The initiation of shoot bud induction occurred at the end of the second week of the culture. It was observed that the number of shoots increased with the increase in the concentration of $AgNO_3$ from 0.1 to 1mg/l. Beyond this level the shoots showed no significant increase in length but turned more hyperhydric. Best response in terms of total number of shoots as well as hyperhydricity per explant was achieved on medium containing 1 mg/l $AgNO_3$ (Fig.6, 7). Results achieved from $AgNO_3$ supplemented medium were better in terms of shoot regeneration and elongation of the shoots per explant than on control, lacking $AgNO_3$.

B.2 Nickel as Nickel Chloride ($NiCl_2$) (Table 4)

Node and shoot tip explants were cultured on MS medium supplemented with 0.5 mg/l BAP, NAA (0.5 mg/l) and different concentrations of $NiCl_2$ (0, 0.1, 0.5, 1 and 1.5 mg/l). The explants showed swelling during the first week of culture. The initiation of shoot bud induction occurred at the end of the second week of the culture. It was observed that the number of shoots decreased with the increase in the concentration of $NiCl_2$. The shoots that even developed up to 0.5 mg/l of $NiCl_2$ were hyperhydric and had abnormally large leaves (Fig. 8, 9). When the concentration of nickel chloride was raised to 1 and 1.5 mg/l $NiCl_2$, the abnormal condition of the shoots also increased.

Table3. Effect of silver nitrate on shoot bud formation from nodal and shoot tips of *Dianthus caryophyllus* cultured on MS supplemented with BAP (0.5mg/l) and NAA (0.5mg/l)

$AgNO_3$(mg/l)	Mean no. of shoot bud ±S.D.	
	Node	Shoot tip
0	4.8±0.9	3.8±1.1
0.1	5.2±1.6	4.1±0.7
0.5	5.5±1.6	4.4±0.6
1	5.9±1.2	4.9±0.9
1.5	4.4±0.9	3.5±0.8

Table4. Effect of nickel chloride on shoot bud formation from nodal and shoot tips of *Dianthus caryophyllus* cultured on MS supplemented with BAP (0.5mg/l) and NAA (0.5mg/l)

$NiCl_2$ (mg/l)	Mean no. of shoot bud ±S.D.	
	Node	Shoot tip
0	4.8±0.9	3.8±1.1
0.1	3.4±0.8	3.1±0.8
0.5	3.0±0.7	2.8±0.7
1	2.7±0.7	2.5±0.6
1.5	2.5±0.8	2.2±0.6

Figure 6

Effect of silver nitrate on shoot bud formation from node of *Dianthus caryophyllus* cultured on MS supplemented with BAP (0.5 mg/l) + NAA (0.5 mg/l)

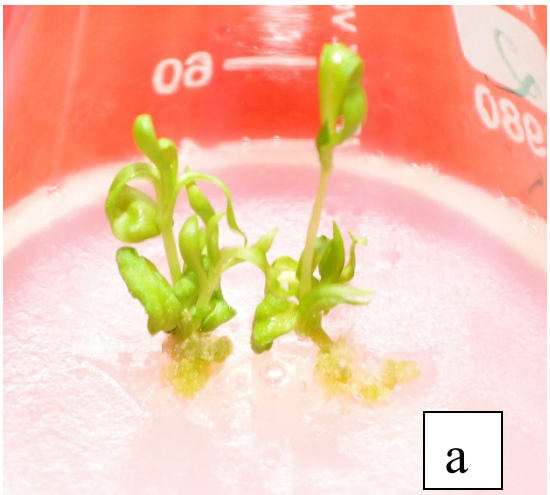
(a) **Without $AgNO_3$**

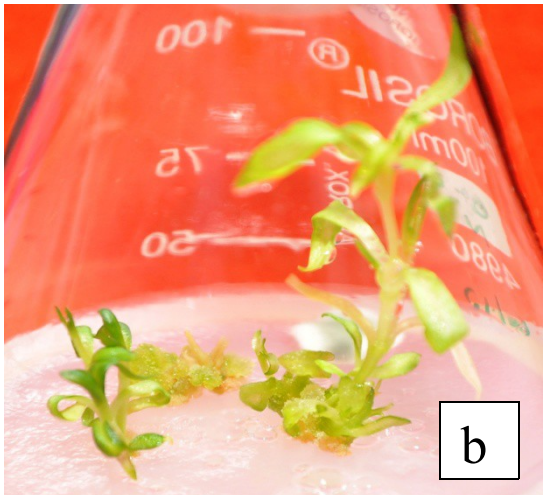
(b) **0.1 mg/l $AgNO_3$**

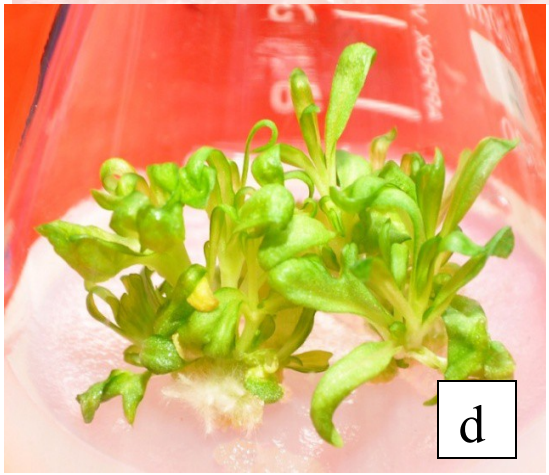
(c) **0.5 mg/l $AgNO_3$**

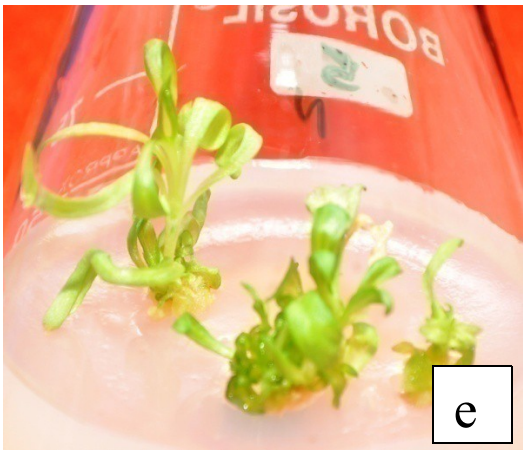
(d) **1 mg/l $AgNO_3$**

(e) **1.5 mg/l $AgNO_3$**









e

Figure 7

Effect of silver nitrate on shoot bud formation from shoot tip of *Dianthus caryophyllus* cultured on MS supplemented with BAP (0.5 mg/l) + NAA (0.5 mg/l)

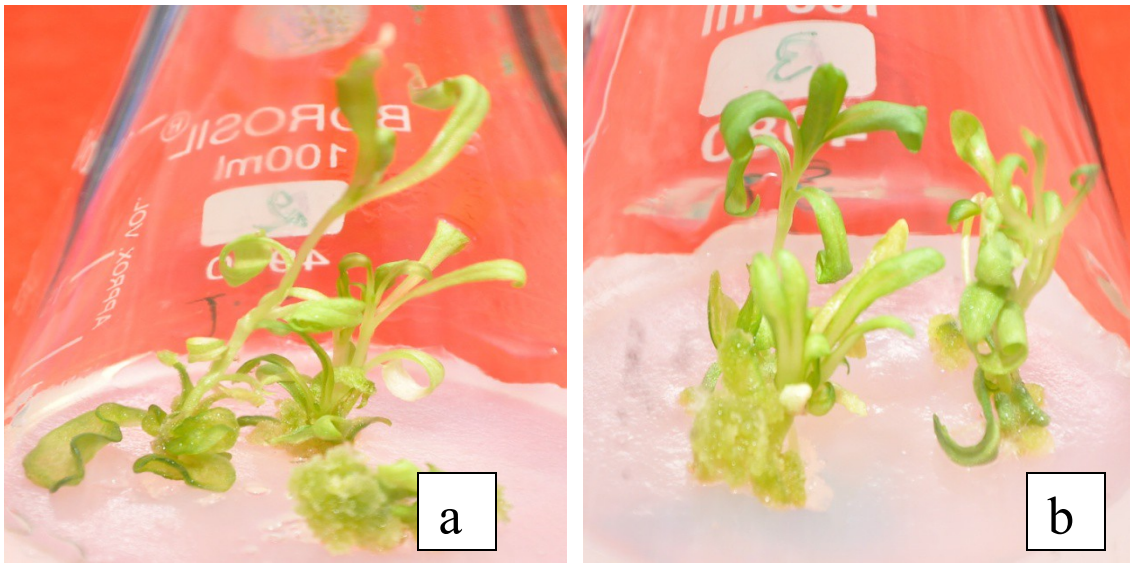
(a) **Without $AgNO_3$**

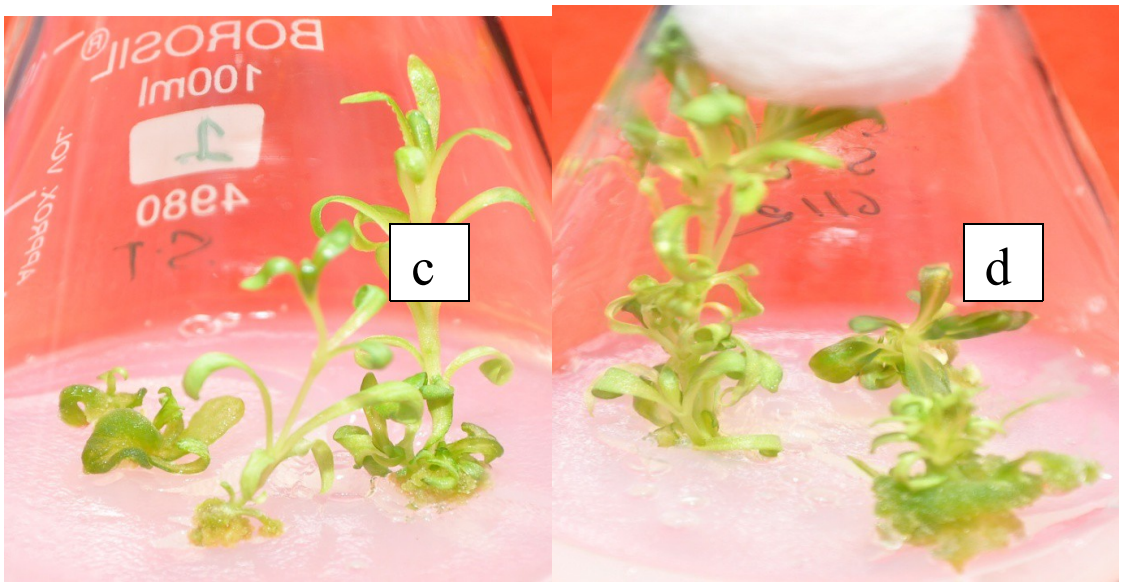
(b) **0.1 mg/l $AgNO_3$**

(c) **0.5 mg/l $AgNO_3$**

(d) **1 mg/l $AgNO_3$**

(e) **1.5 mg/l $AgNO_3$**





e

Figure 8

Effect of nickel chloride on shoot bud formation from node of *Dianthus caryophyllus* cultured on MS supplemented with BAP (0.5 mg/l) + NAA (0.5 mg/l)

(a) **Without $NiCl_2$**

(b) **0.1 mg/l $NiCl_2$**

(c) **0.5 mg/l $NiCl_2$**

(d) **1 mg/l $NiCl_2$**

(e) **1.5 mg/l $NiCl_2$**

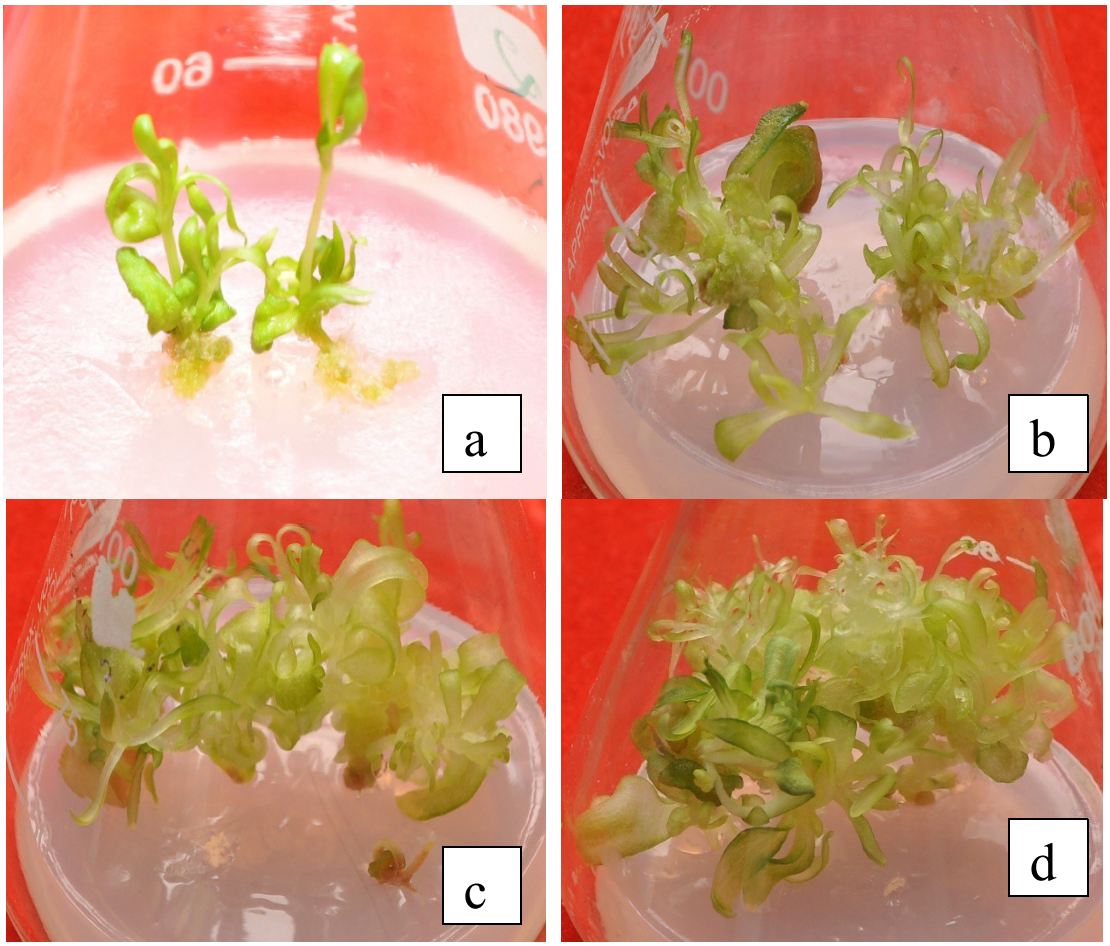

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Figure 9

Effect of nickel chloride on shoot bud formation from shoot tip of *Dianthus caryophyllus* cultured on MS supplemented with BAP (0.5 mg/l) + NAA (0.5 mg/l)

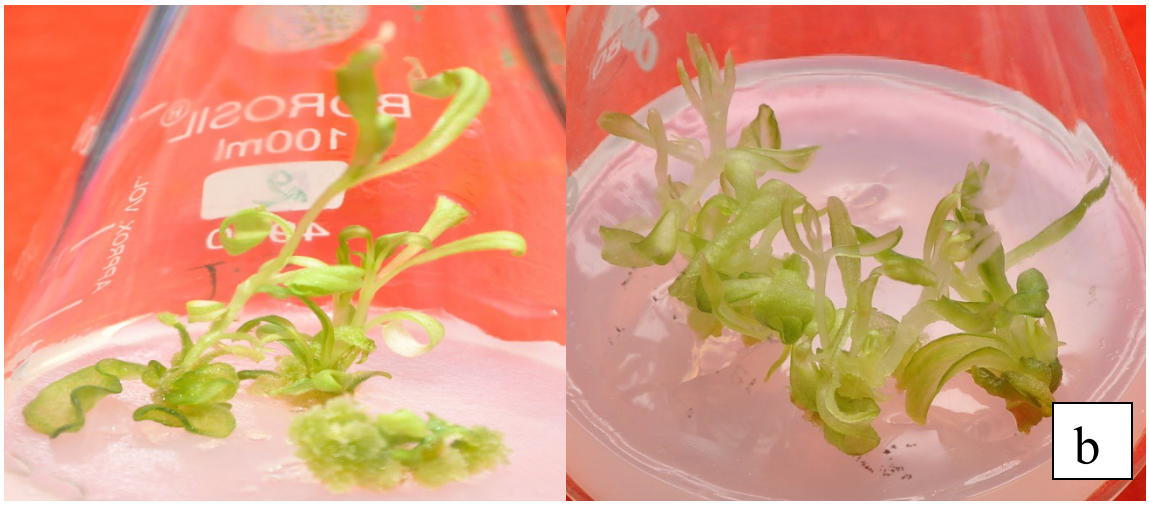
(a) **Without $NiCl_2$**

(b) **0.1 mg/l $NiCl_2$**

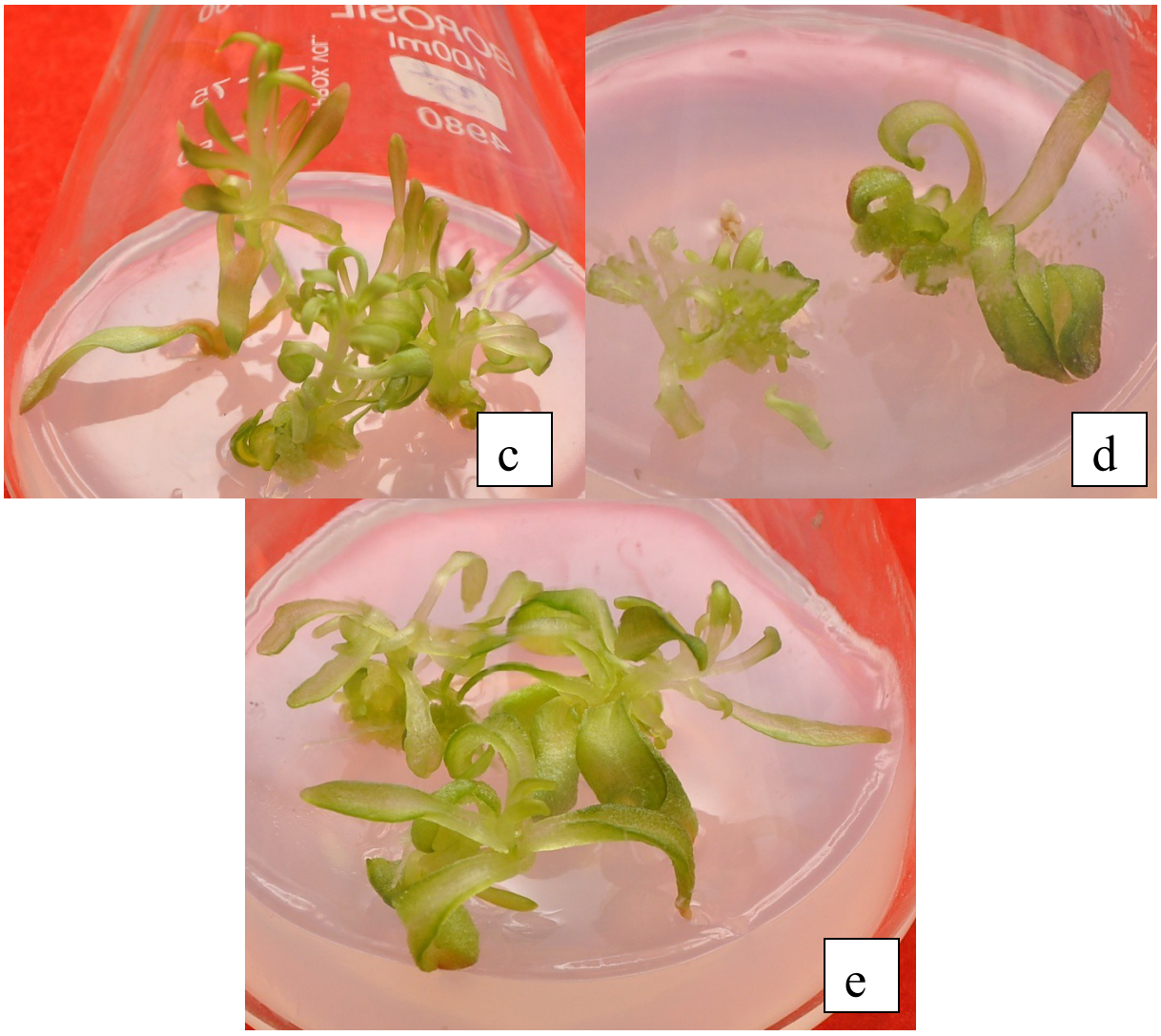
(c) **0.5 mg/l $NiCl_2$**

(d) **1 mg/l $NiCl_2$**

(e) **1.5 mg/l $NiCl_2$**



a



ROOTING OF *IN VITRO* REGENERATED SHOOTS

In vitro regenerated shoots (longer than shoots 2 cm) were excised and transferred into tubes containing solidified MS medium supplemented with various concentrations of auxins viz., IAA, IBA and NAA (0.5 and 1mg/ l) and half strength MS medium. 2-3thin, stout and slender roots induced from cut end of shoot on MS medium fortified with IBA (0.5 mg/l) (Fig. 10a).The addition of NAA (0.5 mg/l) to the MS medium promoted rooting, which was accompanied by callus (Fig. 10b).The MS medium supplemented with IAA (0.5mg/l) did not show any sign morphogenic response. On the other hand, the half strength MS medium showed excellent root formation where roots were long, unbranched and strong (Fig. 10c).

Figure 10

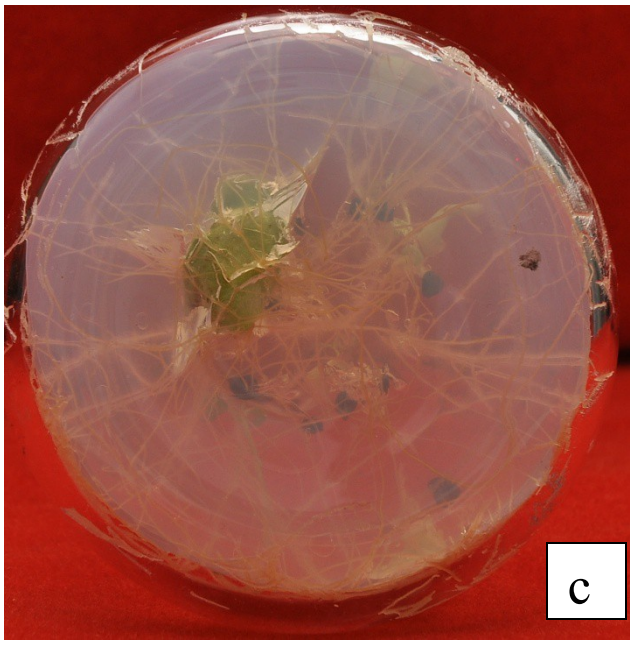
Rooting response of *in vitro* regenerated shoots of *Dianthus caryophyllus* cultured on MS medium supplemented with

(a) IBA (0.5 mg/l)

(b) NAA (0.5 mg/l)

(c) Half strength MS


a
b



DISCUSSION

D. caryophyllus is a valuable plant and has a high potential for export and improvement of floriculture industry. Plant cell and tissue culture have been developed in recent years to such a level that any plant species either endangered or highly recalcitrant can be regenerated *in vitro.* This area of plant biotechnology is mainly concerned with manipulation and subsequent growth of cells, tissues, organs and plant cell. In tissue culture, the use of growth regulator play important role in influencing different plant processes comprising mostly of growth, differentiation and development such as culture establishment, shoot initiation, callogenesis, embryogenesis and rooting (Hobbie, 1998).

To facilitate the yield enhancement and quality improvement of *D. caryophyllus, a* range of *in vitro* tissue culture technique have been developed such as efficient systems have been established for the regeneration of plant from leaf explants and stem segments(Jethwani and Kothari,1996; Watad *et al.*,1996: Kantia and Kothari, 2002).

In present study, the shoot buds induced from the shoot tip and nodes explants were selected for further studies as it proved to be the best respondary explants for shoot regeneration. Nodal and

apical segments have been used widely for shoot morphogenesis reported by Baker and Philips (1962).Similar results were reported by Earle and Langhans, 1975; Jethwani *et al*., 1994; Van Altvorest *et al.*,1995; Van Altvorst and Koehorst, 1995; Kallak *et.al.*,1997; Pareek *et. al*, 2004; H. Saehi, 2006; Danial *et al.*,2008; Duhoky *et al.*, 2008; Ali *et al.*,2008.

Shoot tip and nodal explants were cultured on MS medium supplemented with various concentration of BAP (0.2, 0.5, 1, 2, 3 and 5 mg/l) for shoot regeneration. A maximum number of shoot buds formed per explant was obtained on MS medium supplemented with 0.5 mg/l BAP. This is in agreement with the studies of Lubomski and Jerry (1989) who reported the best shoot formation response of carnation on MS medium supplemented with BAP. Kovac (1995) also reported highest shoot multiplication in carnation in MS medium containing 1.0 mg/l BAP. However, Siddiqui (1993) and Duhoky *et al.,* (2008) reported best shoot induction response in MS medium containing 5.0 mg/l kinetin.In the present investigation BAP in combination with NAA stimulated shoot buds induction and elongation response. It was found that MS medium supplemented with 0.5 mg/l BAP and 0.5mg/l NAA showed maximum shoot elongation and proliferation.

Van (1992) and Yantcheva *et al.*, (1998) reported highest numbers of shoots per explant on MS medium containing 0.9 mg/l BA and 0.3 mg/l NAA. Mangel *et al*., (2002) and Onamu *et al*., (2003) used MS medium supplemented with combination of NAA and kinetin for shoot induction from meristem explant.

Elongated shoots of 2 cm or more in length were excised and placed onto rooting medium which consisted of half strength MS medium and MS medium supplemented with various concentrations auxins (IAA, IBA and NAA).Out of these medium the best response was obtained on half strength MS medium. However Amir *et al* (2009) reported best rooting response on MS medium containing 1.0 mg/l NAA. On the other hand best rooting of shoots was achieved when NAA was included in the culture medium at 0.5 mg/l concentration reported by Daniel *et al.,* (2009).

The composition of MS medium is critical for optimizing the regeneration of plant *in vitro*. Some heavy metals act as microelements play important roles in the regeneration of plant at low concentration but at higher concentration they may cause reduction in growth for the most of plant species (Fernandes and Henrique, 1991; Claire *et al.,* 1991). In our own results, For shoot regeneration explants cultured on induction medium supplemented with various concentration of heavy metals ($AgNO_3$ and $NiCl_2$), we found that shoot regeneration from nodal and shoot tip explants of *D. caryophyllus* on MS medium with $AgNO_3$ (1mg/l) appeared to be the most advantageous in terms of total number of shoots as well as hyperhydricity per explants but with $NiCl_2$ it is not beneficial for shoot regeneration.

Addition of sliver nitrate has been found to be effective in overcoming the recalcitrant behavior. Sliver nitrate act as ethylene inhibitor and used for shoot elongation in *Capsicum* reported by Phillips 1996).It acts as inhibitor of 1-aminocyclopropane -1-carboxylic acid which is a precursor of ethylene in biosynthetic pathway (Palmer, 1992). However Mohiuddin *et al.,* (1997) achieved increased number of shoot in cucumber by incorporation of sliver nitrate. MS medium supplemented with sliver nitrate enhanced the frequency of shoot regeneration in *Brassica* species (Eapan and George, 1997). Gutiérrez-Miceli *et al.,* (2010) reported additive effect of heavy metals in stimulating morphogenesis in callus redifferentiation has been reported using kinetin and $AgNO_3$ in *D. caryophyllus.* There has been many reports of the inhibitory effects of Ni on germination and growth in plants. Madhava *et al.*, (2000) reported the germination of pigeon pea was decreased by addition of 1.5 mM Ni solution. Similar results were reported by Gajewska *et al*., (2006), who observed the inhibitory effect of 0.2 mM nickel on shoot growth of wheat. The roots of *Nicotiana tabacum* became dark brown within 7 to 10 days of exposure to 0.43 mM Ni and growth of the plants was severely inhibited (Boominathan *et al.,* 2002). In the present study lower concentration of nickel chloride had deleterious effect on shoot induction and still higher concentrations of nickel chloride proved toxic.

Plant tissue culture is one of the most commonly used methods for induction of shoot buds. We have demonstrated here that providing optimal concentration of BAP (0.5mg/l) and NAA (0.5mg/l) in MS medium which is induced shoot buds from the explants of *D. caryophyllus.*

Addition of silver nitrate in the medium is applicable to improve the quality and quantity of the regenerated shoots while on the other hand adding nickel to the medium proved detrimental.

SUMMARY

The present work deals with interactive effect of different plant growth regulator and heavy metals on shoot morphogenesis of *Dianthus caryophyllus.* This plant is a member of the family caryophyllaceae and is the most common cultivated species of this family which is characterized by their excellent keeping quality, wide range of forms, ability to withstand long distance transportation and remarkable ability to rehydrate after continuous shipping. It shows high level of heterogeneity in seed population which act as constraint to propagation of agronomic traits and for commercial seed production. Considering the benefits of this plant and to fulfill the world's demand for carnation, the technique of micropropagantion has been widely used. Tissue culture offers an opportunity to access clean planting material and provides a start in production of good quality carnation cut flowers that can adequately compete in the international markets.

The seeds of *D. caryophyllus* were obtained from Namdhari Seed Agency, Bengaluru.After proper sterilization seeds were germinated on half strength MS medium solidified with 0.8% (w/v) agar. Various explants were taken for the studies; shoot tip and nodal explants showed better response in term of shoot regeneration. The explants cultured on MS medium supplemented with various concentration of BAP (0.2-5mg/l). The maximum number of shoot buds was obtained on MS medium supplemented with BAP (0.5mgl/) and NAA (0.5mg/l). The shoot buds induced and proliferated on medium supplemented with BAP (0.5mg/l) and NAA (0.5mg/l) were selected for further studies as it proved to be the best concentration for shoot induction. The shoots were elongated on MS supplemented with BAP (0.5mg/l) and NAA (0.5mg/l), prior to rooting on half strength MS medium. Various auxins (IBA, IAA and NAA) were tried for rooting. The best response was shown by half strength MS medium.

Effect of heavy metals ($AgNO_3$ and $NiCl_2$) was investigated on shoot morphogenesis in *D. caryophyllus*. Node and shoot tip explants were cultured on induction medium (0.5 mg/l BAP + 0.5 mg/l NAA) supplemented with various concentrations of $AgNO_3$ (0, 0.1, 0.5, 1 and 1.5 mg/l). It was observed that the number of shoots increased with the increase in the concentration of $AgNO_3$ from 0.1 to 1mg/l. Beyond this level the shoots showed no significant increase in length but turned more hyperhydricity. Effect of $NiCl_2$ was found to be not advantageous for shoot morphogenesis in *Dianthus*.

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