

EFFECT OF DIFFERENT FACTORS ON VITRIFICATION IN CARNATION

Thesis

by

PUSHPA KUMARI

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for the degree of*

MASTER OF SCIENCE

in

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**COLLEGE OF HORTICULTURE
Dr Yashwant Singh Parmar University
of Horticulture and Forestry, Nauni,
Solan - 173 230 (H.P.), INDIA
2011**

Dr S.K. Sharma
Professor

Department of Biotechnology
College of Horticulture
Dr. Y.S. Parmar University of Horticulture and
Forestry, Nauni, Solan (H.P.) - 173 230

CERTIFICATE-I

This is to certify that the thesis entitled, **“Effects of different factors on vitrification in carnation”** submitted in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE in BIOTECHNOLOGY** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) is a record of bonafide research work carried out by **Ms. Pushpa Kumari** **(H-2009-06-M)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigations has been fully acknowledged.

Place : Nauni, Solan
Dated:

(Dr. S.K. Sharma)
Chairperson
Advisory Committee

CERTIFICATE-II

This is to certify that the thesis entitled, “**Effect of different factors on vitrification in carnation**” submitted by **Ms. Pushpa Kumari (H-2009-06-M)** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE in BIOTECHNOLOGY** has been approved by the Student’s Advisory Committee after an oral examination of the same in collaboration with the external examiner.

Dr. S. K. Sharma
Professor of Biotechnology
Chairman, Advisory Committee

Dr. D. R. Sharma
(Dean, Shoolini University)
Solan (H.P.)
External Examiner

Members of the Advisory Committee

Dr. D. K. Srivastava
Professor
(Deptt. of Biotechnology)

Dr. (Mrs.) Neerja Rana
Assistant Professor
(Deptt. of Basic Sciences)

Dr. R.K.Dogra
Assistant Professor
(Deptt. of Fruit Science)
Co-opted member

Dr. S.V.S. Chaudhary
Associate Professor
(Deptt. of Floriculture
and Landscaping)

Dean’s Nominee

Professor and Head
Department of Biotechnology

Dean
College of Horticulture

CERTIFICATE-III

This is to certify that all the mistakes and errors pointed out by the external examiner have been incorporated in the thesis entitled, **“Effect of different factors on vitrification in carnation”** submitted to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) by **Ms. Pushpa Kumari (H-2009-06-M)** in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE in BIOTECHNOLOGY**.

Dr S. K. Sharma
Professor
Chairperson, Advisory Committee

Dr S.V. Bhardwaj
Professor and Head
Department of Biotechnology
Dr. Y.S. Parmar, University of Horticulture and Forestry,
Nauni-173 230, Solan (H.P.)

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"Words once they are printed have life of their own"

To err is human.

Date :

Place :

(Pushpa Kumari)

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LIST OF ABBREVIATIONS

%	Percent
µg	Microgram
µg l ⁻¹	Microgram per litre
µM	Micro molar
2, 4-D	2,4 –dichlorophenoxy acetic acid
BA	6- Benzyl adenine
BAP	6-benzyl amino purine
CD	Critical difference
cm	Centimeter
CO ₂	Carbon dioxide
CRD	Completely Randomized Design
cv.	Cultivar
cvs.	Cultivars
DMSO	Dimethyl Sulfoxide
FYM	Farm Yard Manure
g	Gram
g l ⁻¹	Gram per litre
GA ₃	Gibberelic acid
h/day	Hours per day
HCl	Hydrochloric Acid
HgCl ₂	Mercuric Chloride
Hr	Hour
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kin	Kinetin
lbs/inch ²	Pound per square inch
lx	Lux

M	Molar
mg	Milligram
mg l ⁻¹	Milligram per litre
min	minute
ml	Millilitre
mm	Millimeter
mol.wt.	Molecular weight
MS medium	Murashige and Skoog's medium
NAA	a- Napthalene acetic acid
°C	Degree Celsius
ppm	Parts per million
rpm	Rotation per minute
TDZ	Thidiazuron
UV	Ultraviolet
w/v	Weight by volume
wk	Week
wt.	Weight

Chapter-1

INTRODUCTION

Carnation (*Dianthus caryophyllus* L.) is one of the world's most popular cut flower. It is one of the major floriculture crops in many countries of world with high ornamental and commercial value (Burchi *et al.*, 1996). It was named Dianthus by Theophrastus about 300 B.C. Carnation belongs to the family Caryophyllaceae, the genus Dianthus and species caryophyllus. The importance of this cut flower is due to its beauty, diversity of colors, excellent keeping quality and wide range of different forms (Ali *et al.*, 2008, Kanwar and Kumar 2009).

It is an herbaceous perennial plant growing to 80 cm tall. The leaves are glaucous greyish green to blue-green, slender, up to 15 cm long. The flowers are produced singly or up to five together in a cyme; they are 3–5 cm diameter, and sweetly scented; the original natural flower color is bright pinkish-purple, but cultivars of other colors, including red, white, yellow and green, have been developed.

Due to its excellent keeping quality and ability to rehydrate after continuous shipping carnation is preferred by growers to rose (Laurie *et al.*, 1986) and chrysanthemum in several exporting countries. Border carnations are among the most popular flowers, whether in gardens or as cut flowers, including in floral arrangements and corsages.

The wild carnations also provide a value for the ecosystem, having a mutualistic relationship with various insect pollinators. While this ecological relationship has allowed the plant to survive in its limited native areas around the Mediterranean, the benefit given to humans has led to its widespread cultivation worldwide and the development of numerous cultivars, with variety in flower size, color, petal number, and so forth.

Besides aesthetic value carnation flowers are considered to be cardiotonic, diaphoretic and alexiteric. Because of its popularity carnation is cultivated widely

on a large scale in Italy, Spain, U.S.A., Columbia, Kenya, Srilanka, Canary, France, Holland, Germany. Not only western countries the development of standard type of carnation has revolutionized its trade in the Indian flower market also.

Because of its popularity carnation is cultivated widely on a large scale in different countries of the world .The natural climate for carnation lies near 30° North or South latitude . In India the specific climatic requirements of the crop are met with at places like Himachal Pradesh, Punjab, Delhi, West Bengal and Kashmir.

Carnation can be propagated both by asexual and sexual methods. Flowering carnations are multiplied vegetatively by means of stem cuttings, while seed propagation is normally practiced in raising plants of marguerite carnations and for the purpose of hybridization (Bose *et al.*, 2003). To meet the growing demand of flower industry propagation of carnation is done on large scale through tissue culture.

Tissue culture has made a great progress in the development of carnation industry. Carnation is propagated on a large scale through micropropagation. Tissue culture has number of advantages in carnation multiplication. The most important factor which makes tissue culture techniques important is rapid multiplication as compared to the conventional methods and also to have true to type plant material. Remarkable progress in *in vitro* regeneration of carnation was made, involving organogenesis from various explants sources (Frey and Janick 1991; Van Altvorst *et al.*, 1992; Messeguer *et al.*, 1993; Zuker *et al.*, 1995). There has been reported problem of vitrification during tissue culture.

Vitrification , a physiological and morphological disorder in regenerated plants, is one of the most drastic problems during micropropagation of carnation (Mii *et al.*,1990; Ziv1991).This abnormality affects the production at commercial level (Yadav *et al.*, 2003). In vitrified plantlets, leaves have large vacuolated mesophyll cells, few stomata and less photosynthetic capacity. Their stem and leaves are rigid, thick and breakable (Sharma and Mohan 2006; Kevers *et al.*,

2004; Leshem 1983). Vitrified plants cannot survive transplanting to the soil which seriously restricts the use of long term propagation for industrial production (Navatel 1982).

Several hypothesis were offered for vitrification such as increase in ammonium concentration (Riffaud and Corns 1981), high concentration of cytokinins (Delbergh 1983), calcium deficiency (Kreutmeier *et al.*, 1984). Addition of activated charcoal provokes vitrification in some plants (Dencso 1987). It has also been demonstrated (Delbergh *et al.*, 1981) that vitrification rate is always higher in liquid than in solid medium.

It has been reported that vitrification is related with the characteristic of explants and the plantlet environment during *in vitro* culture, such as injury due to the prior dissection of the explants, typical fluxes of Ca and K ions, high relative humidity of the flask atmosphere, ammonium and cytokinin content of the media, and ethylene accumulation (Lai *et al.*, 2005).

The abnormal physiological process on hyperhydric plantlets almost in all cases decreased phenotypic performance such as loss of organogenic potential which may correspond or be more advanced form of diminished capacity to organize well structured stem, and leaf formation and multiplication rate (Winarto *et al.*, 2004).

The undesirable effects of hyperhydricity due to high relative humidity and water potential can be lowered by increasing the agar concentration, applying slanted agar surface (Leshem 1983; Bottcher *et al.*, 1988) and using different types and concentrations of gelling agent in the medium (Delbergh *et al.*, 1981). Other studies used looser than tighter closures or ventilated culture vessels (Hakkart and Versluijs 1983; Majada *et al.*, 2000). Applications of permeable closures combined with gelling agents have high potential to be applied in reducing the problem.

Vitrification results in abnormal shoots characterized by a bushy growth habit, stems which fail to elongate and thicken and result in translucent leaves

(Gersani *et al.*, 1986). It has been reported that vitrification results in somaclonal variations (Leshem, 1986). Vitrification is also affected by the MS macrosalts concentration (Choudhary and Prakash 1991).

The most important use of vitrification is the method of cryopreservation of different biological materials. Cryopreservation is the method of choice for long-term preservation of valuable germplasm of plants. Cryopreservation of carnation shoot tips has been done by encapsulation-vitrification. (Halmagyi and Deliu 2007).

A number of other species also has been cryopreserved by means of vitrification. Wang *et al.* (2009) demonstrated the cryopreservation of rose *in vitro* shoot tips by vitrification. Cryopreservation of *Chrysanthemum morifolium* (Halmagyi *et al.*, 2004) has also been done by means of vitrification. A number of factors have been reported which are responsible for vitrification.

The present investigation has been undertaken with the following objective:

- ❖ To standardize the various factors affecting vitrification in carnation (*Dianthus caryophyllus* L.)

Chapter-2

REVIEW OF LITERATURE

The large scale use of tissue culture began in 1960, when Moral reported rapid as well as virus free clonal multiplication of orchids by shoot tip culture. The technique allowed production of millions of genetically identical plants from a single bud in a period of one year.

The relevant literature pertaining to the factors affecting vitrification in carnation have been reviewed under the following headings:

- 2.1 Surface sterilization of explants**
- 2.2 Explant type**
- 2.3 Shoot regeneration**
 - 2.3.1 Callus induction and shoot regeneration**
 - 2.3.2 Organogenesis**
 - 2.3.3 Direct regeneration of adventitious shoots**
- 2.4 Vitrification**
- 2.5 Rooting and Hardening**

2.1 SURFACE STERILIZATION OF EXPLANTS:

Calcium hypochlorite (5%) was successfully used for the surface sterilization of young pedicels of carnation for 20 min (Rosest and Bokelmann 1981) and shoots for 10 min (Sangwan *et al.*, 1987).

Miyazaki and Tashiro (1978) reported that the stem segments sterilized in 0.1% HgCl₂ for 2.5 min at room temperature remained free of microorganisms and produce many shoots in carnation.

Ghosh and Mohan Ram (1986) suggested that carnation explants (axillary buds) sterilized in a solution containing teepol followed by sterilization in a laminar flow cabinet with a three minutes treatment of 2.5% sodium hypochlorite for 20 minutes. Finally the explants should be washed two or three times with

sterile distilled water containing 2 ppm Ambistryn-S (a commercial antibiotic streptomycin sulphate).

Kaveri *et al.* (2007) standardize surface disinfectants for sterilization of explants in carnation. Shoot tips of carnation were immersed in various concentrations of mercuric chloride (0.05, 0.10, 0.20 and 0.25%) or ethanol (70, 80 and 90%) for 3 minutes and 45 seconds, respectively, or pre-treated with Bavistin (0.1%) and streptomycin (0.1%) for 3 min each, then immersed in mercuric chloride (0.1%) or ethanol (80%) for surface sterilization. The highest level of contamination (81.4%) and lowest percentage of survival (15.6%) were recorded for the control, whereas the lowest percentage of contamination (15.5%) and highest percentage of survival (84.6%) were registered for explants pre-treated with Bavistin and streptomycin, then immersed in mercuric chloride solution.

Sharma *et al.* (2010) reported *in vitro* callus multiplication and shoot regeneration in carnation cv. Raggio de Sole. Leaf explant surface sterilized with 0.2 % bavistin (6 min) and 0.1 % HgCl₂ (2 min) yielded 91.44 % uncontaminated leaf cultures at the end of two weeks.

2.2 EXPLANT TYPE

Different explants like nodal segments, intermodal segments, leaves, apical meristem can be used for regeneration in carnation.

Mubarak *et al.* (1991) obtained plant regeneration from leaf segments of carnation. Leaf explants (2 to 3 cm long) obtained from *in vitro*-derived shoots of carnation cultivars Shocking Pink, Arthur Sim and William Sim were subcultured in MS media supplemented with 0.5 mg l⁻¹ 2,4-D, 0.5 mg l⁻¹ NAA, 0.5 mg l⁻¹ 2,4-D + 0.5 mg l⁻¹ NAA, for callus induction. In all 3 cultivars, 2, 4-D + NAA resulted in early callus formation whereas NAA + BA delayed callus formation.

Miller *et al.* (1991) reported shoot regeneration from fragmented flower buds of carnation. Adventitious shoots were regenerated from fragmented flower

buds, individual petals and receptacles in a number of different carnation cultivars. The regeneration medium contained 8 μ M BA and 4 μ M IAA.

Miller *et al.* (1991) reported adventitious shoot regeneration in carnation from axillary bud explants. Adventitious shoots were regenerated from axillary bud explants of 15 cultivars of carnation. The use of leaf and stem explants was not successful due to explant senescence in the presence BA, kinetin and, to a lesser extent, zeatin. For axillary bud explants, a suitable optimum adventitious regeneration medium contained Murashige-Skoog basal medium solidified with Gelrite and supplemented with 15 μ M BA and 0.5 μ M NAA.

Frey *et al.* (1992) reported callus formation from internodal segments in three cultivars of carnation viz. Scania, Improved White Sim and Sandra. Callus was initiated on liquid MS medium supplemented with 3 μ M 2, 4-D. Petal, calyx, node, internode and leaf explants of carnation cvs ('Scania', 'Improved White Sim', and 'Sandra') were grown at 25°C with a 16 hr photoperiod on Murashige and Skoog (MS) medium (pH 5.8) containing 87.6 mM sucrose, 0, 0.5 or 5.0 μ M TDZ and NAA.

Nakano *et al.* (1994) reported adventitious shoot regeneration from cultured petal explants of carnation. Adventitious shoot regeneration was compared among leaf, stem and petal explants of carnation cv. Scania on Murashige and Skoog medium containing different concentrations of BA and NAA. High frequency regeneration was obtained only from petal explants on media containing 5-10 μ M BA with or without 5 μ M NAA.

Sankhla *et al.* (1995) used different explants like petals, sepals, leaves and nodes of heat tolerant carnation cultivar German Red to evaluate their regeneration potential by placing the explants on MS medium containing 2 μ M NAA and various concentrations of TDZ or BA. TDZ was more effective than BA in inducing shoot formation. Petal and sepal explants exhibited good regeneration potential but the shoots produced from these explants invariably developed flower buds *in vitro*.

Narita *et al.* (2003) reported regeneration in carnation line no 156 from 243 lines by using stems and leaves as materials. The base of leaf explants had a higher frequency regeneration than stem explants. Medium consisting of MS salts with 0.2 mg l⁻¹ NAA and 0.5 mg l⁻¹ TDZ was suitable for plant regeneration.

Kumar *et al.* (2006) reported *in vitro* shoot regeneration from leaf segments of carnation via indirect organogenesis. Leaf segments were cultured on MS medium supplemented with different concentrations and combinations of growth regulators. Among the combinations tried, highest frequency of callus induction was obtained with 1.0 mg l⁻¹ 2, 4-D+2.2 mg l⁻¹ TDZ+0.12 mg l⁻¹ zeatin combination.

Ali *et al.* (2008) reported shoot formation in carnation. For shoot formation both apical and nodal meristems were used. MS medium containing BAP alone or in combination with kinetin was tested. Best shoot formation response was obtained after 6 days of inoculation from apical meristem and after 7 days of inoculation from nodal meristem on MS medium supplemented with 4.0 mg l⁻¹ BAP.

Kanwar and Kumar (2009) studied the influence of growth regulators, explants and their interaction on *in vitro* shoot bud formation from callus was studied in carnation. The leaf and internodes explants were cultured on Murashige and Skoog (MS) medium containing different concentrations of growth regulators. The highest callus induction was observed with 2 mg l⁻¹ 2, 4-D and 1 mg l⁻¹ BA.

Kanwar *et al.* (2010) reported direct regeneration from internode explants in carnation cv. Tempo. The highest direct adventitious shoot bud induction and regeneration was achieved with 1 mg l⁻¹ TDZ and 1 mg l⁻¹ NAA. Shoots elongated and multiplied on MS medium supplemented with 1 mg l⁻¹ BA and solidified with 10 g l⁻¹ agar. *In vitro* raised shoots were rooted in 1/2 strength MS medium supplemented with 2 mg l⁻¹ IBA and 2 g l⁻¹ activated charcoal and hardened successful.

Maitra *et al.* (2011) conducted an experiment to study the effect of kinetin and NAA on regeneration in carnation from shoot tip explants. An experiment was undertaken involving 14 different regeneration media to identify effective media composition to get carnation plantlets *in-vitro* from shoot-tip explants. Results from two consecutive years revealed that medium supplemented with NAA (1 mg l⁻¹) + Kinetin (2.5 mg l⁻¹) and NAA (1 mg l⁻¹) + Kinetin (3.5 mg l⁻¹) were equally effective for production of proliferating cultures (99.45%) whereas response to other media were not satisfactory.

2.3 SHOOT REGENERATION

2.3.1 Callus induction and shoot regeneration

Callus culture of carnation has been established from a range of vegetative explants such as leaves, stem segments, hypocotyls, apical meristem, shoot tips etc.

Delbergh (1972) reported induction of callus from stem segments of carnation cv. Leena. Carnation stem segments, cv. Lena, were grown in Murashige and Skoog basic medium supplemented with several auxins, either alone or together with other growth substances. Callus formation at the basal end of the explants was induced by media containing 2, 4-D (1 mg l⁻¹). IAA (0.25 mg l⁻¹) + kinetin (0.25 mg l⁻¹) induced callus at both ends of the explants.

Lumboski and Jerzy (1989) obtained callus by culturing internodal explants of carnation on half strength MS medium. Stem internodes of cv. Mini Pinky were divided into segments 2-3 mm in length (without axillary buds) and placed on half-strength MS medium containing 0.1 µM BA and 0.01µM IAA. After 5 weeks, adventitious shoots were transferred to a medium containing kinetin and IAA for shoot multiplication. Most of the explants cultured without BA and IAA died; those that survived produced only callus. Shoot regeneration was greatest with 1BA + IAA, whereas root regeneration was greatest with IAA alone.

Mubarack *et al.* (1991) obtained callus in three varieties of carnation viz. Shocking Pink, Arther Sim and William Sim using leaf segment (2-3cm long) as explants. Different hormonal concentrations of 2, 4-D, NAA, kinetin and BA were tried for callus induction. For all varieties, 0.5 mg l^{-1} 2, 4-D + 0.5 mg l^{-1} NAA gave earlier callus initiation and the treatment having NAA and BA (0.5 mg l^{-1} each) delay the callus initiation.

Frey *et al.* (1992) reported callus formation from internodal segments in three cultivars of carnation viz. Scania, Improved White Sim and Sandra. Callus was initiated on liquid MS medium supplemented with $3 \mu\text{M}$ 2, 4-D. Petal, calyx, node, internode and leaf explants of carnation cvs 'Scania', 'Improved White Sim', and 'Sandra' were grown at 25°C with a 16 hr photoperiod on Murashige and Skoog (MS) medium (pH 5.8) containing 87.6 mM sucrose, 0, 0.5 or $5.0 \mu\text{M}$ TDZ and NAA. Viable callus was subcultured on MS medium with $0.5 \mu\text{M}$ TDZ and $5 \mu\text{M}$ NAA. Shoots were rooted on half-strength MS medium lacking plant growth factors. Rooted shoots were transferred to soil:peat:perlite (1:2:2 v/v/v) with 60 mg/kg each of superphosphate and magnesium sulfate (pH 6). Only petals, calyxes and nodes were regenerative from all 3 cultivars. Maximum proliferation occurred with petals on MS medium with $0.05 \mu\text{M}$ TDZ and $0.5 \mu\text{M}$ NAA.

Thakur *et al.* (2002) conducted an experiment for *in vitro* culture of carnation cultivars Cabaret and Feyenoord using 2, 4-D. The explants were cultured on MS medium supplemented with 0.5-2.0 ppm NAA, 1.0-2.0 ppm kinetin, and 0.5-2.0 ppm 2, 4-D (alone or in combination with NAA). MS medium supplemented with 0.5 ppm each of 2, 4-D and NAA induced friable and pale yellow callus, but was optimal for callus induction and multiplication. The callus induced on medium supplemented with 2, 4-D alone or in combination with NAA was unable to regenerate shoots when transferred directly to the regeneration medium. The calluses with differentiated shoot primordia when transferred in the medium containing 2.0 ppm kinetin and 0.5 ppm NAA was the best for inducing shoot formation in differentiated shoot buds producing an average shoot number of 20.0 and 13.0 in Cabaret and Feyenoord, respectively.

2.3.2 Organogenesis

In carnation organogenesis can occur from various explants, including hypocotyls (Petru and Landa, 1974), leaves (Takeda, 1978) internodes (Rodojevic *et al.*, 1989).

Mubarack *et al.* (1991) obtained plant regeneration from leaf callus of carnation. Leaf explants (2- to 3-cm-long) obtained from *in vitro* derived shoots of carnation cultivars Shocking Pink, Arthur Sim and William Sim were subcultured in MS media supplemented with 0.5 mg l⁻¹ 2,4-D, 0.5 mg l⁻¹ NAA, 0.5 mg l⁻¹ 2,4-D + 0.5 mg l⁻¹ NAA, for callus induction. In all 3 cultivars, 2, 4-D + NAA resulted in early callus formation whereas NAA + BA delayed callus formation. When the resulting Shocking Pink calluses were subcultured in MS regeneration media, the media supplemented with 0.5 mg kinetin + 0.1 mg NAA l⁻¹ and 0.25 mg 2,4-D + 0.5 mg NAA + 0.5 mg BA l⁻¹ gave best results with respect to leaf morphogenesis and regeneration after 4 months of incubation. The other 2 cultivars did not respond to the regeneration media. Darkness did not seem to have any effect on the regeneration capabilities of explants.

Choudhary and Chin (1995) obtained callus from carnation cultivar White Sim. Shoot tissue were grown in Murashige-Skoog basal medium containing 3% sucrose and 0.6% Difco Bacto agar supplemented with 5, 10, 15 or 20 µM 2,4-D for callus induction in the dark. Calli were either transferred to the same medium or used to initiate suspension cell cultures by transfer of 20-25 days old friable calli (2-3 g fresh wt.) to 50 ml of liquid MS medium with 3% sucrose and lacking plant growth factors. Cultures were incubated at 23°C in continuous light with agitation at 133 rpm. Suspension cells were subcultured and divided every 7-10 days on fresh MS medium. Somatic embryogenesis and plantlet regeneration were obtained from 60-75 day old cell cultures of carnation on removal of 2, 4-D from the medium. The somatic embryos originated from single cells and their early development proceeded normally and with clearly defined apical and root meristems and were acclimatized to *ex vitro* conditions.

Sankhla *et al.* (1995) evaluated the regeneration potential of petals, sepals, leaves and nodes of heat tolerant carnation cultivar German Red by placing the explants on MS medium containing 2 μ M NAA and various concentrations of TDZ or BA. TDZ was more effective than BA in inducing shoot formation. Prolonged growth on TDZ containing medium however resulted in hyperhydricity. Petal and sepal explants exhibited good regeneration potential but the shoots produced from these explants invariably developed flower buds *in vitro*. Such plantlets were of poor quality for micropropagation. Shoots derived from nodal explants or leaves did not produce flower buds and rooted with almost 10% success *in vitro* or *ex vitro*.

Yantcheva *et al.* (1998) reported somatic embryogenesis and plant regeneration from leaf explants of carnation cultivars Leena, Bulgarian Spray, Nasslada, Yanita, Regina and Line 84. For direct induction of embryoids without an additional callus phase MS liquid medium supplemented with 1 mg 2, 4-D and 0.2 mg l⁻¹ BA was used. The first globular structures were observed after 20 days of cultivation. Their further development to the torpedo stage was correlated with the presence of polyethylene glycol (PEG 6000). Somatic embryo maturation was promoted by casein hydrolysate (1000 mg l⁻¹) in MS liquid media. The percentage conversion of embryos and polyembryos to whole plants varied between 10 and 75% among studied cultivars. Plantlets regenerated by this procedure were morphologically identical to the donor material and developed normally in a greenhouse.

Narita *et al.* (2003) selected line no 156 from 243 lines by using stems and leaves as materials. The base of leaf explants had a higher frequency regeneration than stem explants. Medium consisting of MS salts with 0.2 mg l⁻¹ NAA and 0.5 mg l⁻¹ TDZ was suitable for plant regeneration. Four youngest leaves grown *in vitro* were suitable for shoot formation. Light conditions did not affect shoot regeneration. Good shoot elongation was observed on growth regulators free MS medium.

Kumar *et al.* (2006) reported *in vitro* shoot regeneration from leaf segments of carnation via indirect organogenesis. Leaf segments were cultured on

MS medium supplemented with different concentrations and combinations of growth regulators. Among the combinations tried, highest frequency of callus induction was obtained with 1.0 mg l^{-1} 2, 4-D + 2.2 mg l^{-1} TDZ + 0.12 mg l^{-1} zeatin combination. Maximum frequency of shoots was noticed on MS medium containing 0.6 mg l^{-1} TDZ and 1.2 mg l^{-1} zeatin and also gave rise to 27.0 shoot buds per callus mass. Regenerated shoots were elongated on medium with 0.5 mg l^{-1} GA₃.

Mehta *et al.* (2007) reported shoot regeneration in carnation. For the establishment of callus cultures of carnation internodal segments were used as explants. Maximum percentage of callus induction was obtained on Murashige and Skoog (MS) medium supplemented with 2.0 mg l^{-1} NAA and 2.0 mg l^{-1} kinetin. Whereas, maximum shoot regeneration was obtained on medium supplemented with 2.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA.

Ali *et al.* (2008) reported shoot formation in carnation. For shoot formation both apical and nodal meristems were used. MS medium containing BAP alone or in combination with kinetin was tested. Best shoot formation response was obtained after 6 days of inoculation from apical meristem and after 7 days of inoculation from nodal meristem on MS medium supplemented with 4.0 mg l^{-1} BAP. Apical meristem showed more pronounced effect for shoot formation than nodal meristem. Well-developed shoots were shifted for their multiplication. Maximum number of multiple shoots was obtained on MS medium containing 1.0 mg l^{-1} BAP. Addition of Kinetin to BAP failed to show good shoot multiplication response.

Karami *et al.* (2008) reported proliferation of embryogenic callus, induction of somatic embryo and plant regeneration in carnation. The aim of study was proliferation of embryogenic calli followed by regeneration and production of healthy plants in two carnation cultivars, Nelson and Spirit. MS medium used for embryogenic callus formation was supplemented with 30 g l^{-1} sucrose, 2 mg l^{-1} 2, 4-D and 0.2 mg l^{-1} BA. Increasing the number of calli subcultures, increased the rate of embryogenesis. Proliferated calli were

transferred to hormone free MS medium containing 15, 30, 60, 90 and 120 g l⁻¹ mannitol.

Kanwar and Kumar (2009) studied the influence of growth regulators, explants and their interactions on *in vitro* shoot bud formation from callus in carnation. The leaf and internodes explants were cultured on Murashige and Skoog (MS) medium containing different concentrations of growth regulators. The highest callus induction was observed with 2 mg l⁻¹ 2, 4-dichlorophenoxy acetic acid (2, 4-D) and 1 mg l⁻¹ benzyl adenine (BA). Out of twenty seven shoot regeneration media tested, only 2 mg l⁻¹ thidiazuron (TDZ) and zeatin alone or in combination with naphthalene acetic acid (NAA) and/or indole acetic acid (IAA) could differentiate calli. The highest average number of shoots was observed with 2 mg l⁻¹ TDZ and 1 mg l⁻¹ IAA. Significant differences were observed in calli producing shoots and number of shoots per callus in the explants of leaf and internode. The shoots were elongated and multiplied on MS medium supplemented with 1 mg l⁻¹ BA and solidified with 1% agar. The shoots were rooted and hardened with 76% survival success in pots after six weeks of transfer to the pots.

Sharma *et al.* (2010) reported *in vitro* callus multiplication and shoot regeneration in carnation cv. Raggio de Sole. Surface sterilized explants of leaf, petals and internodal segments were inoculated on MS medium supplemented with various combinations of cytokinins and auxins as growth regulators for the establishment of callus cultures. Friable green callus was obtained on 2.0 mg l⁻¹ NAA and 0.5 mg l⁻¹ 2, 4-D from leaf explants only. The calli formed shoots after subculturing on the shoot regeneration medium containing different concentration of auxin and cytokinin. Maximum shoots were obtained on the MS medium in combinations of 0.5 mg l⁻¹ NAA and IAA each.

Kanwar *et al.* (2010) reported shoot regeneration from callus in carnation cv. Tempo. The highest callus induction was achieved on Murashige and Skoog (MS) medium supplemented with 2 mg l⁻¹ 2,4-D in both explants. The hormone-free MS medium was not effective in inducing shoots. A significant increase in the percent calli producing shoots was observed when 2 mg l⁻¹ zeatin was used in

combination with 1 mg l⁻¹ IAA. 2 mg l⁻¹ TDZ in combination with 1 mg l⁻¹ IAA produced the maximum number of shoots per callus. The highest direct adventitious shoot bud induction and regeneration was achieved with 1 mg l⁻¹ TDZ and 1 mg l⁻¹ NAA in both explants. More shoots were produced through callus phase as compared to direct adventitious shoots. The number of shoots per explant was higher in leaf than in internodal explants. Shoots elongated and multiplied on MS medium supplemented with 1 mg l⁻¹ BA and solidified with 10 g l⁻¹ agar. *In vitro*-raised shoots were rooted in ½ strength MS medium supplemented with 2 mg l⁻¹ IBA and 2 g l⁻¹ activated charcoal and hardened successfully.

2.3.3 Direct regeneration of adventitious shoots

Miller *et al.* (1991) reported shoot regeneration from fragmented flower buds of carnation. Adventitious shoots were regenerated from fragmented flower buds, individual petals and receptacles in a number of different carnation cultivars. The regeneration medium contained 8 µM BA and 4 µM IAA. The major site of shoot formation was the sub epidermal cells at the proximal end of the petals. The yields of shoots from a single flower bud were high, ranging between 70 and 275, for the 11 cultivars tested. Single shoots were removed from the explant and transferred to plant growth factor-free solidified basal medium. At each subculture, excessively vitrified or petaloid leaves were removed from the elongating shoots. After 12 wk, 10 adventitious shoots were transferred to MS basal medium supplemented with 2 µM IBA for root induction. The preferred regeneration protocol appears highly suited for the development of gene transfer systems.

Miller *et al.* (1991) reported adventitious shoot regeneration in carnation from axillary bud explants. Adventitious shoots were regenerated from axillary bud explants of 15 cultivars of carnation. The use of leaf and stem explants was not successful due to explant senescence in the presence of BA, kinetin and, to a lesser extent, zeatin. For axillary bud explants, a suitable optimum adventitious regeneration medium contained Murashige-Skoog basal medium solidified with

Gelrite and supplemented with 15 μM BA and 0.5 μM NAA. Adventitious primordia arose from the cut basal end of bud explants, emerging as individual shoots after 2-3 wk incubation. The axillary bud size and the subculture interval influenced the production of adventitious shoots. Transfer of regenerated shoots onto a medium solidified with agar minimized visible signs of vitrification.

Messeguer *et al.* (1993) reported adventitious shoot regeneration in carnation. Leaf, flower base and petal explants from carnation cv. 'Scania' were surface sterilized with dilute commercial bleach (5% w/v active chlorine) for 20 min and grown on Murashige-Skoog (MS) medium containing various concentrations of BA and NAA. Petals and floral segments had high morphogenic capacity in a wide range of plant growth factor treatments. The medium that induced the highest number of shoots also induced floral and abnormal structures. Lowering the BA concentration to 0.01 mg l^{-1} and the NAA concentration to 0.001 or 0.02 mg l^{-1} resulted in the suppression of the floral developmental pattern of petal explants and the regeneration of vegetative shoots that could be well acclimatized. Basal segments were the only part of the leaf that generated shoots when grown in the presence of 0.01 mg l^{-1} NAA and 1 mg l^{-1} BA. Darkness and an agar concentration of 5.5 g l^{-1} improved the percentage of regeneration significantly and the number of shoots per leaf explant. The shoots were normal with a vegetative shape, and could be transferred readily to the greenhouse.

Fisher *et al.* (1993) reported an efficient method for adventitious shoot regeneration from cultured carnation petals. A highly efficient procedure for adventitious shoot regeneration from various parts of carnation cvs. White Sim, Scania, Ceris Royalette, Sandrosa and Lisa) petals using liquid media was developed. Petals excised from young flower buds were cultured in 2 ml liquid Murashige-Skoog medium supplemented with 0.5 mg l^{-1} BA + 2 mg l^{-1} NAA or with 1 mg l^{-1} BA + 1 mg l^{-1} NAA + 2 mg l^{-1} gibberellin. The culture vessels were continuously agitated (100 rpm) at 24-26°C under a 15 hr photoperiod. In contrast to standard agar-containing medium in which shoots regenerate from about half of the petals, shoot regeneration was obtained from most (over 90%)

of the petal explants. Similar results were obtained for both liquid media. These shoots regenerated from various parts of the petals, and up to 7 adventitious shoots were obtained per petal in cv. White Sim. The adventitious origin of the regenerated shoots was confirmed by scanning electron microscopy. The use of liquid media did not affect the extent of vitrification, it being similar to that observed on agar.

Nakano *et al.* (1994) reported adventitious shoot regeneration from cultured petal explants of carnation. Adventitious shoot regeneration was compared among leaf, stem and petal explants of carnation cv. Scania on Murashige and Skoog medium containing different concentrations of BA and NAA. High frequency regeneration was obtained only from petal explants on media containing 5-10 μM BA with or without 5 μM NAA. Among the cytokinins tested, N-2-chloro-4-pyridyl-N'-phenylurea and TDZ were more effective than BA, kinetin, isopentenyladenine and zeatin on regeneration from petal explants. Although high frequency shoot regeneration was obtained from all petal explants harvested from various developmental stages of buds, a significant decrease in regeneration capacity was observed in the explants obtained from fully-opened flowers. High frequency (over 80%) shoot regeneration was also obtained from petal explants of cvs. Coral, Leena, Nora and White Sim, and an interspecific cv. Eolo, on basal medium containing 5 μM NAA and 5 μM TDZ.

Brar *et al.* (1995) reported *in vitro* shoot multiplication of carnation axillary buds and nodes. The response of carnation cv. Barlo II Nora) node culture and isolated axillary bud culture to shoot multiplication treatments was studied on Gamborg's B5 medium containing 100 mg l^{-1} myo-inositol, 50 mg l^{-1} casein hydrolyzate, 0.1 mg l^{-1} pantothenic acid, 1 mg l^{-1} nicotinic acid, 1 mg l^{-1} pyridoxine, 1 mg l^{-1} thiamine, 2 mg l^{-1} glycine, 30 g l^{-1} sucrose, and 8 g l^{-1} agar, supplemented with 0, 0.02 and 0.002 mg l^{-1} NAA and 2.5, 5, 10, 15 and 20 mg l^{-1} BA. The cultures were maintained at a 10 hr photoperiod and 23°C. After 6 wk, shoot length and shoot number per explant were determined and found to be influenced by the concentration of the plant growth factors. Medium containing 0.02 mg l^{-1} NAA and 5 mg l^{-1} BA appeared to induce a high rate of shoot

multiplication. Axillary bud culture resulted in higher shoot multiplication than from node culture.

Altvorst *et al.* (1995) reported that cells within the nodal region of carnation shoots exhibit a high potential for adventitious shoot formation. Leaf, stem and axillary bud explants from greenhouse-grown carnation cuttings (*Dianthus ornamental* cvs. Mediterranean, Sim, Spray, Diantini, Micro and interspecific hybrids) were cultured on Murashige-Skoog basal medium with 0.5 g l⁻¹ casein hydrolyzate, 30 g l⁻¹ sucrose and 8 g l⁻¹ agar supplemented with various plant growth factors (MS-30) at 25°C with a 16 hr photoperiod. For each cv., cuttings were used for the isolation of explants: the 3 most distal leaf explants were cultured in MS-30 medium with 4 µM BA and 1.4 µM NAA; the 4 most distal leaf explants were cultured on MS-30 medium with 8.8 µM BA and 4.8 µM NAA; and all axillary buds with a size of 0.5-5 mm were cultured on MS-30 medium with 13.3 µM BA and 4.8 µM NAA. Using axillary bud explants, shoot propagation efficiency was independent of the size of the bud and of its original position on the plant. In contrast, shoot propagation from stem and leaf explants was strongly dependent on their original position in the plant.

Sharnouby *et al.* (2005) studied the effect of BA, NAA on the production of three cultivars of carnation through tissue culture. This investigation was carried out on three cultivars of carnation (Medea, Candela and Picaro) cultured on solid MS medium supplemented with BA (0.5, 1.0, 2.0 or 3.0 mg l⁻¹) in combination with NAA (0.0, 0.25, 0.50 and 1.0 mg l⁻¹). After 2 months, the optimum concentration of BA was chosen and explants were subcultured on shoot regeneration medium (MS) enriched with BA at 2.0 mg l⁻¹. The results indicated that maximum number of shoots of Medea and Candela were produced on MS medium enriched with 2.0 and 3.0 mg l⁻¹ BA without NAA, whereas the maximum number of shoots of Picaro was obtained on medium enriched with 2.0 mg l⁻¹ BA alone followed by 2.0 mg l⁻¹ BA+0.25 mg l⁻¹ NAA. The cultivar Medea formed significantly longer shoots compared with the other two cultivars. Explants from Medea and Picaro grown on MS medium containing 2.0 mg l⁻¹ BA+0.25 mg l⁻¹ NAA gave the longest shoots.

Wankhede *et al.* (2006) carried out study to identify the procedure for propagation of carnation cv. Supergreen *in vitro*. Different explants i.e. shoot tips, axillary bud and stem segments, of Supergreen were used for culturing in the basal MS and Gamborg's media fortified with the growth hormones BAP, NAA and IBA in different concentrations and combinations. The axillary bud explant responded well compared to the shoot tip and stem segment explants. The treatment Gamborg + BAP at 1.0 mg l⁻¹+NAA at 1.0 mg l⁻¹ treatment was found to exhibit good performance for four traits, i.e. response of explant and shoot initiation to culture media, number of shoot buds per culture and number of shoots elongated in the shoot tip explant. The same treatment showed good performance in the axillary bud explant for four traits, i.e. days to shoot initiation, response of shoot initiation to culture media, number of shoot buds per culture, and number of elongated shoots. The ½ MS+IBA at 0.80 mg l⁻¹, ½ MS+IBA at 1.00 mg l⁻¹ and ½ MS+IBA at 0.05 mg l⁻¹ treatments were found to be superior for root initiation.

Bora *et al.* (2007) reported an efficient method for *in vitro* plant regeneration in carnation. Carnation cv. Lipstick was micropropagated using shoot tips of greenhouse-grown plants and nodal segments of *in vitro*-grown shoots cultured in MS medium supplemented with various growth regulators. High rates of multiplication of shoot tips were recorded in the medium containing basal MS salts supplemented with 0.5 mg NAA, 1.5 mg kinetin and 0.5 mg GA₃. High rates of nodal segment multiplication was observed in the MS medium supplemented with 0.5 mg NAA, 0.5 mg kinetin and 0.5 mg GA₃. Rooting of the micro shoots was most pronounced in the MS medium containing 0.5 mg l⁻¹ IBA. The rooted plantlets were transferred to ambient conditions using different substrates. Vermiculite resulted in a high survival percentage of 90%; the lowest survival percentage was recorded for the substrate soil. The hardened plantlets were planted in beds inside the greenhouse

Kaveri *et al.* (2007) reported regeneration of plantlets from shoot tip culture in carnation. Shoot tips (meristems) of carnation were cultured in

Murashige and Skoog's (MS) medium supplemented with BAP ($20.0 \mu\text{M l}^{-1}$) + NAA (0.5 , 1.0 , or $2.5 \mu\text{M l}^{-1}$) or kinetin ($20.0 \mu\text{M l}^{-1}$) + NAA (0.5 , 1.0 or $2.5 \mu\text{M l}^{-1}$) at 25°C and 16 hr photoperiod. The number of multiple shoots increased with increasing NAA and BAP concentrations. The length of shoots increased up to $1.0 \mu\text{M}$ NAA, then decreased at $2.5 \mu\text{M}$ NAA. The optimum medium for shoot multiplication was that supplemented with $20.0 \mu\text{M}$ BAP + $1.0 \mu\text{M}$ NAA. BAP was more effective in the enhancement of shoot multiplication when combined with NAA than with kinetin. Micro shoots (more than 2.0 cm long) were cultured in half-strength MS medium with various concentrations of IBA or NAA (1.0 , 5.0 and $10.0 \mu\text{M l}^{-1}$) for root induction. The highest rooting percentage (90.0%) and average number of roots (11.33) were obtained with the medium containing $5.0 \mu\text{M l}^{-1}$ IBA.

Wankhede *et al.* (2007) reported *in vitro* propagation of carnation cv. Gaudina. Explants from shoot tips and axillary buds of carnation cv. Gaudina were *in vitro*-cultured on MS and Gamborg's basal media fortified with BA (0.5 and 1.0 mg l^{-1}) and NAA (0.1 and 1.0 mg l^{-1}), alone and in combinations. The multiple shoots were transferred to half-strength MS media supplemented with 0.01 , 0.05 , 0.10 , 0.20 , 0.40 , 0.60 , 0.80 and 1.00 mg l^{-1} IBA for rooting. Successful *in vitro* propagation of Gaudina was achieved by inoculating axillary bud explants in Gamborg basal medium + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} NAA l^{-1} for shoot induction and proliferation followed by transferring the shoots to half-strength MS medium + 0.80 mg l^{-1} IBA for rooting.

Kanwar *et al.* (2010) reported direct regeneration from internode explants in carnation cv. Tempo. The highest direct adventitious shoot bud induction and regeneration was achieved with 1 mg l^{-1} TDZ and 1 mg l^{-1} NAA. Shoots elongated and multiplied on MS medium supplemented with 1 mg l^{-1} BA and solidified with 10 g l^{-1} agar. *In vitro* raised shoots were rooted in $\frac{1}{2}$ strength MS medium supplemented with 2 mg l^{-1} IBA and 2 g l^{-1} activated charcoal and hardened successfully.

Maitra *et al.* (2011) conducted an experiment to study the effect of kinetin and NAA on regeneration in carnation from shoot tip explants. An experiment was undertaken involving 14 different regeneration media to identify effective media composition to get carnation plantlets *in-vitro* from shoot-tip explants. Results from two consecutive years revealed that medium supplemented with NAA (1 mg l⁻¹) + Kinetin (2.5 mg l⁻¹) and NAA (1 mg l⁻¹) + Kinetin (3.5 mg l⁻¹) were equally effective for production of proliferating cultures (99.45%) whereas response to other media were not satisfactory. A composition of MS medium supplemented with NAA (0.5 mg l⁻¹) + Kinetin (0.5 mg l⁻¹) and NAA (1 mg l⁻¹) + Kinetin (0.5 mg l⁻¹) showed inhibitory effect on proliferation. Earliest sign of proliferation (3.97 days) was noticed using a culture medium containing NAA (1 mg l⁻¹) and Kinetin (2.5 mg l⁻¹). Explants cultured on the same medium also produced longest shoots (6.60 cm) and highest number of average leaves (33.07). Maximum number of shoots (6.73) was obtained from the explants cultured on MS medium supplemented with NAA (1 mg l⁻¹) and Kinetin (3.5 mg l⁻¹).

2.4 VITRIFICATION

Kevers and Gaspar (1985) studied the vitrification of carnation *in vitro* and changes in ethylene production, ACC content and capacity to convert ACC to ethylene. Carnation tissue was allowed to vitrify in a liquid medium, and ethylene production, ACC content and capacity to convert ACC to ethylene were compared in this tissue and a control developing normally on solid medium. Flask atmospheres of the liquid cultures accumulated ethylene at a higher rate during the first four days. Ethylene production decreased later but always remained above that in the control when subcultured daily to fresh medium. Explants and microsomal preparations from vitrifying carnations converted ACC to ethylene at a higher rate from the first day in liquid medium. ACC level also increased markedly in vitrifying tissues during the first two days of liquid culture. Raising the level of ethylene in the atmosphere of solid cultures did not induce vitrification nor did the use of inhibitors of ethylene biosynthesis in liquid cultures prevent the process.

Kevers and Gaspar (1985) studied soluble membrane and wall peroxidases, phenylalanine ammonia lyase, and lignin changes in relation to vitrification of carnation tissues cultured *in vitro*. Vitrification of carnation tissues by passage from a solid to liquid medium was accompanied by decrease in lignin content. The higher lignin level of normal tissues corresponded to a higher phenylalanine ammonia lyase activity. It also paralleled a much higher activity of soluble and wall peroxidases.

Kevers and Gaspar (1986) reported the changes in water contents, extracellular space, air volume and ion levels. Nodal explants with entire leaves were cultured for 9 days on solid and liquid medium containing 0.5 mg kinetin and 0.1 mg l⁻¹ NAA. Growth on the solid medium remained normal but 100% vitrification occurred within 9 days in the liquid cultures. The total water content remained stable at about 85.5% fresh weight for the first 2 days and diminished slightly thereafter, in control cultures on solid medium. The first signs of vitrification were visible between days 4 and 6. The tissues of vitrifying explants had greater extracellular space and air volume than those of normal explants; the extra water was located in the extracellular space, outside the protoplast. Explants in both media lost Ca and gain K ions with time, but this was much more marked in liquid medium.

Gersani *et al.* (1986) observed impaired polarity in abnormal plant development. Cultured shoot tips of various species often undergo a stable change resulting in abnormal shoots characterized by a bushy growth habit, stems which fail to elongate and thicken, and translucent leaves (vitrification). Normal and abnormal plantlets were grown from axial buds of virus-free carnation plants (cv. Cerise Royale). The distribution of radioactivity in 1 mm slices of 5 mm leaf sections placed upright or inverted on agar impregnated with ¹⁴C-IAA was studied. Normal leaf sections showed the expected polar transport towards the roots in the inverted sections, with only 50% of the total radioactivity being present in the 1 mm slice closest to the IAA source and 18% present in the most distant slice, compared with 87 and 0.7%, respectively, in the upright sections. Abnormal sections demonstrated very little polarity with 90% of the radioactivity

being present in the 1 mm slice closest to the source in both upright and inverted sections. The cellular structure of normal and abnormal leaves was compared; the abnormal leaves were distinguished by limited though variable vascular differentiation, large epidermal and mesophyll cells with no clear axis of elongation and few mature stomata. It is suggested that loss of cell polarity, expressed by the relative absence of oriented auxin transport, is largely responsible for the characteristics of abnormal plants indicating a major role for cell polarization in the development of most mature tissues.

Leshem (1986) reported carnation plantlets from vitrified plants as a source of somaclonal variation. About one half of explants from lateral buds of the cv. Cerise Royale, cultured on a medium containing Murashige and Skoog salts and amended with growth regulators (NAA, BA, myo-inositol), produced abnormal vitrified plants (translucent plantlets with short stems and thick fragile leaves). The apical 2-3 mm of the shoots of vitrified plants were subcultured. Normal shoots did appear on these plants although not as a direct continuation of the shoot tips. These shoots produced healthy plants after they were rooted and grown in a greenhouse but the flowers they produced were pink to light pink, unlike the dark pink of this cultivar. In 2 other trials with the culture of axillary buds or petals (basal parts) some vitrified plants were also obtained in each case. The findings are briefly discussed in relation to somatic mutation.

Dencso (1987) advocated the effect of micro element content of the nutrient medium and ratio of KIN: NAA on vitrification of carnation. About 19 varieties of carnation were cultured on the medium supplemented with 10 mg l⁻¹ KIN and 0.5 mg l⁻¹ IAA to study glassiness in carnation. Different rate of vitrification and growth of meristems was observed in the same variety. Reduction of micro element level to one tenth lowered the vitrification rate of cv. Scania. However, reduced micro- element was not enough to reduce vitrification of cv. Dark Lena. High rate of vitrification was observed on Earle- Langhans medium containing KIN 2 mg l⁻¹ and NAA 0.02 mg l⁻¹.

Prat and Gaspar (1987) reported changes in cell wall mechanical properties, cellulose and lignin content. Vitrification of internode explants was induced by culture in liquid medium. Cell wall extensibility of these internodes was kinetically followed and compared with that of normal plants using the constant stress method. Liquid culture medium induced an increase in the immediate and total deforming capacities of the walls from the second day. Measurements indicated that these deformation capacities involved plastic rather than elastic properties. These changes were paralleled by decreased level of cellulose and lignin in the vitrifying explants. The result supports the hypothesis that the hyperhydric malformations of vitreous tissues could result from a deficiency in cell wall synthesis and hence cell wall expansion through turgor pressure.

Werker and Leshem (1987) studied the structural changes during vitrification of carnation plantlets. Vitrified and normal plantlets of cv. Cerise Royalette, at various stages of development were compared. Vitrified plantlets showed an acropetal reduction in the length of time during which cells in the developing parts of the shoots remained meristematic. Early cessation of the activity in the stem and leaves may cause various structural abnormalities and early cessation of shoot apex activity resulting in loss of apical dominance may be responsible for the bushy habit of vitrified plantlets. Cell hypertrophy, loss of clear axis of elongation, and the presence of defective cell walls which disintegrate at certain locations after they are formed to produce cavities, were also observed in vitrified plantlets.

Kevers *et al.* (1988) studied composition of the walls of stem and leaves of vitrifying carnation. The vitrification of stem explants was induced by culturing in liquid medium. Cellulose and lignin levels were lower in vitrified stems and leaves, and isolated cell walls of vitrified tissues were characterized by a low Ca content, a low Ca^{2+} uronic acid ratio, and a low uronic acid: neutral sugar ratio (due to increased amounts of the latter). These characteristics may together account for the high wall plastic potential previously measured in vitrifying internodes.

Kim *et al.* (1988) studied physiological and morphological characteristics of glaucous and vitreous carnation plantlets obtained *in vitro*. The shoots of vitreous plantlets of cultivars Sarinah and Scarlet Bell produced *in vitro* showed thick, broad leaves which were translucent, wrinkled and/or curled and brittle. These shoots had lower chlorophyll, lignin, epicuticular wax and water contents than normal shoots and larger cells and intercellular spaces; leaf palisade and stem vascular tissues were poorly developed. Compared with greenhouse plants, all *in vitro* produced plants had greater cortical regions and less epicuticular wax.

Kim *et al.* (1988) studied effects of ABA and agar in preventing vitrification of carnation plantlets cultured *in vitro*. When cultured on MS medium with 0.7% agar, carnation (cvs. Sarinah and Scarlet Bell) tissue cultures produced vitreous plantlets characterized by tender, yellow green, translucent leaves. These plantlets did not survive transfer to soil. Use of a higher concentration of agar ($> 15 \text{ g l}^{-1}$) in the MS medium and/or the addition of ABA (10^{-6}M) produced normal glaucous plantlets. The best results were obtained when cultures on a solid MS medium of pH 5.5 containing $20\text{--}30 \text{ g l}^{-1}$ sucrose were illuminated with 1700 lx white fluorescent light for 16 h/day. Growth retardants inhibited growth, and GA_3 encouraged growth, but neither encouraged the production of glaucous plants. Glaucous plants survived transfer to soil conditions.

Leshem *et al.* (1988) studied the effect of cytokinins on vitrification in carnation and melon. In melon the anatomical traits of vitrification induced high levels of cytokinin (0.7 mg l^{-1} BA) were similar to those previously described for carnation grown on a higher concentration of auxin (1.0 mg l^{-1} NAA). In carnation the plants treated with high concentration of cytokinin (0.5 mg l^{-1} BA) became bushy but the anatomical structures remained normal.

Choudhary and Prakash (1991) studied the effect of different levels of agar and MS macrosalts on the production of hardened carnation *in vitro*. Carnation cv. Scania Red axillary buds were cultured in MS medium at pH 5.7 with 0.6, 0.8, 1.0, 1.2 or 1.4% agar and concentrations of KNO_3 , NH_4NO_3 and

CaCl₂. The percentage of vitrified shoots decreased with increasing agar concentration but at agar concentration above 1.2%, apices developed into small shoots instead of proliferating. The proportion of vitrified plants, compared with the control medium, was reduced by lowering the KNO₃ and NH₄NO₃ but was increased when the CaCl₂ concentrations was doubled.

Matsubra *et al.* (1991) cultured shoot apices of carnation varieties Natyla and Pink Casino on three media consisting of the same basal medium (Hyponex, NH₄NO₃, MgSO₄.7H₂O, Na-EDTA, pyridoxine-HCl, kinetin, NAA and sucrose) but supplemented with either 0.6% agar, 0.2% gelrite or a paper bridge. Shoot apices were excised from the main and lateral shoots, plated on the media and cultured for 3 months at 25°C. Normal shoots developed from 87-93% of apices cultured on agar medium, but 100 and 53% of Natyla and Pink Casino apices, respectively, cultured on gelrite medium formed vitrified multiple buds. A total of 20 and 73% of apices from the 2 varieties, respectively, developed normally on the paper bridge medium. Shoots grown on agar medium had the highest rate of dry weight production although fresh and dry weight was greatest in shoots cultured on gelrite medium.

Tannoury *et al.* (1991) reported a new process for cryopreservation of carnation shoot tips. Axillary shoot tips from 8-wk-old plants were cultured on culture medium supplemented with 1 µM BA and 0.1 M gibberellin. Apical domes with pairs of leaf primordia were covered in alginate beads. After overnight preculture in medium enriched with 0.75 M sucrose, trapped shoot tips were transferred into medium which was progressively enriched with sucrose up to a concentration of 6 g sucrose/4 g water. They were then loaded for 5 hr at 0°C with ethylene glycol at a concentration of 6 g sucrose/4 g water/6 g ethylene glycol. Beads containing 1-3 shoot tips were then cooled by direct immersion into liquid nitrogen, or by a 2-step procedure in which beads were first frozen to -150 °C at different cooling rates (0.5-20 °C/min) and then immersed in liquid nitrogen. The resistance of shoot tips to liquid nitrogen was high whatever the cooling rate: it ranged from 73% (20°C/min) to 93% (0.5°C/min). After direct cooling (200 °C/min), the survival rate was 83%.

Mosquera *et al.* (1992) reported the effects of the interaction between GA₃ and kinetin on the elongation and vitrification of *in vitro* grown plantlets derived from petal tissue of carnation variety Nohra barlo were investigated in order to improve the production of somaclones for the development of the resistance character of this variety to *F. oxysporum* f.sp. *dianthi*. Culture media containing GA₃ (1 mg l⁻¹), IAA (0.01 mg l⁻¹) and kinetin (2 mg l⁻¹) was most effective in allowing plantlet elongation with a low incidence of vitrification. No flower colour variation was observed in the somaclones produced but there was a slight fading in the original pink of the variety.

Hagimori *et al.* (1993) recovered vitrified carnation shoots using Bacto-Peptide and its subfractions. Vitrified shoots regenerated from carnation cv. Scania immature petals were recovered by culturing them at 25°C under a 16 hr photoperiod in modified Kakehi medium containing 3 g l⁻¹ Bacto-peptide, 3 g l⁻¹ Hyponex 6.5-6-19, 30 g l⁻¹ sucrose and 0.8% agar. Wax structures not found on vitrified shoots developed on the abaxial surface of the leaves of recovered shoots and on those of normal leaves. Recovered shoots were rooted in Murashige-Skoog medium with 30 g l⁻¹, 0.2% Gelrite and no plant growth factors, and acclimatized in Perlite at 15-30 °C in an 80% shaded mist room of controlled relative humidity (100% and 60% for the 1st and 2nd weeks, respectively) for 4 wk. Plantlets were then grown in soil in the greenhouse. The Bacto-peptide solution was fractionated and the efficiency of each fraction for the recovery of vitrification was examined. Only basic, non high mol. wt. fractions whose mol. wt. was less than 10,000 were effective. When Bacto-peptide was substituted with other poly peptones, only polypeptide- P1 (enzymatically hydrolyzed animal meat) had slightly higher activity than Bacto-peptide.

Ku and Tsay (1994) observed the effects of medium composition on the vitrification of carnation plantlets cultured *in vitro*. Shoot tips of carnation cv. Opale showed less vitrification when cultured on a solid medium than on a liquid medium. Vitrification occurred more often using the gelling agent Gelrite than Difco agar. Vitrification with Difco decreased with increasing Difco concentration. The addition of BA or kinetin increased vitrification. This increase

was greater at high cytokinin concentrations and with BA. The best medium was half strength MS medium supplemented with 3% sucrose, 0.9% agar and 0.5 ppm BA. Histological examination showed that the leaves of vitrified plants lacked clear differentiation between the palisade layer and spongy mesophyll cells. The stem also showed reduced differentiation, with enlarged cells in the cortex and pith.

Tsay and Drew (1998) reported the combined effects of medium composition (0-1.5% agar; 0-4 ppm BA; and different NO₃:NH₄ ratios) and subculture generation on vitrification of carnation cv. Pinky shoots were investigated. Incidence of vitrification was significantly higher with a reduced agar concentration, an increased BA concentration. Similar results were obtained with shoot tips from the first and fourth subculture, although vitrification was more severe in shoot tips from the later subculture generation.

Genkov and Ivanova (1995) examined the effects of different types of cytokinins on the growth of *in vitro* cultured carnation. NAA alone produced neither multiplication nor elongation. Combined with BAP, NAA increased multiplication. The biological activity of 4-PU-30 and especially that of thidiazuron was considerably higher than BAP's activity. In equal concentrations with BAP thidiazuron increased more than 6 times the number of shoots and about 5 times the number of explants per culture in cv. White Sim. All tested concentrations of 4-PU-30 and thidiazuron increased the fresh weight per culture in cv. Red Lena, more than BAP. Application of 4-PU-30 and thidiazuron induced changes in all fractions of peroxidase but soluble peroxidase had highest effect. New bands only are seen after application of both 4mM and 0.4mM 4-PU-30. SOD activity was stimulated by BAP. All studied concentrations of thidiazuron and 4-PU-30 also increased SOD activity. Strongest increase in SOD activity is in the highest concentration of thidiazuron.

Mujib and Pal (1995) reported highest number of shoots when shoot tips of carnation cvs. Royal Crimson and Candy Sim were cultured on medium supplemented with BA (0.5 mg l⁻¹). However, an increase in shoot height was observed when MS medium containing BA or KIN was supplemented with NAA.

Highest number of vitrified shoots was observed on media supplemented with BA.

Li *et al.* (1997) investigated the influence of certain factors on the vitrification of tissue cultured carnation plantlets and observed that it is highly influenced by plant bioregulators and agar concentration in the culture medium as well as type of culture vessel. MS medium containing 1.5 and 2 mg l⁻¹ of 6-BA and lower agar (0.3%) recorded the highest number of vitrified shoots; however, 0.5 µg 6-BA and 0.2 µg l⁻¹ IAA with 0.6% agar in the medium when culture vessels were sealed with five layers of gauze and no craft paper had the lowest number of vitrified shoots.

Chen *et al.* (1998) studied the effect of BA concentration, strength of MS salts, type of auxin and different vessel closures for improving *in vitro* hardening on growth and rooting of carnation. Half MS salts supplemented with 0.5 mg l⁻¹ BA and use of high ventilation dispenses paper as the vessel closure produced the highest number of normal plantlets with optimal growth.

Correll and Weathers (2001) studied the effect of light, CO₂ and humidity on carnation growth, hyper hydration and cuticular wax development in a mist reactor. Plant survival *ex vitro* requires functioning stomata, adequate cuticular wax composition and deposition and normal morphological environment. Light intensity, CO₂ and relative humidity were altered inside an acoustic window mist reactor to study their effects on carnation growth, stomata development, hyperhydration and epicuticular wax content. Increasing the light intensity and enrichment of gas phase increased the percentage dry weight of normal plants from 17% to 25% and reduced the number of hyperhydrated plants from 75% to 25%. Lowering the relative humidity (70%) surrounding the plants during the mist off phase for the last two weeks of culture reduced the number of hyperhydrated plants from 70 to 9% and also increased the percentage of dry weight of normal plants from 16% to 25%. The stomata on plants grown in conditions of high light or low humidity had smaller apertures and appeared sunken when compared to stomata from plants grown in low light and high

relative humidity. In addition, very different wax profiles were observed from plants grown in reactors with altered CO₂ and light intensities.

Radmann *et al.* (2001) studied the influence of the luminous flux density on the quality of micro propagated *Gypsophila paniculata* L. plants. The effect of luminous flux density on the multiplication of *Gypsophila paniculata* and its influence on rooting and acclimatization were evaluated. MS nutrient medium supplemented with sucrose (30g l⁻¹), meso-inositol (100 mg l⁻¹) and agar (10 g l⁻¹) was used. The explants used were bud microcuttings that were subjected to different radiations (15.705, 41.88 and 83.76 Wm²), for a 16-hr photoperiod and at 23±2°C. The number and length of shoots, number of internodes/shoot, color and incidence of vitrification were evaluated. There were no significant differences observed among the flux densities for the numbers of shoots and internodes. However, plantlets cultivated under low flux density presented a higher shoot length. No vitrification in the shoot leaves was observed, irrespective of the luminous flux density used. Shoots being cultivated under low luminous flux density showed a brilliant green color, while those developed under 41.88 and 83.76 Wm² were opaque green. For the rooting phase and acclimatization, shoots at least 2.0 cm in length were dipped in 100.0 mg l⁻¹ IBA solution for 3 minutes and later transferred to burn rice husk substrate. The rooting percentage was evaluated 30 days after transferring by measuring the weight of fresh and dry matter of the aerial part and roots. The shoots presented 100% rooting, irrespective of the luminous flux density used. Shoots under 83.76 and 41.88 Wm² presented a higher weight of fresh and dry matter.

Majada *et al.* (2001) reported air exchange rate affects the *in vitro* developed leaf cuticle of carnation. The leaf surfaces of carnation cv. Nelken plants cultured *in vitro* in either airtight or ventilated vessels were examined using scanning electron microscopy (SEM). The resultant hyperhydrated (vitrified), non-hyperhydrated and acclimatized plants were compared for stomatal density, cuticular wax development and stomatal function. The leaf surfaces of *in vitro* cultured plants were basically the same as those of acclimatized plants but less wax deposition was observed on their leaves. Stomata were found both open or closed after transfer of plants *ex vitro*.

However, stomata of *in vitro* leaves grown in ventilated culture vessels were more functional than those of plants grown in other conditions. Acclimatized plants had a normal leaf epidermal surface, and were wholly covered with waxes; their stomatal density being similar to that of highly ventilated plants but lower than that of less ventilated plants. Leaves of plants grown in airtight culture vessels or under a low number of air exchanges per hour had less waxes than plants grown at a higher number of air exchanges per hour or than acclimatized plants. In contrast, hyperhydrated plants had abnormal, malformed stomata and no wax deposition was detected. The adaxial surface of non-hyperhydrated leaves seemed more normal than the abaxial, especially in the most ventilated vessels, and this may be due to the former receiving lighter and so developing in a more favourable microenvironment.

Jo *et al.* (2002) observed the effects of sealing materials and photosynthetic photon flux of culture vessel on growth and vitrification in carnation plantlets *in vitro*. Best growth and development of plantlets were obtained from explants cultured on a modified MS medium containing 0.05 mg l^{-1} NAA, 0.01 mg l^{-1} BA, and 2.5 g l^{-1} gelrite. Culture conditions were 16 hr photoperiod. A cotton lid compared with aluminium foil and silistopper showed dramatically healthy growth of the plantlets.

Piqueras *et al.* (2002) studied the changes in polyamines level and patterns in response to hyperhydricity in micro propagated carnation plantlets. Hyperhydric carnation leaves showed high peroxidase activity, low lignification and high malondialdehyde content, suggesting oxidative damage. The most predominant fraction of polyamine corresponded to free polyamine in hyperhydric leaves as well as in control non hyperhydric leaves. Regarding individual amines, hyperhydricity brought about an almost complete depletion of free 1, 3- diaminopropane, a rise in the conjugated form of this amine, and a great reduction in bound spermidine in relation to non- hyperhydric leaves. A very high percentage of reverted shoots was obtained by maintaining hyperhydric carnation shoots in a culture chamber with a bottom cooling device that lowered the relative humidity inside the culture jars. Reversion of hyperhydricity was

associated with change in polyamine patterns. Thus, compared with both non-hyperhydric and hyperhydric leaves, reverted plants showed a drastic reduction in free polyamine. The polyamine profile in non hyperhydric and hyperhydric plants could indicate stress condition and a more suitable physiological situation in reverted plants.

Yadav *et al.* (2003) developed suitable protocol to overcome hyperhydricity in carnation during micropropagation. Hyperhydricity during micropropagation of carnation was reduced by media modifications. Three commercial varieties (White sim, Exquisite and Scania) tested, varied in optimal growth without hyperhydricity. Increased concentration of iron and/or magnesium reduced hyperhydricity with 0.7-0.8% agar. At some concentrations, hyperhydricity was reduced to 0% and shoot multiplication was increased. All non hyperhydrified micro propagated plantlets survived in a glasshouse during acclimatization.

Winarto *et al.* (2004) observed the effect of permeable vessel closure and gelling agent on reduction of hyperhydricity *in vitro* culture of carnation. The effects of permeable vessel closures (cotton wool, plastic wrap, parafilm and aluminium foil) and gelling agents(bacto agar, phytigel, swallow agar and type 900 agar)on the incidence of hyperhydricity and on the multiplication and acclimatization of recovered shoots of carnation cv. Maldives cultured *in vitro* were studied. The upper nodes of recovered shoots were cultured in MS medium containing 0.1 mg BA+ 0.02g l⁻¹NAA. Type 900 agar combined with plastic wrap and cotton wool resulted in the lowest incidence of hyperhydricity (26%). The first combination recorded the highest chlorophyll content and produced 4.2 shoots per explants whereas the second combination increased the chlorophyll content by 57% and registered the highest number of shoots per explants (9.2). Plastic wrap was more economical and easier to use on a commercial scale than cotton wool. The recovered shoots produced 1-3 healthy axillary shoots and easily rooted on half strength MS medium. The concentrations of NAA and BA had no significant effect on shoot multiplication.

Saher *et al.* (2005) reported Pectin methyl esterases and pectins in normal and hyperhydric shoots of carnation cultured *in vitro*. Control and hyperhydric micro propagated plantlets from three carnation cultivars have been used to study their pectin composition and the activity of pectin methyl esterases (PMEs; EC 3.1.1.11). Pectins are a highly heterogeneous group of polymers that contribute to cell adhesion, cell wall architecture, and cell wall mechanical strength. Pectins control cell wall porosity and cell wall ionic status and are implicated in intercellular space development. The degree of esterification of pectins is controlled by the activity of cell wall PMEs; their different actions can affect the properties of the cell wall, which have been considered important with respect to controlling the development of hyperhydricity. The total pectins of hyperhydric leaves of the three cultivars were significantly reduced compared with the control. The pectate fraction was significantly increased in hyperhydric leaves of all cultivars while soluble pectins and protopectins were significantly lower. The PME activity of hyperhydric leaves was higher (4-10 times) compared to controls of the 3 cultivars. Isoelectric focusing of PME isozymes revealed the presence of 3 isoforms; neutral PME activity was the major isozyme in control and hyperhydric leaves of the 3 cultivars, whilst a decrease in the activity of the acidic isoforms was observed in hyperhydric leaves. The different PME activities could regulate some of the structural changes related to hyperhydricity in micro propagated carnation plants.

Ahmed *et al.* (2006) reported the effect of TDZ on shoot multiplication from somatic tissues. Adding TDZ to the regeneration medium improved the regeneration efficiency. Of the TDZ concentrations tested, 5 μ M proved to be the optimum for the induction of maximum number of shoot buds and shoots per explant. However, continuous culture on TDZ containing media induced abnormal bud primordia, which failed to grow into plantlets. For normal shoot development, 10 days limited exposure to TDZ containing media was the optimal treatment. The induced shoot buds were transferred onto the plant growth regulator (PGR) free medium for shoot multiplication and elongation. *In vitro* rooting in elongated micro shoots was obtained on MS medium supplied with 1.0 μ M NAA on which 85-90% of cultured shoots produced healthy roots. The *in*

vitro regenerated plantlets grew well, flowered and attained maturity under greenhouse conditions.

Joung *et al.* (2007) observed the vitrification of gladiolus shoot tips from cormels. Gladiolus shoot tips were excised from *in vitro* and green house grown cormels of cultivar “Peter Pears”. The major factors that affected growth of shoot tips following vitrification were size and dormancy of the excised shoot tip cultivar. The highest survival of shoot tips was achieved using either greenhouse grown Peter Pears (77%) or field grown 02-943A (55%). Recovery of viable shoot tips was best with incubation times of 30 and 60 minutes.

DeFeng *et al.* (2007) conducted an experiment to study the causes of vitrification and the reuse effect of protocorm like body in cultivation of *Phalaenopsis amabilis*. Results show that the extent of vitrification for protocorm-like bodies aggravates with hormone concentration increasing and subculture time prolonging. The vitrification rates of two culture media with 5 mg l⁻¹ NAA and 10 mg l⁻¹ BA rose to 71% and 65%, respectively, when subculture to the ninth generation. The proliferation rate and regeneration rate of vitrification protocorm (2.2 and 183 individuals per bottle) were distinctly lower than those of formal protocorm (5.3 and 1297 individuals per bottle). The vitrification was significantly reduced after 2 or 3 generations of cultivation using culture medium without hormone, and the number of vitrification protocorm could decrease to 0.84%. The differentiation ability of the restored protocorm and the state of plant growth were consistent with those of formal protocorm, and the restored protocorm could be continually applied to tissue culture.

Budiarto (2008) reported that the incident of hyperhydricity was a common problem in propagated carnation during *in vitro* culture. Due to its possible relations with the decrease in phenotypic performance of plantlets, the observation on the occurrence of hyperhydricity was conducted on several *in vitro* conserved carnation cultivars. The research was conducted from July 2007 to August 2008 at The Indonesian Ornamental Crops Research Institute, Cianjur, and West Java. A complete factorial experiment with 24 replications was

designed to accomplish the combination of two factors. The first factor was six commercial carnation cultivars, namely light pink candy, Malaga, opera, white candy, liberty and pink maladi, while the second dealt with type of conservation media, i.e. $\frac{1}{2}$ MS+DMSO 3%, +3% sucrose and control ($\frac{1}{2}$ MS+3% sucrose). The results showed that the percentage of hyperhydric plantlet and plantlet viability after *in vitro* conservation were varied among carnation cultivars. Single treatment of sucrose had the least capacity in inducing plantlet resistance to low temperature conditions during *in vitro* conservation. Supplemental DMSO postponed the occurrence of hyperhydricity and with the existence of sucrose, higher plantlet viability was achieved.

Wang *et al.* (2009) demonstrated the cryopreservation of rose *in vitro* shoot tips by vitrification. The cryopreservation of shoot tips of rose cultivars (Gold Medal, Pink Maya, Han Hong and Bo) by vitrification was studied. The shoot tip survival rate was highest for Golden Medal. The effects of preculture, loading and vitrification solution on survival rate were studied in Pink Maya shoot tips. Shoot tips approximately 1.0 cm long were precultured for 5-6 days in the MS medium supplemented with 0.3 mol sucrose l^{-1} . Dissected shoot apices approximately 1.5 mm long were cultured in solution A (MS medium supplemented with 2.0 mol glycerol + 0.4 mol sucrose l^{-1}) for 30 minutes or solution B (60% PVS2 + 40% MS medium supplemented with 0.15 mol sucrose l^{-1}) for 40 minutes at room temperature. These excised shoot tips were sufficiently dehydrated in a highly concentrated plant vitrification solution (PVS2 supplemented with 300 g glycerol + 150 g ethylene glycol + 150 g dimethyl sulfoxide + 0.4 mol sucrose l^{-1} + MS) for 40 minutes at 0°C and then maintained in liquid nitrogen for 24 h. After rapid thawing in water at 40°C for 90 seconds, the tips were rinsed with sucrose (1.2 mol l^{-1}) solution and plated in MS medium supplemented with 1.0 mol 6-BA and 0.1 mol IBA l^{-1} . The survival rate of shoot tips reached 53.3%.

Kharrazi *et al.* (2011) evaluated the effect of plant growth regulators on *in vitro* shoot multiplication, vitrification and rooting of two carnation cultivars (Eskimo Mogr and Innove Orange Bogr). Isolated axillary buds were cultured on

MS medium supplemented with different levels of BAP or kinetin in combination with 0.2 mg l⁻¹ NAA and shoot multiplication and vitrification rate were tested. Then different concentrations of NAA or IBA on root induction of regenerated shoots were surveyed. Shoot regeneration and vitrification were influenced by the cultivar, kind of cytokinin and its concentration. BAP-containing media had more shoot multiplication and vitrification than Kin and by increasing the concentration of cytokinin, shoot multiplication and vitrification increased. There was a negative correlation between plant height and internode length with vitrification (-77% and -85%, respectively). By considering the amount of multiplication and rate of vitrification for obtaining the highest number of normal shoots, MS medium containing lower concentration of BAP (1mg l⁻¹ for Eskimo Mogr and 2 mg l⁻¹ for Innove Orange Bogr) and 0.2 mg l⁻¹ NAA is recommended. Rooting of regenerated shoots was dependent on the cultivar. The highest root percentage for Eskimo Mogr (83%) was achieved on the medium supplemented with 0.5 mg l⁻¹ NAA, while for Innove Orange Bogr (98%) it was obtained on the medium containing 1.5 mg l⁻¹ IBA.

2.5 ROOTING AND HARDENING

Beruto *et al.* (1991) reported *in vitro* root culture of two carnation cultivars. Carnation cv. Ausonia and Tagik roots, with high and low rooting ability, respectively, were cultivated *in vitro* for several weeks on modified culture medium supplemented with NAA or its ester, ethyl 1-naphthylacetate, at 1 or 3 µM. Tagik root development was good at 1 µM, while growth was not exhibited by either cultivar in the absence of auxin. Both NAA and ethyl 1-naphthylacetate induced Tagik root elongation to the same extent at 1 µM.

Choudhary and Prakash (1992) obtained roots on 0-5 µM IBA and observed that the number of days for rooting decreased with increasing sucrose concentration. Number of roots and root length increased with increasing sucrose concentration. IBA alone and the interaction between IBA with sucrose had no significant effect on root formation.

Yin *et al.* (1995) studied the effect of ABT (aminobenzotriazate) on root formation in carnation. However, when the shoots were treated with 100 mg l⁻¹ ABT for 1 hr, the rooting frequency was 90.7 percent, which was significantly higher than that obtained for IBA, NAA or the control.

Cuzzuol *et al.* (1996) reported that carnation could be rooted on MS medium supplemented with IAA, NAA or IBA each at 0.25, 0.5 or 1 mg l⁻¹. Root development was similar in all media with different growth regulators as well as in control.

Manreet *et al.* (2000) reported effect of gamma ray irradiation on *in vitro* multiple shoot formation and establishment of carnation plants. Elongating shoots were transferred to half-strength MS medium containing 20 g sucrose and 0.5 mg IBA l⁻¹ for rooting. Plantlets were transferred to pots containing sterilized sand and eventually hardened and planted in the field.

Ahmed *et al.* (2006) obtained *in vitro* rooting in elongated microshoots of carnation cv. Finest Mix on MS medium supplemented with 1.0 µM NAA in which 85-90 per cent of cultured shoots produced healthy roots. The *in vitro* regenerated plantlets grew well, flowered and attained maturity under greenhouse conditions.

Salehi (2006) conducted an investigation to find best shoot proliferation and rooting medium for 13 cultivars of carnation. For rooting, *in vitro* shoots were cultured on the best rooting medium. Transferring to a mixture of sand, leaf mold and vermiculite (1:1:1) successfully acclimatized the plantlets.

Wankhede *et al.* (2006) reported *in vitro* propagation of carnation cv. Supergreen. Different explants i.e. shoot tips, axillary bud and stem segments, of Supergreen were used for culturing in the basal MS and Gamborg's media fortified with the growth hormones BAP, NAA and IBA in different concentrations and combinations. The ½ MS + IBA at 0.8 mg l⁻¹, ½ MS + IBA at 0.1 mg l⁻¹ and ½ MS + IBA at 0.05 mg l⁻¹ treatments were found to be superior for root initiation.

Bora *et al.* (2007) reported an efficient method for *in vitro* plant regeneration in carnation cv. Lipstick. The best rooting of the microshoots was obtained on MS medium with 0.5 mg l⁻¹ IBA. The rooted plantlets were transferred to the ambient conditions using different substrates. Vermiculite recorded a high survival percentage of 90% while the lowest was recorded in the substrate soil. The hardened plants were finally planted in beds inside greenhouse.

Ahmad *et al.* (2008) reported effect of growth regulators viz., NAA, IAA and IBA on the rooting of carnation shoots under *in vitro* conditions which revealed that IBA @ 0.5 mg l⁻¹ significantly influenced all the parameters studied. Maximum rooting (90.66%) number of roots formed (4.70 per explants) and root length (1.47 cm per explants) was achieved in shoots cultured in MS medium supplemented with 0.5 mg l⁻¹ IBA.

Ali *et al.* (2008) reported shoot formation, their multiplication and rooting in carnation. Shoots after attaining the size of 5.0 cm were shifted for rooting. Best response was observed on MS medium containing 1.0 mg l⁻¹ NAA. Well rooted plants were shifted to glasshouse for hardening and acclimatization and were shifted to natural climatic condition.

Talukdar *et al.* (2009) studied the effect of substrates, poly-tunnels and growing conditions on hardening of *in vitro* raised carnation plantlets. Seven different substrates viz., vermiculite, cocopeat, sand, soil, vermiculite+cocopeat (1:1), vermiculite+sand (1:1) and vermiculite+soil (1:1) were used as the *ex vitro* growing media. The plantlets were kept under poly-tunnels made of polyethelene of three grades of 200, 140 and 50 micron thickness for two weeks. The plantlets, under the poly-tunnels, were placed in three different conditions viz., at room temperature, in building courtyard (full shade) and under agro-shade net (75%). Out of the seven substrates, maximum plant height, number of leaves and survival percentage were recorded in the substrate vermiculite followed by cocopeat, while, among the poly-tunnels, 140 micron thickness showed better growth and survival of the plantlets. Of the three growing, conditions, the

building courtyard and room temperature proved to be better for hardening of the *in vitro* raised plantlets.

Kharrazi *et al.* (2011) evaluated the effect of plant growth regulators on *in vitro* shoot multiplication, vitrification and rooting of two carnation cultivars (Eskimo Mogr and Innove Orange Bogr). Rooting of regenerated shoots was dependent on the cultivar. The highest root percentage for Eskimo Mogr (83%) was achieved on the medium supplemented with 0.5 mg l⁻¹ NAA, while for Innove Orange Bogr (98%) it was obtained on the medium containing 1.5 mg l⁻¹ IBA

Chapter-3

MATERIALS AND METHODS

The present investigation on the factors affecting vitrification in carnation was carried out in the Department of Biotechnology, Dr. Y. S. Parmar University of Horticulture and Forestry. The plant material of commercial cut flower carnation (*Dianthus caryophyllus* L. cv. Yellow Dot Com) procured from Department of Floriculture and Landscaping.

The methodology followed to carry out the investigation has been described as given below:

- 3.1 Plant material
- 3.2 General procedures followed
- 3.3 Shoot regeneration
- 3.4 Different factors affecting vitrification
 - 3.4.1 Effect of agar concentration
 - 3.4.2 Effect of growth regulators
 - 3.4.3 Effect of culture vessel
 - 3.4.4 Effect of nutrient medium
 - 3.4.5 Effect of sucrose concentration
- 3.5 *In vitro* rooting
- 3.6 Hardening
- 3.7 Statistical Analysis

3.1 PLANT MATERIAL

The studies on the factors affecting vitrification in carnation started using already multiplied shoots of carnation in the Department of Biotechnology , U.H.F. Nauni, Solan (H.P.).

3.2 GENERAL PROCEDURES FOLLOWED

3.2.1 Surface Sterilization

Surface sterilization was carried out by using different concentrations of bavistin(0.1% and 0.2%). The explants were washed thoroughly under running

tap water for one hour. After that they were treated with bavistin for half an hour containing a drop of Tween- 20. This was followed by washing with distilled water. The explants were then surface sterilized using 0.1% HgCl_2 for 1-3 minute; finally they were washed 2-3 times with autoclaved distilled water to remove any traces of sterilants. The surface sterilized explants were subsequently cultured on Murashige and Skoog (1962) medium.

3.2.2 Cleaning of Glassware

The new glasswares were cleaned by washing in a dilute solution of teepol in hot water with the help of a brush. The glassware was then soaked in chromic acid solution overnight and rinsed thoroughly with hot water. Finally rinsed with double distilled water and dried at 150°C in a hot air oven for one hour.

The used glassware with spent up media and contaminated cultures were first autoclaved to kill the microorganisms. The medium was allowed to cool to a semi molten form and disposed off to empty the culture vessels. The culture vessels were washed with hot water and teepol and rinsed with double distilled water followed by sterilization in an oven at 150°C for one hour.

3.2.3 Preparation of culture medium

The composition of media was based on Murashige and Skoog (1962) abbreviated as MS medium (Appendix-1). Separate stock solutions of inorganic nutrients, vitamins and growth regulators were prepared, kept in the refrigerator and used within one month. The chemicals used were of analytical grade and obtained from reliable firms.

During media preparation each stock (after bringing its temperature to room temperature) was added one by one in required quantity. After addition of sucrose (30g l^{-1}), vitamins and growth regulators, the pH of media was adjusted to 5.7. Thereafter, agar-agar (8g l^{-1}) was added and homogenized by heating the media. The media was finally dispensed into desired culture vessels (culture tubes/ flasks) for aseptic manipulations. However, for dispensing in petriplates

the medium was first autoclaved and then poured in the sterilized petriplates under laminar flow chamber before it was solidified. After dispensing the media, culture vessels were plugged with cotton plugs wrapped in muslin cloth and autoclaved at a pressure of 15 lbs per inch² and a temperature of 121.6°C for 15-20 min for proper sterilization of culture media. The media were stored in dark at 25±2°C and used after 5-7 days of preparation.

3.2.4 Aseptic manipulations and culture conditions

All the operations were carried out aseptically in a laminar flow chamber fitted with ultra violet (UV) light. Before starting with aseptic manipulations the laminar airflow chamber was thoroughly wiped out with rectified spirit and then culture vessels containing medium, culture equipments, spirit, spirit lamp, autoclaved petri dishes were kept inside the chamber and switched on UV light for 15-20 min.

Thereafter, the air flow was followed to run and UV light switched off after five min. During aseptic manipulations hands were frequently wiped out with rectified spirit. All metallic tools were autoclaved before use, inserted in a glass beads sterilizer and flame sterilized during the time of use. The rims of culture vessels were quickly flame sterilized before and after inoculation. In all the experiments the cultures were incubated in culture room at 25±2°C under 16 hr photoperiod. Dark conditions were maintained by wrapping the culture vessels with carbon paper or keeping the cultures in a closed cardboard box.

3.3 SHOOT REGENERATION

The nodal explants were inoculated on MS basal medium supplemented with different concentrations of cytokinins and auxins alone or in combinations. The cultures were incubated under dark conditions for two weeks and subsequently transferred to 16 hr photoperiod for two more weeks. After four weeks, the observations were recorded for an average number of shoots per explants.

3.4 DIFFERENT FACTORS AFFECTING VITRIFICATION

3.4.1 Effect of agar concentration:

The regenerated shoots were inoculated on the MS medium supplemented with the different concentrations of agar. The culture medium was supplemented with agar concentrations like 0.8%, 1% and 1.2% (Table1).

Table 1. Different concentrations of agar used to study the effect of agar concentration on vitrification in carnation

S. No	Medium Code	MS basal medium supplemented with different concentrations of agar (%)
1	A1	0.8%
2	A2	1%
3	A3	1.2%

The cultures were maintained under 16 hr photoperiod for four weeks and the observations were recorded for:

- (i) No. of normal shoots
- (ii) No. of vitrified shoots.

3.4.2 Effect of culture vessel

Different type of culture vessels were used for inoculating the shoots on the culture medium. The culture vessels used for culturing were conical flasks (100ml); culture tubes and glass jars.

The regenerated shoots were cultured on the medium present in the different vessels. After this the cultures were followed by incubation at 25±2°C under 16 hr photoperiod. After four weeks the observations were recorded (Table 2):

- (i) No. of normal shoots
- (ii) No. of vitrified shoots

Table 2. Different culture vessels used to study the effect of culture vessel on vitrification in carnation

S. No	Medium Code	Culture vessel used
1	B1	Conical flask(100 ml)
2	B2	Culture tubes
3	B3	Plastic jars

3.4.3 Effect of nutrient medium

The nutrient medium used was MS basal medium. Variable strength MS basal medium like $\frac{1}{2}$ strength, full strength and $\frac{1}{4}$ strength MS basal medium was used.

Table 3. Different strength MS basal medium used to study the effect of nutrient medium on vitrification in carnation

S. No	Medium code	Medium Strength
1	S1	Full strength
2	S2	$\frac{1}{2}$ Strength
3	S3	$\frac{1}{4}$ strength

The concentrations of the different macrosalts used in the MS basal medium were also changed. The concentrations of ammonium, calcium and potassium nitrate ions were changed. The shoots were cultured on the MS basal medium with variable strength and different concentrations of macrosalts. The cultures were then maintained in the culture room and observations were recorded after four weeks for:

- (i) No. of normal shoots
- (ii) No. of vitrified shoots

Table 4. Different concentrations of macrosalts used to study their effect on vitrification in carnation

S. No	Medium Code	MS basal medium supplemented with different concentration of macrosalts(mg l ⁻¹)		
		NH ₄ NO ₃	KNO ₃	CaCl ₂ .2H ₂ O
1	C ₀	1650.00	1900.00	440.00
2	C1	825.00	1900.00	440.00
3	C2	3300.00	1900.00	440.00
4	C3	1650.00	950.00	440.00
5	C4	1650.00	3800.00	440.00
6	C5	1650.00	1900.00	220.00
7	C6	1650.00	1900.00	880.00

3.4.4 Effect of sucrose concentration

Different concentrations of sucrose were used in the culture medium. The concentrations of sucrose used were 20g l⁻¹, 30g l⁻¹, 40g l⁻¹ and 60g l⁻¹ in the medium. The shoots were cultured on the medium supplemented with different concentrations of sucrose and the cultures were kept in the culture room and the observations were taken after four weeks for:

- (i) No. of normal shoots
- (ii) No. of vitrified shoots

Table 5. Different concentrations of sucrose used to study the effect of sucrose concentration on vitrification in carnation

S. No	Medium code	MS medium supplemented with different concentrations of sucrose (g l ⁻¹)
1	D1	20
2	D2	30
3	D3	40
4	D4	60

Table 6. Different concentrations of agar and sucrose used to study the effect of agar concentration on vitrification in carnation

S. No.	Medium Code	Concentration of sucrose(g l ⁻¹)	Concentration of agar(g l ⁻¹)
1	S1	20	8
2	S2	20	10
3	S3	20	12
4	S4	30	8
5	S5	30	10
6	S6	30	12
7	S7	40	8
8	S8	40	10
9	S9	40	12
10	S10	60	8
11	S11	60	10
12	S12	60	12

3.4.3 Effect of growth regulators

The shoots were inoculated on the MS medium supplemented with different concentrations of growth regulators in the culture medium. Different concentrations of auxins viz. IAA, IBA, NAA and cytokinins viz. TDZ, zeatin, BA and kinetin were used alone or in the combinations in the culture medium. The cultures were maintained under 16 hr photoperiod in the culture room and the observations were recorded after four weeks regarding:

- (i) No. of normal shoots
- (ii) No. of vitrified shoots

Table 7. Different concentrations of auxins and cytokinins alone or in combination used to study effect of growth regulators on vitrification in carnation

S. No.	Medium Code	MS medium supplemented with growth regulators (mg l ⁻¹)						
		BA	KIN	Zeatin	TDZ	IAA	IBA	NAA
1	E1	-	-	2.0	-	1.0	-	-
2	E2	-	-	1.0	-	1.0	-	-
3	E3	-	-	-	2.0	1.0	-	-
4	E4	-	-	-	1.0	1.0	-	-
5	E5	-	-	1.0	-	-	-	-
6	E6	-	-	2.0	-	-	-	-
7	E7	-	-	-	1.0	-	-	-
8	E8	-	-	-	2.0	-	-	-
9	E9	-	-	-	-	1.0	-	-
10	E10	-	-	-	-	2.0	-	-
11	E11	-	1.0	-	-	-	-	-
12	E12	-	1.5	-	-	-	-	-
13	E13	-	2.0	-	-	-	-	-
14	E14	-	-	-	-	-	1.0	-
15	E15	-	-	-	-	-	1.5	-
16	E16	-	-	-	-	-	2.0	-
17	E17	-	-	-	-	-	-	1.0
18	E18	-	-	-	-	-	-	1.5
19	E19	-	-	-	-	-	-	2.0
20	E20	1.0	-	-	-	-	-	-
21	E21	1.5	-	-	-	-	-	-
22	E22	2.0	-	-	-	-	-	-

3.5 *IN VITRO* ROOTING

In vitro raised shoots of 2.5-3.0 cm length were cut and rooted on half strength MS medium supplemented with 1.0 mg l⁻¹ and 2.0 mg l⁻¹ IBA, IAA and NAA with 0.2% activated charcoal.

After four weeks the observations were recorded for:

- (i) No. of shoots producing roots
- (ii) No. of roots per shoot.

Table 8. Different concentrations of auxins used for rooting of *in vitro* grown shoots of carnation

S. No	Medium Code	$\frac{1}{2}$ MS basal medium supplemented with growth regulators(mg l ⁻¹)		
		IAA	IBA	NAA
1	RM ₁	-	-	-
2	RM ₂	1.0	-	-
3	RM ₃	2.0	-	-
4	RM ₄	-	1.0	-
5	RM ₅	-	2.0	-
6	RM ₆	-	-	1.0
7	RM ₇	-	-	2.0

3.6 HARDENING

After four weeks of incubation on MS rooting medium the roots of well established plantlets were washed thoroughly to remove sticking medium. The plantlets were dipped in 0.01% carbendazim (bavistin) solution for 15-20 min and transferred to plastic cups containing cocopeat. For initial 15 days the plants were covered with polythene to maintain humidity. Acclimitization to external environmental conditions was done by removing the polythene cover to reduce humidity gradually.

After four weeks, well established plantlets were transferred directly to earthen pots containing sand, soil and FYM as potting mixture in the ratio of 1:1:1. Plants were nourished by adding 10 ml of 1/10 MS basal medium (devoid of sucrose on alternate days). After four weeks, the observations were recorded on for percent survival of plantlets upon transfer to plastic cups followed by their transfer to earthen pots for which observations were recorded after six weeks.

3.7 STATISTICAL ANALYSIS

The data recorded for different parameters were subjected to completely randomized design (CRD) (Cochran and Cox, 1963; Gomez and Gomez, 1984). The statistical analysis based on the mean values per treatment was made using analysis of variance technique for CRD.

Chapter-4

EXPERIMENTAL RESULTS

The results obtained during present investigation on “Effect of different factors on vitrification in carnation (*Dianthus caryophyllus* L.) cv. Yellow Dot Com have been presented under the following sections:

4.1 Different factors affecting vitrification

- 4.1.1 Effect of agar concentration
- 4.1.2 Effect of growth regulator concentration
- 4.1.3 Effect of culture vessel
- 4.1.4 Effect of nutrient medium
- 4.1.5 Effect of sucrose concentration

4.2 *In vitro* rooting

4.3 Hardening

4.1.1 Effect of agar concentration:

The experiment was carried out to study the effect of agar concentration on vitrification. Different concentrations of agar were used in the culture medium. Three concentrations of agar i.e. 0.8%, 1% and 1.2% were used in the culture medium. The explants were cultured on the medium supplemented with different concentrations of agar and the observations were recorded after 4 weeks and the results are given in the Table 9.

The data presented in Table 9 showed that maximum percentage of normal shoots were obtained on medium A3 (MS basal+2.0 mg l⁻¹ BA +1.2% agar) i.e. 74.43% which was statistically at par with A2 (MS basal+2.0 mg l⁻¹ BA+1 % agar) i.e.71.47%. The minimum percentage of normal shoots was obtained on the medium A1 supplemented with 0.8% agar concentration i.e.37.97% which was significantly different from rest of the treatments.

Table 9. Effect of different concentrations of agar on vitrification in carnation

Sr. No	Medium Code	MS basal medium supplemented with different concentrations of agar (%)	Percentage of normal shoots	Percentage of vitrified shoots
1	A1	0.8%	37.97(38.04)	60.67(51.16)
2	A2	1%	71.47(57.71)	28.32(32.15)
3	A3	1.2%	74.73(59.83)	24.73(29.82)
		CD _{0.05}	5.32	4.24

Values expressed in the parenthesis are the arc sine transformation of percentage

The maximum percentage of vitrified shoots was observed on the medium A1 (MS basal +2.0 mg l⁻¹ BA + 0.8% agar) i.e. 60.67% whereas the minimum percentage of vitrified shoots was obtained on the medium A3 (MS basal+2.0 mg l⁻¹BA+1.2% agar) i.e. 24.73% which was statistically at par with A2 (MS basal+2.0 mg l⁻¹BA+1 % agar) i.e. 28.32%.

4.1.2 Effect of growth regulators

The experiment was carried out to study the effect of different growth regulators on vitrification. Different concentrations of auxins (IAA, IBA, NAA) and cytokinins (TDZ, zeatin, BA and kinetin) were used alone or in combinations in the culture medium. The observations were recorded after four weeks of culturing and the results obtained are presented in Table 10.

The data presented in Table 10 showed that the maximum percentage of normal shoots was observed on the medium E10 (MS basal+1.0 mg l⁻¹ zeatin+1.0 mg l⁻¹ IAA) i.e. 96.09%.Treatment E13(MS basal+2.0 mg l⁻¹ kinetin) resulted in 66.94% normal shoots which was statistically at par with E11(MS basal+ 1.0 mg l⁻¹ kinetin) i.e. 66.62%, E19(MS basal +2.0 mg l⁻¹ NAA) i.e.66.43%, E22(MS basal +2.0 mg l⁻¹ BA) i.e. 66.40% and E1(MS basal +1.0 mg l⁻¹ IAA)i.e. 66.00% and E18(MS basal +1.5 mg l⁻¹ NAA) 1.e. 60.10% was statistically at par with E14(MS basal +1.0 mg l⁻¹ IBA) i.e. 60.00%.

However, maximum percentage of vitrified shoots was observed on the medium E3 (MS basal+1.0 mg l⁻¹ TDZ) i.e. 96.93%. Medium E17 (MS basal+1.0 mg l⁻¹ NAA) resulted in 46.40% vitrified shoots which was statistically at par

Table 10. Effect of different concentration of growth regulators on vitrification in carnation

S. No.	Medium Code	MS medium supplemented with growth regulators (mg l ⁻¹)							Percentage of normal shoots	Percentage of vitrified shoots
		BA	KIN	Zeatin	TDZ	IAA	IBA	NAA		
1	E1	-	-	-	-	1.0	-	-	66.00(54.33)	32.96(35.04)
2	E2	-	-	-	-	2.0	-	-	44.00(41.55)	56.00(48.44)
3	E3	-	-	-	1.0	-	-	-	0.00(0.00)	96.93(84.11)
4	E4	-	-	-	2.0	-	-	-	19.93(26.51)	79.93(13.39)
5	E5	-	-	-	1.0	1.0	-	-	22.73(28.45)	76.00(60.66)
6	E6	-	-	-	2.0	1.0	-	-	15.96(23.55)	84.03(66.45)
7	E7	-	-	1.0	-	-	-	-	60.02(50.78)	40.03(39.25)
8	E8	-	-	2.0	-	-	-	-	86.00(68.03)	14.30(22.21)
9	E9	-	-	2.0	-	1.0	-	-	79.98(63.42)	19.93(26.51)
10	E10	-	-	1.0	-	1.0	-	-	96.09(78.60)	4.13(11.72)
11	E11	-	1.0	-	-	-	-	-	66.62(54.70)	33.10(35.12)
12	E12	-	1.5	-	-	-	-	-	73.14(58.78)	26.40(30.91)
13	E13	-	2.0	-	-	-	-	-	66.94(54.60)	33.33(35.26)
14	E14	-	-	-	-	-	1.0	-	60.00(50.77)	40.16(39.33)
15	E15	-	-	-	-	-	1.5	-	40.00(39.23)	60.03(50.79)
16	E16	-	-	-	-	-	2.0	-	33.07(35.10)	66.40(54.57)
17	E17	-	-	-	-	-	-	1.0	53.11(46.78)	46.40(42.93)
18	E18	-	-	-	-	-	-	1.5	60.10(50.83)	40.06(39.27)
19	E19	-	-	-	-	-	-	2.0	66.43(54.59)	33.26(35.22)
20	E20	1.0	-	-	-	-	-	-	53.26(46.87)	46.40(42.93)
21	E21	1.5	-	-	-	-	-	-	50.08(45.04)	50.06(45.03)
22	E22	2.0	-	-	-	-	-	-	66.40(54.57)	33.33(35.26)
CD _{0.05}									2.21	4.99

Values expressed in the parenthesis are the arc sine transformation of percentage

with E20 (MS basal+1.0 mg l⁻¹ BA) i.e. 46.40% and E22 (MS basal+2.0 mg l⁻¹ BA) resulted in 33.33% vitrified shoots which was statistically at par with E13 (MS basal+2.0 mg l⁻¹ kinetin) i.e.33.33%, E19 (MS basal+1.5 mg l⁻¹ NAA) i.e. 33.26%, E11(MS basal+1.0 mg l⁻¹ kinetin) i.e.33.10% and E1(MS basal +1.0 mg l⁻¹ IAA) i.e. 32.96%. The minimum percentage of vitrified shoots was observed in E10 (MS basal +1.0 mg l⁻¹ zeatin +1.0 mg l⁻¹ IAA) i.e. 4.13% which was statistically different from all other treatments.

4.1.3 Effect of culture vessel

The effect of culture vessel was studied by using three different type of culture vessels viz. conical flasks (100ml), plastic jars and culture tube .The explants were cultured in the different culture vessels and the observations were recorded after 4 weeks of culturing. The results obtained are presented in the table 11.

The data presented in table 11 showed that out of the three culture vessels used B1 (conical flasks) proved to be best for culturing with maximum percentage of normal shoots (86.00%) followed by B2 (culture tubes) i.e. 76.62% and B3 (plastic jars) i.e. 40.00%. B3 (plastic jars) resulted in minimum percentage of normal shoots i.e. 40.00%.

Table 11. Effect of different culture vessels on vitrification in carnation

S.No	Medium Code	Culture vessel used	Percentage of normal shoots	Percentage of vitrified shoots
1	B1	Conical flask(100 ml)	86.00(68.03)	14.04(22.01)
2	B2	Culture tubes	76.62(61.08)	23.15(28.76)
3	B3	Plastic jars	40.00(39.23)	60.05(50.80)
CD _{0.05}			2.70	1.91

Values expressed in the parenthesis are the arc sine transformation of percentage

The maximum percentage of vitrified shoots was observed in treatment B3 i.e. 60.05% and the minimum percentage of vitrified shoots was observed in B1 i.e. 14.04% which was significantly different from all other treatments.

4.1.4 Effect of nutrient medium

The experiment was carried out to study the effect of nutrient medium on vitrification. For this different strength medium were used. The nutrient medium used were half strength, full strength and $\frac{1}{4}$ strength MS medium. The explant was cultured on different strength medium and the observations were recorded after four weeks of culturing and the results are given in Table12.

The data presented in Table 12 showed that the medium S1 resulted in maximum percentage of normal shoots i.e. 86.63%, S2 resulted in 70.02% normal shoots and the medium S3 resulted in 43.28% normal shoots. All the three treatments were significantly different from each other. However, the maximum percentage of vitrified shoots was observed in S3 i.e. 56.60% whereas S2 resulted in 30.00% vitrified shoots and the minimum percentage of vitrified shoots were observed in medium S1 i.e. 13.28%.

Table12. Effect of different strength MS basal medium on vitrification in carnation

S. No	Medium code	Medium Strength	Percentage of normal shoots	Percentage of vitrified shoots
1	S1	Full strength	86.63(68.55)	13.28(21.38)
2	S2	$\frac{1}{2}$ strength	70.02(56.80)	30.00(33.21)
3	S3	$\frac{1}{4}$ strength	43.28(41.14)	56.60(48.80)
CD _{0.05}			7.31	9.80

Values expressed in the parenthesis are the arc sine transformation of percentage

The concentration of different ions was also changed in the medium. Different concentration of ammonium nitrate, potassium and calcium ions was used in the culture medium.

Three different concentrations of each of the ion were used in the medium. The explant was cultured on the medium and the observations were recorded after four weeks of culturing and the results are presented in Table 13.

The data presented in Table13 showed that the maximum percentage of normal shoots was observed in medium C₀ i.e.86.27%. C5 resulted in 40.03% normal shoots which was statistically at par with C6 (36.59%).Minimum

percentage of normal shoots was observed in C1 i.e. 20.03% which was significantly different from all other treatments.

Table 13. Effect of different concentrations of macrosalts on vitrification in carnation

Medium Code	MS basal medium supplemented with different concentration of macrosalts(mg l ⁻¹)			Percentage of normal shoots	Percentage of vitrified shoots
	NH ₄ NO ₃	KNO ₃	CaCl ₂ .2H ₂ O		
C ₀	1650.00	1900.00	440.00	86.90(68.78)	13.28(21.38)
C1	825.00	1900.00	440.00	20.03(26.59)	79.17(62.85)
C2	3300.00	1900.00	440.00	60.10(50.83)	39.37(39.21)
C3	1650.00	950.00	440.00	46.45(42.97)	53.34(46.92)
C4	1650.00	3800.00	440.00	56.45(48.71)	43.26(41.13)
C5	1650.00	1900.00	220.00	40.03(39.25)	59.77(50.63)
C6	1650.00	1900.00	880.00	36.59(37.22)	63.18(52.65)
CD _{0.05}				3.05	4.65

Values expressed in the parenthesis are the arc sine transformation of percentage

Maximum percentage of vitrified shoots was observed in C1 i.e.79.17%. Medium C4 resulted in 56.45% vitrified shoots which was statistically at par with C2 (60.10%).The minimum percentage of vitrified shoots was observed in C₀ i.e.13.28%.

4.1.5 Effect of sucrose concentration

For this experiment different concentration of sucrose i.e. 20 g l⁻¹, 30 g l⁻¹, 40 g l⁻¹ and 60 g l⁻¹ were used in the culture medium. The explant was cultured on the medium with different concentration of sucrose and the observations were recorded after four weeks of culturing.

The data presented in the Table 14 showed that D2 resulted in maximum percentage of normal shoots (75.03%) statistically different from other treatments. The minimum percentage of normal shoots was observed in D3 (56.00%) which was statistically at par with D1 (56.03%).The maximum percentage of vitrified shoots was observed in the treatment D1 i.e. 44.03% which was statistically at par with D3 i.e. 44.03% whereas the minimum

percentage of vitrified shoots was observed on D2 i.e. 25.03% which was significantly different from rest of the treatments.

Table 14. Effect of different concentration of sucrose on vitrification in carnation

S. No	Medium code	MS medium supplemented with different concentrations of sucrose (g l ⁻¹)	Percentage of normal shoots	Percentage of vitrified shoots
1	D1	20	56.03(48.47)	44.03(41.57)
2	D2	30	75.03(60.02)	25.03(30.02)
3	D3	40	56.00(48.455)	44.03(41.57)
4	D4	60	59.97(50.75)	40.10(39.29)
CD _{0.05}			1.79	1.60

Values expressed in the parenthesis are the arc sine transformation of percentage

In the same experiment different concentration of sucrose was used along with different concentration of agar in the medium and the observations were recorded after four weeks of culturing and data is presented Table 15.

Table 15. Effect of different concentration of sucrose and agar on vitrification in carnation

S. No.	Medium Code	Concentration of sucrose (g l ⁻¹)	Concentration of agar (g l ⁻¹)	Percentage of normal shoots	Percentage of vitrified shoots
1	S1	20	8	32.36(34.67)	68.10(55.61)
2	S2	20	10	64.02(53.15)	36.00(36.87)
3	S3	20	12	56.02(48.46)	44.03(41.57)
4	S4	30	8	38.99(38.64)	61.00(51.36)
5	S5	30	10	27.97(31.93)	72.14(58.14)
6	S6	30	12	75.02(60.01)	25.03(30.02)
7	S7	40	8	40.14(39.30)	59.97(50.75)
8	S8	40	10	60.03(50.79)	40.11(39.30)
9	S9	40	12	56.00(48.45)	44.03(41.57)
10	S10	60	8	32.08(34.50)	68.00(55.55)
11	S11	60	10	51.97(46.13)	48.03(43.87)
12	S12	60	12	59.95(50.74)	40.02(39.24)
CD _{0.05}				1.95	1.45

Values expressed in the parenthesis are the arc sine transformation of percentage

The data presented in Table 15 showed that the medium S6 resulted in maximum percentage of normal shoots (75.02%). Treatment S1 resulted in 32.36% normal shoots which was statistically at par with S10 (32.08%) and S8 (60.03%) was statistically at par with S12 (59.95%). However the maximum percentage of vitrified shoots was observed in the medium S5 i.e. 72.14% and medium S3 resulted in 44.03% vitrified shoots which is statistically at par with S9 i.e. 44.03%. The minimum percentage of vitrified shoots was observed on the medium S6 i.e. 25.03%.

4.2 *IN VITRO* ROOTING

The experiment was carried out to study the effect of different treatments (1.0 or 2.0 mg l⁻¹ each of NAA, IBA, IAA) on rooting of *in vitro* raised shoots. The data presented in Table 16 showed that different concentration of auxins had a significant effect on average number of roots per shoot and average number of shoots producing roots as compared to control which yielded no response. The maximum average number of roots per shoot was observed in RM5 (2.0 mg l⁻¹ IBA) treatment i.e. 8.22 and maximum percent rooting was also observed in RM5 (2.0 mg l⁻¹ IBA) i.e. 90.66% which was statistically at par with RM4 (MS basal+1.0 mg l⁻¹ IBA). Average number of shoots producing roots was found to be 22.00 which were statistically at par with RM4 (1.0 mg l⁻¹ IBA) treatment i.e. 21.00 shoots producing roots and 7.66 roots per shoot (Table 16).

The minimum average number of roots per shoot was achieved with treatment RM6 (1.0 mg l⁻¹ NAA) i.e. 2.92. RM7 (2.0 mg l⁻¹ NAA) resulted in 4.00 roots per shoot which was statistically at par with RM2 (1.0 mg l⁻¹ IAA) i.e. 4.11 and RM3 (2.0 mg l⁻¹ IAA) i.e. 5.00 treatment.

The minimum percent rooting was observed in RM6 (1.0 mg l⁻¹ NAA) i.e. 41.62% which was statistically different from other treatments. The minimum average number of shoots producing roots was observed in RM6 (1.0 mg l⁻¹ NAA) i.e. 9.00 which was statistically at par with RM7 (2.0 mg l⁻¹ NAA).

Table 16. Effect of different rooting media on average number of shoots producing roots and average number of roots per shoot in carnation

Sr. No.	Treatment	Average number of shoots producing roots	Average number of roots per shoot	Percent rooting
1	RM1	0.00	0.00	0.00(0.00)
2	RM2	18.00	4.11	75.10(60.07)
3	RM3	16.00	5.00	66.05(54.37)
4	RM4	21.00	7.66	83.93(66.38)
5	RM5	22.00	8.22	90.60(72.17)
6	RM6	9.00	2.92	41.62(40.18)
7	RM7	11.00	4.00	45.98(42.69)
CD _{0.05}		2.56	2.56	1.14

Values expressed in the parenthesis are the arc sine transformation of percentage

4.3 HARDENING

After four weeks of incubation on MS rooting medium the roots of well established plantlets were washed thoroughly to remove sticking medium. The plantlets were dipped in 0.01% carbendazim (bavistin) solution for 15-20 min and transferred to plastic cups containing cocopeat for the hardening of regenerated plantlets. After four weeks, the observations were recorded for percent survival of plantlets upon transfer to plastic cups.

The results presented in Table 17 showed that the percent survival of plantlets was 70% in cocopeat.

Table 17. Per cent survival of plantlets after four weeks of transfer in cocopeat

Potting mixture	Survival of plantlets (%)
Cocopeat	70

Plate 1. Shoot multiplication of carnation on MS medium supplemented with 1.0 mg l⁻¹ BA in carnation

- a) Explant on zero day of culturing
- b) Shoots after one week of culturing
- c) Shoots after two weeks of culturing
- d) Fully grown shoots after four weeks

PLATE 1

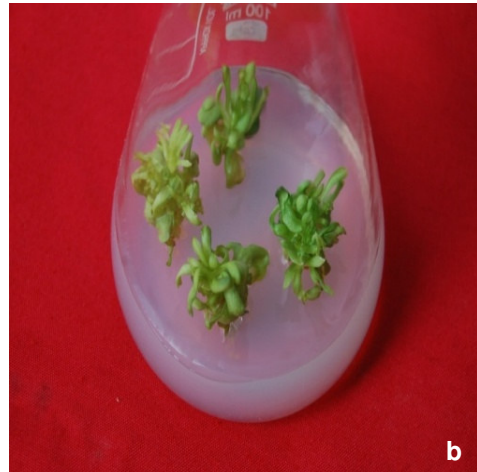


Plate 2. *In vitro* raised shoots of carnation cultured on the MS medium supplemented with different concentrations of agar

- a) Shoots cultured on the MS medium supplemented with 0.8% agar+ 2.0 mg l⁻¹ BA
- b) Shoots cultured on the MS medium supplemented with 1% agar+ 2.0 mg l⁻¹ BA
- c) Shoots cultured on the MS medium supplemented with 1.2% agar+ 2.0 mg l⁻¹ BA

PLATE 2

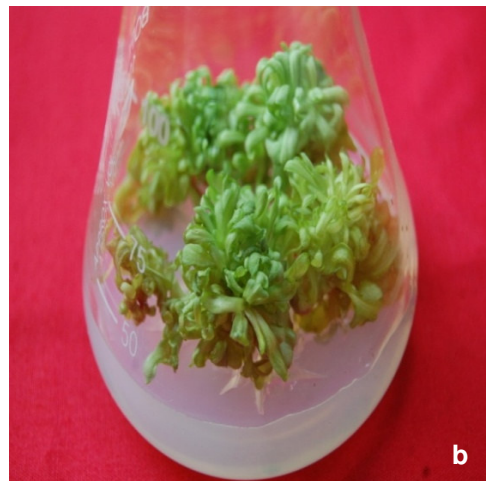


Plate 3. *In vitro* raised shoots of carnation cultured on the MS medium supplemented with different concentrations of growth regulators

- a) Shoots cultured on the medium supplemented with MS basal+1.0 mg l⁻¹ IAA
- b) Shoots cultured on the medium supplemented with MS basal+2.0 mg l⁻¹ IAA
- c) Shoots cultured on the medium supplemented with MS basal+1.0 mg l⁻¹ TDZ
- d) Shoots cultured on the medium supplemented with MS basal+2.0 mg l⁻¹ TDZ
- e) Shoots cultured on the medium supplemented with MS basal+1.0 mg l⁻¹ TDZ + 1.0 mg l⁻¹ IAA
- f) Shoots cultured on the medium supplemented with MS basal+2.0 mg l⁻¹ TDZ + 1.0 mg l⁻¹ IAA

PLATE 3

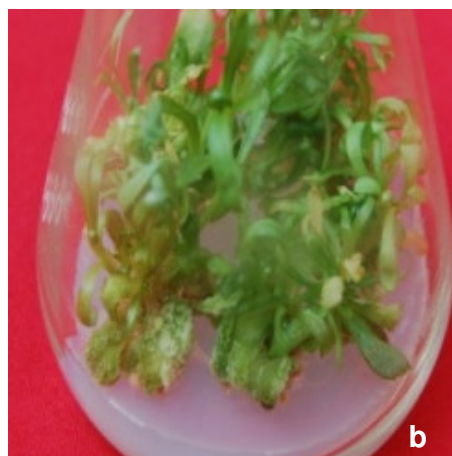


Plate 4. *In vitro* raised shoots of carnation cultured on the MS medium supplemented with different concentrations of growth regulators

- a) Shoots cultured on the medium supplemented with MS basal + 1.0 mg l^{-1} zeatin
- b) Shoots cultured on the medium supplemented with MS basal + 2.0 mg l^{-1} zeatin
- c) Shoots cultured on the medium supplemented with MS basal + 2.0 mg l^{-1} zeatin + 1.0 mg l^{-1} IAA
- d) Shoots cultured on the medium supplemented with MS basal + 1.0 mg l^{-1} zeatin + 1.0 mg l^{-1} IAA
- e) Shoots cultured on the medium supplemented with MS basal+ 1.0 mg l^{-1} kinetin
- f) Shoots cultured on the medium supplemented with MS basal+ 1.5 mg l^{-1} kinetin

PLATE 4



Plate 5. *In vitro* raised shoots of carnation cultured on the MS medium supplemented with different concentrations of growth regulators

- a) Shoots cultured on the medium supplemented with MS basal+2.0 mg l⁻¹ kinetin
- b) Shoots cultured on the medium supplemented with MS basal+1.0 mg l⁻¹ IBA
- c) Shoots cultured on the medium supplemented with MS basal+1.5 mg l⁻¹ IBA
- d) Shoots cultured on the medium supplemented with MS basal+2.0 mg l⁻¹ IBA
- e) Shoots cultured on the medium supplemented with MS basal+1.0 mg l⁻¹ IAA
- f) Shoots cultured on the medium supplemented with MS basal+1.5 mg l⁻¹ IAA

PLATE 5

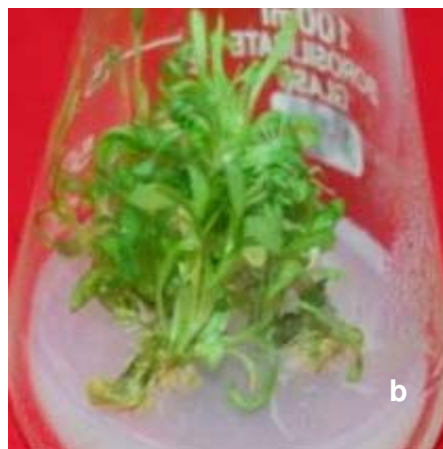


Plate 6. *In vitro* raised shoots cultured on the MS medium supplemented with different concentrations of growth regulators

- a) Shoots cultured on the medium supplemented with MS basal+2.0 mg l⁻¹ IAA
- b) Shoots cultured on the medium supplemented with MS basal+1.0 mg l⁻¹ BA
- c) Shoots cultured on the medium supplemented with MS basal+1.5 mg l⁻¹ BA
- d) Shoots cultured on the medium supplemented with MS basal+2.0 mg l⁻¹ BA

PLATE 6



Plate 7. Different culture vessels used for culturing *in vitro* raised shoots of carnation

- a) *In vitro* raised shoots in conical flask
- b) *In vitro* raised shoots in culture tube
- c) *In vitro* raised shoots in culturing jar

PLATE 7

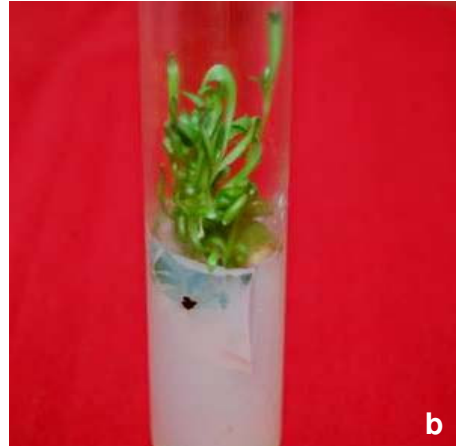


Plate 8. *In vitro* raised shoots of carnation cultured on different strength MS medium

- a) Shoots cultured on full strength MS basal + 1.0 mg l⁻¹ zeatin + 1.0 mg l⁻¹ IAA
- b) Shoots cultured on ½ strength MS basal + 1.0 mg l⁻¹ zeatin + 1.0 mg l⁻¹ IAA
- c) Shoots cultured on ¼ strength MS basal + 1.0 mg l⁻¹ zeatin + 1.0 mg l⁻¹ IAA

PLATE 8



Plate 9. *In vitro* raised shoots of carnation cultured on MS medium with different concentrations of ammonium nitrate

- a) Shoots cultured on MS medium supplemented with 825.00 mg l⁻¹ ammonium nitrate
- b) Shoots cultured on MS medium supplemented with 1650.00 mg l⁻¹ ammonium nitrate
- c) Shoots cultured on MS medium supplemented with 3300.00 mg l⁻¹ ammonium nitrate

PLATE 9



Plate 10. *In vitro* raised shoots of carnation cultured on MS medium with different concentrations of potassium nitrate

- a) Shoots cultured on MS medium supplemented with 950.00 mg l⁻¹ potassium nitrate
- b) Shoots cultured on MS medium supplemented with 1900.00 mg l⁻¹ potassium nitrate
- c) Shoots cultured on MS medium supplemented with 3800.00 mg l⁻¹ potassium nitrate

PLATE 10



Plate 11. *In vitro* raised shoots of carnation cultured on MS medium with different concentrations of calcium chloride

- a) Shoots cultured on MS medium supplemented with 220.00 mg l⁻¹ calcium chloride
- b) Shoots cultured on MS medium supplemented with 440.00 mg l⁻¹ calcium chloride
- c) Shoots cultured on MS medium supplemented with 880.00 mg l⁻¹ calcium chloride

PLATE 11



Plate 12. *In vitro* raised shoots of carnation cultured on MS medium supplemented with different concentrations of sucrose

- a) Shoots cultured on MS medium supplemented with 20 g l⁻¹ sucrose+ 2.0 mg l⁻¹ BA
- b) Shoots cultured on MS medium supplemented with 30 g l⁻¹ sucrose+ 2.0 mg l⁻¹ BA
- c) Shoots cultured on MS medium supplemented with 40 g l⁻¹ sucrose+ 2.0 mg l⁻¹ BA
- d) Shoots cultured on MS medium supplemented with 60 g l⁻¹ sucrose+ 2.0 mg l⁻¹ BA

PLATE 12

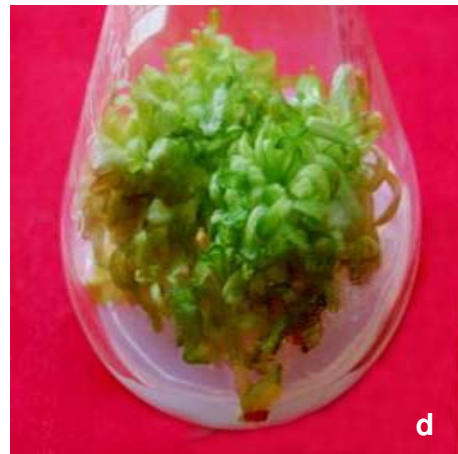


Plate 13. Rooting of *in vitro* raised shoots of carnation

- a) *In vitro* raised shoots on rooting medium (1/2 strength MS basal +2.0 mg l⁻¹ IBA+0.2% activated charcoal)
- b) Rooting of *in vitro* raised shoots on rooting medium (1/2 strength MS basal +2.0 mg l⁻¹ IBA+0.2% activated charcoal)
- c) Rooting of *in vitro* raised shoots on rooting medium (1/2 strength MS basal +2.0 mg l⁻¹ IBA+0.2% activated charcoal)

PLATE 13





Plate 14: Complete Microplant

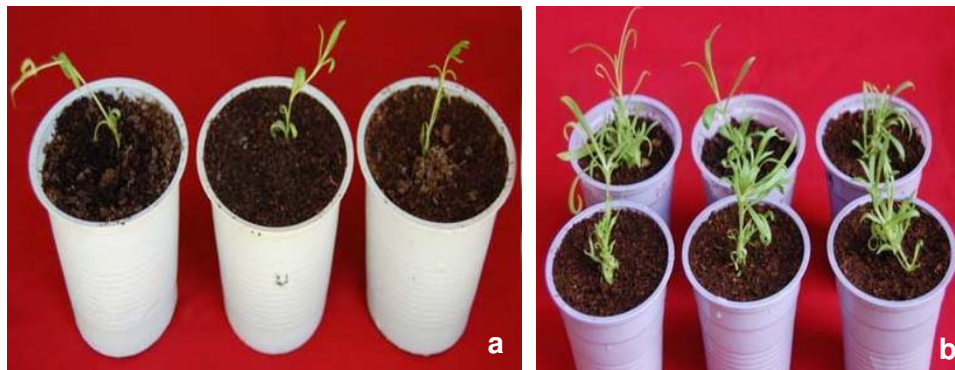


**Plate 15: a) Hardened plants of carnation after one week
b) Hardened plants of carnation after four weeks**



Plate 16. Hardened Plants After Six Weeks

Chapter-5

DISCUSSION

The most extensive and viable application of plant tissue culture is micropropagation, which produces disease free planting material in shorter period from a single individual plant. Propagation of carnation on large scale through tissue culture has successfully been standardized for the rapid multiplication. However, vitrification is still a major problem that causes great loss in both quality and quantity of the produce. Vitrification is detrimental to successful transplanting of these plantlets to field.

In the present investigation attempts were made to standardize the “Various factors affecting vitrification in carnation”. The results of present studies on the factors affecting vitrification in carnation have been discussed in this chapter.

5.1 Factors affecting vitrification

5.1.1 Effect of agar concentration

5.1.2 Effect of growth regulators concentration

5.1.3 Effect of culture vessel

5.1.4 Effect of nutrient medium

5.1.5 Effect of sucrose concentration

5.2 *In vitro* rooting and hardening

5.1 FACTORS AFFECTING VITRIFICATION

5.1.1 Effect of agar concentration

Agar concentration in the medium is one of the important factor which affect vitrification. In present study different concentrations of agar viz. 0.8%, 1% and 1.2% were used in the culture medium. Out of these concentrations 1.2% agar was found to be best concentration resulting in maximum percentage of normal shoots (74.43%). The studies revealed that with an increase in the agar

concentration the rate of vitrification decreases. Similar results were observed by Choudhary and Prakash (1991) in carnation. They reported that percentage of vitrified shoots decreased in culture medium with increasing agar concentration.

Dolev *et al.* (1986) also reported reduction in the number of vitrified shoots with an increase in agar concentration in the medium when they cultured carnation meristem tips in shoot proliferating medium. Delbergh *et al.* (1981) in globe artichoke cultures found decrease in vitrification with an increase in agar concentration. Pasqualetto *et al.* (1986) reported a decrease in percentage of vitrification in “Gala” apple with an increase in concentration of agar or gelrite.

The maximum percentage of vitrification was observed with an agar concentration of 0.8%. The medium supplemented with 0.8% agar resulted in 60.67% vitrified shoots. These results were supported by the results obtained by Kim *et al.* (1988) who observed vitreous plantlets of carnation characterized by tender, yellow green and translucent leaves on a medium containing 0.7% agar in the medium. Li *et al.* (1997) also recorded highest number of vitrified shoots with a reduced or lower concentration of agar i.e. 0.3% agar.

The medium supplemented with 1% agar was also found to be better resulting in 71.47% normal shoots with reduced vitrification. Hakkart and Versluijs (1983) used 1% agar concentration in medium to reduce the glassiness to an acceptable level in carnation cv. Sam’s Pride.

5.1.2 Effect of growth regulators

Plant growth regulators are very important component of the culture medium. The concentrations of auxins and cytokinins in the medium affect the rate of vitrification. Cytokinins have been shown to induce hyperhydricity. Among cytokinins, kinetin led to produce longer shoots as compared to BAP and as level of cytokinins increased the height of shoots decreased. BAP induces the loss of apical dominance and accelerate the growth of lateral buds.

The results of previous studies have shown that application of cytokinins stimulated sprouting and development of higher number of axillary shoots and

apical dominance release was suppressed with increase of cytokinin concentration (Kapchina Toteva and Yakimova 1997).

In present studies different concentrations of auxins (IAA, IBA and NAA) and cytokinins (TDZ, zeatin, BA and kinetin) were used in the culture medium to study their effect on vitrification. The maximum percentage of normal shoots was obtained on the medium supplemented with 1.0 mg l^{-1} zeatin + 1.0 mg l^{-1} IAA followed by the MS basal medium supplemented with 2.0 mg l^{-1} zeatin.

Maximum percentage of vitrification was observed in medium supplemented with TDZ. All the shoots on TDZ containing medium were found to be vitrified. The results were in conformity with the results obtained by Ahmed *et al.* (2006) who reported that vitrification was more acute on TDZ containing medium. The studies also revealed that the medium containing kinetin also resulted in reduced vitrification as compared to BA. The results were supported by the results obtained by Mujib and Pal (1995) on carnation cv. Royal Crimson. They obtained more vitrified shoots with BA as compared to kinetin in the culture medium.

Chen *et al.* (1998) obtained maximum number of glaucous plantlets on half MS medium supplemented with 0.5 ppm BA. Miroshinichenko *et al.* (1998) while studying the effect of different cytokinins concluded that BA promote premature senescence of explants and shoot multiplication. Similar results were found by Pasqualetto (1986) who reported that higher BA level generally increased vitrification.

5.1.3 Effect of culture vessel

Hakkart and Versluijs (1983) reported that size and type of culture vessel, the type of closure affects the production of vitrified plants. Culture vessel is also an important factor which affects vitrification. The looser type of culture vessels (cotton wool, metal caps, steri tops) were found better than the tighter ones (aluminium foil and parafilm). Application of permeable closure successfully reduced hyperhydricity. Cotton wool gave possibility in increasing gas exchange

potential that occurred between inside and outside of culture vessel reducing relative humidity and water potential (Delbergh *et al* 1981, Hakkart and Versluijs (1983).

Increasing the potential of gas exchange between the external and internal atmospheric condition of culture vessel and lowering the high humidity lead to improve CO₂ concentration in the culture vessel. Culture tubes with non absorbent cotton reduced the vitrification and plantlets turned green and developed normally. This is because of the beneficial effect of an evaporation period on vitrification due to an interaction between reduced relative humidity and enhanced gas exchange.

In present studies different type of culture vessels viz. conical flasks, culture tube and plastic jars were used for culturing. Maximum percentage of normal shoots was observed in conical flasks i.e. 86.00%. Culture tubes resulted in 76.62% normal shoots. It was revealed from the present study that the looser closure of culture vessel results in less vitrification as compared to the vessels with tight closure. Maximum vitrification was observed in the plastic jars i.e. 60.05%.

The results obtained from present study were in conformity with the results obtained by Cuzzol *et al.* (1996) who reported that MS medium supplemented with 4.0 g l⁻¹ gelrite + 0.5 mg l⁻¹ NAA in the conical flasks plugged with cotton wool resulted in the production of non vitreous carnation plantlets. Majada *et al.* (2001) cultured carnation cv. Nelkon *in vitro* in air tight or ventilated vessels resulted in the production of hyperhydrated plants.

Jo *et al.* (2002) observed the effect of sealing material of culture vessel on growth and vitrification of carnation plantlets *in vitro*. Best growth and development of plantlets were obtained from explants cultured in the vessel having cotton lid compared with aluminium foil. Winarto *et al.* (2004) observed the effects of permeable vessel closure on reduction of hyperhydricity in *in vitro* culture of carnation. They observed that vessel with looser closure like cotton wool resulted in lowest incidence of hyperhydricity.

5.1.4 Effect of nutrient medium

Nutrient medium is also one of the major factor influencing the rate of vitrification in carnation. In present study the effect of nutrient medium was studied using different strength medium and by changing the concentrations of different macromolecules in the medium. The concentrations of ammonium nitrate, potassium and calcium ions were used in the nutrient medium and their effect was studied. It was revealed from the present study that the nutrient medium with full MS salts gave best results resulting in maximum percentage of normal shoots (86.63%). However, maximum vitrification (56.60%) was observed in ¼ strength nutrient medium.

Maximum vitrification was observed in the medium having double concentration of ammonium nitrate ions (79.17%). The results supported the findings of Beauchesne (1981), and Tsay and Drew (1998) who reported that high ammonium nitrate in particular is responsible for increasing the amount of vitrification. Similar results were obtained by Letouze and Daugin (1983) in *Prunus avium* and *Salix babylonica* with increased concentration of ammonical salts in the culture medium. Large quantities of ammonium ions, as present in MS medium, were found to increase vitrification, mainly of woody species, by causing toxification of the activities of some enzymes involved in the synthesis of lignin (Vieitez *et al.* 1985; Daugin and Leuouze, 1986; Phan and Hegedus, 1986).

The present study revealed that rate of vitrification decreased by lowering the ammonium nitrate concentration but increased when calcium chloride concentration was increased in the medium. The results were supported by the findings of Choudhary and Prakash (1991) who reported that vitrification was reduced by lowering concentration of ammonium nitrate but rate of vitrification increased when concentration of calcium chloride was doubled.

5.1.5 Effect of sucrose concentration

The cultured plant tissues need a continuous supply of carbohydrates from medium to encourage growth and to survive *in vitro*. Therefore, sugar such as

sucrose is added as carbon source. Sucrose concentration also affects the rate of vitrification.

In present studies different concentrations of sucrose were used in the culture medium i.e. 20 g l⁻¹, 30 g l⁻¹, 40 g l⁻¹ and 60 g l⁻¹. Out of the different concentrations used the best concentration was found to be 30 g l⁻¹ resulting in maximum percentage of normal shoots. This concentration was found to be ideal concentration of sucrose in the medium to reduce the vitrification. The results obtained were in conformity with Kim *et al.* (1988) who observed normal carnation plants with a sucrose concentration of 30 g l⁻¹ in the culture medium. Tsay and Drew (1998) also obtained similar results with controlled vitrification in carnation during *in vitro* multiplication when the medium was supplemented with 30 g l⁻¹ sucrose.

While the maximum percentage of vitrification was observed in the medium having a high concentration of sucrose i.e. 40 g l⁻¹ (44.03%) and 60 g l⁻¹ (40.10%). As the concentration of sucrose was increased in the medium rate of vitrification was also increased. Ziv *et al.* (1983) also reported production of glaucous leaf in carnation as a result of increase in sucrose concentration in the medium.

In present studies different concentrations of sucrose (20 g l⁻¹, 30 g l⁻¹, 40 g l⁻¹ and 60 g l⁻¹) along with different concentrations of agar (0.8%, 1% and 1.2%) were used in the culture medium. The maximum percentage of normal shoots (75.02%) was obtained with the combination 30 g l⁻¹ sucrose+ 1.2% agar and maximum vitrification (72.14%) was observed with 30 g l⁻¹ +0.8% agar.

5.2 IN VITRO ROOTING AND HARDENING

Auxins play a major role in root induction through its effect on the first cell division which forms root initial (Farooq *et al.*, 2008). Plant growth regulators significantly influenced the height and number of produced roots.

The *in vitro* raised shoots were rooted on half strength MS basal medium containing 1 or 2 mg l⁻¹ IAA, IBA and NAA alone and their effect on average

number of shoots producing roots and average number of roots per shoot was investigated. The maximum average number of shoots and maximum number of roots per shoot was found with the medium consisting of 2.0 mg l⁻¹ IBA and 0.2 percent activated charcoal. These results are in agreement with Mehta *et al.* (2007) who reported well developed root system on liquid MS medium supplemented with 2.0 mg l⁻¹ IBA and 0.2 percent activated charcoal. Kanwar and Kumar (2009) reported best rooting on MS medium supplemented with 2.0 mg l⁻¹ IBA and 0.2 percent activated charcoal.

Choudhary and Prakash (1992) obtained roots on 0.5µM IBA, whereas, Jagannatha *et al.* (2001) found 20µM IBA as better treatment for rooting of two cultivars of carnation viz. Sterile Dop and IAHS-22. Wankhede *et al.* (2006) found half strength MS basal medium with 0.8, 1.0 mg l⁻¹ IBA as the superior treatment for root initiation in carnation cv. Supergreen. Bora *et al.* (2007) obtained best rooting of the microshoots on MS medium with 0.5 mg l⁻¹ IBA in carnation cv. Lipstick. Ahmad *et al.* (2008) reported maximum rooting (90.66%) in shootlets cultured in MS medium supplemented with 0.5 mg l⁻¹ IBA.

On the other hand Ahmad *et al.* (2006) obtained *in vitro* rooting in elongated microshoots of carnation cv. Finest Mix on MS medium supplemented with 1.0µM NAA. Similarly Ali *et al.* (2008) reported best rooting response in carnation on MS medium supplemented with 1.0 mg l⁻¹ NAA. Kharrazi *et al.* (2011) obtained *in vitro* rooting of two carnation cvs. Eskimo Mogr and Innove Orange Bogr on the MS medium supplemented with 0.5 mg l⁻¹ NAA and 1.5 mg l⁻¹ IBA.

The rooted plantlets were acclimatized initially in the plastic cups containing cocopeat for four weeks and the percent survival was recorded. The results indicated about 60% survival of plants during their acclimatization. Salehi (2006) successfully acclimatized the plantlets of 13 cultivars of carnation in pots containing a mixture of sand, leaf mold and vermiculite in 1:1:1 ratio. Kanwar and Kumar (2009) reported 76% survival success when plantlets of carnation cv. Indios were hardened and transferred to earthen pots containing mixture of sand, soil and FYM in 1:1:1 ratio.

Chapter-6

SUMMARY AND CONCLUSION

The present investigation on “Effect of different factors on vitrification in carnation” was conducted with a view to standardize the factors affecting vitrification in carnation. The important findings of present investigations are summarized below:

1. The shoots regenerated from nodal explant were used as explant for studying the factors affecting vitrification.
2. To study the effect of agar concentration three concentrations of agar viz. 0.8%, 1% and 1.2% were used in culture medium.
3. Maximum percentage of normal shoots was obtained from the medium supplemented with 1.2% agar. Maximum vitrified shoots were obtained from medium containing 0.8% agar.
4. Out of the different culture vessels used conical flasks proved to be the best resulting in the maximum percentage of normal shoots whereas the maximum vitrification was observed in the plastic jars.
5. Different strength medium viz. full strength, $\frac{1}{2}$ strength and $\frac{1}{4}$ strength medium was used. Full strength medium was found to be best resulting in the maximum percentage of normal shoots whereas the maximum vitrification was observed in $\frac{1}{4}$ strength medium.
6. The concentrations of different macrosalts like ammonium nitrate, potassium nitrate and calcium chloride was also changed in the medium. Maximum percentage of normal shoots was observed in the medium having the
7. While studying the effect of sucrose concentration different concentrations of sucrose viz. 20 g l^{-1} , 30 g l^{-1} , 40 g l^{-1} and 60 g l^{-1} was used in the medium. Maximum percentage of normal shoots was observed in the medium having 30 g l^{-1} sucrose and maximum vitrification was observed in the medium having 20 g l^{-1} sucrose.

8. Different concentrations of sucrose were used with different concentrations of agar. The medium having 30 g l⁻¹ sucrose and 1.2% agar resulted in the maximum percentage of normal shoots. Maximum vitrified shoots was observed in the medium having 30 g l⁻¹ sucrose and 0.8% agar.
9. Different concentrations of auxins and cytokinins were used alone and in combination to study the effect of growth regulators. Maximum percentage of normal shoots was observed in the medium having 1.0 mg l⁻¹ zeatin +1.0 mg l⁻¹ IAA. Maximum vitrification was observed in the medium with 1.0 mg l⁻¹ TDZ.
10. To obtain *in vitro* rooting different auxins viz. IAA, NAA and IBA were used in the half strength medium with 0.2% activated charcoal.
11. The maximum percent rooting was observed in the rooting medium having 2.0 mg l⁻¹ IBA and the minimum percent rooting was observed in the medium having 1.0 mg l⁻¹ NAA.
12. Maximum average number of shoots producing roots and maximum average number of roots per shoot was obtained in the medium having 2.0 mg l⁻¹ IBA.
13. After rooting the rooted plantlets were hardened and 70% survival of the plants was recorded after four weeks of hardening.

From the present investigation it can be concluded that different factors viz. agar concentration, sucrose concentration, growth regulators, nutrient medium and culture vessel affect the rate of vitrification. On the basis of present investigation, the medium containing 1.2% agar, 30 g l⁻¹ sucrose and MS full strength medium was found to be the best medium resulting in the decreased rate of vitrification. The vessel with looser closure i.e. conical flask proved ideal culture vessel. Thus it is concluded that by standardizing the different factors affecting vitrification the rate of vitrification can be reduced. This will result in the normal and healthy plantlets which can be easily transplanted to the soil.

Chapter-7

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**DR Y S PARMAR UNIVERSITY OF HORTICULTURE AND FORESTRY
NAUNI, SOLAN 173 230 (H P) INDIA**

DEPARTMENT OF BIOTECHNOLOGY

Title of thesis : “Effect of different factors on vitrification in carnation”
Name of the student : Pushpa Kumari
Admission Number : H- 2009- 06- M
Major Advisor : Dr. S. K. Sharma
Major Field : Biotechnology
Minor Field (s) : i)Floriculture
ii)Biochemistry
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ABSTRACT

The present investigation entitled “Effect of different factors on vitrification in carnation” different factors viz. agar concentration, sucrose concentration, growth regulators concentration, culture vessel and nutrient medium were standardized using already multiplied shoots of carnation. The explant was cultured on the medium with different concentrations of agar (0.8%, 1% and 1.2%). The maximum percentage of normal shoots was obtained with 1.2% agar while maximum vitrification was observed with 0.8% agar. Different concentrations of sucrose (20 g l⁻¹, 30 g l⁻¹, 40 g l⁻¹ and 60 g l⁻¹) alone or in combination with different concentrations of agar were used in the culture medium. The medium containing 30 g l⁻¹ sucrose with 1.2% agar resulted in maximum percentage of normal shoots. Different concentrations of growth regulators were used in the culture medium. The best results with maximum percentage of normal shoots was achieved with medium having 1.0 mg l⁻¹ zeatin + 1.0 mg l⁻¹ IAA. The medium containing 1.0 mg l⁻¹ TDZ resulted in maximum number of vitrified shoots. Out of the three different culture vessels viz. conical flask, culture tube, jar used conical flasks proved best resulting in maximum number of normal shoots. The effect of nutrient medium was studied by using different strength MS medium(Full strength, ½ strength and ¼ strength) and by changing the concentration of different macrosalts in the medium. Full strength MS medium resulted in maximum percentage of normal shoots and the maximum vitrification was observed in ¼ strength MS medium. The normal shoots were rooted on the rooting medium with different concentrations of auxins. The maximum average number of shoots producing roots and maximum average number of roots per shoot was obtained on MS medium supplemented with 2.0 mg l⁻¹ IBA and 0.2% activated charcoal. The rooted plantlets were hardened and 70% survival of plantlets was recorded after 30 days of their transfer to pots.

Signature of Major Advisor

Signature of the Student

Countersigned

**Professor and Head
Department of Biotechnology
Dr. Y.S.P, UHF, Nauni, Solan (H.P.)**

APPENDIX-I

Composition of Murashige and Skoog's (1962) basal medium (MS medium)

Constituents	Amount (mg/l)
A. INORGANIC CONSTITUENTS	
(i) Macronutrients	
NH ₄ NO ₃ (Ammonium nitrate)	1650.00
KNO ₃ (Potassium nitrate)	1900.00
CaCl ₂ .2H ₂ O (Calcium chloride)	440.00
MgSO ₄ .7H ₂ O (Magnesium sulphate)	370.00
KH ₂ PO ₄ (Potassium dihydrogen <i>ortho</i> -phosphate)	170.00
(ii) Micronutrients	
H ₃ BO ₃ (Boric acid)	6.200
MnSO ₄ .4H ₂ O (Manganese sulphate)	22.300
ZnSO ₄ .7H ₂ O (Zinc sulphate)	8.600
KI (Potassium iodide)	0.830
Na ₂ MoO ₄ .2H ₂ O (Sodium molybdate)	0.250
CuSO ₄ .5H ₂ O (Cupric sulphate)	0.025
CoCl ₂ .6H ₂ O (Cobalt chloride)	0.025
C ₁₀ H ₁₄ N ₂ O ₈ Na ₂ .2H ₂ O (Sodium EDTA)	37.300
FeSO ₄ .7H ₂ O (Ferrous sulphate)	27.800
B. ORGANIC NUTRIENTS	
C ₈ H ₁₁ NO ₃ .HCl (Pyridoxine-HCl)	0.500
C ₂ H ₅ NO ₂ (Glycine)	2.000
C ₁₂ H ₁₇ CIN ₄ OS.HCl(Thiamine-HCl)	0.100
C ₆ H ₅ NO ₂ (Nicotinic acid)	0.500
C ₆ H ₁₂ O ₆ (Inositol)	100.00

APPENDIX-II

Analysis of the variance for effect of different concentrations of agar on the percent normal shoots produced in carnation cv. Yellow Dot Com- Table 9

Source	Degree of freedom	Mean sum of square	F value
Treatment	2	433.18	6087.51
Error	6	0.071	
Total	8		

Analysis of the variance for effect of different concentrations of agar on the percent vitrified shoots produced in carnation cv. Yellow Dot Com- Table 9

Source	Degree of freedom	Mean sum of square	F value
Treatment	2	410.95	9102.82
Error	6	0.045	
Total	8		

Analysis of the variance for effect of different concentrations of growth regulators on the percent normal shoots produced in carnation cv. Yellow Dot Com- Table 10

Source	Degree of freedom	Mean sum of square	F value
Treatment	21	847.78	5843.51
Error	44	0.145	
Total	65		

Analysis of the variance for effect of different concentrations of growth regulators on the percent vitrified shoots produced in carnation cv. Yellow Dot Com- Table 10

Source	Degree of freedom	Mean sum of square	F value
Treatment	21	767.32	161.58
Error	44	4.749	
Total	65		

Analysis of the variance for effect of different culture vessels on the percent normal shoots produced in carnation cv. Yellow Dot Com- Table 11

Source	Degree of freedom	Mean sum of square	F value
Treatment	2	677.49	36900.86
Error	6	0.018	
Total	8		

Analysis of the variance for effect of different culture vessels on the percent vitrified shoots produced in carnation cv. Yellow Dot Com- Table 11

Source	Degree of freedom	Mean sum of square	F value
Treatment	2	680.27	74249.18
Error	6	0.009	
Total	8		

Analysis of the variance for effect of different strength MS basal medium on the percent normal shoots produced in carnation cv. Yellow Dot Com- Table 12

Source	Degree of freedom	Mean sum of square	F value
Treatment	2	567.32	422992.76
Error	6	0.001	
Total	8		

Analysis of the variance for effect of different strength MS basal medium on the percent vitrified shoots produced in carnation cv. Yellow Dot Com- Table 12

Source	Degree of freedom	Mean sum of square	F value
Treatment	2	567.44	235535.38
Error	6	0.002	
Total	8		

Analysis of the variance for effect of different concentrations of macrosalts on the percent normal shoots produced in carnation cv. Yellow Dot Com- Table 13

Source	Degree of freedom	Mean sum of square	F value
Treatment	8	635.73	19996.58
Error	18	0.031	
Total	26		

Analysis of the variance for effect of different concentrations of macrosalts on the percent vitrified shoots produced in carnation cv. Yellow Dot Com- Table 13

Source	Degree of freedom	Mean sum of square	F value
Treatment	8	632.27	8571.99
Error	18	0.073	
Total	26		

Analysis of the variance for effect of different concentrations of sucrose on the percent normal shoots produced in carnation cv. Yellow Dot Com- Table 14

Source	Degree of freedom	Mean sum of square	F value
Treatment	3	91.024	10024.84
Error	8	0.009	
Total	11		

Analysis of the variance for effect of different concentrations of sucrose on the percent vitrified shoots produced in carnation cv. Yellow Dot Com- Table 14

Source	Degree of freedom	Mean sum of square	F value
Treatment	3	90.801	12457.44
Error	8	0.007	
Total	11		

Analysis of the variance for effect of different concentrations of sucrose and agar on the percent normal shoots produced in carnation cv. Yellow Dot Com- Table 15

Source	Degree of freedom	Mean sum of square	F value
Treatment	11	229.93	17053.55
Error	24	0.013	
Total	35		

Analysis of the variance for effect of different concentrations of sucrose and agar on the percent vitrified shoots produced in carnation cv. Yellow Dot Com- Table 15

Source	Degree of freedom	Mean sum of square	F value
Treatment	11	231.72	31024.68
Error	24	0.007	
Total	35		

Analysis of the variance for effect of different rooting media on average number of shoots producing roots in carnation cv. Yellow Dot Com- Table 16

Source	Degree of freedom	Mean sum of square	F value
Treatment	6	181.43	84.67
Error	14	2.142	
Total	20		

Analysis of the variance for effect of different rooting media on average number of roots per shoot in carnation cv. Yellow Dot Com- Table 16

Source	Degree of freedom	Mean sum of square	F value
Treatment	6	23.599	11.01
Error	14	2.142	
Total	20		

Analysis of the variance for effect of different rooting media on percent rooting in carnation cv. Yellow Dot Com- Table 16

Source	Degree of freedom	Mean sum of square	F value
Treatment	6	1750.8	4081.61
Error	14	0.428	
Total	20		

CURRICULUM VITAE

Name : Pushpa Kumari
Father's Name : Jagroop Singh
Date of Birth : May 10, 1989
Sex : Female
Marital Status : Unmarried
Nationality : Indian

Educational Qualifications :

Certificate/Degree	Class/Grade	Board/University	Year
Matriculation	First	HPSEB	2003-04
10+2	First	HPSEB	2005-06
B.Sc.(Biotechnology)	First	G.N.D.U.	2006-09

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Central Govt./Univ./SAARC : NA

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other financial assistance received
during the study period

(Pushpa Kumari)