STUDIES ON MICROPROPAGATION OF CARNATION (DIANTHUS CARYOPHYLLUS L.) CVS. ARTHUR SIM AND ALAS RED

A THESIS SUBMITTED TO
ORISSA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY, BHUBANESWAR
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE IN AGRICULTURE
(HORTICULTURE)

By
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BHUBANESWAR
1994

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CERTIFICATE-I

This is to certify that the thesis entitled "**Studies on Micropropagation of carnation (*Dianthus caryophyllus* L.) cvs. Arthur Sim and Alas Red**" submitted in partial fulfilment of the requirement for the award of degree of **Master of Science in Agriculture (Horticulture)** of Orissa University of Agriculture and Technology, Bhubaneswar is a faithful record of bona fide and original research work carried out by Sri Siddharth Kumar Palai under my guidance and supervision and that no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of the investigation have been duly acknowledged.

16/3

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CERTIFICATE-II

This is to certify that the thesis entitled **"Studies on micropropagation of carnation (Dianthus caryophyllus L.) cvs.Arthur Sim and Alas Red"** submitted by Sri Siddharth Kumar Palai to the Orissa University of Agriculture and Technology, Bhubaneswar in partial fulfilment of the requirements for the degree of **Master of Science in Agriculture (Horticulture)** has been approved by the Student's Advisory Committee after an oral examination on the same in collaboration with an External Examiner.

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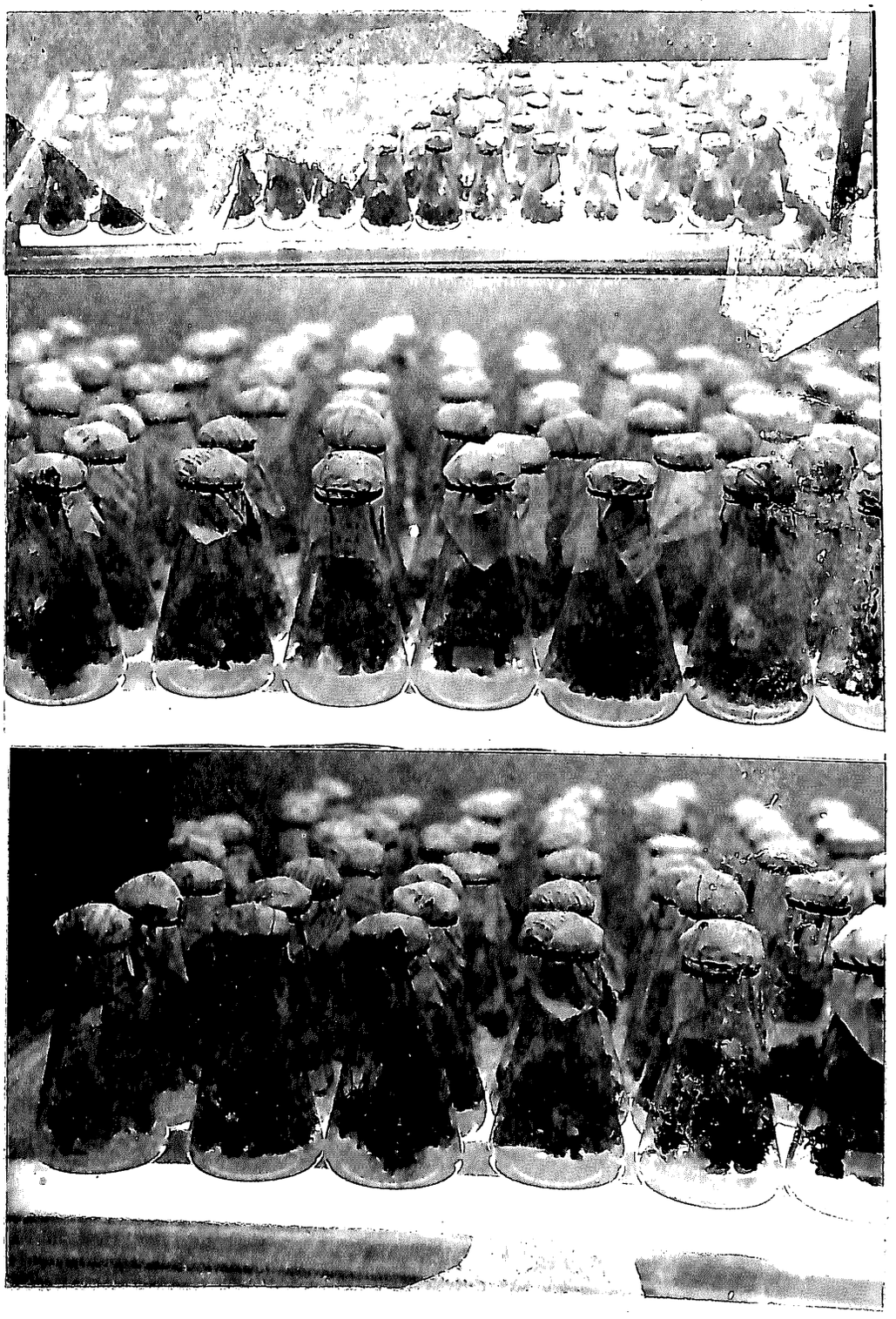
ABBREVIATION

Ads	adenine sulphate
BA	6-benzylaminopurine
cm	centimeter(s)
cv/cvs	cultivar/cultivars
^{o}C	degree centigrade
2,4-D	2,4-dichlorophenoxy acetic acid
g/l	gram(s)/litre
GA_3	gibberellic acid
h/hrs	hour/hours
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
Kn	kinetin
l	litre
u/uM	micron/micrometer
min	minutes
mm	millimeter(s)
mg	milligram(s)
ml	millilitre(s)
MS	Murashige & Skoog (1962) salts
NAA	a-naphthalene acetic acid
v/v	volume/volume
w/v	weight/volume
Zn	zeatin



Micropropagation of carnation (*Dianthus caryophyllus* L.)

C O N T E N T S

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CHAPTER - I
INTRODUCTION

Introduction

Carnation (Dianthus caryophyllus L.) belongs to the family Caryophyllaceae. The genus Dianthus comprises of more than 300 species. Carnation is one of the most important flower crops in the world. It is one of the major contributors to the international cut-flower trade and therefore has been an important target for the classical breeding of new varieties with novel characteristics which may vary with the cultivation system, climate, taste, etc. However, some of the main requirements are novel flower colours such as blue and purple, flowers with a strong fragrance, large flowers with strong stems, double regular flowers without a splitting of the calyx, flowers with long vase life, plants that are highly resistant to diseases, and plants that are tolerant to high and low temperatures. In addition, the breeding for some novel types such as spray and miniature carnations is necessary because both are now becoming popular in Europe and some other countries (Mii et al., 1991).

Carnation varieties are highly heterozygous and are propagated vegetatively. Breeding is done mainly by selecting desirable individuals from seedlings/populations obtained from intervarietal crosses. Therefore, detailed genetic information on the carnation is indispensable to incorporate desirable traits in performing the breeding efficiently. However, genetic studies on carnation to date have concentrated mainly on such flower charac teristics as size, colour and doubloons, and little is known

about other important characteristics such as disease resistance. Because carnation is highly heterozygous, many budsports from the leading varieties have played an important role in carnation breeding. Carnations are also subjected to many bacterial and fungal diseases. Because of the damage caused by virus infection, commercial producers of carnations are more interested to start from virus-free plants.

Carnation is propagated both by seed and stem cuttings. The annual carnation is raised from seed. Carnation needs long growing period to come to flowering. In vitro techniques are being employed in recent years to raise planting material in mass scale for commercial plantings; through this technique pathogen free planting materials can be obtained particularly virus free plants can be raised through meristem culture.

To obtain large number of disease free planting material of carnation, this investigation was taken up on carnation and the objectives of the study were:

(a) to standardise culture media and culture conditions for multiplication of shoots derived from apical and axillary meristems.

(b) to standardise culture media and culture conditions for initiation and proliferation of callus, regeneration of shoot bud from callus.

(c) to induce rooting in the excised shoots and also in the regenerated shoots.

(2) to record the biochemical changes, total carbohydrates and protein during callusing, organogenesis and rhizogenesis.

(3) acclimatization and hardening of the *in vitro* grown plants.

CHAPTER - II
REVIEW OF LITERATURE

REVIEW OF LITERATURE

In carnation, micropropagation has been attempted mainly from shoot apices by varying culture conditions although nodes (Roest and Bokelmann, 1981) and petals (Kakehi, 1979; Gimelli et al., 1983, 1984) are also used.

2.1.Meristem culture and shoot multiplication

Shoot tips were widely used for micropropagation as well as for obtaining virus free plants in various plant species because of their highly regenerative nature (Murashige, 1974). Hackett and Anderson, 1967 first demonstrated propagation of carnation cultivars by multiple shoot formation via shoot tip culture. They placed 0.5mm long shoot apices on a medium containing 5 times White's inorganic salts supplemented with 11uM NAA and obtained the callus tissue that eventually developed dark green meristematic regions. Usually, hormonal combinations with high concentrations of cytokinin (2 to 10uM) and low concentrations of auxin (0.1 to 0.5uM) were effective for multiple shoot formation from the shoot apex (Petru and Landa, 1974; Earle and Langhans, 1975; Hempel, 1979; Dabski et al 1979; Kozak and Hempel, 1979).

The pH of the media ranged from 5.5 (Davis et al., 1977) to 5.8 (Roest and Bokelmann, 1981) while, temperature ranged from

22±2 °C (Davis et al., 1977) to 26 °C (Jelaska and Sutina, 1977). The light intensities also varied from 2,000 to 10,000 lux, and several workers suggested the use of alternating day/night periods such as 16/8h (Jelaska and Sutina, 1977) and 14 hours day/10 hours night (Hempel, 1979). Finally, an increase in both light from 2 to 10 Klux and temperature from 22 to 25 °C was suggested by Davis et al. (1977) from the initial to the multiplication stage. Earle and Langhans (1975) reported that the subculturing of the inoculum was important for shoot multiplication; the rate of shoot multiplication was the maximum within 3-6 weeks of culture in the liquid medium when the cultures were continuously & slowly agitated at 1rpm. An increase of about 60-fold in shoot multiplication was obtained every 6weeks with a higher cytokinin/auxin ratio (9.3uM Kn and 0.11uM NAA). Good quality shoots were obtained consistently when subculturing was done at 20 days interval without much callusing of the shoots.

2.2.Callus culture and plant regeneration

2.2.1.Callus culture

Choudhary and Prakash (1992) reported that among the auxins tested, 2,4-D and NAA were found to be the main inducer of callus. The effects of growth hormones on the callus characteristics were reported. The medium containing 2,4-D (1.0-1.5 mg/l) produced calli that were semi-friable, watery and yellow in colour,

whereas, NAA induced slightly greenish callus with floffy mass around; IAA at higher doses produced greenish brown callus that developed thick roots. The calli induced by higher concentrations of IAA and IBA were hard, compact and chlorophyllus which never regenerated when transferred to the regeneration media (Choudhary and Prakash, 1992).

The genetic control of callus growth and, in particular, differentiation seems to be rather difficult. Kakehi (1979) compared 12 genotypes for shoot differentiation from petals and found frequencies ranging from 0-94%. Differences between genotypes were also observed by Hauzinska (1974) and by Gimelli et al. (1984). The existence of complex interactions among the source of explant, culture medium and genotype in both callus induction and plant regeneration have been reported. The best callus growth was obtained from shoot tips and low callus growth occurred when leaf primordia, root apices, ovaries and pistils were used as primary explants (Kakehi, 1970). Hauzinska (1974) pointed out that the age of initial explant was of great importance for callus proliferation; older tissues were less efficient than younger ones.

The pH and temperature seemed to be of prime importance for callus induction, proliferation and regeneration. The pH values ranged from 5.5-5.7 and temperature from 20 ^{o}C (Hauzinska, 1975) to 26 ^{o}C (Spinsky et al., 1974; Malczewska et al., 1979). Few

published reports on the effects of light are available. Gimelli et al (1983;1984) did not find any difference, between long day (16h/8h) and short day (8h/16h) conditions as far as shoot regeneration from petals was concerned. The different light intensities had great effect on callus proliferation and shoot bud regeneration. The light intensities used ranged from total darkness (Kakehi, 1970) to 1600 lux (Spinsky et al., 1974) or 6000 lux (Malczewska et al., 1979), or continuous light (Engvild, 1972).

Calli were induced readily from a number of sources such as shoot tips (Hackett and Anderson, 1967; Kakehi, 1970), stem or pith sections (Malczewska et al., (1979), hypocotyl (Petru and Landa, 1974), leaf protoplast (Mii and Cheng, 1982) and cell suspension cultures (Engvild, 1972). There is no report on regeneration of plants from callus. Frey et al. (1992), have, however, achived somatic embryogenesis by initiating internodal callus directly in liquid medium with 2,4-D and subsequently regenerating in hormone free medium. Floral tissue that has been used successfully as a source of explants for shoot regeneration included anthers (Villalobos, 1981), ovules (Demmink et al., 1987) and petals (Kakehi, 1979; Gimelli et al., 1984 and Leshem 1986). The most recent work with petals (Frey and Janick, 1991; Miller et al., 1991b; Nugent et al., 1991) has shown to be a reliable and prolific source of adventitious shoots even when a

large assortment of cultivars were used. Miller et al.(1991b) used immature flower buds to petals as the suitable explants, to achive greater shoot regeneration. Shoot formation never occurred from the cut distal surface of the petals, but was mainly confined to the green proximal tissues of individual petal pieces and the attached fragments of the receptacle. Histological examination of regenerating petals showed that shoots arose from actively dividing subepidermal cells (Frey and Janick, 1991; Miller et al. (1991b) Lubomski and Jerzy (1989) reported that the addition of BA (10^{-7} M) and IAA (10^{-5} M) resulted in shoot and root formation within 5 weeks of culture. Subsequently, Messeguer et al. (1993) reported that petals and floral segements of D. caryophyllus L. cv. Scania exhibited a high morphogenic capacity to a wide range of growth regulator treatments. He also indicated that dark conditions and an agar concentration at 5.5gm/l significantly improved the percentage of regenerating leaves and the number of shoots per leaf explant. Nakano et al. (1994) compared adventitious shoot bud regeneration among leaf, stem and petal explants of Dianthus caryophyllus L. cv.Scania on MS medium containing different concentrations of 6-benzyladenine (BA) and a-naphthaleneacetic acid (NAA). He obtained high frequency regeneration only from the petal explant on the media containing 5-10uM BA with or without 5uM NAA. Plant regeneration via somatic embryogenesis was induced from internodal callus of cvs. Scania, Improved White Sim and Sandra carnation. The induction of somatic

embryogenesis was achieved on liquid basal Murashige and Skoog (1962) medium supplemented with 3.0uM 2,4-D followed by a transfer to the liquid basal medium devoid of 2,4-D for embryo development. (Frey et al., 1992).

2.3. Induction of rooting

Regeneration of root was induced on media containing high concentration of auxin (IAA and NAA) and various concentrations of cytokinin (BA or Kn) (Engvild, 1972; Debergh, 1972, 1973; Malczewska et al., 1979; Mii and Cheng, 1982) or in which auxin concentration was reduced after the callus had been cultured on media with auxin alone (Earle and Langhans, 1975). Lubomski and Jerzy (1989) achieved root initiation from isolated shoot in MS medium supplemented with IAA (10^{-5} M). Bull and Garton (1985) noted regeneration of only roots from internodal segments of 5 cultivars of carnation in medium containing IAA (10^{-5} M). Slight addition of kinetin and NAA had positive effect on rooting of shoots obtained from shoot tips (Hempel, 1977). However, early root initiation was recorded with 5.0uM/l of NAA alone.

2.4. Acclimatization and field establishment

There were several studies on acclimatization and field establishment of in vitro raised plants. Plants grown in vitro usually do not possess protective mechanisms against desiccation.

Impaired stomatal function (Marin et al., 1988) and reduced epicuticular waxes (Gaspar et al., 1987, Sutter, 1988) have been noted in these plants. In addition, their photosynthetic competency may be reduced (Preece and Sutter, 1991). Voyiatzis and McGranahan (1994) reported that when the plantlets were transferred to the greenhouse or the field, in vitro grown plantlets are subjected to desiccation due to rapid water loss and may soon die. Ziv et al. (1987) reported that carnation plantlets survived better when the shoot apices were cultured in media with reduced minerals but with elevated Ca^{+2} that helped in development of normal leaves with functional guard cells. He also reported that neither translucent nor succulent plants survived transplanting in the greenhouse and withered, indicating inefficient transpiration control mechanism in the regenerated plants. The leaves showed signs of severe water stress which could be due to mainly of non-functioning of the stomata.

Hayashi et al. (1988) reported that at higher photosynthetic photon flux (PPF) the plantlets contributed to a greater total fresh and dry weight on the 40th day in the acclimatization unit.

CHAPTER - III
MATERIALS AND METHODS

MATERIALS AND METHODS

3.1. Source of explants

The terminal cuttings of two cultivars of carnation (*Dianthus caryophyllus* L.) cvs. Alas Red and Arthur Sim were obtained from the experimental garden of Regional Plant Resource Centre, Bhubaneswar. All large leaves and lateral shoots were removed until there remained only the terminal meristem sustained by leaf primordia and 1-2 pairs of small leaves. Apical meristems and axillary buds, leaf discs were also used as explants.

A wide variety of culture media, viz. B_5 (Gamborg *et al.*, 1968, MS (Murashige & Skoog, 1962), SH (Schenk & Hildbrandt, 1972) and N_6 (Nitsch, 1969) with or without minor alterations were tried. Of these, MS media was most commonly used. The inorganic and organic constituents of these media are listed in Table 1. Three percent sucrose was routinely used as the carbon source. In different experiments both liquid and solid media were used.

The growth regulators and other additives were added to the basal media, either alone or in various combinations, to test their efficiency in inducing, promoting or regulating the process of growth and differentiation.

Table 1. Composition of the stock soultions for Murashige and Skoog's (1962).

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i) Major salts in 100 ml of stock solution.

NH_4NO_3	16.5g
KNO_3	19.0g
$CaCl_2 2H_2O$	4.4g
$MgSo_4 7H_2O$	3.7g
KH_2PO_4	1.7g

(Each component was dissloved separately; then mixed to make up the final voulme to 100 ml)

ii) Minor salts in 100 ml of stock solution

H_3BO_3	620mg
$MnSo_4 4H_2O$	2230mg
$ZnSo_4 4H_2O$	860mg
$Na_2MoO_4 2H_2O$	25mg
$CuSO_4 5H_2O$	2.5mg
$CoCl_2 6H_2O$	2.5mg

iii) Na FeEDTA in 50 ml stock solution
2

Na EDTA 2	373mg
FeSo 7H O 4 2	278mg

iv) Potassium iodide in 100 ml of stock solution

KI	83mg

v) Glycine in 70 ml of stock solution

Glycine	20mg

vi) Vitamins in 100 ml of stock solution

Nicotinic acid	50mg
Pyridoxin-HCl	50mg
Thiamin-HCl	10mg

Nicotinic acid was first dissolved in 50 ml of warm distilled water; after cooling, the other two vitamins were added. The final volume was made upto 100 ml.

1000 ml of MS medium was prepared by adding the stock solution in the following manner.

a) Major salts 10ml

b) Minor salts 1ml

c) Na_2-FeEDTA 5ml

d) KI 1ml

e) Vitamins 1ml

f) Glycine 7ml

g) Meso-inositol 100mg

h) Sucrose 30g

The final volume was made upto 1000 ml. The pH of the medium was adjusted to 5.8 by addition of 0.1N NaOH or 0.1N HCl. This solution was gelled with 8g of Difco-Bacto agar for gelling the medium.

Besides basal components, some other additives were also included in the media whenever necessary. The growth regulators like indole-3 acetic acid (IAA), indole-3-butyric acid (IBA), a-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin, Adenine sulphate (Ads), gibberellic acid (GA_3) and 6-benzylaminopurine (BA), organic growth additives like coconut water and casein hydrolysate were used. Since, the concentration of these substances varied in different treatments they have been mentioned in the "Experimental Findings" chapter at appropriate places and were mostly used in w/v or v/v basis.

The stock solution for plant growth regulators were prepared as follows:

i) IAA, IBA, NAA and 2,4-D (20 mg of each) (BDH, England) were dissolved in the minimum quantity of ethanol, the volume was then made up to 80cc by adding strile distilled water.

ii) BA and Kn (20 mg of each) were first dissloved in the minimum quantity of 0.1N HCl; the volume was made upto 80 cc with sterile distilled water.

(iii) GA_3 (20 mg) was first dissloved in the minimum quantity of absolute alcohol; the final volume was then made upto 80 cc with sterile distilled water.

3.2.Preparation of media

Stock solutions were prepared using analytical grade chemicals and glass-distilled water to prepare the media. The pH of the media was adjusted to 5.8 using 0.1N HCl prior to autoclaving. Appropriate amount of the medium was dispensed into glass culture tubes (25x150 mm) and 250 ml conical flask (Borosil, India) for different experiments. The culture vessels were plugged with non-absorbent cotton wrapped in one layer of cheese cloth, and sterilized by autoclaving at 1.06 kg/cm^2 (121 ^oC) for 15 to 20 minutes. Semi-solid media in the culture tubes were allowed to set for inoculation.

3.3.Raising of cultures

3.3.1.Sterilization of explants

Juvenile shoots (about 2cm) segments were kept in a 2% (v/v) 'Teepol' detergent solution for 5 minutes, followed by washing in running tap water for 15 minutes. A solution of 0.5% sodium hypochloride (Commercial bleach diluted 10-fold and containing 2 drops Tween 20 emulsifer/100 ml) was added to the tubes to cover their contents. After 5 minutes, the disinfectant was decanted and the contents of the tube were rinsed 3 times with sterile distilled water.

3.4. Initiation of cultures

Excision of the shoot apex was performed in a horizontal laminar-air-flow chamber. A streo dissecting microscope (Nikon, Japan), with magnifications of 2.5-7.5 X, was used for the surgical manipulations. The excision was performed in a glass petridish, lined with sterile, moist filter paper to retard desiccation of the tiny explant. Isolation of the meristem dome was accomplished with microscalpels made from razor blade mounted on a Beaver Chuck handle. The explant was lifted on the tip of the knife and transferred to a culture tube. The apical meristem dome measured about 0.1mm in height and 0.2 mm at the base. All aseptic manipulations were done in a laminar-air flow cabinet (Thermadyne, India). All the instruments used were sterilized several times during inoculation by dipping them in 90% alcohol and

flaming them before use. Sterile leaf segments (ca 0.5 x 0.25 cm) were also used as explant source.

3.5.Culture condition

A minimum of 20 replicates were employed for each treatment.All the cultures were kept in the culture room maintained at 25± 2 °C temperature under 16-h photoperiod (3000 lux) provided by cool, white fluorescent lamps (Philips, India). Depending on the experiment, subculturing was done at an interval of 4 weeks.

3.6.Transplantation methods

Three methods were tried for transplanting the *in vitro* raised plantlets to soil.

(a) Plants were transferred to conical flask (100 ml capacity) containing half strength of MS salts. The shoots were placed on paper bridges (Whatman No.1) in such a way that only the roots floated in the medium. The culture tubes were capped with non-absorbent cotton plugs. The cultures were placed under 16-h light (3000 lux) for 7 days, subsequently transferred to pots containing perlite.

(b) Plantlets were transferred from agar medium to a mixture of nutrient solution in culture tubes. All other treatments were same as described earlier.

(c) The plantlets were transferred to earthen pots containing sterlized graden soil, sand and leafmould in the ratio of 2:2:1 (w/v). The pots were placed under a humidified chamber to check desiccation of the plants. The plants were supplied with 1/4th strength solution of MS salts without organics for 3 to 4 days and subsequently watered using tap water.

3.7. Observation of cultures and presentation of results

The cultures were examined periodically and the responses were recorded on the basis of visual observations. Effect of different treatments were quantified on the basis of percentage of cultures showing the response and the degree of response per culture. Rate of shoot multiplication was expressed in terms of the number of propagules available at the end of each passage in 8 weeks. The organogenic differentiation under different treatments was also recorded.

3.8. Estimation of chlorophyll and carotenoids

On the scheduled sampling dates, 100mg of the fresh tissue was collected from *in vitro* grown shoots for chlorophyll estimation. The samples were cut into small pieces by scissors and thoroughly crushed in a glass mortar and macerated with a small amount of 80% acetone. Maceration was done with more acetone until the plant tissues became white. The acetone crude extract was centrifuged and the clear supernatant solution was diluted

to 10ml volume with 80% acetone. With the help of a UV-spectrophotometer (Japan), the chlorophyll-a, chlorophyll-b and carotenoid were determined at wavelengths of 663nm, 645nm and 450 nm respectively. The samples under each treatment were analysed three times. Quantitative analysis of chlorophyll-a, chlorophyll-b and carotenoid (mg/g of fresh tissue) were calculated following the formulae of Jensen (1978).

$$\text{Chl- a (mg/g)} = \frac{12.7\,A_{663} - 2.69A_{645}}{a \times 1000 \times W} \times V$$

$$\text{Chl-b (mg/g)} = \frac{22.9A_{645} - 4.68A_{663}}{a \times 1000 \times W} \times V$$

$$\text{Total chlorophyll (mg/g)} = \frac{20.2A_{645} + 8.02A_{663}}{a \times 1000 \times W} \times V$$

Where a = length of the path light in the cell, usually 1 cm

V = Volume of the extract in ml and

W = fresh weight of sample

$$\text{Carotenoid (C)} = \frac{D \times V \times f \times 10}{2500}$$

Where ,

C = total amount of carotenoid in mg

D = absorbency at 450nm in a 1.0cm

V = volume of the original extract in ml

f = dilution factor and

2500 = average extinction coefficient of the pigments

3.9. Estimation of total carbohydrate

The total carbohydrate content was estimated quantitatively by the method described by Chattopadhyay (1976). Fresh tissues (100 mg) were extracted with 30% ethanol and kept at 60 ^{o}C for 30 minutes. The volume was maintained at 10ml with ethanol during the period of extraction. The extract was made colourless with activated charcoal and then filtered. The residual tissue after extraction with ethanol was treated with 10 ml of 70% perchloric acid in order to remove the starch. Finally, the ethanol extract and that of acid extract were made up to the volume of 100 ml separately with the addition of distilled water.

The aliquota was taken separately and to each of the aliquota, 4 ml of 0.1% anthrone reagent was added immediately and mixed thoroughly with a glass rod and allowed to cool on water bath. After 10-15 minutes, it was measured with the help of UV-spectrophotometer at 610 nm. The blank used contained a mixture of 1ml distilled water and 4 ml 0.1% anthrone reagent and treated in an identical manner. The total carbohydrate content was expressed in

terms of ug glucose unit per ml fresh weight of tissue by comparing the observed values with the standard curve made from analar grade glucose (BDH).

3.10. Estimation of protein

Protein estimation was done using tissues of the same age obtained from different cultures at regular intervals. The tissues (100mg) were homogenised with 0.5M phosphate buffer (pH-6.5) and then centrifused. To an aliquota of the supernatant, 30% trichloroacetic acid (TCA) was added to precipitate the protein which was dissolved in 0.3N NaoH and measured colorimetrically following the method of Lowery et al. (1951). Bovine serum albumin (Sigma, fraction-V) was used as the standard and the values were expressed in ug/ml of fresh tissues.

CHAPTER - IV
EXPERIMENTAL FINDINGS

EXPERIMENTAL FINDINGS

4.1. Meristem Culture:

MS medium was the most suitable medium for bud proliferation among different basal nutrient media tried. In the absence of auxin and cytokinin, the apical meristem dome though survived did not show appreciable growth; all other explants, however, failed to survive in the culture. Inclusion of both BA or Kn with NAA in the nutrient medium enabled 58% of the explants to develop shoots. Presence of the youngest pair of leaf primordia was even inadequate; only a few explants gave rise to plants. Of the various combinations tested; the BA (0.5-2.0 mg/l) alongwith 0.25-0.5 mg/l NAA helped in proliferation of shoots in both the cultivars (Figs. 1 & 2). In most of the treatments, a feeble callus developed at the basal end. Sometimes, roots developed at the base of the explants (Tables 2 and 3) . Growth of the meristem slowed down in the medium containing Kn alongwith NAA. In many cases, the friable and brown coloured calli were formed at the base of the meristem. These experiments proved that both BA and NAA were necessary for shoot bud proliferation.

At higher concentrations of either BA or Kn or both Kn and BA, abnormal shoots developed that were condensed and had vitrified leaves. In most of the cases, the plantlets were bushy having multiple shoots, in contrast to the single dominant main shoot normally found (Figs.3 -6).

Figs. 1 & 2 Proliferation of shoot primordia of Dianthus caryophyllus cv. Alas Red after 8-12 days of inoculation. (3.5X magnification).

Table 2. Effect of cytokinins and auxin on shoot multiplication of Dianthus caryophylls cv. Alas Red after 4 weeks of culture.

MS + growth regulators (mg/l)			No.of explants cultured	No.of explants responded	Av.no.of multiple shoots/explant * ±S.E.
BA	KN	NAA			
0	0	0	60	0	0
0.5	0	0.25	70	22	22.4 ± 0.3
1.0	0	0.25	65	44	32.6 ± 0.8
1.5	0	0.25	70	58	38.2 ± 0.9
2.0	0	0.25	82	54	44.4 ± 0.6
0.5	0	0.5	80	11	22.3 ± 0.2
1.0	0	0.5	75	24C	24.6 ± 0.3
1.5	0	0.5	80	32C	28.2 ± 0.4
2.0	0	0.5	76	28C	34.6 ± 0.1
2.5	0	0.5	80	26C	32.7 ± 0.2
0	0.5	0.25	76	14C	14.2 ± 0.9
0	1.0	0.25	80	23	29.3 ± 0.4
0	1.5	0.25	72	38	31.8 ± 0.6
0	2.0	0.25	74	46	34.2 ± 0.3
0	2.5	0.25	76	48	30.4 ± 0.7

* Mean of two repeated experiments; 20 replicates/treatment

c- indication of callus.

Table 3. Effect of cytokinins and auxin on shoot multiplication of *Dianthus caryophyllus* cv. Alas Red after 4 weeks of culture.

MS + growth regulators (mg/l)			No.of explants cultured	No.of explants responded	Av.no.of multiple shoots/explant * ±S.E.
BA	KN	NAA			
0	0	0	82	0	0
0.5	0	0.25	84	18	18.4 ± 0.3
1.0	0	0.25	90	52	34.2 ± 0.8
1.5	0	0.25	92	63	41.8 ± 0.7
2.0	0	0.25	86	56	48.2 ± 0.8
0.5	0	0.5	92	24C	16.7 ± 0.7
1.0	0	0.5	85	36	23.6 ± 0.4
1.5	0	0.5	86	43	31.4 ± 0.6
2.0	0	0.5	80	52	42.2 ± 0.7
2.5	0	0.5	72	58	46.4 ± 0.3
0	0.5	0.25	76	11C	18.4 ± 0.6
0	1.0	0.25	77	26	18.3 ± 0.2
0	1.5	0.25	78	32	29.2 ± 0.4
0	2.0	0.25	82	36	31.4 ± 0.1
0	2.5	0.25	86	42	37.6 ± 0.24

* Mean of two repeated experiments ;20 replicates/treatment.

c - indication of callus.

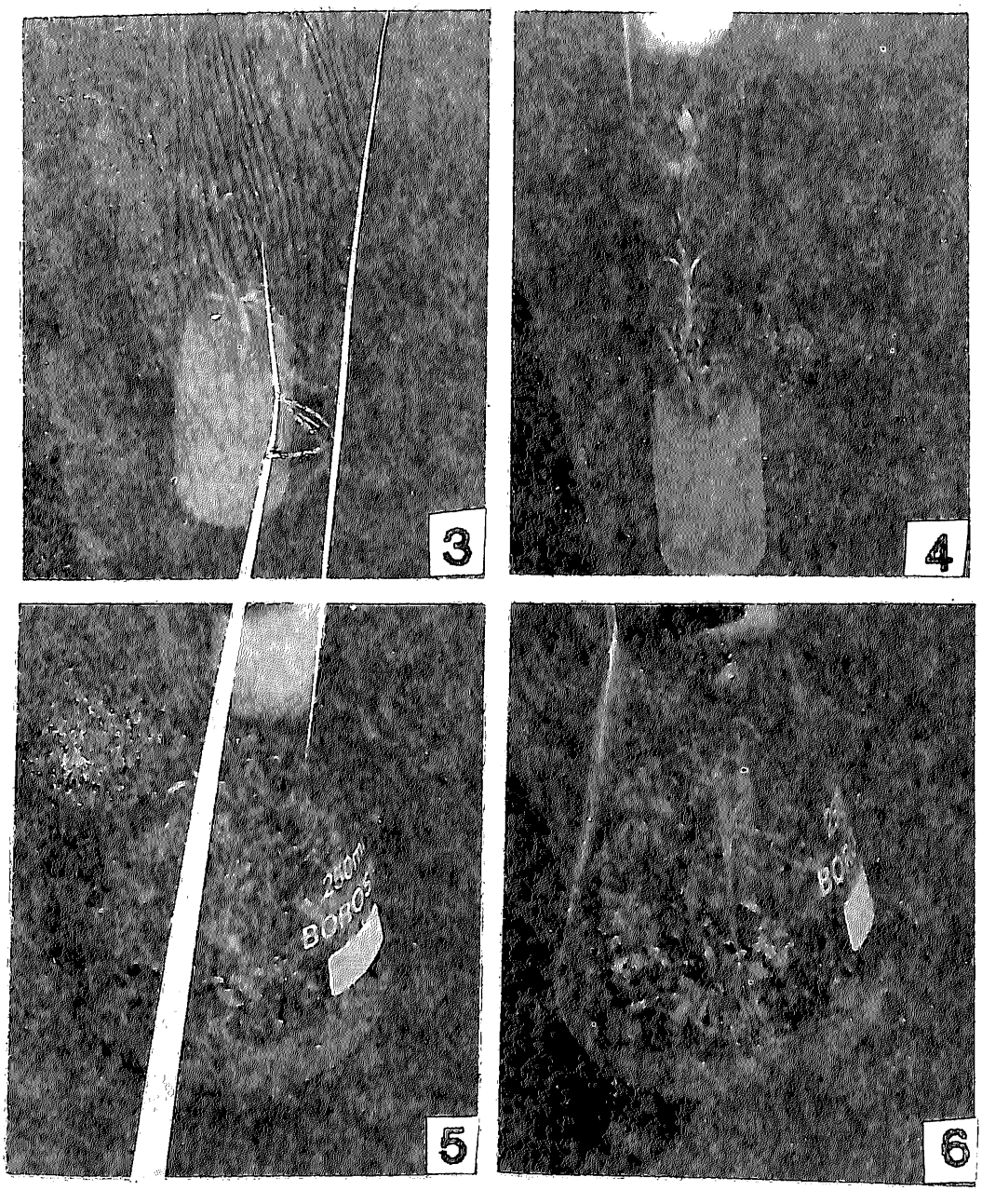
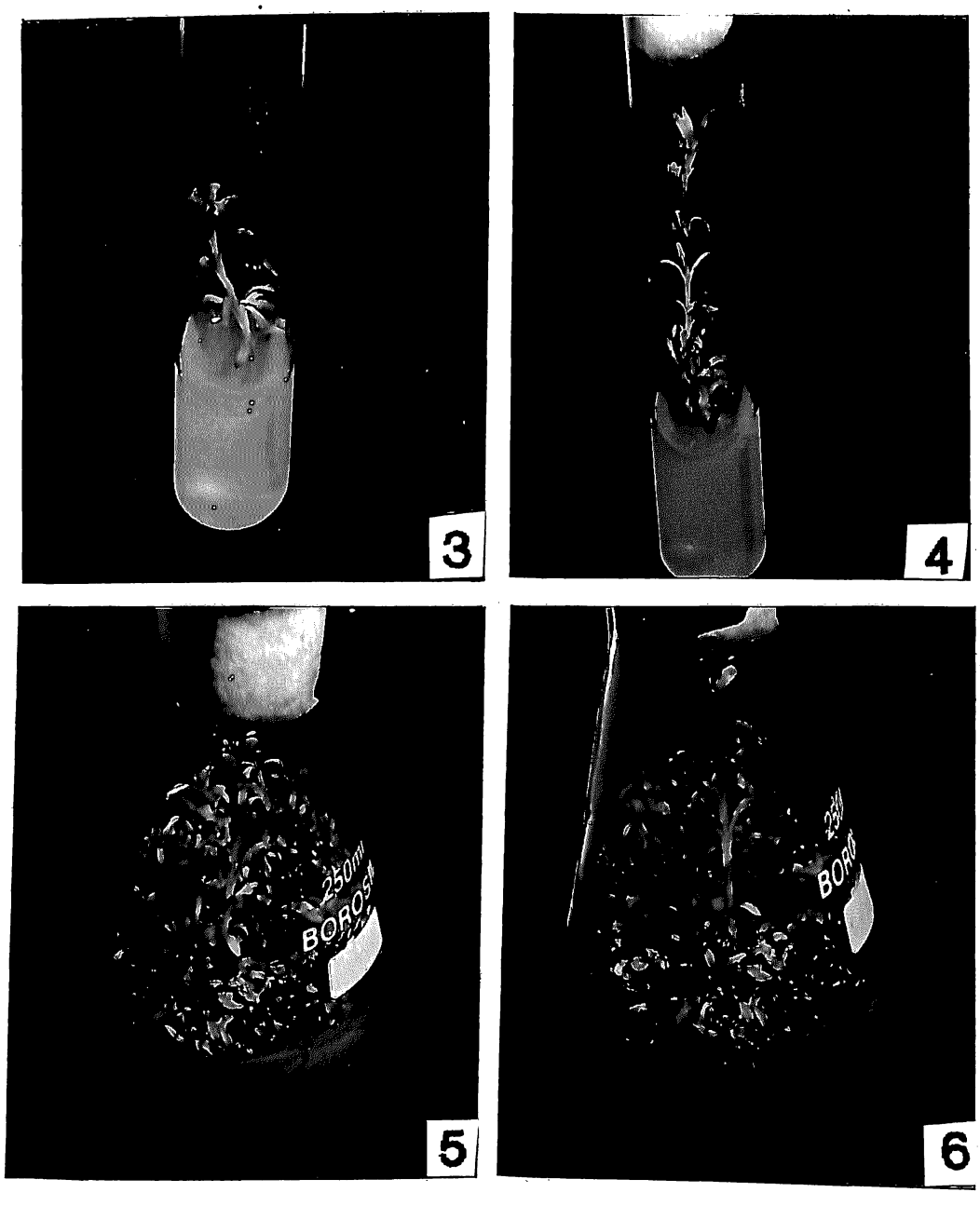


Fig. 3 & 4 Shoot elongation of Dianthus caryophyllus cv. Arthur Sim after 8-10 days (Fig.3) & 16-18 days of inoculation (Fig.4).

Figs. 5 & 6. Shoot proliferation and multiplication of Dianthus caryophyllus cv. Arthur Sim (Fig.5) and D.caryophyllus cv. Alas Red (Fig.6) cultured on semi-solid 1/2 MS medium supplemented with 1.5-2.0 mg/l BA + 0.5 mg/l NAA + 3% (w/v) sucrose.



Figs.3 & 4 Shoot elongation of Dianthus caryophyllus cv. Arthur Sim after 8-10 days (Fig.3) & 16-18 days of inoculation (Fig.4).

Figs 5 & 6.Shoot proliferation and multiplication of Dianthus caryophyllus cv. Arthur Sim (Fig.5) and D.caryophyllus cv. Alas Red (Fig.6) cultured on semi-solid 1/2 MS medium supplemented with 1.5-2.0 mg/l BA + 0.5 mg/l NAA + 3% (w/v) sucrose.

4.1.1. Effect of subculture

Tables 4 and 5 show the effects of subculture on shoot multiplication and elongation in two cultivars of carnation. The response of both the cultivars "Alas Red" and "Arthur Sim" showed a remarkable difference with regard to bud proliferation and multiplication of shoots. The rate of shoot multiplication was higher during the 3rd and 4th subculture and decreased thereafter. The number of shoot buds/culture varied from 10.28 to 42.36 depending on the subculture period. The average height of the shoots also declined from the 4th to the 10th subculture.

4.1.2. Effect of solid and liquid media

Fig.A shows the effect of solid and liquid media on shoot bud proliferation in both the cultivars of Dianthus caryophyllus. The liquid medium was more suitable than agar-gelled medium for multiplication and subsequent elongation of shoots . In liquid culture, many axillary shoots were usually developed. Most of them eventually broke off and floated free in the medium. These shoots in turn formed new axillaries showing rapid growth in the liquid medium. One explant produced 20 shoots /culture in 4 weeks on transfer to the agar-gelled medium compared to 32 shoots/culture in liquid medium. Several shoots were developed from a single explant which grew upto 1-2 cm; the shoots, however did not grow further even when cultured for prolonged period. The rate of shoot multiplication was higher in cv. Alas Red than cv. Arthur Sim.

AS - Arthur Sim AR - Alas Red

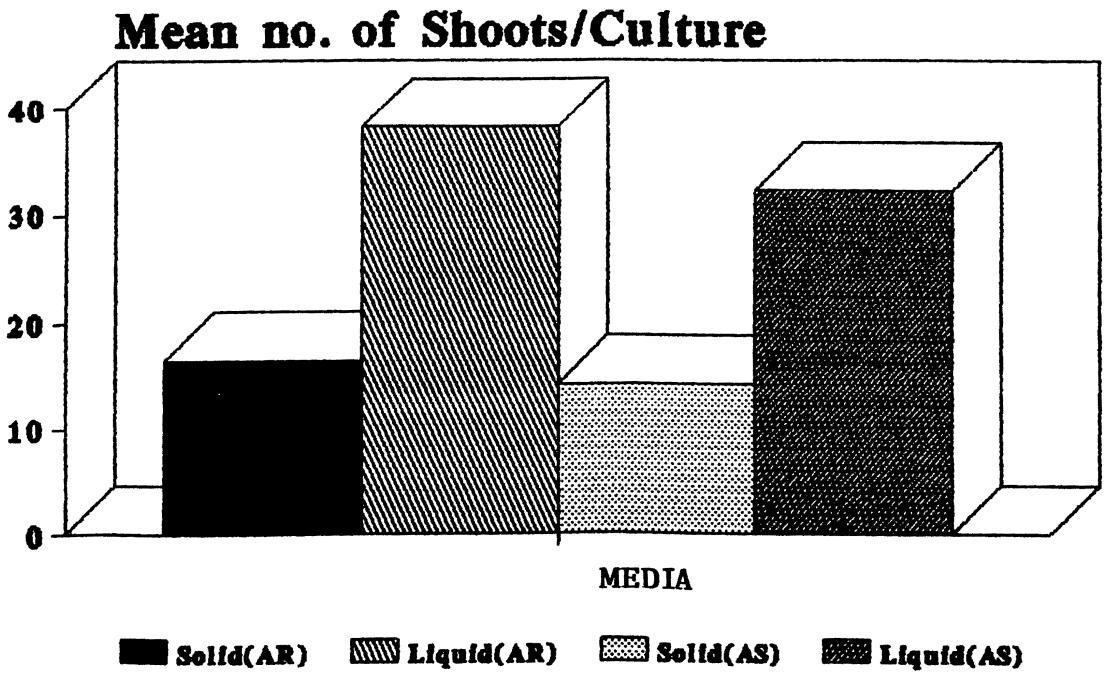


FIG. A Effect of solid & liquid media ($\frac{1}{2}$ MS + 1.5 mg/l) BA + 0.25 mg/l NAA + 3% (w/v) sucrose) on shoot multiplication of Dianthus caryophyllus L. cvs. Alas Red & Arthur Sim after 4 weeks of culture.

Table 4. Effect of subculture on development of multiple shoots/culture of Dianthus caryophyllus L. cv. Alas Red on 1/2 MS + 1.5 mg/l BA + 0.25 mg/l NAA + 3% sucrose (w/v)

No. of subcultures (4 week interval)	Mean no. of shoots/culture * ± S.E.	Mean height of the shoots/culture * ± S.E (in cm)
1	18.23 ± 0.56	1.23 ± 0.61
2	22.36 ± 0.23	1.42 ± 0.14
3	34.21 ± 0.34	1.13 ± 0.21
4	42.36 ± 0.18	0.96 ± 0.34
5	29.41 ± 0.48	0.83 ± 0.71
6	24.23 ± 0.31	0.75 ± 0.62
7	19.42 ± 0.32	0.77 ± 0.51
8	16.48 ± 0.72	0.62 ± 0.32
9	13.33 ± 0.21	0.64 ± 0.46
10	10.28 ± 0.52	0.58 ± 0.32

* 20 replicates/ treatment; repeated twice, plant less than 0.5 cm were not counted.

Table 5. Effect of subculture on development of multiple shoots/culture of Dianthus caryophyllus L. cv. Arthur Sim on 1/2 MS + 1.5 mg/l BA + 0.25 mg/l NAA + 3% sucrose (w/v).

No. of subcultures (4 week intervals)	Mean no. of shoots/culture * ± S.E.	Mean height of the shoots/culture * ± S.E (in cm)
1	18.44 ± 0.23	1.34 ± 0.28
2	23.81 ± 0.44	1.41 ± 0.31
3	36.23 ± 0.51	1.13 ± 0.71
4	44.21 ± 0.16	0.02 ± 0.73
5	32.32 ± 0.22	0.91 ± 0.61
6	26.12 ± 0.51	0.82 ± 0.42
7	18.37 ± 0.72	0.62 ± 0.32
8	15.28 ± 0.36	0.56 ± 0.22
9	14.76 ± 0.41	0.61 ± 0.23
10	9.48 ± 0.32	0.52 ± 0.41

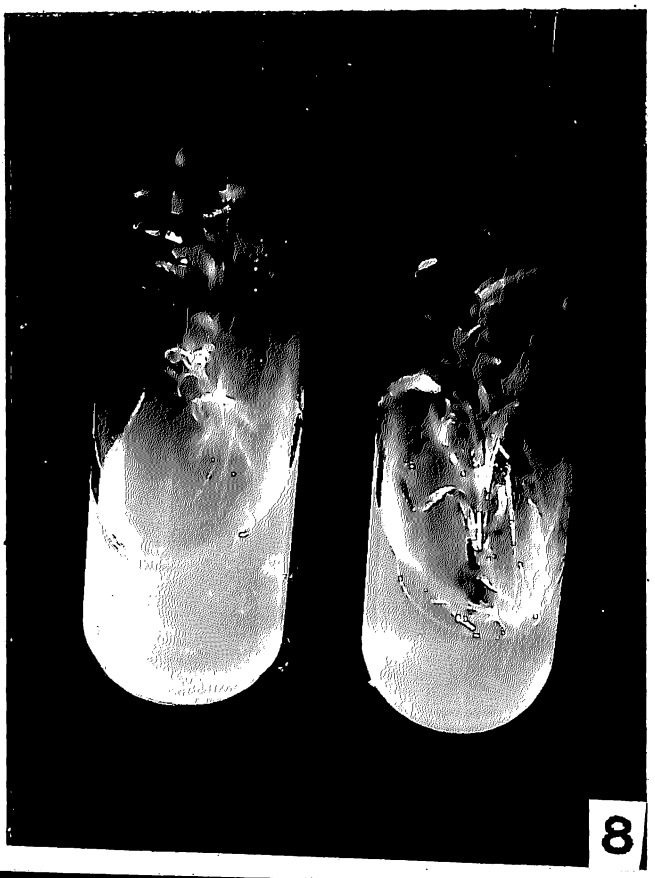
* 20 replicates/ treatment; repeated twice, height less than 0.5 cm is not counted.

4.1.3. Effect of agar concentration on shoot proliferation and multiplication

Various concentrations of agar were tested for shoot bud proliferation and further multiplication of carnation. The shoots became succulent in nature at low concentration (0.6%) of agar in the culture medium (Table 6). Agar concentrations of 0.8, 1.0 and 1.2% in the culture medium influenced development of more number of normal shoots. The lowest number of succulent shoots/culture was observed in the medium containing 1.2% of agar-agar within 4-weeks of culture. In case of Alas Red, the number of succulent shoots/culture varied from 6 to 22 depending upon the concentration of agar in the culture medium.

4.1.4. Effect of growth regulators on rooting

Elongated shoots were transferred to various media containing NAA (0.1, 0.25 and 0.5 mg/l) and IBA (0.1, 0.25 and 0.5 mg/l) with 2% (w/v) sucrose for induction of roots. The maximum number of shoots were rooted in 1/2 strength liquid/semi-solid MS medium supplemented with either 0.25 mg/l of NAA or IBA (Figs.7-9). Liquid media with similar supplements had spectacular effect on rooting in both the cultivars. The shoots became very weak and the percentage of rooting was inhibited in the media devoid of growth regulators, increase in the concentration of NAA or IBA (0.5-1.0 mg/l), however, had adverse effects on root induction (Tables 7 & 8). The rooting response varied from 10 to 16 in cv. Arthur Sim.



Figs. 7 & 8 Induction of rooting from growing shoots of Dianthus caryophyllus cv. Alas Red cultured on liquid and semi-solid 1/2 MS medium supplemented with 0.25 mg/l IBA + 2% (w/v) sucrose.

Fig.9 Rooted plantlets for transplantation.

Table 6. The effect of concentration of agar and duration of culture on *D. caryophyllus* L. cv. Alas Red, *in vitro*.
*
(Mean of two experiments ±S.E.).

Agar concentration (%)	Duration of growth (week)	Av.No.of normal shoots/ explant	Av.No. of succulent shoots/ culture.
0.6	1	28 ± 1.2	14 ± 1.2
0.6	2	32 ± 2.3	16 ± 1.8
0.6	3	38 ± 3.2	22 ± 1.4
0.6	4	43 ± 2.2	24 ± 2.1
0.8	1	30 ± 1.0	13 ± 2.1
0.8	2	33 ± 1.5	14 ± 1.4
0.8	3	40 ± 2.6	18 ± 1.2
0.8	4	45 ± 2.1	19 ± 2.1
1.0	1	33 ± 2.0	10 ± 1.6
1.0	2	36 ± 1.2	7 ± 1.7
1.0	3	43 ± 1.8	8 ± 1.8
1.0	4	52 ± 1.4	10 ± 1.4
1.2	1	34 ± 1.2	8 ± 1.2
1.2	2	37 ± 2.4	7 ± 2.1
1.2	3	45 ± 1.8	6 ± 1.4
1.2	4	54 ± 1.0	9 ± 1.8

* - Each treatment contained 25 cultures.

Table 7. Effect of IBA and NAA on rooting of Dianthus caryophyllus L. cv. Alas Red.

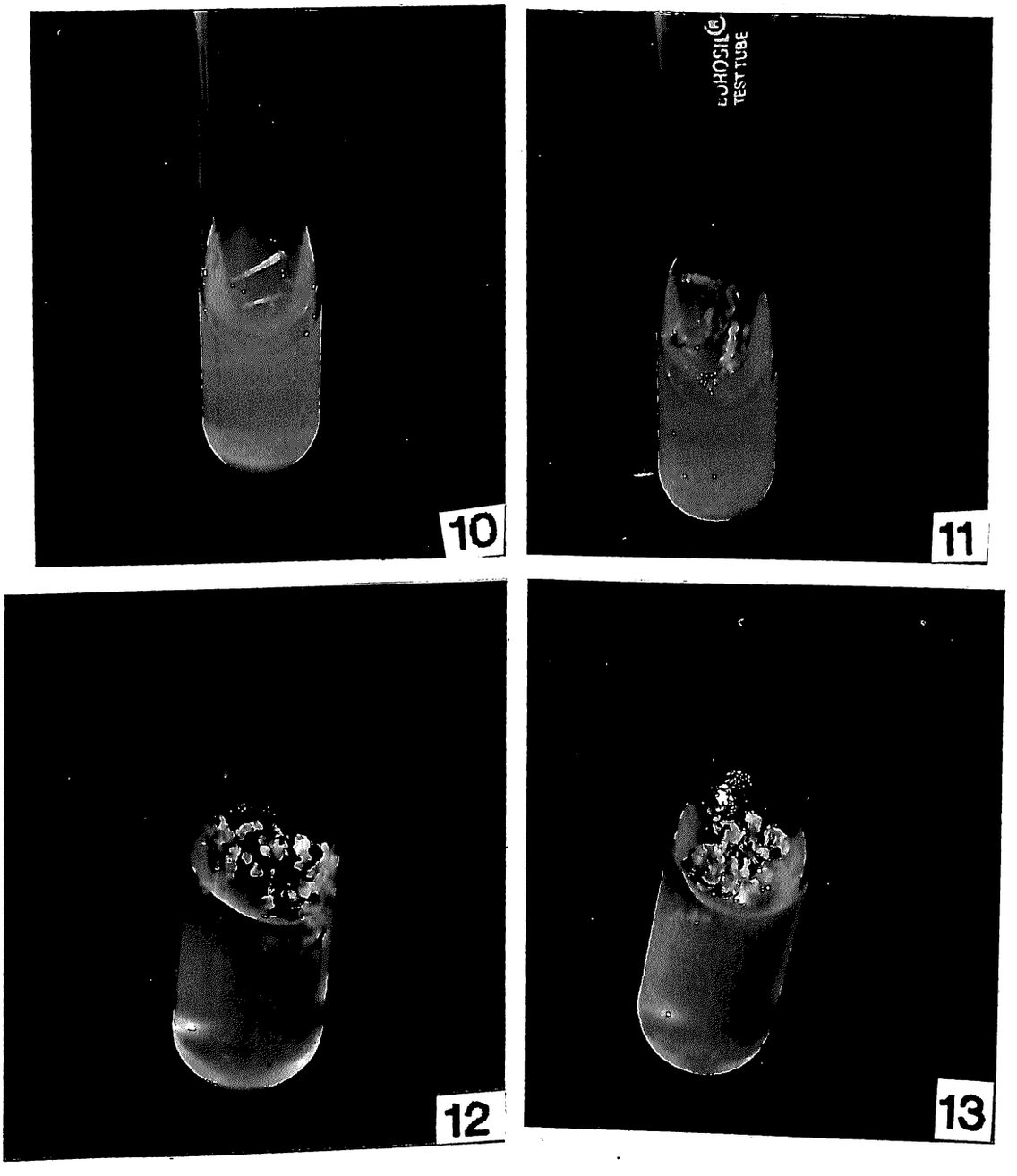
1/2MS + growth regulator(mg/l)		Days to rooting	% of rooting	Average no.of roots/shoot ± S.E.*
IBA	NAA			
0	0	0	0	0
0.10	0	10-11	75-80	16.1 ± 0.8
0.25	0	11-12	70-75	14.2 ± 0.6
0.50	0	15-16	55-60	10.3 ± 0.3
1.0	0	Callusing	0	0
1.5	0	Callusing	0	0
0	0.10	12-13	70-75	15.2 ± 0.6
0	0.25	14-15	70-76	14.4 ± 0.4
0	0.50	16-18	60-65	11.2 ± 0.8
0	1.00	Callusing	0	0
0	1.50	Callusing	0	0

* - 40 replicates/treatment; repeated twice.

Table 8. Effect of IBA and NAA on rooting of *Dianthus caryophyllus* L. cv. Arthur Sim.

1/2MS + growth regulator(mg/l)		Days to rooting	% of rooting	Average no.of roots/shoot * ± S.E.
IBA	NAA			
0	0	0	0	0
0.10	0	11-12	72-78	13.2 ± 0.7
0.25	0	13-14	74-76	16.4 ± 0.2
0.50	0	17-18	47-51	11.2 ± 0.9
1.0	0	Callusing	0	0
1.5	0	Callusing	0	0
0	0.10	10-11	70-78	14.4 ± 0.4
0	0.25	12-13	72-77	15.3 ± 0.8
0	0.50	16-18	43-54	10.2 ± 0.4
0	1.00	Callusing	0	0
0	1.50	Callusing	0	0

* - 40 replicates/treatment; repeated twice.



Figs.10 & 11. Cultures of stem explant (Fig.10) and leaf explants (Fig.11) of Dianthus caryophyllus cv. Alas Red on MS semi-solid medium supplemented with 0.5 mg/l BA + 2.0 mg/l NAA.

Figs.12 & 13 Proliferation of callus from leaf explant of Dianthus caryophyllus cv. Arthur Sim (Fig. 12) & cv. Alas Red (Fig.13) after 2-3 weeks of culture.

Table 9. Effect of differnt cytokinins on callus initiation from leaf and stem explants of D. caryophyllus L. after 4 weeks of culture.

MS+ regulators (mg/l)	(Rate of callus growth) cv. Alas Red		cv. Arthur Sim	
	leaf	stem	leaf	stem
0	0	0	0	0
BA 0.5	+	0	+	0
BA 1.0	+	+	+	+
BA 1.5	++	++	++	+
BA 2.0	++	+++	+++	++
BA 2.5	++	+++	+++	++
Kn 0.5	0	0	0	0
Kn 1.0	+	0	+	0
Kn 1.5	++	+	++	+
Kn 2.0	++	+++	+++	++
Kn 2.5	++	+++	+++	++
Zn 0.5	0	0	0	0
Zn 1.0	0	0	0	0
Zn 1.5	+	0	+	0
Zn 2.0	++	++	++	+
Zn 2.5	+	+	+	+

20 replicates per treatment ; repeated twice.

+ - low; ++ - moderate; +++ - good; ++++ - excellent.

4.2.2. Effect of auxins on callus growth

Different types of auxins were tested for callus initiation and proliferation from the stem and leaf explants of Dianthus caryophyllus cvs. Arthur Sim and Alas Red. A significant morphological response on callus growth was observed in both the cultivars of Dianthus (Table 10). Of all the auxins tested, 2,4-D or NAA was found to be the main inducer of callus growth. Higher concentrations of 2,4-D or NAA, repressed the callus growth. The medium devoid of growth regulators did not induce callus formation from leaf and stem explants of both the cultivars. The results further revealed the gross inhibition of callusing by IBA and delaying action of IAA. A significant interaction was noticed between growth hormones and their concentration so far fresh weight of callus was concerned. The maximum weight of fresh callus was obtained at 1.0 - 1.5 mg/l 2,4-D; the callus was semi-friable, watery and yellowish in colour. The explants cultured on medium containing NAA, induced friable callus greenish in colour and IAA at higher doses produced greenish brown callus with thick roots. The time taken for callus initiation varied with the cultivars. The percentage of explants showing callus initiation and proliferation was higher in cv. Alas Red as compared to cv. Arthur Sim on medium containing MS basal salts supplemented with 1.5 mg/l 2,4-D. The rate of growth and proliferation of callus was enhanced when the cultures were subcultured on medium with similar compositions at 4-week intervals.

Table 10. Effects of different auxins on the induction of morphogenic callus from leaf explants of D. caryophyllus L. cv. Alas Red.

Growth regulators	Days to callus induction					Texture of callus					% of callus
	0.0	0.5	1.0	1.5	2.0	0.0	0.5	1.0	1.5	2.0	
2,4-D	0	11	10	9	9	0	CY	CY,F	CY,F	CY,F	92
NAA	0	13	9	8	8	0	GW	GW,F	GW,F	GW,F	90
IBA	0	NC	16	14	13	0	N,G	GW,N	DR,GW	GW,F	42
IAA	0	NC	14	12	11	0	G,N	G,N	Necrosis		Necrosis

NC - No callus

G - Greenish

CY - Creamy yellow

GW - Greenish white

N - Nodular

DR - Direct rhizogenesis

F - Friable

4.2.3. Effect of auxins and cytokinins on callus growth

The results of the present investigation showed that, the presence of BA or Kn in combination with NAA or 2,4-D induced callus formation from both stem and leaf explants of Dianthus caryophyllus cvs. Alas Red & Arthur Sim. The rate of callus growth and further proliferation was faster on medium containing BA or Kn + 2,4-D, than the BA or Kn + NAA. In the medium containing BA or Kn + 2,4-D, the callus became greenish-white in colour; greenish-brown calli were obtained on medium containing BA or Kn +NAA. In most of the cases, small green shoot-buds like structures developed directly in the surface of the leaf or stem explants on medium containing low concentrations of cytokinin and auxin. The morphological response reduced with the increase in auxin and cytokinin concentration in the culture medium.

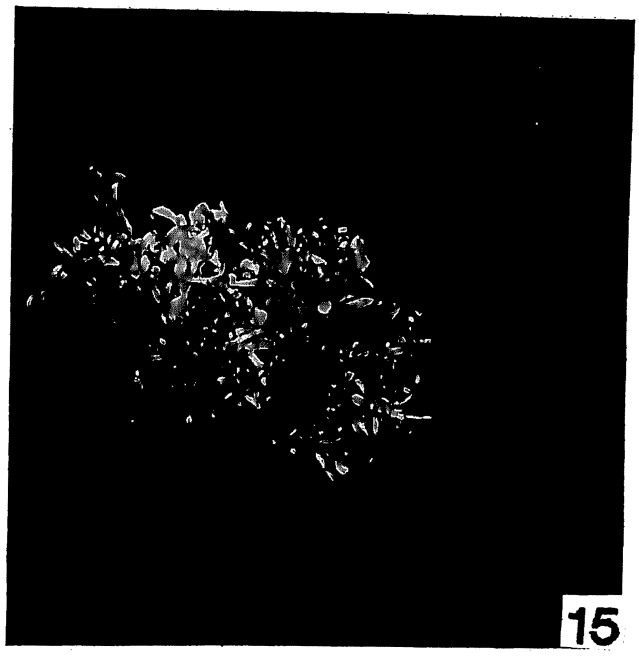
4.2.4. Shoot bud regeneration

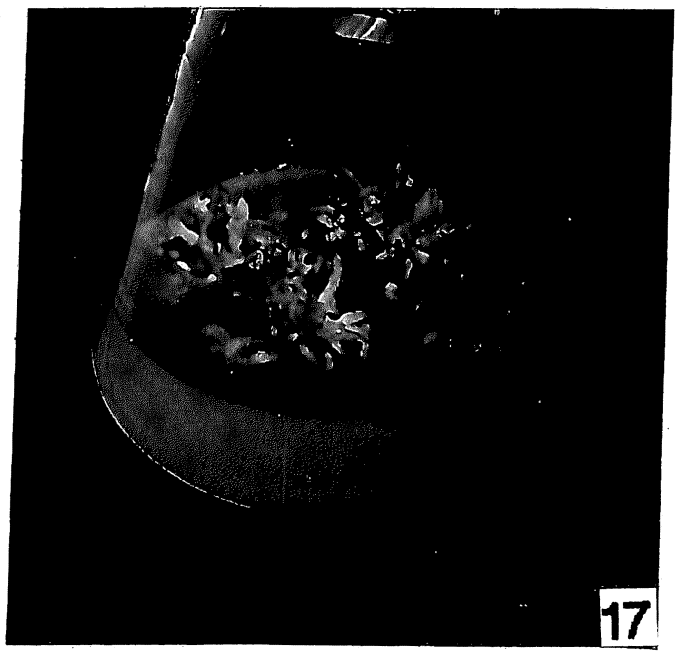
Four-week-old friable calli derived from both stem and leaf segments were transferred to various regeneration medium containing different concentrations of BA in combination with NAA (Tables 11 & 12). No regeneration of adventitious buds was observed in any of the cultures supplemented with BA or Kn alone. The medium containing BA or Kn + 2,4-D at different concentrations did not help in regeneration of any adventitious shoot bud but rhizogenesis. Inclusion of BA + NAA in the culture medium induced small green meristematic buds on the surface of the

callus which subsequently gave rise to numerous shoot primordia within two weeks of culture. Shoot bud regeneration was obtained on MS basal media supplemented with the factorial combination of 0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l BA and 0.1, 0.25, 0.5 and 3.0 mg/l Kn and 0.1, 0.25, 0.5 and 1.0 mg/l NAA. The optimum regeneration was achieved on media containing 1.5 -2.0 mg/l BA and 0.25 mg/l NAA in both the cultivars of Dianthus caryophyllus. The average percentage of shoot bud differentiation were 75% and 62%, in case of stem calli and 86% & 67% in case of leaf calli of Dianthus caryophyllus cvs. Arthur Sim and Alas Red respectively. The number of shoot buds/200 mg fresh calli varied in the two cultivars (Figs.14 & 15). At higher concentrations of auxin & cytokinin, the shoots became stunted.

4.2.5. Effect of light/dark on shoot bud regeneration

The cultures were incubated both in the light (16-h-photoperiod) and in the dark for induction of shoot-bud regeneration. The percentage of shoot bud regeneration was enhanced when the cultures were incubated in the light at 16h photoperiod. The rate of shoot bud regeneration declined in the dark and the shoots became vitrified within 2 weeks of culture (Figs.16 & 17). The cultures incubated in the dark followed by a transfer to 16-h photoperiod also showed shoot bud differentiation; the cultures incubated in the light for one week followed by transfer to the dark condition did not elicit any positive response for shoot bud regeneration.





Figs. 14 & 15 Regeneration of normal shoots from leaf callus of Dianthus caryophyllus cv. Alas Red (Fig.14) & cv. Arthur Sim (Fig.15) after 3 weeks of culture.

Figs.16 & 17 Regeneration of vitrified shoots from leaf callus of Dianthus caryophyllus cv.Alas Red (Fig.16) Arthur Sim (Fig.17). after 3 weeks of culture.

Table 11. Effect of different treatments on shoot bud differentiation from stem calli (200 mg fresh wt. basis) of *Dianthus caryophyllus* cv. Arthur Sim. (Data represent the mean number of shoot buds per callus ± S.E.

MS+Growth regulators (mg/l)		% of shoot bud regeneration		No. of shoots/culture * ± S.E.	
BA	NAA	stem	leaf	stem	leaf
0	0	0	0	0	0
0.5	0.1	0	0	0	0
1.0	0.1	10.0	11.0	4.2 ± 0.1	10.5 ± 0.8
1.5	0.1	12.0	18.0	8.4 ± 0.8	16.2 ± 0.6
2.0	0.1	30.0	36.0	10.2 ± 0.2	14.4 ± 0.9
2.5	0.1	25.0	28.0	18.5 ± 0.3	24.2 ± 0.6
3.0	0.1	20.0	22.0	14.2 ± 0.2	20.8 ± 0.8
1.0	0.25	42.0	44.0	18.6 ± 0.4	22.4 ± 0.4
1.5	0.25	76.0	80.0	42.3 ± 0.8	51.2 ± 0.6
2.0	0.25	75.0	86.0	50.2 ± 0.6	57.8 ± 0.7
2.5	0.25	67.0	60.0	38.4 ± 0.8	47.2 ± 0.6
3.0	0.25	60.0	62.0	41.2 ± 0.6	51.5 ± 0.8
1.0	0.5	20.0	18.0	14.3 ± 0.5	24.4 ± 0.6
1.5	0.5	28.0	30.0	26.5 ± 0.9	22.2 ± 0.8
2.0	0.5	32.0	34.0	25.2 ± 0.6	26.5 ± 0.6
2.5	0.5	26.0	28.0	18.8 ± 0.7	16.2 ± 0.8
3.0	0.5	10.0	0	4.8 ± 0.7	0
2.0	1.0	0	0	0	0
2.5	1.0	0	0	0	0
3.0	1.0	0	0	0	0

(20 replicates/treatment); repeated thrice

Table 12. Effect of different treatments on shoot bud differentiation from leaf and stem callus of *D.caryophyllus* cv. Alas Red.(Data represent the mean number of shoots per callus ± S.E.)..

MS + Growth regulators(mg/l)		% of shoot regeneration		Av.no.of shoots/culture ±S.E.*	
BA	NAA	Stem	Leaf	Stem	Leaf
0	0	0	0	0	0
0.5	0.1	0	0	0	0
1.0	0.1	10.0	14.0	6.2 + 0.6	8.4 + 0.8
1.5	0.1	12.0	19.0	10.5 ± 0.8	12.2 ± 0.6
2.0	0.1	18.0	22.0	11.2 ± 0.6	16.4 ± 0.7
2.5	0.1	20.0	26.0	17.2 ± 0.5	18.6 ± 0.2
3.0	0.1	26.0	28.0	22.4 ± 0.6	24.5 ± 0.6
0.5	0.25	10.0	12.0	14.8 ± 0.9	18.2 ± 0.7
1.0	0.25	25.0	28.0	27.2 ± 0.8	20.4 ± 0.6
1.5	0.25	57.0	61.0	58.2 ± 0.6	67.2 ± 0.4
2.0	0.25	62.0	67.0	64.4 ± 0.9	52.4 ± 0.8
2.5	0.25	60.0	58.0	52.9 ± 0.2	41.2 ± 0.3
3.0	0.25	42.0	38.0	28.5 ± 0.3	22.4 ± 0.6
1.0	0.5	18.0	20.0	20.3 ± 0.6	18.2 ± 0.5
1.5	0.5	24.0	26.0	28.2 ± 0.8	22.8 ± 0.6
2.0	0.5	22.0	28.0	21.8 ± 0.6	31.2 ± 0.7
2.5	0.5	23.0	24.0	22.6 ± 0.4	24.6 ± 0.8
3.0	0.5	28.0	25.0	23.2 ± 0.6	22.8 ± 0.1

* Experiments repeated thrice, 20 replicates/treatment.

4.2.6 Effect of subculture on shoot bud regeneration

Subculturing of callus had great influence on shoot bud differentiation from stem and leaf calli of Dianthus caryophyllus cvs. Arthur Sim and Alas Red. To begin with the rate of shoot bud regeneration was declined in the calli derived from the stem explants. Leaf callus was more responsive for shoot bud regeneration and the regeneration rate was maintained upto 7th subculture after which there was a sharp decline in the regenerative ability.

4.3 Induction of rooting from organogenic shoots

The growing shoots were excised (Fig.18) and cultured on various medium containing 1/2 strength basal MS salts supplemented with different concentrations of NAA, IAA and IBA. About 75-85% of the shoots were rooted within 10-12 days of culture (Fig.19) on 1/2 MS supplemented with 0.1-0.25 mg/l NAA or IBA. (Table 13) When the concentration of IBA was increased beyond 0.25 mg/l, the shoots developed roots with callus at the basal end of the shoot. Increase in the concentration of NAA from 0-25 to 0.5 mg/l, also induced the callus at the basal end of the shoots. The percentage of rooting was more in the liquid medium than the solid medium. The root primordia developed quickly in the liquid medium and growth was faster in the subsequent culture period.

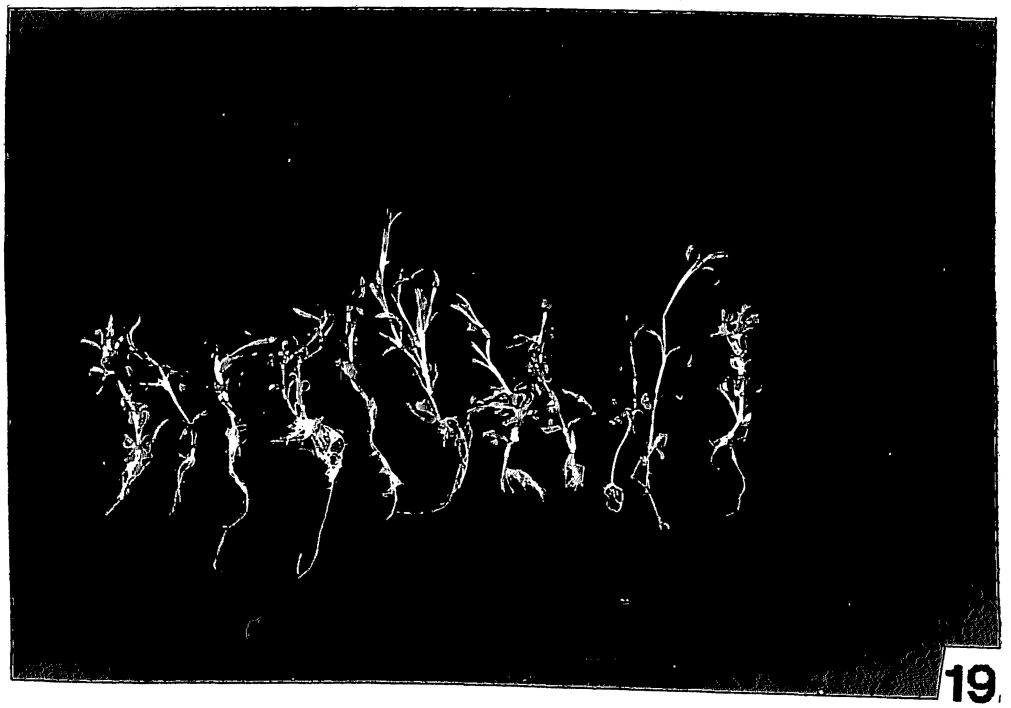


Fig.18 Isolated shoots derived from leaf calli of Dianthus caryophyllus cv.Alas Red.

Fig.19 Rooted plantlets for transplantation.

Table 13. Effect of NAA and IBA on root induction from excised shoots of *Dianthus caryophyllus* cv. Alas Red.

1/2MS+growth regulators		Days to rooting	No.of roots/ shoot ±S.E.	% of rooting
IBA	NAA			
0	0	26-28	3.2 ± 0.36	25-30
0.10	0	10-11	12.3 ± 0.11	75-85
0.25	0	13-14	8.6 ± 0.36	65-70
0.50	0	16-18a	6.4 ± 0.2	40-45
1.0	0	30a	2.1 ± 0.1	20-30
0	0.10	11-12	9.2 ± 0.3	75-80
0	0.25	13-14	8.2 ± 0.8	66-72
0	0.50	18-20a	6.4 ± 1.2	37-42
0	1.00	28a	3.1 ± 0.9	20-25

a - callusing at the basal end; experiments repeated twice; 20 replicates per treatment.

4.4 Acclimatization and field establishment

For hardening and acclimatization, the rooted plantlets were grown in 1/2 strength basal MS medium without growth regulators for 15 days and subsequently transferred to sterile compost containing sand: soil (3:1). About 80-85% of plantlets survived in the greenhouse (Figs. 20 & 21). Subsequently, the plantlets grew well and developed flower buds (Fig. 22). When the plantlets were rooted in semi-solid medium, the rate of survival reduced to 50-60%. In another experiment, the in vitro grown shoots were directly transferred to sterile soil containing sand:soil (3:1) and kept under the green house conditions for rooting; about 40-50% of shoots rooted in the greenhouse but most of the shoots died after being transplanted to the pots.

4.5 Biochemical changes during growth and development

During callus proliferation, the levels of chlorophyll-a, chlorophyll-b declined. Protein and carbohydrate contents increased as compared to the initial explants (Tables 14-16). The chlorophyll-a, chlorophyll-b, protein and carbohydrate contents increased during the shoot bud regeneration but the carotenoids were very negligible. At induction of roots, the protein and carbohydrate content were less pronounced as compared to stages of callus proliferation and shoot-bud differentiation. The protein and carbohydrate were 22.82 $\pm$ 0.236 & 28.6 $\pm$ 1.36 respectively during rooting of the in vitro derived shoot.

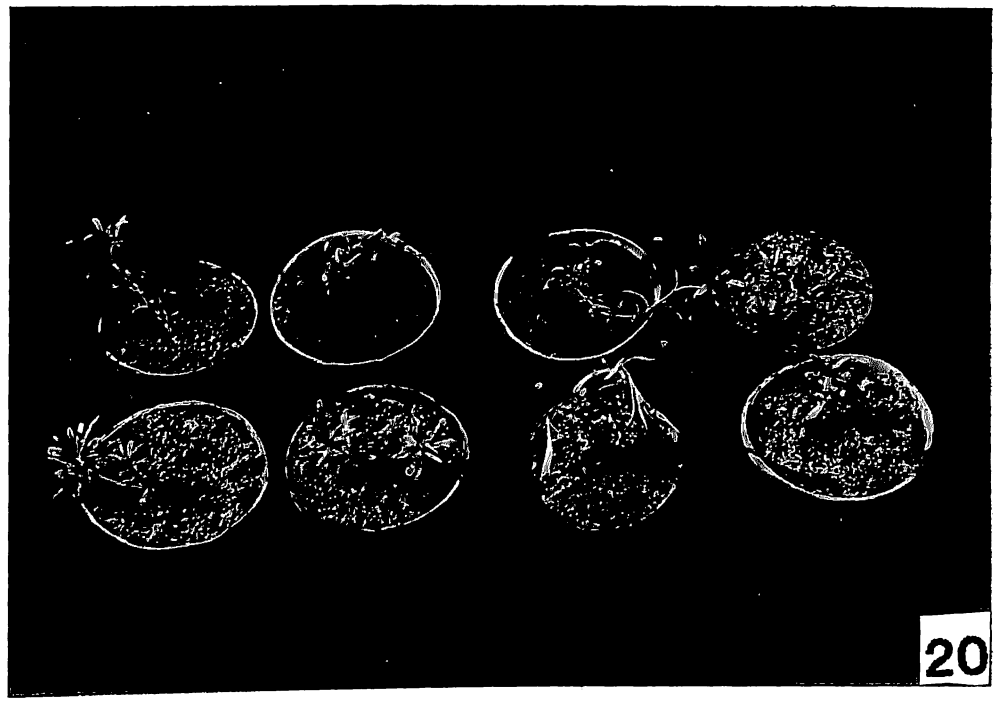


Fig.20. Micro-Plantlets in 2 inch earthern pots.

Fig.21.Plants after 50 days of transplantation.

Table 14. Pigment concentration at different stages of growth and development of Dianthus caryophyllus cv. Alas Red.

Sources of explant /tissue	Pigment content (mg/g) on fresh weight basis ± S.D.*			
	Chlorophyll-a	Chlorophyll-b	Total Chlorophyll(a+b)	Carotenoid
Leaf explant	0.138 ± 0.016	0.211 ± 0.041	0.349 ± 0.057	0.086 ± 0.013
Callus Proliferation	0.098 ± 0.036	0.102 ± 0.029	0.200 ± 0.065	0.036 ± 0.018
Shoot-bud differentiation	1.012 ± 0.019	1.059 ± 0.051	2.071 ± 0.070	0.028 ± 0.059
Root induction	0.026 ± 0.023	0.081 ± 0.041	0.107 ± 0.064	0.041 ± 0.017

* - 10 replicates per experiment; repeated thrice.

Table 15. Carbohydrade content (ug/ml) at different stages of growth and development of Dianthus caryophyllus cv. Alas Red.

Source of explants/tissue	Carbohydrate content on fresh weight basis (ug/ml) Mean ± S.D. *
Leaf explant	22.6 ± 1.16
Callus proliferation	43.8 ± 2.26
Shoot-bud differentiation	76.4 ± 1.42
Root induction	28.6 ± 1.36

* - 10 replicates/experiment ; repeated thrice.

Table 16. Protein content (ug/ml) at different stages of growth and development of Dianthus caryophyllus cv.Alas Red.

Source of explants/tissue	Protein content on fresh weight basis (ug/ml) Mean ± S.D. *
Leaf explant	18.32 ± 0.141
Callus proliferation	17.48 ± 0.782
Shoot-bud differentiation	47.27 ± 0.418
Root induction	22.82 ± 0.236

* - 10 replicates/experiment; repeated thrice.

Fig.22.Plant with flower buds.

CHAPTER - V
DISCUSSION

Discussion

Studies were under-taken for improving the clonal multiplication of carnation by the use of different growth hormones and the culture conditions.

5.1. Meristem culture

Bud proliferation and elongation of shoot occurred in about one week after culture on MS + 1.5 mg/l BA + 0.25 mg/l NAA; on an average 48 new shoot buds emerged from a single meristematic dome in 4 weeks of culture, on the other hand, only few new shoots were produced on the same (MS) medium supplemented with high concentrations of Kn or BA + NAA. At higher concentration of BA or Kn along with NAA the proliferation of shoot-bud was slow; the shoots had callus at the basal end. Similar observation was noticed in other cultivars tested earlier (Ghosh & Mohan Ram, 1986). The present study revealed that the media containing 1.5-2.0 mg/l BA with 0.25 mg/l NAA proved to be the most effective for shoot-bud multiplication. High rate shoot proliferation in both the cultivars was noted due to the synergistic effects between BA and NAA. These findings are in accordance with the earlier findings of Choudhary(1991). When NAA concentration was increased beyond 0.25 mg/l; the shoots became vitrified and small callus formed at the base of the shoots. In most of the cases, the shoots became abnormal in appearance.Similar type of observation was reported by Leshem and Sachs(1985).

Liquid medium was superior to the semi-solid medium with regard to the rate of shoot multiplication. The rate of multiplication was enhanced in liquid base than semi-solid medium with similar composition within 4-week of culture. Earle and Langhans (1975) reported that the rate of multiplication was enhanced two to three times within four weeks of culture period with the inclusion of 10^{-5} M BA + 10^{-6} M NAA in the culture medium. The addition of aminoacids and vitamins in B_5 media significantly enhanced the rate of shoot multiplication in other carnation cultivars (Choudhary, 1991). Shoot bud continued to multiply upto 120 days and thereafter it declined there after in both the cultivars.

Growth of the normal shoots were fully dependent on the concentration of the agar. As the concentration of agar was increased beyond 0.8%, the number of succulent shoots/culture decreased. The rate of development of normal shoots/ culture increased. This might be due to the reduction in the mobility of nutrient solution. The rapid growth rate noticed on 0.6% (w/v) agar concentration but, the shoots became abnormal with broad and thick leaves having higher turgidity. The abnormal leaves were due to the excessive uptake of water and nutrients, as reported by Leshem, (1983).

5.2. Induction of rooting

To induce rooting, the isolated shoots were placed on both semi-solid medium and on sterilized filter paper bridges in the liquid medium with growth regulators and 2% (w/v) sucrose. The results of the present investigation indicates that the liquid medium was advantageous than the semi-solid medium for quick induction of root. The rooting was better in the liquid than the semi-solid medium due to quick penetration of growth regulators and other additives (Hempel, 1979). In the media without growth regulator, the shoots became very weak and the rooting was inhibited. However, the increased concentration of NAA or IBA (>0.5 mg/l) had adverse effects on root induction. Similar responses were reported earlier in other cultivars (Hempel and Gabry Szewska, 1983; Hempel, 1979).

5.3 Induction of callus

The higher levels of 2,4-D or NAA repressed the initiation of callus. The 2,4-D or NAA along with BA was found to be the main inducer of callus. All the treatments containing BA + 2,4-D and BA + NAA favoured early initiation of callus; the treatment containing BA + 2,4-D was, however, less effective than other treatments tested. Within 4 weeks of culture, the callus became friable and yellowish-green in colour. The rate of callus growth was faster on subculturing in the same medium.

5.4. Regeneration of shoot-bud

Regeneration was found to be very poor on medium containing BA + NAA. The maximum rate of shoot bud regeneration was observed in the medium containing 1.5-2.0 mg/l BA + 0.25 mg/l NAA in both the cultivars tested. High cytokinin and low auxin ratio influencing shoot-bud regeneration was reported earlier in carnation (Sastry, 1963).

More number of shoot buds/callus was observed in calli derived from leaf than stem explants in both the cultivars. Liquid medium was more effective than semi-solid medium for shoot-bud regeneration. This may due to direct penetration of growth regulators with nutrients to the callus systems. Moreover, high yield of adventitious shoots per callus varied with BA & NAA concentration (Kozak & Hempel, 1979). Higher percentage of regeneration were obtained by increasing the BA or Kn concentration, but the regenerated shoots became vitrified. Similar observations were recorded by Messeguer et al. (1993); Frey & Janick (1991) and Kakehi (1971).

Light treatment strongly enhanced the regeneration of shoot buds from leaf and stem calli derived from both the cultivars of Dianthus caryophyllus . Continuous light or dark did not have any positive effect on shot bud regeneration. At higher concentrations of agar (1.0 to 1.2%) vitrification could be prevented and

shoots rooted normally produce rootable shoots. These results confirm the observations of Ziv et al. (1987); Kevers & Gaspar (1986); and Shabde & Murashige (1977). The rate of regeneration was poor in case of cv. Arthur Sim as compared to cv. Alas Red. Halperin (1986) reported that the growth regulators acted as receptors and were essential for growth and development of carnation. Lack of such receptor in cv. Arthur Sim could be one of the reason as the diverse concentrations and types of growth regulators tried might have been irrelevant for differentiation. The cells might have lost genetically controlled features (Totipotency) to take advantage of the in vitro system. Such operation of genetic factors for organogenesis in vitro have been reported by several authors in different species (Bingham et al., 1975 and Reisch & Bingham, 1980). Variation of shoot-bud regeneration between the two cultivars may be due to physiologically controlled factors in which the cell may be having genetic potential to show differentiation but not to be expressive under that particular in vitro system (Skvirsky et al, 1982).

5.5. Induction of roots from regenerated shoots

Regenerated shoots were rooted on half-strength MS medium supplemented with 0.25 mg/l IBA or NAA. About 75% of the shoots were rooted within 10-12 days of culture. When IBA concentration was increased beyond 0.25 mg/l, the regenerated shoots developed callus at the basal portion of the cut end. These results are

consistent with the earlier findings of Hempel (1979). Liquid medium with filter bridges helped better rooting than semi-solid medium because, the rate of absorption of growth regulators and other additives by the shoots were quicker in liquid than in solid medium. Similar results were reported by Leshem & Sachs (1985); Leshem (1983) and Choudhary (1991).

5.6. Acclimatization and field establishment

For hardening and acclimatization, the rooted plantlets were grown in liquid medium without growth regulators for 15 days and subsequently transferred to sterile compost containig sand:soil (3:1). About 80-85% of plantlets were survived in greenhouse conditions. Subsequently, the plantlets grew well and developed flower buds. When the plantlets were rooted in semi-soild medium, the rate of percentage of survival was reduced to 50-60%. This was due to the lack of physical and physiological disorder of the transpiration system. Earle & Langhans (1975) reported that the plantlets survived better in vermiculite : perlite (1:1) (v/v) kept at 20 ^{o}C in a growth chamber with 16h photoperiod at the light intensity of 19,000 lux for 15 days.

In an another experiment, the growing *in vitro* shoots were directly transferred to sterile soil containing sand:soil (3:1) and kept in a greenhouse condition for rooting. About, 35-40% of shoots were rooted in the greenhouse and most of the shoots died due to lack of a functional connection between the roots and the shoot formed *in vivo*.

5.7. Biochemical changes during growth and development

The chlorophyll-a, chlorophyll-b, protein and carbohydrate content increased sharply during shoot-bud regeneration but the carotenoid content gradually declined and became negligible. During root initiation, the protein and carbohydrate content declined as compared to earlier stages. The content of protein and carbohydrate were 22.82 $\pm$ 0.236 and 28.6 $\pm$ 1.36 during rooting phase.

CHAPTER - VI
SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Mass cloning of Dianthus caryophyllus through in vitro technique was attempted by manipulation of growth regulators and culture conditions. Two cultivars namely 'Alas Red' and ' Arthur Sim' were used for micropropagation. Apical, axillary meristems, stem and leaf explants were used as explant sources for shoot multiplication and callus culture. Bud proliferation and multiplication were achieved in both the cultivars on medium containing MS basal salts supplemented with 1.5 mg/l BA plus 0.25 mg/l NAA. The rate of multiplication was highly pronounced in the liquid medium than the semi-solid medium. The multiplication rate also varied between the two cultivars. Induction of roots from isolated shoots were achieved in both the cultivars. The frequency of rooting was maximum in the liquid medium than solid medium. The root proliferation was faster in liquid medium containing half strength basal MS salts supplemented with 0.1-0.25 mg/l IBA and 2%(w/v) sucrose.

Callus induction from stem and leaf explants were achieved in MS medium supplemented with BA + NAA. The rate of callus proliferation was better during subculture in the medium containing similar supplements. Callus was yellowish-green in colour during proliferation. Shoot-bud regeneration was achieved from both leaf and stem calli of D. caryophyllus cvs. Alas Red and Arthur Sim. Maximum shoot buds per callus was observed on MS medium containing 1.5-2.0 mg/l BA plus 0.25 mg/l NAA in both the

cultivars. The cultures incubated in the light recorded high percentage of shoot-bud differentiation than the dark treatment. Higher concentration of either BA or NAA, inhibited shoot-bud differentiation in both the cultivars. Regenerated shoots were rooted in liquid medium containing half strength basal MS salts supplemented with 0.1 to 0.25 mg/l IBA, 2% (w/v) sucrose. Rooted shoots were transferred to 1/2 strength MS basal salts devoid of groth regulators for hardening. About 80-85% of plantlets survived in the greenhouse.

Pigments, carbohydrate and protein were estimated during callus induction, shoot bud differentiation and rooting of D. caryophyllus cv. Alas Red. The pigment content was maximum at the shoot bud differentiation stage as compared to the callus-induction and rooting stages.

The plantlets were successfully hardened and grown under field condition; very high percentage of the micropropagated plants survived transplanting. The plants were healthy and uniform in growth and showed synchronous flowering.

Methods were standardized for mass cloning of two improved cultivars for use in commercial flower production. The protocols can be employed for inducing somaclonal variations to develop new cultivars.

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