CLONAL PROPAGATION OF *NASTURTIUM* - AN ORNAMENTAL PLANT THROUGH TISSUE CULTURE

A

Thesis submitted

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BY

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CERTIFICATE

This is to certify that the thesis entitled, **"Clonal Propagation of *Nasturtium* - An Ornamental Plant Through Tissue Culture"** submitted by Rajneesh Kumar in partial fulfilment of the requirement for the award of the degree of Master of Science in Biotechnology, to Thapar Institute of Engineering and Technology, Patiala, is an authentic record of his own work carried out by him during the period of six months from January 2005 to June 2005, under my supervision and guidance. This report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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Dated: **(RAJNEESH KUMAR)**

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CONTENTS

ABBREVIATIONS

A^{O}	Angstrom
BAP	Benzylaminopurine
BMS	Basal Murashige and Skoog's medium
^{O}C	Degree Celsius
CH	Casein hydrolysate
CM	Coconut milk
2, 4-D	2, 4-dichlorophenoxy acetic acid
IAA	Indole- 3-acetic acid
IBA	Indole-3-butyric acid
2-ip	2-isopentenyl adenine
Kn	Kinetin
NAA	Naphthalene acetic acid
ppm	Parts per million
W	Watt

ABSTRACT

The present investigation was carried out on a herbaceous angiosperm namely *Tropaeolum majus* belonging to family *Tropaeolaceae*. It is commonly known as "Garden Nasturtium" and is grown as an ornamental in the gardens. Different vegetative parts like root stem, leaves, and shoot apices were excised from both the *in vivo* and *in vitro* raised plants and thereafter planted on Murashige and Skoog's medium supplemented with various growth adjuvants for callus induction, organogenesis and multiple shoot formation.

Multiple shoot proliferation was effected from shoot apices on MS medium supplemented with various growth adjuvants in different combinations. Multiple shoot formation occurred on MS supplemented with BAP (0.5 – 4 ppm) either alone or in combination with NAA (0.5 ppm). Best results, however, were obtained on MS + BAP (4 ppm) where 25 – 35 shoots regenerated from a single shoot apex. It was also observed that initial treatment with BAP (2 – 4 ppm) for 2 - 3 weeks followed by transfer to lower concentration of BAP (0.01 - 0.1 ppm) or simple MS medium proved most effective in terms of new shoot production and shoot growth rate. The shoots thus formed were excised and transferred to different root inducing media.

Roots, stem and leaves were planted on variously supplemented MS medium for organ regeneration and callus induction. *Nasturtium* exhibited a high propensity of rooting from all the vegetative parts of the plant. Prolific root regeneration directly from these explants was observed on MS supplemented with NAA (1 - 4 ppm) with or without Kinetin. Best results, however, were obtained on MS + NAA (4 ppm) only.

Direct Shoot regeneration was effected from stem explants of both *in vivo* and *in vitro* raised plants. Shoot regeneration directly from stem

explants was observed on simple MS medium, MS + 2, 4-D (2 ppm) + CM 15 %, and MS + 2, 4-D (2 ppm) + Kinetin (1 ppm) + CM 15 %. Best results were however, obtained on simple MS medium, where 15 – 20 shoots regenerated from the stem explant. The regenerated shoots were transferred to MS medium having IAA or IBA for adventitious root initiation. After shoot and root development, attempts were made to establish regenerated plantlets into soil through a series of hardening stages.

Callus induction was effected from petiole and stem segments of both *in vivo* and *in vitro* reared plants. Synergistic action of 2, 4-D (2 ppm), and CH (2g/l) was demonstrated for the initiation and good growth of Callus .Calli obtained from petiole and stem explants on MS + 2,4-D (2 ppm) + CH (2g/l) was yellowish, solid and slow growing.

Cell types studied in various calli showed their heterogeneous nature with wide variations in the size and shapes of cells. Histogenetic differentiation in the form of tracheids was observed in all the calli. Tracheids occurred singly or in groups and possessed scalariform thickenings on their walls.

No differentiation of roots, shoots or whole plantlets from the calli could be effected under the present cultural conditions employed

INTRODUCTION

Plant tissue culture has emerged as a potential tool and forms the backbone of plant biotechnology. Tissue culture techniques are widely applied for the improvement of field crops, forests, horticulture and plantation crops for increased agricultural and forestry production. This technique has been commercialized globally and contributed significantly towards the enhanced production of high quality planting material.

Clonal propagation of selected phenotypes is an essential step in most of the plant breeding programmes. It is a faster method of asexual reproduction in comparison to propagation through seeds. Plants raised through seeds are highly heterozygous and one has to select plants from a wide population which have the best qualities. Owing to heterozygosity, the seed raised plants show high variation in growth, habit, and yield and they may have to be discarded because of the poor quality of their flowers and fruits for commercial release. Likewise majority of the plants propagated by vegetative means contain systemic bacteria, fungi and viruses which affect the yield, quality and appearance of selected plants. Moreover majority of plants are not amenable to vegetative propagation through cuttings, budding and grafting, thus limiting multiplication of desired cultivars.

In the recent years, tissue culture has emerged as a promising technique to obtain genetically pure elite populations under *in vitro* conditions. *In vitro* propagation also called micropropagation is infact the miniature version of conventional propagation, which is carried out under aseptic conditions. The advent of *in vitro* tissue culture technique has offered a new approach to the morphogenetic investigations. It allows a living system to be studied under controlled environmental conditions. This enables a study of the complex biological phenomenon in parts. Moreover these partial processes are amenable to controlled investigations.

Plants raised through micropropagation are:

- of uniform quality
- Pathogen free
- Can be produced much more rapidly as new cultivars could become commercially available with in 2 to 3 years from development rather than 5 to 10 years needed using conventional propagation.
- Produce uniformly superior seeds.
- Show improved vigor and quality

Major Steps in *In-Vitro* Clonal Propagation are:-

Stage – I

Initiation and establishment of aseptic culture:-

This involves explant isolation, surface sterilization and establishment on an appropriate culture medium. Cultures are initiated from explants of several organs but shoot tips and axillary buds are most often used for commercial micropropagation.

Stage – II

Micropropagation of plants can be achieved through any one of the following four methods:-

- Multiplication through calli raised from different organs and tissues and their subsequent subculturing leading to organogenesis or somatic embryogenesis.
- Multiplication through the direct induction of shoots on the explants.
- Multiplication through growth and proliferation of existing apical shoots excised from the parent plant.

- Multiplication through induction of adventitious shoots of existing meristems within axillary buds which proliferate after removal from the parent plant.

Stage – III

Rooting of regenerated shoots in *in vitro* conditions:-

This stage is characterized by preparation of stage II shoots or shoot clusters for successful transfer to the soil. The process may involve:-

- Elongation of shoots prior to rooting.
- Rooting of individual shoots or shoot clumps.
- Pre - hardening of cultures to increased survival.

Stage – IV

Transfer to natural environment:-

The ultimate success of shoot culture depends upon the ability to transfer and reestablish vigorously growing plants from *in vitro* to green house conditions. Micropropagated plants are difficult to transplant for two primary reasons:-

- A heterotrophic mode of nutrition.
- Poor control of water loss.

To overcome these limitations, plantlets should be transplanted into a well drained sterile growing medium and maintained initially at high relative humidity (90 %) and reduced light at 20 to 27^0C for the first 10 - 15 days by keeping them under mist or covering them with clear plastic bags. After spending few days under high humidity the plants should be moved to the green house bench. Transplants should then acclimatized by gradually lowering the relative humidity over 1 to 4 weeks period. Plants are gradually moved to higher light intensities to promote vigorous growth.

Though abundant work has been done on herbaceous plants, more information is required concerning differentiation and morphogenesis in unattempted materials to gain broad understanding of the physiology of development. The main objective of the present investigation was to develop a dependable protocol for large scale clonal multiplication of Nasturtium under *in vitro* conditions.

REVIEW OF LITERATURE

The growth of all the living organisms – plants as well as animals begins with a single fertilized cell i.e. zygote. This cell is evidently the repository of all the information necessary for its subsequent growth into a multicellular, highly organized, complex but co-ordinated system. This tiny totipotent cell conceals the potential for differentiation. The differentiated somatic cells in a plant carry out specialized activities and appear to have surrendered their totipotency in the bargain.

The idea of totipotency of plant cells was put forward by G. Haberlandt, the great German physiologist who in 1902 suggested that "one could successfully cultivate artificial embryos from vegetative cells". He isolated cells from a number of higher plants and maintained them alive in a viable state in simple nutrient solutions for about 10 days. During this period, cell swelling and wall thickening occurred, but the cells failed to divide. Haberlandt's attempt to grow vegetative cells in an artificial medium did not succeed due to lack of proper techniques and unfortunate choice of highly specialized materials but it opened up new vistas in morphogenesis.

Although Haberlandt failed, Gautheret, Nobecourt and White in 1939 reported the formation of continuous callus cultures in carrot and tobacco independently of each other. Although the significance of Haberlandt's idea was not lost on botanists, the demonstration of totipotency consumed half a century. In 1958 Steward *et al*. demonstrated the totipotency of higher plant cells in unambiguous terms with their success in forming somatic embryos from cultured root phloem cells. Haberlandt's hypothesis has now, flowered into a vigorous discipline – "Tissue culture". This broad term refers to the growth of cells, tissues and organs in artificial medium under aseptic conditions.

Floriculture is fast developing as a flourishing industry to meet the growing demands of cut and ornamental flowers. In order to meet the growing demands, there will be an increasing need of improved quality and shortened rotation. Most

of the plant raised through seeds are highly heterozygous and show great variations in growth, habit and yield and may have to be discarded because of poor quality of flowers and fruits for their commercial release.

Likewise majority of the plants are not amenable to vegetative propagation through cutting and grafting, thus limiting multiplication of desired cultivars. Moreover many plants propagated by vegetative means contain systemic bacteria, fungi and viruses which may affect the quality and appearance of selected items. During the last few years micropropagation technique has emerged as a promising technique for rapid and large scale propagation of vascular plants.

Micropropagation is a complex multistep process and it is in effect the miniature version of conventional propagation which is carried out under aseptic conditions. The ease with which plants can be micropropagated varies from species to species. Mostly seeds, seedlings and juvenile plant parts are used as starting materials since they are easier to propagate and hence form the basis of development of suitable medium and methods for multiplication of new species. Since, desirable characters in plants are identifiable only after their maturity, their studies although of profound academic interest are not that effective for immediate use in plant improvement programmes.

Micropropagation can be achieved by any of the three approaches:

1) Enhanced axillary bud breaks / shoot multiplication.

2) Production of adventitious buds.

3) Somatic embryogenesis.

Multiplication by apical and axillary shoots:

Micropragation through apical and axillary shoot proliferation is the most reliable technique for mass multiplication since it ensures genetic stability of clones. Apical and axillary shoots contain active meristems. Shoot tips cultured on basal medium containing no growth hormones typically develop into single

seedling like shoot with strong apical dominance. On the contrary when the shoots of the same explant material are grown on culture medium containing cytokinins or other growth adjuvants, axillary shoots develop clusters of secondary and tertiary shoots. These clusters can be further subdivided into smaller clumps of shoots or separate shoots which, in turn, will form similar clusters when subcultured on a fresh medium. This subdivision process may continue indefinitely provided the basic nutrient formulations are adequate for normal growth.

The multiplication rates through this technique vary with genotype and the cytokinin requirement has been extremely variable. Mehra and Cheema (1980) reported formation of multiple shoots by the activation of immature axillary buds in *Populus* on MS + BAP medium (1×10^{6}m). Rout *et al*., (1989) reported multiple shoot formation on nodal explant of *Rosa hybrida* on MS medium supplemented with BAP, GA_3 and casein hydrolysate. Gurel and Gulshan (1998) reported multiple shoot proliferation from shoot tips of *Amygdalus communis* on MS medium with the combination of 0.1 mg/l IBA and 1.0 mg/l BAP. Multiple shoot formation were reported from shoot tips (1 – 2 cm) of field grown plants of *Paederia foetida* and *Cantella asiatica* on MS medium supplemented with BAP (1.0 mg/l) with in 7 days of culture (Singh *et al*., 1999). In *Bixa orellana*, Sharon and D'sauza (2000) regenerated plants from shoot apex and nodal explants on MS medium supplemented with 2-ip (2-isopentenyl adenine 1.0 mg/l). Addition of BAP (0.3 mg/l) and kinetin (0.2 mg/l) has been found to give a good response of shoot proliferation in *Withania somnifera* with a regeneration of 85 % (Kulkarni *et al.,* 2000). Amin *et al*., (2002) reported axillary shoot formation on nodal segments of *Ixora fulgens* on MS medium with the combination of 0.5 mg/l BA + 0.1 mg/l NAA. MS medium with growth regulators such as BAP (0.5 mg/l) in conjunction with NAA (0.01 mg/l has been reported to give optimum results in *Utleria salcifolia* (Gangaprasad *et al*., 2003).

Likewise greatest efficiency of shoot formation from meristem was reported in *Origanum vulgare* by Goleniowski *et al*., 2003. In *Crataeva magna*, rapid multiplication was achieved on MS + BAP (8.8 µm) (Benniaamin *et al*.,

2004), whereas in *Peltophorum pterocarpum*, highest number of multiple shoots was observed on MS + kinetin (2.0 mg/l) + NAA (0.5 mg/l) (Uddin *et al*., 2005).

Multiplication by adventitious shoots:

Adventitious shoots arise naturally on plant tissues located in sites other than at the normal leaf axil regions. Many ornamental and horticultural species have been successfully propagated *in vitro* by adventitious shoot initiation. New adventitious shoots can develop directly from the explants like root, stem, petiole, leaf lamina, flower parts or indirectly from the callus cultures obtained from these explants. The initiation of adventitious shoots is dependent on two factors a) choice of explant; b) hormone regime to which plant is subjected.

Mehra and Cheema (1980) reported multiple shoots (75 – 80 per culture) on immature lamina discs of female *Populus ciliata* on MS medium with optimal amount of BAP in dark. Chen *et al*., (2000) reported adventitious shoot formation from the stem internode explants of *Adenophora triphylla*-an important medicinal plant. Kantia and Kothari (2002) reported adventitious shoot bud formation directly on the surface of the leaf explants in *Dianthus chinesis.* Koroch *et al*., (2002) reported multiple shoot regeneration and Agrobacterium mediated transformation of *Echinacea purpurea leaf explants*. Martin *et al*., (2003) reported direct shoot regeneration from lamina explants of two commercial cut flower cultivars of *Anthurium andraeanum*. Uddin *et al*., (2005) established a protocol for rapid multiplication of shoots from cotyledonary node of *Peltophorum*.

Organogenesis through callus cultures has been reported in a number of herbaceous plants. Callus and cell suspension derived from organs or single cell can be induced to form roots, shoots or embryoids under defined physical / chemical conditions. For instance, the somatic cells of *Nicotiana tabacum* (Skoog and Miller, 1957; Vasil and Hildebrandt 1967), *Convolvulus arvensis* (Earle and Torrey, 1965), *Cichorium endivia* (Vasil and Hildebrandt, 1966), *Pterotheca falconeri* (Mehra and Mehra, 1972), *Mesembryanthemum floribundum* (Mehra and Mehra, 1972), *Petunia hybrida* (Colizn *et al*., 1979), *Dimorphotheca ecklonis* (Anand and Mehra, 1983), *Pelargonium graveolens*

(Sreedhar, 1999), *Withania somnifera* (Manickam *et al*., 2000), *Coleus forskohlii* (Reddy *et al*., 2001) can give rise to whole plantlets, thus demonstrating the totipotency of higher plant cells in unambiguous terms.

As far as clonal propagation is concerned, the plants obtained from calli may not be true elites because of considerable morphological, physiological and genetic variations found within the callus and there are relatively high incidences of aneuploidy and polyploidy associated with callus cells and plants obtained from it. Another disadvantage of the method of shoot multiplication involving a callus phase is that it is not applicable to many important crop species. Where applicable, the initial plant regeneration capacity of the tissues may decline with the passage of time and is eventually lost. But still callus constitutes one of the unique materials for rapid multiplication of plants, since thousands of plants can be obtained from a small tissue.

Somatic embryogenesis: -

It involves the formation of a bipolar structure containing both shoot and root meristems, and developing in a manner similar to zygotic embryos. These embryoids can develop into fully functional plants under appropriate conditions. Steward *et al*. (1958) and Reinert (1959) firstly reported somatic embryogenesis from phloem cells of roots in *Daucus carota*. Embryoid formation has been reported in tissue and organ culture of a number of plant species derived from petiole, floral parts, mesophyll cells, nucellar tissue and peduncle. Complete plant regeneration by embryogenesis was observed on different explant cultures of *Dendrophthoe falcata* (Johri and Bajaj , 1962), *Ranunculus sceleratus* (Konar and Nataraja,(1969); Konar *et al*., (1972); Thomas *et al*., 1972), *Citrus aurantifolia* (Mitra and Chaturvedi, 1972), *Macleaya cordata* (Kohlenbach,1977), *Coffea species* (Monaco *et al*.,1977;Sondahl *et al*.,1979), *Santalum album* (Bapat and Rao, 1979), *Mangifera indica* (Durzan, 1985), *Muntingia calabura* (Raut *et al*., 1996), *Allium sativum* (Abo EL-Nil, 1997), *Sapindus* (Sinha *et al*., 2000), *Kaempferia galanga* (Rahman *et al.,* 2004) and

many more plants. Nakano *et al.,* (2004) reported somatic embryogenesis and plant regeneration from callus cultures of several species of *Tricyrtis.*

MATERIAL AND METHODS

Choice of Material:

Tropaeolum majus: A herbaceous plant of family *Tropaeolaceae* was selected as the experimental material. It is commonly known as Indian Cress or Canary Bird Flower. It is an annual herbaceous plant which can be easily grown from seeds. It grows best on light and sandy soils and good flowering occurs in full sunlight (Fig 1). It is used for edging flower-beds, in widow-boxes, patio containers or hanging baskets. The abundant colorful flowers can be cut for use as an elegant entrée garnish or salad decoration. The leaves which contain good dose of Vitamin-C can be used to add peppery flavor to fresh salads. The flower petals are also edible.

The following criteria favored its choice:

- Plants are of easy cultivation and seeds are readily available.
- The growth of plant is rapid
- A scrutiny of literature revealed that the plant has not been previously worked out for tissue culture studies.

Glassware:

The glassware used for culture work comprised of 6”x1” Riviera and Borosil test tubes, 100 ml, 250 ml, 500 ml, and 1000 ml corning and Borosil flasks, pipettes, and measuring cylinders (100 ml, 500 ml). Before use, glassware were thoroughly brushed with alkaline detergent teepol and then washed in running water. These were then treated with hot Chromic acid (mixture of $K_2Cr_2O_7$ + H_2SO_4 + H_2O) followed by thorough washing with tap water. The glasswares were then inverted in a clean tray and left to dry in the oven. Plugs for the tubes and flasks were made out of absorbent surgical cotton wrapped in muslin. 5 - 10 ml water was then poured into every culture vessel which was tightly plugged. The

glasswares were then steam sterilized in an autoclave at a pressure of 15 lb/in^2 at 121^o C for 15 - 20 minutes.

Culture Medium:

The media formulation described as Murashige and Skoog (1962) referred as MS medium was selected as the optimal culture medium. Stock solutions of generally 4 times major elements, 1000 times minor elements, 100 times organic constituents were prepared. These stock solutions were stored in a freeze chest at - 4^{o}C and were mixed in desired proportions only before use. None of the stock solutions were stored for more than 15 days.

MURASHIGE AND SKOOG's MEDIUM (1962):

Constituents	Amount (mg/l)
Major Elements	
1. NH_4NO_3	1650
2. KNO_3	1900
3. $CaCl_2.2H_2O$	440
4. $MgSO_4.7H_20$	370
5. KH_2PO_4	170
6. Na_2EDTA	37.31
7. $FeSO_4.7H_2O$	27.81
Minor Elements	
1. H_3BO_3	6.2
2. $MnSO_4.4H_2O$	22.3
3. $ZnSO_4.7H_2O$	8.6
4. KI	0.33
5. $Na_2MoO_4.2H_2O$	0.25
6. $CuSO_4.5H_20$	0.025
7. $CoCl_2.6H_2O$	0.025
Organic Constituents	
1. Glycine	2.0
2. Myo-inositol	100
3. Nicotinic Acid	0.5
4. Pyridoxine HCl	0.5
5. Thiamine HCl	0.1
6. Sucrose	30,000
7. Agar	10,000

The reagents used were of Analytical Reagent Grade. Each salt was dissolved separately one after one to avoid precipitation. Coconut milk (liquid endosperm) when used was extracted from young green coconuts and stored at - 4^{o}C.

All the constituents except agar were mixed and then the pH of the solution was adjusted to 5.5 - 5.8. Later, agar was added and the medium was heated to boil so as to homogenize agar.

Following are some of the supplements which were used either singly or in combination for the induction of callus, differentiation and multiple shoot formation.

i. Basal Medium (BM)
ii. BM + NAA, IAA and 2, 4-D (0.5-4 ppm each)
iii. BM + K (0.5-4ppm) + NAA, IAA and 2, 4-D (0.5-4 ppm each)
iv. BM + BAP (0.5-4 ppm) + NAA, IAA, and 2, 4-D (0.5-4 ppm each)
v. BM + BAP (0.5-4 ppm)
vi. BM + K (0.5-4 ppm)
vii. BM + CM (10-20% V/V) +NAA, IAA and 2, 4-D (0.5-4 ppm each)
viii. BM + CM (10-20% V/V) + K (0.5-2 ppm) + NAA, IAA, 2, 4-D (0.5-4 ppm each)
ix. BM + CM (10-20% V/V) + BAP (0.5-2 ppm) + NAA, IAA, 2, 4-D (0.5-4 ppm each)
x. BM + 2, 4-D (2-4 ppm) + NAA, IAA + CH (2g/l)

After the preparation of the medium, water was poured out of the autoclaved glassware. Definite aliquots of the medium were then added depending upon the capacity of the culture vessel. Generally 25 ml, 50 ml, 100 ml of the medium was distributed into the test tubes, 100 ml and 250 ml flasks respectively. After plugging the glassware with cotton plugs, media ware steam-sterilized at 15 lb/in^2 (121^{o}C) for 15 - 20 minutes. After

autoclaving, tubes were placed in slanting stands to prepare the slants. These were then left to cool and solidify.

Inoculations:

All the experimental manipulations were carried out under strictly aseptic conditions in laminar air flow bench fitted with a bactericidal U. V. tube (15 W, peak emission 2637 A^{o}). The floor of the chamber was thoroughly scrubbed with cotton dipped in alcohol. The surface of all the vessels and other accessories such as instruments (spatula, forceps, scalpels, blade etc.), gas burner, lighter, tube containing absolute alcohol etc were also cleaned with alcohol. The fresh material to be inoculated was kept in a petri dish covered with a piece of black paper in order to protect it from the harmful effects of U. V. rays. Alcohol was then sprayed in the chamber with the help of an atomizer. The chamber was then sterilized with U.V. rays continuously on for one hour.

The explants like stems, leaves and shoot apices were taken from the plants growing under the *in vivo* conditions. The stem pieces, leaves, shoot apices were placed in different bottles and covered with net and washed for 30 minutes under running tap water to remove all the adhering dust particles and microbes from the surface. The explants were then washed with liquid detergent (teepol) for another 15 minutes and then washed properly to remove the detergent. The explants were then treated with Bavistin (fungicide) for another 20-30 minutes to remove the fungus and then washed properly to remove the fungicide.

Fresh seeds of *Nasturtium* were used to raise the seedlings under sterile conditions. Seeds with testa were presoaked overnight and the seeds which settled down were selected and taken to be viable. Like explants, seeds were washed under running tap water, then liquid detergents and finally under Bavistin to remove dust particles, microbes and fungi.

Hands and arms which were to be used inside the inoculation chamber were scrubbed with alcohol before inoculation. The rims of the test tubes and the sides of the plugs were flame sterilized. Instruments (like forceps, scalpels, spatula etc.) were all sterilized by dipping in the alcohol and flaming a number of times. Care was taken to cool the instruments before putting into operation. The explants taken from field borne plants were treated with 0.01 - 0.1% mercuric chloride solution for 5-10 minutes respectively depending upon the explants. Shoot apices of *Nasturtium* were treated with 0.1% mercuric chloride for 4 - 5 minutes. The explants like stems and leaves were treated with 0.1% $Hgcl_2$ for 5 - 6 minutes. The explants were then thoroughly washed (4 - 5 washings) with sterilized distilled water to remove the traces of $Hgcl_2$. Fresh cuts were given to the stem explants after sterilization to remove undesirable or dead portions. The explants were then planted on variously augmented MS medium.

Seeds were surface sterilized with 0.1% $Hgcl_2$ for 7 - 8 minutes. Constant shaking was done during this period to get thorough sterilization. Rinsing with sterile distilled water 4 - 5 times was necessary for the removal of sterilant from the seeds. These were then planted on Basal MS medium for germination. Various parts like root, stem, leaves and shoot apices were excised from 4 weeks old seedlings and transferred separately to different experimental media.

Cultural conditions:

All the cultures were maintained in an air conditioned culture room at a temperature of 25 ± 4^{o}C. The source of illumination consisted of 2.5 feet wide fluorescent tubes (40 watt) and incandescent bulb (25 watt). The intensity of illumination was 3500 lux at the level of cultures and a 12 hour light regime was followed by 12 hour darkness.

OBSERVATIONS AND RESULTS

The seeds of *Tropaeolum majus* were collected from the National Nursery, Patiala and were planted in the soil beds for raising plants. Shoot apices , leaves and stems were excised from these *in vivo* raised plants and used for experimental work .Likewise seeds were grown in test tubes to raise *in vitro* seedlings .They were firstly washed for 30 minutes under running tap water to remove all the dust particles from the surface .The seeds were then washed with liquid detergent (teepol) for another 15 minutes and then washed properly to remove the detergent .The seeds were then treated with Bavistin (fungicide) for about 20 minutes and then washed thoroughly to remove the traces of fungicide. In the laminar flow the seeds were then treated with 0.1 % mercuric chloride for 7 - 8 minutes and planted on basal MS medium supplemented with 3 % sucrose. The seeds started germinating after 7 - 10 days of inoculation and complete seedlings were formed within 3 - 4 weeks (fig - 2). The presoaking of seeds in water for overnight prior to inoculation facilitated an early germination. The various parts of seedling i.e. roots, stem and leaves were excised and planted on variously supplemented MS medium for callus induction, organgenetic differentiation and multiple shoot formation.

Shoot tip culture:

Fresh shoot apices 1 - 2 mm in length were excised from field grown mature plants. They were surface sterilized with 0.1% $HgCl_2$ for 4 - 5 minutes and were cultured on MS medium supplemented with various growth regulators. Proliferation of axillary shoots was induced from the shoot tip explant on MS medium supplemented with different concentrations of BAP (0.5 - 4 ppm) alone or in combination with NAA. Shoot tips cultured on MS medium supplemented with different concentrations of BAP alone showed the best results for shoot proliferation after 8 - 10 days of culture. 4 - 5 shoot buds proliferated on MS + NAA (0.5 ppm) + BAP (1 ppm) (fig - 3), whereas 6 - 7 shoots regenerated on lower concentrations of BAP (0.5 - 2 ppm) alone (figs - 4 & 5). Highest number of

shoot buds per culture was obtained on the medium with 4 ppm BAP where shoot tip explants produced nearly 25 - 35 axillary shoot buds. (fig - 6). Transfer of these shoots buds to lower concentrations of BAP (0.01 – 0.1 ppm) or to simple MS medium proved most effective in increasing shoot growth rate (fig 7 & 8).

Multiple shoot formation from shoot apices on MS medium supplemented with various concentrations of BAP and NAA either alone or in various combinations is shown in (fig - 10).

Rooting of shoots:

The regenerated shoots were very carefully rescued from the culture tubes, placed on a sterile petridish and were given the cut from the basal end of the shoots. Then each of these shoots was planted on rooting medium which consisted of MS salts and different concentrations of IAA, NAA and IBA for root initiation. Among the various growth regulators tested, IAA (1 ppm) showed the best results where roots initiated after 15 days of culture. Figure 9 shows complete plantlets with elongated shoot and root systems ready to be transferred to the soil.

Transfer to the soil:

After shoot and root development, attempts were made to establish regenerated plantlets into soil. Sufficiently rooted plantlets were transplanted to small plastic glasses for hardening prior to their final transfer to the soil. The rooted plantlets were gently removed from the culture tubes keeping the roots intact by using forceps with extreme care to avoid any mechanical damage to the plantlets. Plantlets were thoroughly washed with tap water to remove any remaining medium possibly on them. Further, plantlets were given Bavistin treatment (1 gm/l) for 10 minutes to protect the plant from any near future fungal attack. The regenerants were then transferred to plastic glasses containing soil and agropeat (2:1 ratio) (fig 11). Plants were thoroughly watered and kept in poly house under humidity range of 70 -90 % for about one week. After this period in

poly house these plantlets were transferred to shade house in which they were kept under the humidity range of 60 – 70%.

Root culture:

Roots of the seedlings grown under sterile conditions were white and smooth. These were cut into 4 - 5 mm segments and planted on BMS supplemented with various growth regulators used in various combinations.

Profuse growth of lateral roots from the original explant took place in the presence of NAA (1 – 4 ppm). Addition of 15 % CM to the above medium further enhanced the formation of lateral roots (fig 12). Likewise addition of kinetin to the auxin supplemented medium had a stimulating effect on the rooting (fig 13). The roots formed were short, branched and bore thick crop of root hairs. A little callus was also formed which, however, was soon overcome by the profuse rooting of the explant. No regeneration of multiple shoots could be effected under the cultural conditions employed.

Stem culture:

Stem explants 3 -5 mm length were excised from 4 - 5 week old *in vitro* raised seedlings and were cultured on MS containing different growth regulators used either alone on in conjunction with each other.

Stem explants showed proliferation of roots on NAA supplemented medium either alone or in conjunction with kinetin (figs 14 and 15). Best results were however, obtained on higher concentration of NAA (4 ppm) with or without kinetin (fig 16), where profuse rooting occurred from all over the surface of the explant. Fig 17 shows the frequency of rooting on different concentrations of NAA with or without kinetin

Stem explants also showed proliferation of roots on MS supplemented with 2, 4-D (4 ppm) + kinetin (1 ppm) (fig 18), but rooting was less extensive then on NAA and kinetin supplemented medium.

Multiple shoot proliferation:

Stem explants of both *in vitro* and *in vivo* raised seedlings showed multiple shoot proliferation (15 – 20) on simple MS medium (figs 19 and 20) and 4 – 5 shoots on simple MS + 2, 4-D (2 ppm) + kinetin (1 ppm) + CM (15 %) (fig 21). Multiple shoot proliferation from stem explant on Basal MS medium and MS medium supplemented with various concentrations of 2, 4-D or kinetin either alone or in various combinations is shown in table 1.

Callusing:

Stem explants from both *in vivo* and *in vitro* raised seedlings showed very little callusing on MS + 2, 4-D (2 – 4 ppm) + kinetin (1 – 2 ppm).

Leaf culture:

Leaf explants 4 – 5 cm in length were excised from 4 weeks old *in vitro* raised seedlings and planted on MS supplemented with various additives. On MS + NAA (1 – 4 ppm), profuse growth of lateral roots occurred from the explant after 8 – 10 days of culturing. Best results were obtained on higher concentration i.e. 4 ppm of NAA (fig 22).

Multiple shoot formation:

No multiple shoot proliferation from leaf explants could be effected under cultural condition employed.

Callusing:

The auxin (2, 4-D) and kinetin (cytokinin) caused callusing of the *in vivo* and *in vitro* raised explants but did not show active growth. Incorporation of casein hydrolysate along with 2, 4-D (2 – 4 ppm) considerably enhanced the callus growth. Out of auxins (2, 4-D, NAA, IAA) tested for the purpose of establishing callus, 2, 4-D in combination with casein hydrolysate proved most effective. On MS + 2, 4-D (2 ppm) + CH (2 gm/l), the petiole explant swelled and 75 % of them callused (fig 23) either at the cut ends or along the entire surface. Fig 24 shows 4 week old petiole callus on MS + 2, 4-D (2 ppm) + CH (2 gm/l).

The callus was yellowish brown, solid and slow growing and did not show sustained growth when transferred to fresh medium.

The response of petiole segments to callusing with various growth regulators is summarized in table – 2.

Study of callus:

The callus was solid and thus had to be teased with needles to study its cell types. Cell types studied in various calli showed their heterogeneous nature with wide variations in size and shapes of cells. The cells were spheroidal, ovoid, elongated and of different sizes.

Differentiation

Xylogenesis:

Histogenetic differentiation in the form of tracheids was observed in all the calli. Tracheids occurred singly or in groups and possessed scalariform thickenings on their walls (figs 25 and 26).

No differentiation of roots, shoots or whole plantlets from the calli could be effected under the present cultural conditions employed.

Table 1:-

S.no.	Hormone concentration	Type of explant	Number of shoots per explant
1.	Basal MS medium	Stem	15-20
2.	MS + 2, 4-D (1 ppm) + Kn (1 ppm) + CM (15%)	Stem	2-3
3.	MS + 2, 4-D (2 ppm) + Kn (2 ppm) + CM (15%)	Stem	4-5

Table 2:-

S.no.	Hormone concentration	Percentage explants forming callus	Callus growth
1.	Basal MS	0	-
2.	Basal MS + NAA (2-4 ppm)	30	+
3.	Basal MS + 2, 4-D (2-4 ppm) + Kn (1 ppm)	40	++
4.	Basal MS + 2, 4-D (2-4 ppm) + CH (2 gm/L)	75	+++

- No response + Poor callus ++ Better growth

+++ Good growth

Fig 1. Tropaeolum majus

Fig. 2 Two weeks old seedling of *Nasturtium* on MS medium.

Fig 3. Swollen shoot apex showing the formation of 4 – 5 adventitious shoot buds on MS + NAA (0.5 ppm) + BAP (1 ppm).

Fig 4. Initiation of multiple shoot proliferation on MS + BAP (0.5 ppm) from shoot apex.

Fig 5. Initial development of adventitious shoot buds from shoot apices on MS + BAP (1 ppm) and BAP (2 ppm) respectively after two weeks of culture.

Fig 6. Multiple shoot proliferation on MS + BAP (4 ppm) from shoot apex after two weeks of culturing.

Fig 7. Proliferation of green leafy shoots on simple MS medium.

Fig 8. Further elongation of shoots on simple MS medium.

Fig. 9 Adventitious root formation from regenerated shoots on MS + IAA (1 ppm).

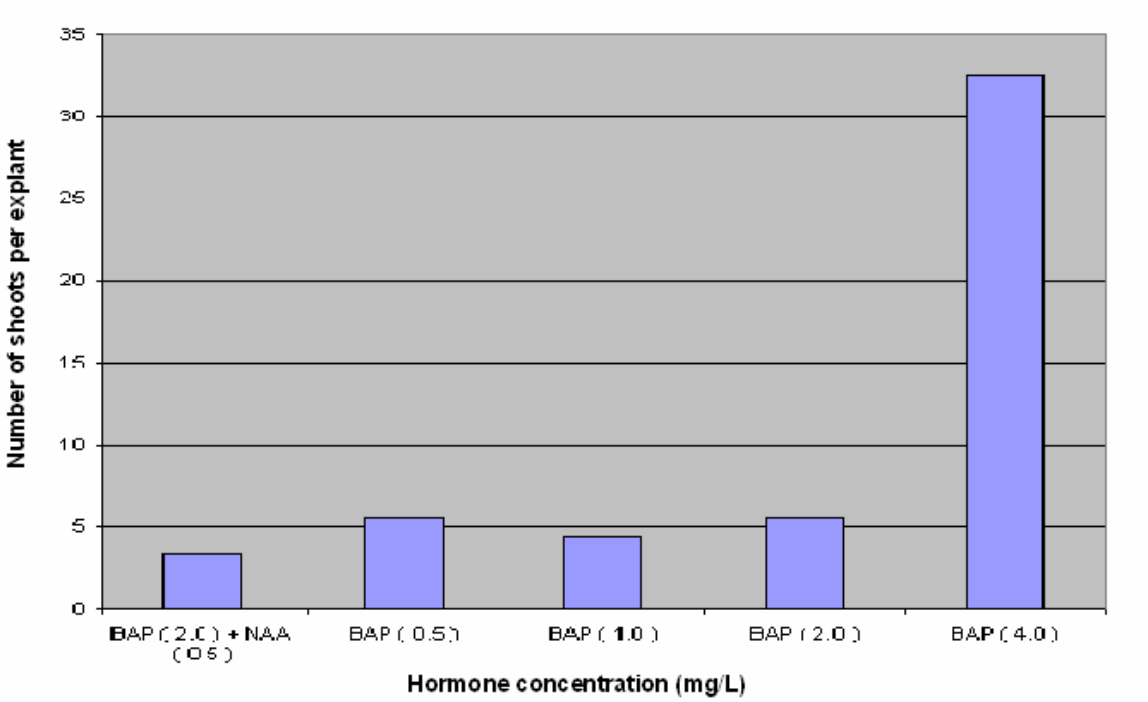

Fig 10. Frequency of multiple shoots formed from shoot tip explants cultured on different concentrations of BAP alone or in combination with NAA.

Fig.11 In vitro formed plantlets transferred to potting mixture.

Fig 12. Root proliferation on MS + NAA (2 ppm) + CM (15%) from root explant.

Fig 13. Root proliferation on MS + NAA (2 ppm) + Kn (1 ppm) from root explant.

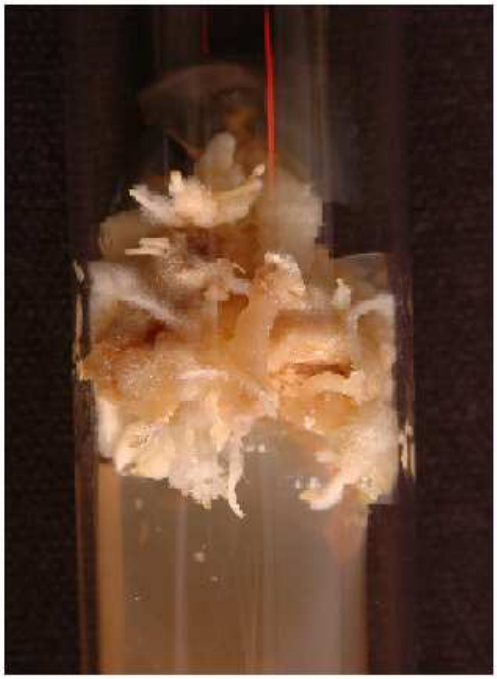

Fig 14. Initial stage of root proliferation on MS + NAA (2 ppm) from stem explant.

Fig 15. Initial stage of root proliferation on MS + NAA (2 ppm) + Kn (1 ppm) from stem explant.

Fig 16. Root proliferation on MS + NAA (4 ppm) + Kn (1 ppm) from stem explant.

Fig 17. Root proliferation on MS + NAA (2 ppm), MS + NAA (4 ppm), MS + NAA (2 ppm) + Kn (1 ppm), MS + NAA (4 ppm) + Kn (1 ppm) from stem explan

Fig 18. Root proliferation and shoot regeneration on MS + 2, 4-D (4 ppm) + Kn (1 ppm) from stem explant.

Fig 19.Initial stage of multiple shoot formation on Basal MS medium from stem explant.

Fig 20. Multiple shoot formation on Basal MS medium from stem explant after two weeks of culturing.

Fig 21. Shoot proliferation and root regeneration on MS + 2, 4-D (2 ppm) + Kn (1 ppm) + CM (15 %) from stem explant.

Fig 22. Root proliferation on MS + NAA (4 ppm) from leaf explant.

Fig 23. Callus induction along the entire surface of petiole segment on MS + 2, 4-D (2 ppm) + CH (2gm/L).

Fig 24. Four week old petiole callus on MS + 2, 4-D (2 ppm) + CH (2gm/L).

Fig 25. Group of tracheids isolated from stem callus.

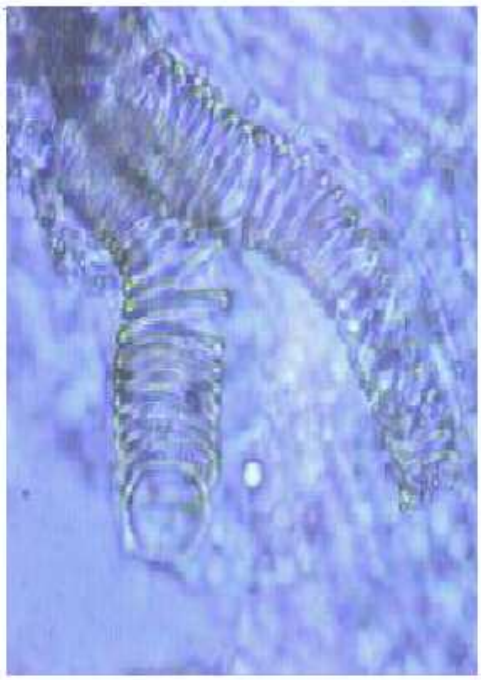

Fig 26. Single tracheids magnified showing scalariform thickenings on their Walls.

DISCUSSION

The present investigation was undertaken on an important ornamental plant, *Tropaeolum majus* with a view to develop a reliable protocol for its clonal propagation under *in vitro* conditions.

In *Nasturtitium*, multiple shoot proliferation from shoot apices taken from both *in vitro* and *in vivo* raised plants occurred on MS medium supplemented with different concentrations of BAP either alone or in combination with lower concentrations of NAA. However, best results were obtained on higher concentrations of BAP. For shoot proliferation growth regulators especially cytokinins are one of the most important factors affecting the response (Lane, 1979; Stolz, 1979; Bhozwani, 1980; Garland and Stolz, 1981). A wide range of cytokinins like kinetin, BAP, 2-ip and zeatin have been employed in shoot proliferation (Bhozwani and Razdan, 1982). Murashige (1974), Hussey (1978) and Sharon and D'sauza (2000) described 2-ip as more effective than either kinetin or BAP. A number of plants such as *blueberries* (Cohen, 1980), *garlic* (Bhozwani, 1980), *annatto* (Sharon and D'sauza, 2000) and *Gardenia jasminoides* (Chuenboonngarm, et al., 2001) have been successfully multiplied by using 2-ip. However, a wider survey of literature suggests that BAP is the most reliable and effective cytokinin. Gurel and Gulsan (1998) reported shoot multiplication from the shoot tip of *Amygdalus communis* on MS medium supplemented with 1 mg/l BAP. Amin *et al.* reported shoot proliferation from the shoot tip of *Ixora fulgens* on MS medium supplemented with 0.5 mg/l BAP with low concentrations of NAA 0.1 mg/l. Kalamani *et al.* (2002) reported multiple shoot bud formation from the young shoot tip explant of *Clitoria ternatea* on 0.5 mg/l BAP with lower concentrations of NAA or IAA.

In the present study shoot proliferation occurred on BAP (0.5 - 4 ppm) but better response was obtained on higher concentrations of BAP (4 ppm), where 25 – 35 shoots were formed per culture. Likewise Uddin *et al.* (2005) reported highest percentage of shoot tip proliferation in *Peltophorum pterocarpum* on MS medium having 4 mg/l of BAP. It was also observed that initial treatment with

BAP (2 – 4 ppm) for 2 - 3 weeks followed by transfer to lower concentration of BAP 0.1 mg/l or simple MS proved most beneficial for the induction of shoot multiplication. This observation is an agreement with that of Mishra and Dutta (1999) who showed in *Tagetes erecta* that initial treatment with BAP at the concentration of 5 mg/l for seven days followed by transfer to 50 times lower concentration (1.0 mg/l) proved most beneficial for the induction of shoot multiplication. The results can be explained on the basis that different plants and even different organs of the same plant are characterized not only by their unique intrinsic biochemical make-up but also by the sensitivity of the endogenously supplied chemical stimuli.

Stem explants produced adventitious shoots directly on the surface without formation of intervening callus on variously supplemented MS medium. Both *in vitro* and *in vivo* raised stem explants showed best results on MS medium supplemented with BAP (1 – 4 ppm) and simple MS medium where 15 – 20 shoots were regenerated from a single explant. Likewise Archana *et al*. (2002) reported adventitious bud regeneration from the entire leaf surface of *Dianthus chinensis* on BAP (3 ppm) and NAA (0.5 ppm). Chen *et al.* (2001) reported adventitious bud formation from the stem internode explant of *Adenophora triphylla* on MS medium supplemented with BAP (2.22 - 35.51 µM) and NAA (0.54 µM). Koroch *et al.* (2002) reported 100% shoot regeneration from leaf explants of *Echinacea purpurea* on MS medium supplemented with BAP (4.4 µM) and NAA (0.054 µM).

Induction of roots at the base of *in vitro* grown shoots is essential and indispensable step to establish tissue culture derived plantlets to the soil. The most effective auxins for rooting are IBA and NAA (Perik., 1987; Uddin *et al*., 2005). Likewise Amin *et al*. (2002) reported root initiation on *in vitro* raised shoots of *Ixora fulgens* on ½ MS supplemented with 0.2 mg/l IBA. Chen *et al.* (2000) reported root initiation on *in vitro* raised shoots of *Adenophora triphylla* on ¼ MS medium supplemented with 5.37 µM NAA within 15 days.

In the present case, however IAA (1 ppm) proved better than IBA for induction of adventitious roots from the base of regenerated shoots. Likewise Archana *et al.* (2002) reported root formation on shoots of *Dianthus chinensis* on ½ MS supplemented with IAA 0.5 mg/l.

Callus induction was effected from petiole and stem segment of both *in vitro* and *in vivo* raised plants. The callus growth was however very slow from the stem segments. Majority of plant tissues growing *in vitro* require exogenous hormones in the nutrient medium. The reaction of isolated tissues to auxins depends upon their endogenous auxin level at the time of excision and their genetic capacity for its synthesis. Those tissues which do not require an external supply meet their auxin requirement endogenously by biosynthesis. Rahman *et al.* (2004) reported callus induction from leaf base segments of *Kaempferia galanga* on 1.5 mg/l 2, 4-D with 0.1 mg/l BAP. Likewise, similar combination of auxin with cytokinin for callus induction has been reported in the past by Malamug (1991) on *ginger*, Vincent *et al.* (1992) on *Kaempferia galanga*, Muthukumar *et al.* (2000) on *Datura metel*. Likewise, Mehra and Anand (1983) reported callus induction from cotyledon explant of *Cryptomeria japonica* on MS medium supplemented with 22.8 µM IAA.

In the present work MS medium when supplemented with growth regulating substances registered callus growth. 2, 4-D initiated callusing from the explants but could not sustain its further growth. Addition of CH to the 2, 4-D supplemented medium proved good for callus growth.

Many plant tissues don't grow satisfactorily unless the medium is supplemented with natural complex growth substances such as casein hydrolysate, yeast extract, beef extract, fruit juices and sometimes the extracts of some plant organs. Extracts of immature *Zea* grains, gelatinous materials in immature *Juglans* and *Aesculus* fruits, liquid endosperm of *Cocos nucifera* which exhibit substances analogous to cytokinin activity have also been abundantly tried. These supplements may support additional growth and proliferation by triggering on many metabolic processes.

In the present study synergistic action of CH with 2, 4-D was demonstrated for the initiation and growth of callus. Likewise synergistic action of CH with NAA favored callus growth in *Cosmos sulphureus* (Anand, 1983). It is quite clear that CH makes up the deficiency of certain essential nutrients whose synthesis is impaired or suppressed in the normal tissues.

Both stem and leaf calli showed variability in cell shape. According to Steward et al. (1963) “even in the most uniform environment, the two daughter cells from a single clone were rarely identical”. A similar observation was made in carrot, endive parsley, lettuce and spinach callus by Kant and Hildebrandt (1969) and in *Pterotheca falconeri* by Mehra and Mehra (1971).

Histogenetic differentiation in the form of tracheids occurred in the callus cultures.Tracheids were mostly elongated, occurred singly or in groups and had scalariform thickenings on their walls. Tracheids were seen in the early stages of callus formation. Firstly only few tracheids could be seen which multiplied with the active proliferation of the callus. It seems there is a correlation between cell division and vascular differentiation. This contention gets support from other reports which suggests that cell division must precede the formation of vascular elements and that no vascular differentiation occurs in the absence of cell division.

In the present work it has not been possible to effect organogenesis (except the direct shoot regeneration from the stem segments) or differentiation of whole plants from the calli. Control of differentiation has been based on hypothesis of Skoog and Miller (1957) who showed that differentiation of root and shoot is a function of interaction between two plant growth regulators – auxin and cytokinin. A relatively high auxin and low cytokinin causes root formation while the reverse favors shoot formation. This is true in the case of many herbaceous angiosperms though it is not universally accepted.

It is opined that while the cells in plant are undoubtedly totipotent but in the present case we have not been able to trigger them to activity either to organ formation or to give rise to plantlets.

The ability of auxins and cytokinins to induce differentiation in other system means that they are generally implicated in differentiation, though in incompetent systems, like the present one, they may not be the limiting factors. Some vital hormonal and/or nutritional factor or their combination for differentiation in the *Tropaeolum majus* could not be discovered by us during the stipulated period of this project.

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