In vitro cloning of *Antirrhinum majus* – an important ornamental plant

A

Dissertation

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for award of the degree of

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IN

BIOTECHNOLOGY

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CERTIFICATE

This is to certify that the dissertation entitled "***In vitro* cloning of *Antirrhinum majus* – an important ornamental plant"** submitted by **Kiranbir Kaur** (301201006) in partial fulfillment of the requirements for the award of degree of Master of Science in Biotechnology, Thapar University, Patiala, is a record of students' own work carried out by her under my guidance and supervision. The 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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I, hereby declare that the work presented in the dissertation entitled **"*In vitro* cloning of *Antirrhinum majus* – an important ornamental plant"** in partial fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology, Department of Biotechnology, Thapar University, Patiala, is an authentic record of my own work during the period of six months from Jan 2014 to July 2014, under the guidance of Dr. Manju Anand, Associate Professor, Thapar University, Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

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ABBREVIATIONS

BAP	Benzyl amino purine
MS	Murashige and Skoog’s medium
CM	Coconut milk
IAA	Indole-3- Acetic acid
IBA	Indole-3-Butyric acid
NAA	Naphthalene acetic acid
2,4-D	2,4-dicholorophenoxy acetic acid
Kn	Kinetin
mg/l	Milligrams per litre
µM	Micromolar
mM	Millimolar
^{o}C	Degree celsius

ABSTRACT

Antirrhinum majus is an attractive herbaceous plant cultivated as ornamentals for its aesthetic view. Shoot tips, nodal segments, stem and leaves were excised from field grown mature plant and transferred to variously supplemented Murashige and Skoog's medium with various hormones for multiple shoot proliferation, callus induction and *de novo* adventitious root and shoot formation directly from the explant or indirectly from the callus raised from the explants.

Antirrhinum majus also exhibited multiple shoot proliferation from nodal segments. Out of all the cytokinins tested, multiple shoot formation was observed on MS medium supplemented with BAP (4 mg/l) resulting in the formation of 12-14 shoots after 6 weeks of culturing. Acceptable multiplication rates were obtained from shoot tips when transferred to MS medium supplemented with Kn(2 mg/ml) where a maximum of 15-16 shoots were formed. MS medium supplemented with BAP (4 mg/l) also promoted multiple shoot proliferation leading to the formation of 12-14 shoots after 6 weeks. A noticeable feature observed was the induction of roots along with the multiple shoot proliferation. Once the clusters of shoots were formed, small clumps of 2-3 shoots were excised and transferred onto fresh multiplication medium where they exhibited continuous shoot proliferation.

The regenerated shoots thus formed were excised and transferred to different root inducing media to form complete plantlets. Among the various growth regulators tested, NAA (2 mg/l) showed the best results where roots appeared after 2 weeks which elongated further forming numerous roots after 4-5 weeks.

The plant exhibited good degree of propensity for *de novo* adventitious root formation directly from the stem and leaf segments. Direct root regeneration from the stem segment was observed on different concentrations of NAA (0.5-2 mg/l) where as *de novo* root formation from leaf segment was observed only from NAA (4mg/l). The roots were thin, white, long and bore profuse root hairs.

The present plant material did not exhibited *de novo* adventitious shoot formation in any of the media tried.

Callus induction from stem and leaf explants was observed on MS medium supplemented with 2,4-D in combination with cytokinins such as BAP and Kn. Auxin such as NAA used in combination with BAP and Kn also promoted callus formation.

The calli obtained from leaf and stem segments was hard, compact and whitish green in color.The callus was heterogenous in nature being composed of ovoid, oblong, semicircular cells or those with aberrant shapes. Histogenetic differentiation in the form of tracheids was observed. Tracheids occurred singly or in groups and possessed reticulate thickenings on their walls.

No oraganogenetic differentiation could be effected from the calli on the various media tried.

LITERATURE CITED

Altvorst A.C., Koehorst H., Bruinsma T., Custers J., Jong J., Dons J. (1992). Adventitious shoot formation from *in vitro* leaf explants of carnation (*Dianthus caryophyllus*). Scientia horticulturae 51(3): 223-235.

Altvorst V., Koehorst H., Bruinsma T., Dons J. 1994, Improvement of adventitious shoot formation from Carnation leaf explants. Plant cell, Tissue and Organ Culture. 37: 87-90.

Atkinson J., Newbury H. J., Ford-Lloyd B. V. (1991). *In vitro* adventitious root induction in *Antirrhinum majus* L. Plant Cell. Tissue and Organ culture 27: 77-79

Bhojwani S. and Razdan M. (1982). Plant cell Tissue culture theory and practice. Elsevier Sci. Publ. Amsterdam, 1-520.

Brar M. (1996). Effect of Thidiazuron and Benzylaminopurine on *in vitro* shoot proliferation of carnation (*Dianthus caryophyllus* L.). 31(3, Part 2): 61A.

Casas J., Olmos E., Piqueras A. (2010). *In vitro* propagation of Carnation (*Dianthus caryophyllus* L.), Protocols for *in vitro* propagation of ornamental plants 589: 109-116.

Chen T., Tsung J., Chang W. (2004). Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum* orchids. Plant cell, Tissue and Organ Culture 76: 11-15.

Emek Y., Erdag B. (2007). *In vitro* propagation of *Gladiolus anatolicus* (boiss.) stapf, Pak.J. Bot., 39(1): 23-30.

European Commission, General for agriculture and rural development, 2006.

Frey and Janick (1991), Organogenesis in Carnation, J. Amer Soc. Hort. Sci. 116(6): 1108-1112.

Gonzalez-Benito M., Tapia J., Rodriguez N., Iriondo J. M. (1996). Micropropagation of Commercial and Wild Genotypes of Snapdragon (*Antirrhinum* spp.). The Journal of Horticultural Science & Biotechnology 71(1): 11-16.

Haouala and Salhi (2012). Axillary budding and rooting of Gladiolus (*Gladiolus grandiflorus* Hort.) in salt stress conditions, Afr. J. Hort. Sci. 6: 101-110.

Kantia A., Kothari S. (2002). High efficiency adventitious shoot bud formation and plant regeneration from leaf explants of *Dianthus chinensis* L. Scientia Horticulturae 96: 205-212.

Kanwar J.K., Kumar S. (2009). Influence of growth regulators and explants on shoot regeneration in carnation 4:140-146

Karami O., Deljou A., Kordestani G. (2007). Secondary somatic embryogenesis of Carnation (*Dianthus caryophyllus* L.). Plant Cell, Tissue and Organ culture 92: 273-280.

Kharrazi M., Nemati H., Tehranifar., Bagher A., Sharifi A. (2011). *In vitro* culture of Carnation (*Dianthus caryophyllus* L.) focusing on the problem of Vitrification, J. Biol. Environmental Sciences, 5(13): 1-6.

Mahoney (2014). Home Guides by demand media.

Mani T., Senthil K. (2011). Multiplication of *Chrysanthemum* through somatic embryogenesis. Asian J. Pharm. Tech. 1(1): 13-16.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiology 15: 473-497.

Muthukumaram K. (2009). Indian floriculture Industry: Opportunities and Challenges.

Muthukumaran K. (2008). Indian floriculture industry and challenges.

Newbury, H.J. (1986). Multiplication of *Antirrhinum Majus* L. by Shoot-tip Culture. Plant Cell, Tissue and Organ Culture 7: 39-42.

Poirier-Hamon, Rao P. S., Harada H. (1974). Culture of mesophyll protoplasts stem segments of *Antirrhinum majus* (snapdragon): growth and organization of embryoids. Journal of Experimental Botany 25(4): 752-760.

Ruud. L.M., Nico. S.P. (2010). Floriculture World Wide; production, trade and consumption patterns.

Saha A. (2005). Export Growth and Prospect of Floriculture in India.

Sangwan, Harada (1975). Chemical regulation of callus growth, organogenesis, plant regeneration and somatic embryogenesis in *Antirrhinum majus* tissue and cell culture. Environmental Horticulture, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida.

Sharma A., Agrawal V. (2012). Tissue culture aspects of ornamental plants**.** CIBTech Journal of Biotechnology.1: 40-48.

Shirdel M., Azar A., Matloobi M., Nahandi F. (2012). Effect of nodal position and growth regulators on *in vitro* growth of Dog Rose (*Rosa canina*). Journal of Ornamental and horticulture plants 2251-6433.

Singh A., Ahlawat T. (2014). Floriculture as an upcoming industry in India: Present scenario and future prospects. Indian Farmer 1(1): 28-32.

Sudhagar S. (2013). Production and Marketing of Cut flower (Rose and Gerbera) in Hosur Taluk. International Journal of Business and Management Invention 2(5): 15-25.

Tanaka K., Kanoo Y., Kudo S., Suzuki M., (2000). Somatic embyogenesis and plant regeneration in chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura), Plant Cell Reports 19: 946-953.

Udom N., Kanchanapoom K., Kamnoon K., (2009). Micropropagation from cultured nodal explants of rose (*Rosa hybrid*a L.). J. Sci. Technology 31(6): 583-586.

Vantu S. (2006). Organogenesis in *Chrysanthemum morifolium* Ramat (Cultivar "Romica") callus cultures 71-76.

Waseem K., Jilani M.S., Khan M.S.(2009). Rapid plant regeneration of chrysanthemum (*Chrysanthemum morifolium* L.) through shoot tip. African Journal of Biotechnology 8(9): 1871-1877.

Waseem K., Khant M., Jaskant Q., Jilani M., Sohail M. (2009). Effect of different auxins on the regeneration capability of *Chrysanthemum* leaf discs. International Journal of agriculture and biology. 11-4: 468-472.

Xing W., Bao M., Qin H., Ning G. (2010). Micropropagation of *Rosa rugosa* through axillary shoot proliferation, Acta Biologica Cracoviensa Series Botanica 52/2: 69-75.

Chapter 6

Literature Cited

DISCUSSION

The present investigation was carried out on an important ornamental plant *Antirrhinum majus* with an aim to develop an efficient, reliable and reproducible protocol for its clonal propagation under *in vitro* conditions.

Micropropagation through enhanced axillary branching is a reliable technique for clonal propagation as it enables to retain the clonal fidelity and prevents somaclonal variations in the culture unlike the callus tissue. In the present investigation, multiple shoot proliferation was achieved using shoot tip and nodal segment. The axillary shoot proliferation from the cultured explants was remarkably influenced by the type and concentration of the growth regulator used. For shoot proliferation growth regulators especially cytokinins are one of the most important factors affecting the response (Sterk *et.al.,* 2003, Mishra *et.al.,* 2005, George *et.al.,* 2008, Warrier *et.al.,* 2010). A wide range of cytokinins like Kn, BAP, Zeatin and Thidiazuron have been employed in shoot proliferation (Bhojwani and Razdan, 1982 Viji *et.al.,* 2010). However a wide survey of literature suggested that BAP is the most reliable and effective cytokinin for shoot proliferation. A number of plants such as *Dianthus* (Pareek *et.al.,* 2004), *Rosa indica* (Hameed *et.al.,* 2007), *Rosa canina* (Shirdel *et.al.,* 2013), *Gladiolus grandiflorus* (Haula *et.al.,* 2012), *Dianthus caryophyllus* (Brar *et.al.,* 1996; Daniel *et.al.,* 2009) have been successfully multiplied using BAP.

In the present investigation, axillary shoots were induced from the nodal segments and shoot apices on MS medium supplemented with BAP or Kn. For nodal explants best results were obtained on BAP (4 mg/l), where a maximum of 12-14 shoots were formed after 6 weeks of culturing.

Multiple shoot proliferation from shoot tips was achieved on MS medium supplemented with different combinations of BAP (0.5-4 mg/l) or Kn (0.5-4 mg/l). Highest shoot proliferation occurred on BAP (4 mg/l), where a maximum of 12-14 shoots were formed after 6 weeks of culturing and on Kn (2 mg/l), where 15-16 shoots were formed after 6 weeks of culturing. Our results are in agreement with other workers who have reported multiple shoot proliferation from shoot tips and nodal segments in *Antirrhinum majus* using MS medium supplemented with BAP (Atkinson *et.al.,* 1989). Earlier Newbury, 1986 used

nine varieties of *Antirrhinum majus* in a study of *in vitro* multiplication of plants using shoot tip culture. The reported works were done on the shoot tips taken from the seedlings raised from seeds under *in vitro* conditions where as in our case the results are achieved from shoot tips and nodal explants taken from of healthy, mature field grown plants. González-Benito *et.al.* (1996) also reported multiple shoot proliferation from nodal segments of *Antirrhinum majus* on MS medium augmented with BAP (1mg/l) but in combination with NAA (0.5 mg/l).

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. In the present study the *in vitro* regenerated shoots were carefully excised and transferred to basal MS medium alone or supplemented with various auxins for root initiation. Best root formation occurred on 2 mg/l NAA after 15-20 days where a bunch of well developed roots were formed at the base of stem. On the other hand, rooting was achieved in *Antirrhinum majus* when shoots were cultured in the absence of plant growth regulators as reported by Sangwan and Harada, 1975; Pfister Widholm, 1984; González- Benito *et.al.*, 1996.

Attempts are underway to acclimatize the plantlets and transfer them to the field conditions.

De novo adventitious shoot formation through direct regeneration is considered to be a reliable and safe method of micropropagation since in this method genetic uniformity is maintained among the progenies which are less prone to contamination because total number of stages of culturing are reduced significantly. New adventitious roots or shoots can develop directly from the explants like root, stem, petiole, leaf and floral parts. Choice of explants and hormone regime to which the explants are subjected to, are two important factors for their initiation.

The present material exhibited prolific growth of roots directly from leaf and stem segments. Root induction in leaves was observed on MS medium supplemented with NAA (4 mg/l). The roots were thin, long and white in color with profuse root hairs. In case of stem segments the root induction was observed on different concentrations of NAA (0.5-2 mg/l) with 2 mg/l NAA showing the best response where highest number of roots were formed per culture. Similarly Sangwan and Harada (1975) reported the development of abundant roots

from the stem segments of *Antirrhinum majus* seedlings on IAA or NAA supplemented medium. Atkinson *et.al.*(1991) reported *in vitro* adventitious root induction in *Antirrhinum majus* on the shoots formed from seeds. Our is perhaps the first report demonstrating adventitious root induction from the mature explants taken from *ex vitro* plants.

De novo shoot formation directly from leaf or stem segments was not observed on any of the medium tried in the present investigation. However Sangawn and Harada (1975) have reported the regeneration of whole plant from stem explants of seedlings through the interaction of IAA and Kn.

Majority of the plant tissues growing *in vitro* require exogenous hormones in the nutrient medium for dedifferentiation. The reaction of an isolated tissue to an auxin depends upon its endogenous auxin level at the time of its excision and its genetic capacity for its synthesis. In the present investigation, callusing of the leaf and stem segments occurred very rapidly after culturing on optimal media compositions. Best callus formation was observed on 2,4-D in combination with BAP or Kn. Our work is in conformity with the work reported earlier by other authors. Sangwan and Harada, 1975 reported callus formation from stem explants of seedlings in *Antirrhinum majus* on MS media supplemented with 2,4-D where soft friable callus was formed. Rao *et.al.* (1976) also reported callus formation in *Antirrhinum majus* using leaf and stem segments cultured on MS medium supplemented with 2,4-D (1mg/l). Pfister and Widholm (1984) reported the formation of callus from stem internode and seedling root on Sangwan and Harada's medium (1975) augmented with 0.25 mg/l Naphthoxyacetic acid.

The calli formed from stem and leaf segments on the same media were more or less identical in morphology. The calli were heterogenous being composed of ovoid, oblong, semicircular cells with aberrant shapes. According to steward *et.al.* (1963) "even in the most uniform environment, 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 was observed in all the calli. Tracheids occurred singly or in groups and possessed reticulate thickenings on their walls. Tracheids

were seen in the early stages of callus formation. Firstly only a 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.

Poirier-Hamon (1974) reported the formation of embryoids via callus of stem internode of *Antirrhinum majus* on media supplemented with 2,4- D (0.5-2 mg/l). Sangwan and Harada (1975) also reported the formation of embryogenic green callus from internode stem segments of *Antirrhinum majus* on Nitsch *et.al.* (1967) medium supplemented with 0.25-5 mg/l naphthoxyacetic acid or 0.5 mg/l 2,4-D.The shoot differentiation from root callus of *Antirrhinum majus* was reported at low auxin concentration i.e. 0.25 mg/l naphthoxyacetic acid or 0.4 mg/l 2,4-D by Pfister and Widholm, 1984.

In conclusion a reliable and reproducible protocol has been established for the multiplication of *Antirrhinum majus* through forced axillary branching. It has not been possible to induce direct or indirect shoot oragnogenesis from the explants and callus tissue. It is opined that cells in plant are undoubtedly totipotent but some hormonal and nutritional factor or their combination for differentiation could not be discovered by us during the stipulated period of the project.

Chapter 5

Discussion

RESULTS AND OBSERVATIONS

Different vegetative parts like nodal segments, shoot apices, leaf and stem segments were excised from mature, healthy, field grown plant of *Antirrhinum majus* and used for experimental work.

Nodal explant and shoot tip culture

Nodal explants (5 mm long) holding one dormant lateral bud each and young healthy shoot tips were collected from mature, healthy plant of *Antirrhinum majus.* After sterilization, the damaged internodal tissue on both sides of the nodal segment were cut off and the nodal segments 3-4 mm in size and shoot apices were cultured on MS medium supplemented with different concentrations of BAP (1-4 mg/l) or Kn (1-4 mg/l) either alone or in combination with NAA (0.5–2 mg/l). The axillary shoot proliferation was remarkably influenced by the type and concentration of the cytokinin used.

Multiple shoot proliferation from nodal segments

Out of various growth regulators used, the best results were obtained on MS medium supplemented with BAP (4mg/l). The initial bud break from nodal segment occurred after 10 days of inoculation (Fig.1) and 3-4 shoots were formed after 15 days (Fig.2). These shoots multiplied further leading to the formation of a maximum of 12-14 shoots after 6 weeks of inoculation in about 60% cultures (Fig.3). A change in the concentration level of BAP below the optimum value (4 mg/l) resulted in decline in the number of shoots formed.

Fig 1: Nodal Bud Break on MS+BAP (4 mg/l) after 10 days of inoculation

Fig 2: Formation of few shoots on MS+BAP (4 mg/l) after 15 days

Fig 3: Development of numerous shoots on MS+BAP (4 mg / l) after 6 weeks

Shoot tip culture

Multiple shoot proliferation from the shoot apices was effected on both BAP and Kn supplemented medium. Young and healthy shoot apices were collected from mature field grown plant of *Antirrhinum majus* and cultured on MS medium supplemented with different concentrations of BAP and Kn. Out of various concentrations of BAP (0.5-4 mg/l) tested, maximum sprouting occurred on BAP (4mg/l) supplemented medium where 5-6 shoots were formed after 15 days (Fig.4) leading to further proliferation of shoots forming 12-14 shoots after 6 weeks (Fig.5). Once the clusters of shoots were formed, small clumps of 2-3 shoots were excised and transferred onto fresh multiplication medium where they registered further increase in the number of shoots (Fig.6). A noticeable feature observed was the induction of roots along with shoot multiplication on subculturing on BAP (4mg/l).

Fig 4: Regeneration of shoots from shoot tip on MS+BAP (4 mg/l) after 15 days

Fig 5: Numerous shoots formed on MS+BAP (4 mg/l) after 6 weeks

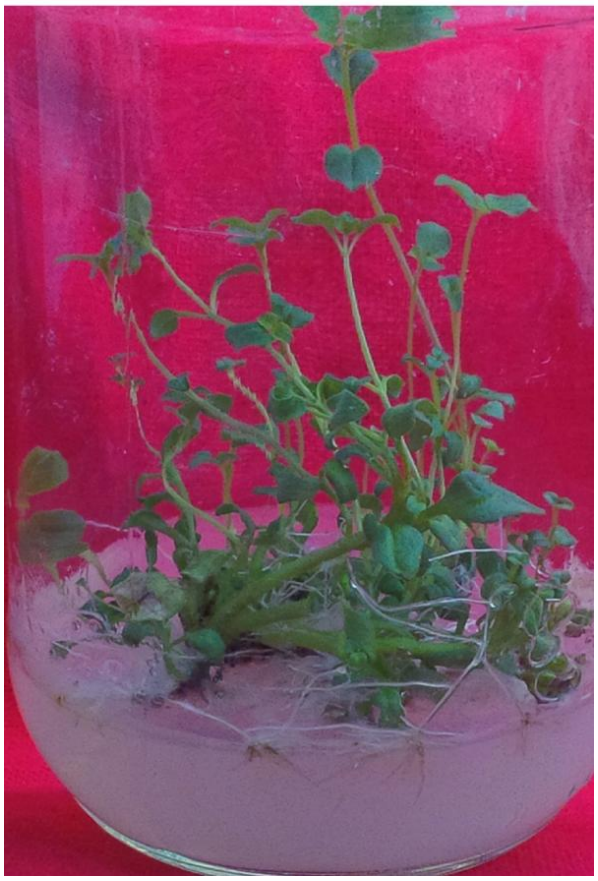

Fig 6: Multiplication of shoots after subculturing on MS + BAP (4mg/l)

Best shoot proliferation, however, occurred on 2mg/l Kn, where 5-6 shoots were formed after 15 days (Fig.7) further leading to the formation of 8-10 shoots after 25 days (Fig.8). The shoots proliferated further forming long, well developed shoots (15-16) with numerous leaves after 6 weeks (Fig.9). The shoots thus formed were further subdivided into smaller clumps having 3 shoots and subcultured onto fresh multiplication medium supplemented with Kn (2mg/l) where the shoots further increased in number along with the formation of roots (Fig.10).

The %age response regarding axillary shoot proliferation from shoot tip on various concentrations of BAP and Kn is depicted in Fig.11 and Table 2. Effects of different concentrations of BAP and Kn on multiple shoot proliferation from the shoot tip is depicted in Fig.12.

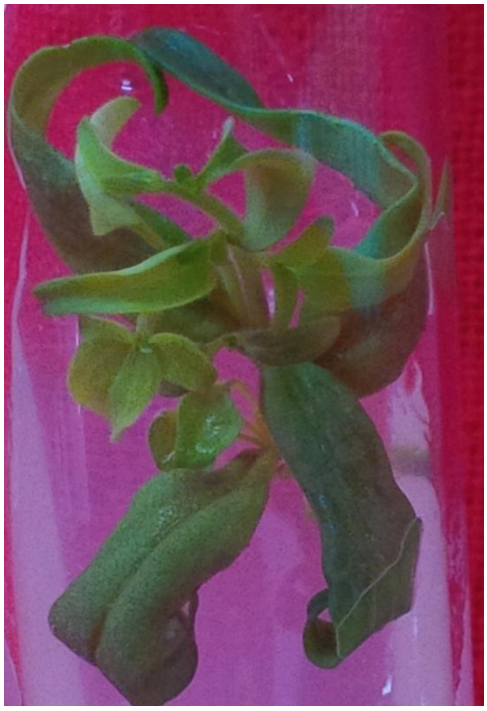

Fig 7: Initiation of multiple shoots on MS+Kn (2 mg/l) after 15 days

Fig 8: Increase in number of shoots on MS+Kn 2 mg/l) after 25 days

Fig 9: Formation of numerous shoots on MS+Kn (2 mg/l) after 6 weeks

Fig 10: Further proliferation after subculturing on MS+Kn (2mg/l)

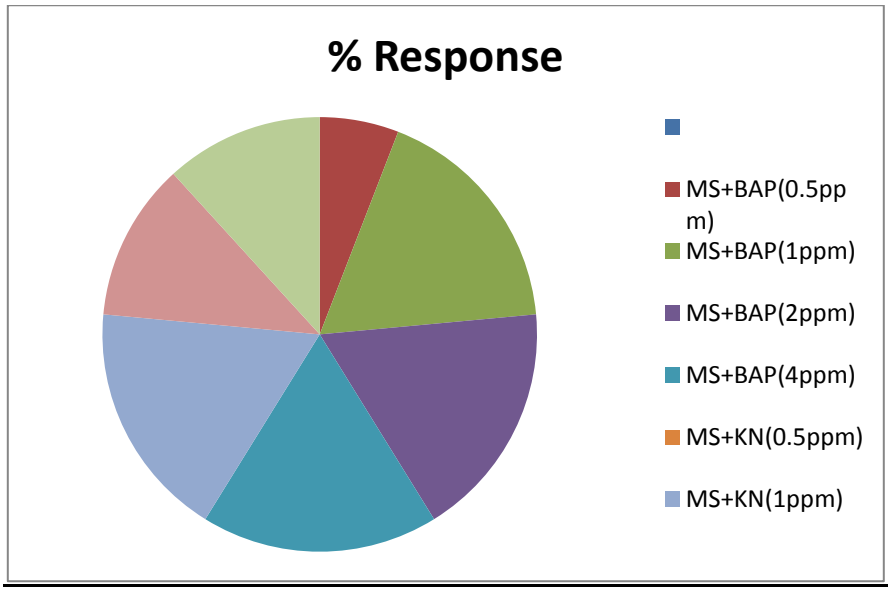

Fig 11: Percentage response regarding shoot proliferation on different media

Table 2: Percentage response and number of shoots formed on different media composition

Hormone Conc.	% Response	No. of shoots formed after 40 days	Mean no. of shoots
MS+BAP(0.5ppm)	20	7 to 8	7.5
MS+BAP(1ppm)	60	8 to 10	9
MS+BAP(2ppm)	60	8 to 10	9
MS+BAP(4ppm)	60	12 to 14	13
MS+KN(0.5ppm)	-	-	-
MS+KN(1ppm)	60	6 to 8	7
MS+KN(2ppm)	40	15 to 16	15.5
MS+KN(4ppm)	40	8 to 10	9

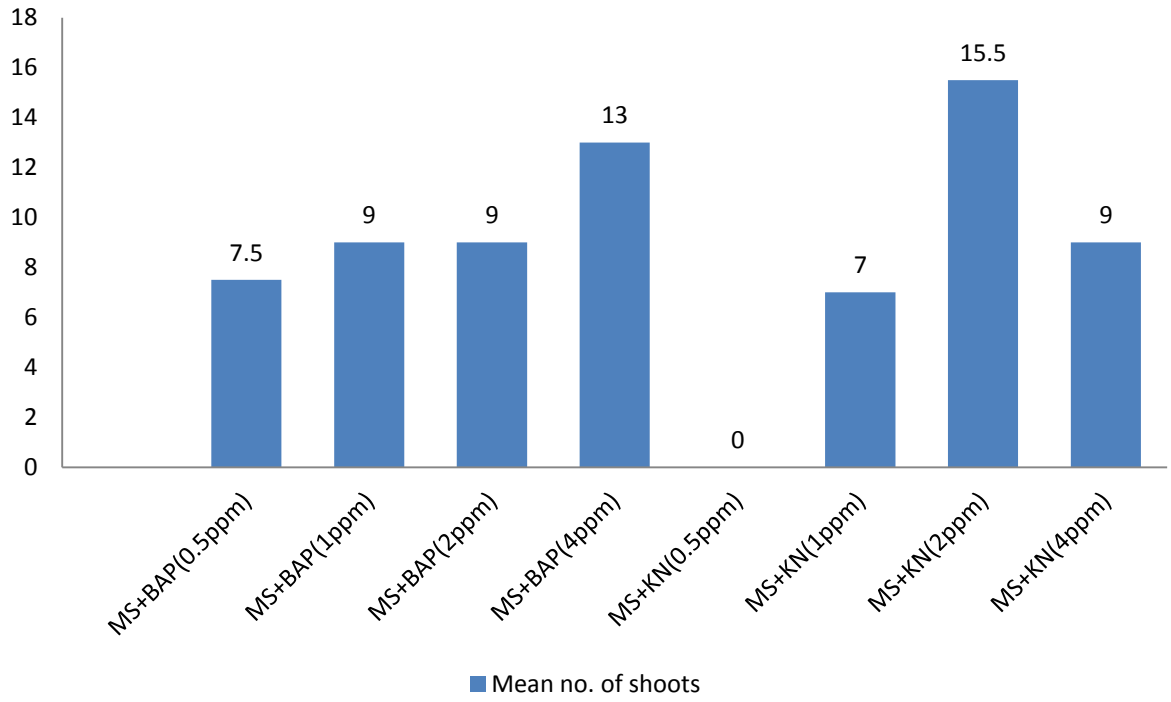

Fig 12: Effects of different concentrations of BAP and Kn on multiple shoot proliferation

Fig 13: Formation of thin, long roots on MS+NAA (2 mg/l) giving rise to complete plantlet

Rooting of microshoots-

Regenerated microshoots were carefully rescued from bottles in laminar air flow on sterile glass plate. These shoots were carefully isolated and inoculated upright in MS medium for root initiation. The shoots were cultured on basal MS medium and MS medium supplemented with different concentrations of NAA (0.5-2 mg/l). Root initiation occurred at the base of the shoot on NAA (2 mg/l) supplemented medium after 2 weeks of culturing forming well developed bunch of roots after 4-5 weeks. The roots were long, white and bore profuse root hairs (Fig.13).

Stem culture:

Direct *de novo* adventitious root formation-

The stem segments 3-4 mm in length were inoculated on basal MS medium supplemented with different concentrations of auxins such as IAA, IBA and NAA for *de novo* adventitious root formation. Out of the various auxins tested, direct rooting from the stem segment was observed only on MS medium supplemented with different concentrations of NAA (0.5-2 mg/l).

Direct rooting was observed on MS medium supplemented with NAA (0.5mg/l), where 2-3 roots were formed after 20 days of culturing (Fig.14). The number increased to 5-6 roots after 30 days (Fig.15). The roots formed were white and bore root hairs.

Fig 14: Root induction from stem segment on MS+NAA (0.5 mg/l) after 20 days

Fig 15: Formation of 5-6 roots on MS+NAA (0.5 mg/l) after 30 days

Maximum rooting was obtained on higher concentrations of NAA i.e. 1-2mg/l. MS medium supplemented with NAA (1mg/l) induced root formation resulting in the formation of 3-4 shoots after 20 days of culturing(Fig.16). The number increased to 6-8 after 30 days (Fig.17) forming 8-10 roots after 40 days of culturing (Fig.18). The roots formed were long, elongated and green or white in color. The roots bore root hair.

Figure 19 depicts the initiation of few thin roots on 2 mg/l NAA which multiplied further covering the entire stem segment after 40 days (Fig.20).

Fig 16: Root induction from stem segment on MS+NAA (1 mg/l) after 20 days

Fig 17: Formation of numerous, long roots on MS+NAA (1 mg/l) after 4 weeks

Fig 18: Increase in number of roots on MS+NAA (1 mg/l) after 6 weeks

Fig 19: Root induction from stem segment on MS+NAA (2 mg/l) after 20 days

Fig 20: Further increase in number of roots on MS+NAA (2 mg/l) after 6 weeks

***De novo* adventitious shoot formation-**

No adventitious shoot formation was observed in cultures of stem segments.

Callusing-

For callus induction, stem segments (4-5 mm) were transferred on MS medium supplemented with different growth regulators. Best callus growth took place on MS medium supplemented with 2,4-D (2 mg/l) and BAP (1mg/l) where callus initiated in 10-12 days, grew further (Fig.21) and within 4 weeks, the entire stem segment was converted into a mass of callus (Fig.22). The callus was whitish green, hard and compact in nature. Likewise, synergistic action of NAA (2-4 mg/l) with BAP or Kn was also found to be effective in inducing callus from the stem explants (Figs. 23 - 26). The callus was compact, non friable and greenish white in color.

Fig 21: Formation of whitish green callus on MS+2,4-D (2mg/l) + BAP (1mg/l) after 20 days

Fig 22: Mass of callus on MS+2,4-D (2mg/l) + BAP (1mg/l) after 4 weeks

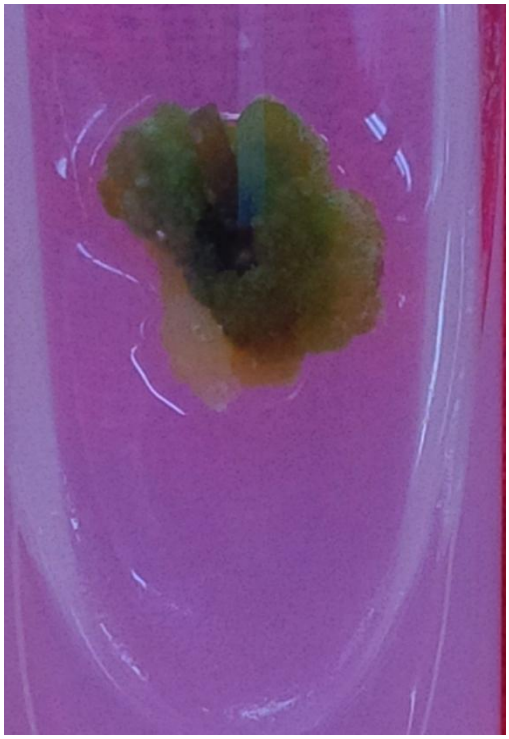

Fig 23: Initiation of callusing son MS+NAA (4mg/l) + Kn (1mg/l) after 10-12 days

Fig 24: Formation of greenish white callus on MS+NAA (4mg/l) + Kn (1mg/l) after 4 weeks

Fig 25: Callus induction on MS+NAA (4mg/l) + BAP (1mg/l) after 10 days

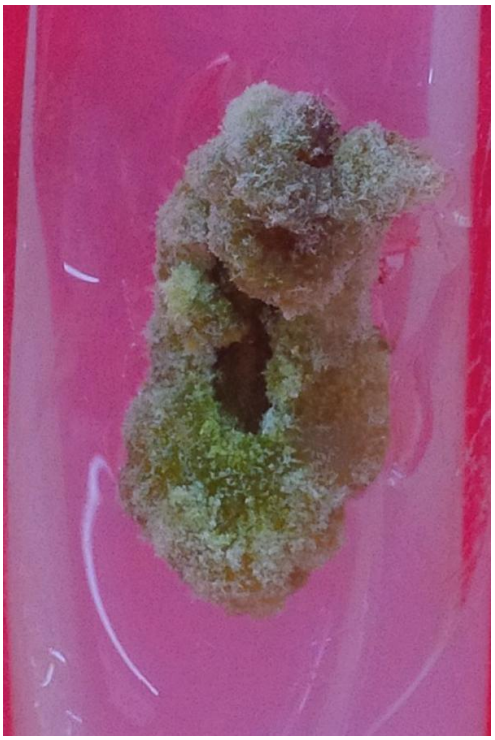

Fig 26: Formation of mass of callus on MS+NAA (4mg/l) + BAP (1mg/l) after 4 weeks

Study of callus-

The callus formed was extremely hard and non friable thus had to be teased properly with needles to study its cell types. The callus was heterogenous in nature comprising of cells of different shapes like ovoid, elongated and spherical. Four week old stem callus revealed differentiation of tracheids which occurred either singly or in groups possessing reticulate thickenings on their walls (Fig. 27).

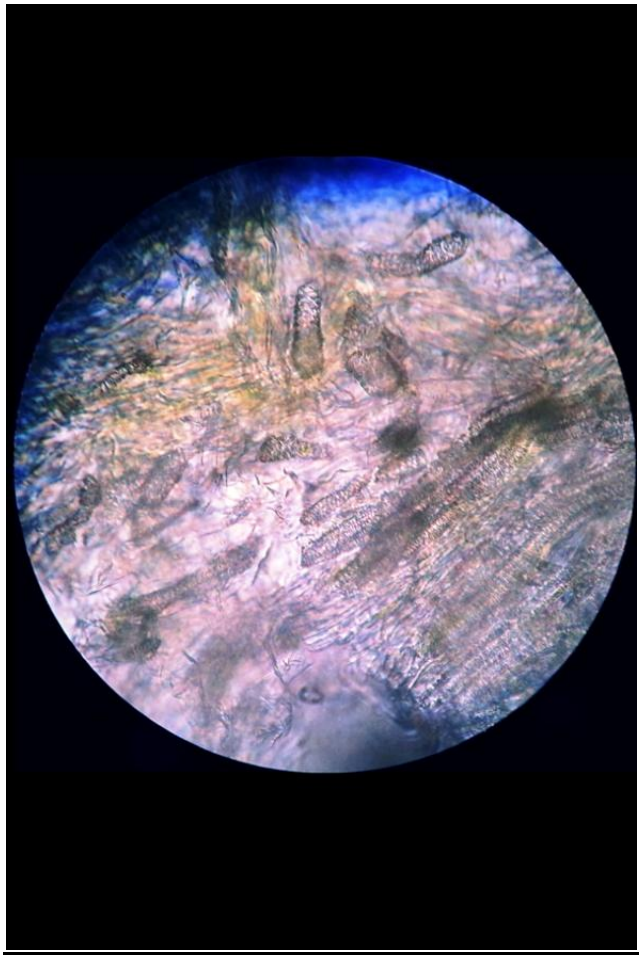

Fig 27: Tracheids with reticulate thickenings on their walls.

Leaf culture

***De novo* adventitious root formation**

Leaf segments 4-5 mm in length were excised and planted on basal medium supplemented with various auxins to promote root induction. Direct rooting from leaf explants was observed only on higher concentration of NAA (4 mg/l). Rooting initiated at one end of the leaf lamina after 20 days of culturing forming 2-3 roots (Fig.28). The number of the roots increased to 8-10 after 4 weeks (Fig.29) and ultimately 15-16 roots were formed after 6 weeks of culturing (Fig.30). The roots formed were long, white and bore profuse root hair.

Fig 28: Root induction on MS+NAA (4 mg/l) after 20 days

Fig 29: Increase in number of roots on MS+NAA (4 mg/l) after 4 weeks

Fig 30: Further proliferation on long, thin profuse roots on MS+NAA (4 mg/l) after 6 weeks

Callusing

Among the various growth regulators tested for establishing callus from the leaf explants, 2,4-D (4 mg/l) in combination with BAP (1mg/l) proved most effective. The callus was hard, compact and whitish green in color (Fig.31). Callus induction from leaf explants also occurred on NAA (2-4mg/l) in combination with BAP or Kn but the growth of callus was slow (Fig.32-34).

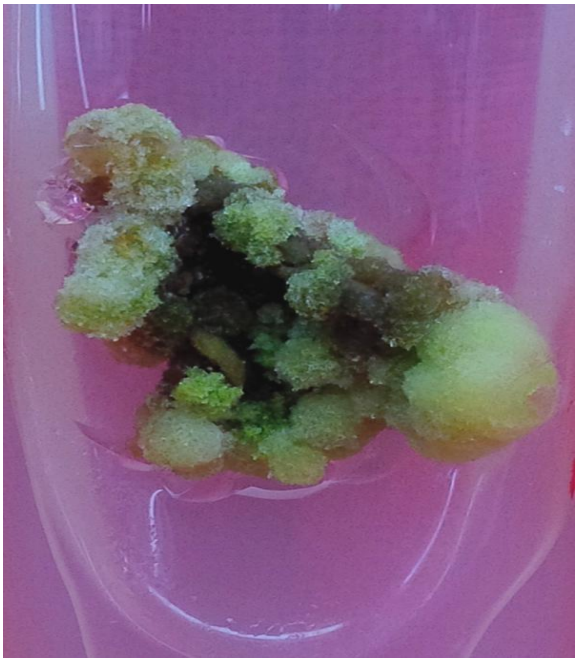

Fig 31: Formation of callus from the cut ends on MS+2,4-D (4mg/l) + BAP (1mg/l) after 4 weeks

Fig 32: Mass of callus formed on MS+NAA (4mg/l) + BAP (1mg/l) after 4 weeks

Fig 33: Formation of whitish green callus on MS+NAA (2mg/l) + Kn (1mg/l) after 2-3 weeks

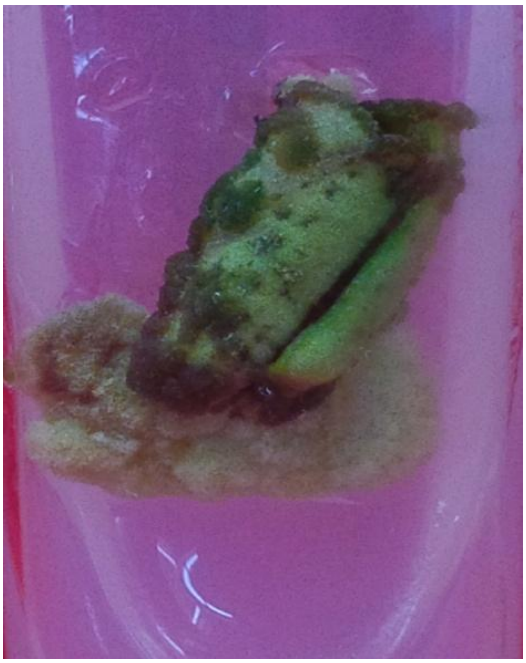

Fig 34: Small mass of callus formed on MS+NAA (4mg/l) + Kn (1mg/l) after 20-25 days

Study of callus

The callus was hard and thus had to be teased with needles to study its cell types. Cell types studied in various calli showed their heterogenous nature with wide variations in size and shapes like ovoid, spherical and elongated. Histogenetic differentiation in the form of tracheids was observed in the calli. Tracheids occurred singly or in groups and possessed reticulate thickenings on their walls (Fig.35).

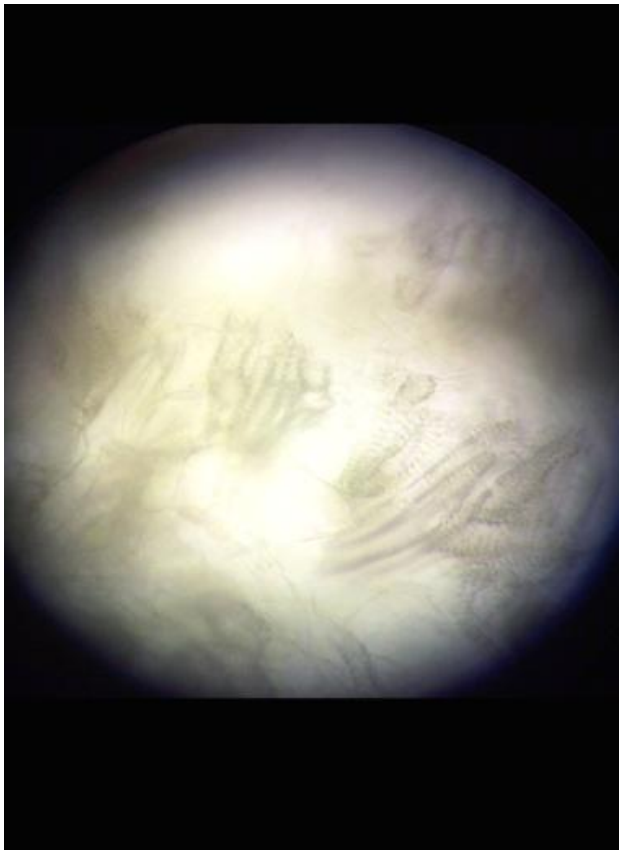

Fig 35: A group of Tracheids showing reticulate thickenings on their walls

No differentiation of root or shoots could be effected from the leaf or stem calli in any of the media tested.

Chapter 4

Results and Observations

MATERIAL AND METHODOLOGY

Experimental Material:

Antirrhinum majus belonging to family Scrophulariaceae was selected as the experimental material. It is known by the name of dog flower in India and snapdragon in other places. *Antirrhinum majus* is important commercially as a cut flower, annual bedding plant, and potted plant. Children love opening the jaw of the flower and watching it snap shot. Dog flowers are the most familiar flowers brightening the cottage gardens.

Habitat:

Old walls, rocks, and dry places

Distribution:

The plant is native originally to North Africa, Spain and along the Mediterranean to Italy. Snapdragons have become naturalized in temperate regions in North America and India (Floridata.com).

Morphology:

Snapdragon is an herbaceous perennial plant, growing to 0.5-1 m tall, rarely up to 2 m. The leaves are spirally arranged, broadly lanceolate, 1-7 cm long and 2-2.5 cm broad. The flowers are produced on a tall spike, each flower is 3.5-4.5 cm long, zygomorphic, with two lips closing the corolla tube (Figure 1). Wild plants have pink to purple flowers, often with yellow lips. The fruit is an ovoid capsule 10-14 mm diameter, containing numerous small seeds. The plants are pollinated by bumblebees and the flowers close over the insects when they enter and deposit pollen on their bodies (Wikipedia, the free Encyclopedia). Antirrhinum is an anthocyanin found in *A. majus*. It is the 3-rutinoside of cyanidin (Scott-Moncrieff, R (1930).

FIGURE 1

***Antirrhinum majus* (Snapdragon/Dog flower)**

Cultivation: *A. majus* cultivated as a biennial or annual plant, particularly in colder areas where it may not survive the winter.

Soil: Snapdragons need a very well drained soil and can grow in heavy clay soils and nutritionally poor soils. It can tolerate acidic, slightly alkaline and basic pH.

Light: Snapdragons do best in full sun. It can grow in semi shade or no shade.

Moisture: Snapdragons need frequent watering for the First Couple of weeks after transplanting (daily watering). Once established, water when the top 1 in (2.5 cm) of soil feels dry to the touch.

Hardiness: USDA Zones 4-11. Snapdragons perform best in cool weather, and most cultivars can tolerate frost and an occasional light freeze. They don't do well in summer heat, and in zones 9-11. Snapdragons are grown in the winter.

Pests and Diseases:

Rust causes brown pustules surrounded by yellowed tissue on the leaves. Plants may bloom prematurely, have small flowers, and die early.

Anthracnose attacks the leaves and stems in late summer. On older stems the spots are sunken, oblong, yellowish green to gray with a narrow brown border. On the leaves, the

spots are yellowish green turning dirty white with a narrow brown border. When the stem is girdled the plant dies.

Gray mold causes flower spikes to wilt and light brown areas form on the lower stem of the flower cluster. Infected plants break over below the flowers. The disease is worst in wet weather.

Stem rot can be detected by the presence of cottony growth on stems of infected plants near the soil line. Infected plants die (Gilman, 2011).

Uses:

Snapdragons are usually planted as bedding annuals, often with petunias or pansies. The short and dwarf snapdragon cultivars are excellent for border edges, raised beds and rock gardens. Snapdragons make great cut flowers, and the tall varieties are often grown in the cutting garden for use in bouquets. The dwarf and trailing cultivars Intermediate varieties are best for bedding and mixed border.

Edible Uses: An oil that is little inferior to olive oil is said to be obtained from the seeds. The report also says that the plant has been cultivated in Russia for this purpose (Grieve, 1931)

Medicinal Importance: The leaves and flowers are antiphlogistic, bitter resolvent and stimulant (Chiej, 1984). They have been employed in poultices on tumours and ulcers (Grieve, 1931). It is effective in the treatment of all kinds of inflammation and is also used on haemorrhoids. The plant is harvested in the summer when in flower and is dried for later use (Chiej, 1984).

Other Uses: A green dye is obtained from the flowers, it does not require a mordant. Dark green and gold can also be obtained if a mordant is used.

Glassware:

The glassware used for culture work comprised of 25X125, 25X150 mm borosil or corning test tubes, 250ml, 500ml and 1000ml borosil or corning flasks. Other glassware includes graduated measuring cylinder, Petri dishes, beakers and a range of pipettes. All the glassware was thoroughly washed and cleaned prior to use. It was brushed with alkaline detergent and then washed in running tap water. It was then treated with hot chromic acid (mixture of $K_2Cr_4O_7 + H_2O$) followed by thorough washing with tap water.

All vessels were then allowed to dry. Copper distilled water (5-10ml) was then poured into every culture vessel which was tightly plugged. Plugs were made out of absorbent surgical cotton wrapped in muslin. Glassware was then steam sterilized in an autoclave at a pressure of 15 lb\in^2 (121^{o}C) for 15 to 20 minutes.

Culture Media:

Murashige and Skoog's (1962) medium was used as basal medium. Stock solutions of generally 8-10 times major elements, 1000 times minor elements and 100 times organic constituents were prepared. These stock solutions were stored at 4^{H}C and were mixed in desired proportions only before use. None of the stock solutions were stored for more than 15 days.

Composition of Murashige and Skoog's medium (1962):

Ingredient	**Amount (mg/l)**
Major elements-	
$(NH_4)NO_3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
$FeS0_4.4H_20^*$	27.8
Na_2EDTA	37.3

*$FeSO_4$ was not used alone but in combination with Na_2EDTA.

Minor elements-	
$MnS0_4.4H_20$	22.3
$ZnS0_4.7H_20$	8.6
H_3BO_3	6.2
KI	8.3

$Na_2Mo0_4.2H_2O$	0.25
$CuS0_4.5H_2O$	0.025
$CoCl_2.6H_20$	0.025

Organic constituents-

Myoinositol	100
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Sucrose	20000
Agar-agar	10000

Weighed quantities of all the constituents except agar were mixed and volume was adjusted using distilled water. The pH of solution was adjusted to 5.8 ± 0.2 using 0.1N NaOH or HCl depending upon high or low.

The basal medium was supplemented with various auxins such as NAA, IBA, IAA, 2,4-D and cytokinins like, BAP, Kn in different concentrations. These hormones were used singly or in combinations according to the growth requirements.

Following are some of the supplements, which were used either singly or in combinations:

1. Basal Medium (BM)
2. BMS + NAA, IAA, IBA or 2,4-D (0.5- 4 mg/l)
3. BMS + BAP or Kinetin (1- 4 mg/l)
4. BMS + BAP or Kn (1- 4 mg/l) + NAA (0.5 mg/l)
5. BMS + NAA (0.5- 4 mg/l) + BAP or Kn (1 mg/l)
6. BMS + 2, 4-D (2- 4 mg/l) + BAP or Kn (1 mg/l)
7. BMS + NAA or 2, 4-D (2- 4 mg/l) + BAP or Kn (1 mg/l)

Inoculation:

All the experimental manipulations were carried under aseptic conditions in an inoculation chamber fitted with a bactericidal ultraviolet tube. The floor of the chamber was thoroughly wiped with cotton dipped in alcohol. The surface of all the vessels and other accessories such as instruments (spatula, forceps, needles and scalpel etc.), spirit lamp, matchbox, 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 ultraviolet rays. Alcohol was then sprayed in the chamber with the help of an atomizer. The chamber was then sterilized with ultraviolet tube kept continuously on for one hour.

Surface sterilization of inoculum:

Plant materials to be inoculated are also required to be surface sterilized before placing over the media. Explants like leaves, stem and nodal explants were taken from plants growing under the *in vivo* conditions. These were placed in different bottles and covered with net and kept under running tap water for 30 minutes to remove all the adhering dust particles and microbes from the surface. The explants were then washed with liquid detergent for another 15 minutes and then again washed properly with water to remove detergent. The explants were then treated with bavistin for another 20-30 minutes to remove fungus and then washed properly to remove fungicide. Under the sterile conditions in laminar air flow chamber, the explants were treated with 0.1% $HgCl_2$ solution for 5-10 minutes depending upon the explants. The explants like stem and nodal explants were treated with 0.1% $HgCl_2$ for 3-4 minutes. Similarly explants like leaves were treated with 0.1% $HgCl_2$ for 1-2 minutes. The explants were then thoroughly washed with sterilized distilled water to remove traces of $HgCl_2$. Fresh cuts were given to the stem explants after sterilization to remove undesirable or dead portions.

Cultural Conditions:

All the cultures were maintained in an air conditioned culture room at a temperature of 25 ± 4^{o}C. Illumination was provided by cool white fluorescent tubes (Philips India Limited, Mumbai) and the intensity of illumination was 50µm m-2s-1 lux at the level of cultures and 16 hour light regime was followed by 8 hours of darkness.

Chapter 3

Material and Methodology

REVIEW OF LITERATURE

Ornamental plants are produced primarily for their aesthetic value, thus the propagation and improvement of quality attributes and the creation of novel variation are important economic goals for floriculturists. Micropropagation, clonal reliability and conservation are important aspects to be considered. These partial processes are amenable to controlled investigations and successful *in vitro* propagation of ornamental plants is now being used for commercialization. The advent of tissue culture technique has offered a new approach to the morphogenetic variations (Ameeta and Agrawal, 2012).

Micropropagation of horticultural crops and ornamental plants is today a reliable technology applied commercially worldwide, which allows large-scale plant multiplication, production and supply of selected plants. It is an aseptic method of clonal propagation. The technique of tissue culture is based on the concept of totipotency as proposed by Haberlandt, that is, the genetically based ability of a non embryonic organ or cell to develop along a pathway similar to that of a zygote, leading to the formation of entirely new plant identical to the original one. The use of tissue culture for cloning ornamentals is expensive and presently limited to a certain number of species. However, the introduction of some additional new techniques may possibly reduce the cost and broaden the range of plants that can be propagated economically *in vitro*. Micropropagation protocols have been developed for a wide range of ornamental plants like *Gladiolus* (Priyakumari *et. al.*, 2005; Emek *et. al.*, 2007), *Orchids* (Chugh *et. al.*, 2009), *Dianthus caryophyllus* (Duhoky *et. al.*, 2009; Casas *et. al.*, 2010), *Rosa* (Pati *et. al.*, 2010; Xing *et. al.*, 2010), *Chrysanthemum* (Nencheva 2010; Mani *et. al.*, 2011), and many more.

Micropropagation is a complex process which can be achieved by any of the following three approaches:

- Multiplication by axillary bud and apical shoots
- Multiplication by adventitious buds
- Somatic embryogenesis

The first two approaches lead to plantlet formation via organogenesis through production of unipolar shoots which must then be further multiplied, followed by rooting in a multistage process. In contrast, somatic embryogenesis leads to the formation of bipolar embryos through steps that are often similar to zygotic embryogenesis.

All the above three techniques have been adopted for the production of ornamental plants under *in vitro* conditions.

Multiplication by Apical and Axillary Shoots:

Axillary and apical shoots contain quiescent or active meristems depending on the physiological state of the plant. However, only limited numbers of axillary meristems have the capacity to develop *in vivo* if the type of branching of a particular species display apical dominance. A shoot tip and an axillary bud when cultured in medium supplemented with high cytokinin concentration, develop axillary shoots which further proliferate to form clusters of secondary and tertiary shoots. This technique is the most reliable technique as it ensures genetic stability of clones.

The multiplication rate through this technique varies with genotype and cytokinin requirement. Karim *et. al.* (2003) reported multiple shoot proliferation from the nodal explants of *Chrysanthemum morifolium* on MS medium containing BAP + sucrose 30mg/l + agar 6g/l and pH 5.5-6.0. Udom *et. al.* (2009) reported multiple shoot proliferation from nodal explants of *Rosa hybrida* on MS medium containing several concentrations of BA and NAA. Multiple shoot formation of up to 3 shoots was obtained on MS medium supplemented with 3 mg/l BA and 0.003 mg/l NAA. Waseem *et.al.* (2009) established a protocol for *in vitro* propagation of *Chrysanthemum* from shoot tips on IAA (0.1mg/l) where maximum shoot initiation (86.6%) was recorded. Xing *et.al.* (2010) reported multiple shoot proliferation in *Rosa rugosa* from nodal segments on MS medium supplemented with BA 2.2 µM, NAA 0.054 µM, GA_3 2.0µM and 3% sucrose. Kharrazi *et.al.* (2011) evaluated the effect of different plant growth regulators for multiple shoot proliferation from axillary buds in *Dianthus caryophyllus*. The highest number of shoots was formed on medium supplemented with 4.4 µM BAP + 1.47 µM NAA.

Newbury (1986) reported multiplication of *Antirrhinum majus* L. by shoot tip culture. Nine varieties of *Antirrhinum majus* were used in the study of *in vitro* multiplication of plants using shoot tip culture. Acceptable multiplication rates were obtained in several media with only variety Victory showing significantly lower rates of shoot production. Wounded shoots of this variety produced callus in the absence of added auxin and some of this callus produced prolific roots.

Atkinson *et. al.* (1989) reported multiple shoot formation in *Antirrhinum majus* from shoot tips. Shoot induction occurred in MS medium supplemented with 1 mg/l BAP.

Gonzalez-Benito *et. al.* (1996) reported multiple shoot formation from nodal segments of *Antirrhinum majus* when grown on MS medium containing 0.5 mg/l NAA + 1 mg/l BAP.

Multiplication by adventitious buds:

Many ornamental plants 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 and flower parts or indirectly from calli obtained from these explants. Choice of explants and hormone regime to which the explants are subjected to, are two important factors in the initiation of adventitious shoots.

Indirectly through callus:

Kantia and Kothari (2002) reported adventitious shoot bud formation on BAP (2.2µM) and 2,4-dichlorophenoxyacetic acid (4.87µM) supplemented MS medium along with excessive callus formation on the surface of the leaf explants in *Dianthus chinensis*. Dillen *et.al.*(1996) reported shoot regeneration in long term callus culture derived from mature leaves as explants of *Cyclamen persicum*. Ghanati *et. al.* (2012) developed an efficient procedure for the development of callus and shoot formation from different explants especially leaf explants of *Eustoma grandiflorum*. MS medium containing 14.4 µM IAA, 0.465 µM Kn, 22.05 µM NAA was the best medium reported for the induction of callus. Smaranda *et. al.* (2006) reported indirect micropropagation of *Chrysanthemum morifolium* through callus cultures obtained from stem and leaf explants. Callus cultures induced from stems have a greater shoot differentiation than those from leaves.

Sangawn and Harada (1975) reported chemical regulation of callus growth, organogenesis, plant regeneration and somatic embryogenesis in *Antirrhinum majus* tissue and cell cultures. Excised stem explants of *Antirrhinum majus* were grown to investigate factors influencing bud and root development, callus induction and somatic embryogenesis. Auxins such as IAA limited callus development where as 2,4-dichlorophenoxyacetic acid promoted soft friable callus with embryos and occasional development of thick abnormal roots. 2-Naphthoxyacetic acid and coconut milk together induced friable green callus and differentiation of small globular embryos which developed into plantlets after transfer to auxin free mineral medium containing sucrose.

Rao *et. al.* (1976) reported callus formation from leaf and stem segments of *Antirrhinum majus* on Ms medium containing 1 mg/l 2,4-D.

Atkinson *et. al.* (1979) reported induction of callus from shoot tips of *Antirrhinum majus* when cultured on MS medium supplemented with 1 mg/l BAP.

Atkinson *et. al.* (1988) reported regeneration of plants from callus cultures using three types of expolant tissues in Antirrhinum majus callus. Regeneration from mature stem internode - derived callus was extremely poor. Callus derived from seedling shoot tips could be induced to form new shoots. Regeneration was most effective using MS medium containing 0.25mg/l naphthoxyacetic acid + 10% coconut milk.

Directly from the explant:

Altvorst *et. al.* (1992) established a protocol of adventitious shoot formation from *in vitro* leaf explants of *Dianthus caryophyllus*. The highest number of adventitious shoots was obtained on medium containing 4.4 µM BA and 2.205 µM NAA. Kantia *et.al.*(2002) established a protocol of high efficiency adventitious shoot bud induction from leaf explants of *Dianthus chinensis* cultured on MS medium supplemented with 6-BAP (13.2 µM) and 1-NAA (7.35 µM). Chen *et.al*. (2004) developed a protocol of high efficiency plant regeneration through direct shoot formation from leaf cultures of *Paphiopedilum* orchids. The three treatments (4.52 µM 2,4-D, 22.71 µM TDZ, 4.52 µm 2,4-D plus 4.54 µM TDZ) gave a higher response of mean numbers of shoots per explant with intact leaf explants.

Waseem *et. al.* (2009) demonstrated the effect of different auxins on the regeneration capability from leaf discs of *Chrysanthemum.*

Somatic embryogenesis:

It involves the formation of 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.

Kumar *et. al.* (2002) reported heat shock induced somatic embryogenesis in callus cultures of gladiolus in the presence of high sucrose. Tanaka *et.al.*(2002) demonstrated high efficiency of somatic embryogenesis from leaf explants of *Dendranthema grandiflorum (Chrysanthemum*) on MS medium supplemented with 57.08 mM IAA + 0.465 nM Kn. No embryogenesis was observed in the absence of kinetin. Mandal *et. al.* (2005) reported direct somatic embryogenesis from ray floret explants of *Chrysanthemum* on MS medium supplemented with 2,4-D and BA. The best sucrose concentration in this medium was found to be 60 g dm^{-3} where 70% explants responded while 55% embyogenic response was obtained on medium supplemented with 400 mg dm^{-3} inositol. Karami *et. al.* (2006) established an effective protocol of somatic embryogenesis of *Dianthus caryophyllus* and studied the effect of sucrose concentrations on somatic embryogenesis. The maximum frequency of embryogenic callus was obtained on medium containing 9% and 12% sucrose supplemented with 9 µM 2,4-D and 0.8 µM BA. Emek *et.al.*(2007) reported somatic embryogenesis in *Gladiolus anatolicus* using leaves of *in vitro* grown shoots as explants. The highest rate of callus formation was obtained on MS medium supplemented with NAA (36.75 µM) in darkness and this creamy white and friable calli produced numerous somatic embryos on MS medium supplemented with BA (0.44 µM) within 4 weeks of culturing. Frey *et. al.* (2009) developed a procedure for somatic embryogenesis in carnation. The optimum protocol for induction of somatic embryogenesis included initiation of callus reported on basal MS supplemented with 3 µM 2,4-D. Mani *et.al.*(2011) reported indirect somatic embryogenesis in *Chrysanthemum* via callusing from leaf explants on MS medium containing 4.87 µM 2,4-D and the best friable calli were subjected to suspension culture in MS medium supplemented with 4.4 µM BAP for somatic embryogenesis. Xu *et. al.* (2012) established a protocol for somatic embryogenesis from leaf and stem segments of

Chrysanthemum. Somatic embryogenesis was more readily obtained from leaf where 100% regeneration rate was observed on MS medium supplemented with 0.6 mg/l BA and 1.8 mg/l NAA.

Sangwan and Harada, 1975 reported somatic embryogenesis in stem explants of *Antirrhinum majus* on 2,4-D supplemented medium.

Chapter 2

Review of Literature

INTRODUCTION

Ornamental Plants – An Overview:

Plants have always been an important part of human existence. An ornamental is grown for decoration rather than food and other by products. They are intentionally planted for aesthetic appeal and to beautify the surroundings (Mahoney 2014).

Flowers are inseparable from the social fabric of human life. Flowers being adorable creation of God, befits all occasions, be it birth, death or marriage. In the past, flowers were not of much economic importance. One would grow flowers to fulfill his desire aesthetic desire. With the passage of time drastic changes have come about in the lifestyle of the people leading to commercialized cultivation of plants. They play an important role in the decoration of living houses and office establishments. The science and art of commercial floriculture has been recognized as an economic activity with potential for generating employment and earning valuable foreign exchange (Sudhagar, 2013).

Apart from use as beautifying item, flowers are also an important means of communication. Flowers have been an instrument for celebrating special occasions of the moment and to express emotions. Garlands of olive were worn by the roman soldiers and lotus blossoms decorated the Egyptian royalty. The presence of bright multicolored flowers can intensify feelings of contentment and can produce joyful emotions. Studies have also revealed that flowers also have healing properties. They are used for the extraction of various essential oils which are used in the preparation of scents, perfumes etc.

Flowering plants are sold in pots for indoor use. The major flowering plants are orchids, chrysanthemum, and poinsettias. Cut flowers are usually sold as bunches or as bouquets with cut foliage. Foliage plants are also sold in pots and hanging baskets for indoor use. They are used for the decoration of restaurants, office and hotel interiors. Among the non flowering plants, ferns and asparagus are most preferred because of their need in flower arrangements and decoration. Some ornamental foliage have a striking appearance created by lacy leaves or long needles while otherornamentals are grown for distinctively colored leaves such as silvery gray ground covers and bright red grasses (Wikipedia, the free encyclopedia).

Ornamental plants are used in landscapes and throughout the homes to enhance the beauty of the surroundings. Colorful flowering ornamental plants break up the browns and the greens that occur naturally outside. Even fruit and vegetable trees and plants are sometimes used ornamentally when the plants lend themselves in some way to improving the visual appeal of the landscape. Many ornamental plants are chosen because they appeal to the sense of smell, in addition to their visual appeal. Lavender is widely regarded for its pleasing fragrance. Roses are another type of flower well known for their pleasing scent. Ornamental plants provide nutrition and shelter for many wildlife species. Entire gardens of ornamentals are often dedicated to attracting butterflies. Keeping ornamental houseplants has been shown to improve indoor air quality. The healthier a plant is, the more effective it is at removing harmful toxins from the air (Mahoney 2014).

Some ornamental plants are grown for showy foliage. Their foliage may be deciduous, turning bright orange, red, and yellow before dropping off in the fall, or evergreen, in which case it stays green year round. Some ornamental foliage has a striking appearance created by lacy leaves or long needles, while other ornamentals are grown for distinctively colored leaves, such as silvery-gray groundcovers and bright red grasses, among many others (European Commission, General for agriculture and rural development, 2006).

Other ornamental plants are cultivated for their blooms. Depending on the types of plants being grown, the flowers may be subtle and delicate, large or showy, with some plants producing distinctive aroma.

Floriculture or flower farming is a discipline of Horticulture and is the study of growing and marketing flowers and foliage plants. Floriculture includes cultivation of flowering and ornamental plants for sale or for use as raw materials in cosmetic and perfume industry and the pharmaceutical sector. India has a blooming future in floriculture industry. Enormous genetic diversity, varied agro climatic conditions, versatile human resources etc offer India a unique scope for judicious employment of resources (Mathur and Pachpande, 2013).

The production of floriculture products has acquired much importance in the recent times due to their increasing demand. Changing lifestyles and inclination towards healthier and luxurious lifestyle has given boost to overall horticulture along with the flower industry.

Commercial floriculture has emerged in the form lucrative flora industry as Government of India has identified it as a sunrise industry. As per APEDA, commercial floriculture has been viewed as high growth industry and hi-tech activity taking place under controlled climatic conditions inside greenhouse and is gaining importance from the export angle (Singh and Ahlawat, 2014).

Production and Consumption of Ornamental Plants:

Floriculture is growing as a global industry both in developed and developing countries. The world market for floricultural product has been changing constantly. In Europe the most ornamentals are produced. The Netherlands is known for cut flowers and potted plants, perennials and annuals. Germany has a name in nursery and garden plants. In Italy flowers and potted plants are produced. Denmark is famous for its potted plants. The total production value is about 10 billion dollars. The countries with largest share in cut flower production are Germany (11%), Italy (18%) and Netherlands (35%). In North and South America ornamental production mainly consists of flowers and cuttings. In North America, 80% of the flowers and potted plants are grown in USA and Canada. California and Florida are the most important production regions. In South America, Columbia and Mexico have developed rapidly over the last decade. Production in Africa has also increased over the last decade with Kenya on the top followed by Tanzania, South Africa and Uganda. Production in countries like Zimbabwe and Ivory Coast has decreased due to political conditions. Japan is traditionally a producer of specialities in ornamentals. South Korea, Thailand and India are coming up as strong producers (Ruud et.al. 2010)

Global Scenario:

Floriculture is nowadays fast emerging industry. The world production of floriculture is growing at a rate of 10 percent per year. There are over 50 countries that are actively involved in floriculture production on large scale. The Netherlands, the United States, Japan, Italy, Germany and Canada are the largest producers of cut flowers and plants. Europe, the US and Japan are the major consumers of floriculture products.

The global exports of floriculture products stood at about 17 billion US $ in 2007. Fresh cut flowers and foliage accounted for around 49.1 percent (8.31 billion US $), and live plants, bulbs and cuttings accounted for 50.9 percent of the total cut flower industry trade. More than 90 percent of the world trade is accounted by developed countries in Europe, America and Asia. The world imports of floriculture products stood at 16.7 billion US $ in 2007. Germany (2.59 billion US $) was the largest importer followed by the United Kingdom (1.89 billion US $), the USA (1.81 billion US $), the Netherlands (1.55 billion US $) and France (1.43 billion US $) (Muthukumaran, 2009).

Indian scenario:

India is bestowed with diverse agro-climatic and ecological conditions, which are favourable to grow all kinds of commercially important flowers generally found in different parts of the world. Tamil Nadu is the leading state in area and production of flowers in the country. The area under flower crops was 20,801 ha and the production was 1.24 lakhs million tones of loose flowers during 1999-2000 (Sudhagar, 2013).

In our country, flowers are grown in around 233,000 ha land, with the production of loose flowers around 1729,000 MT and that of cut flowers 76732 lac numbers (2012-2013), as per NHB 2013 database. Loose flowers like marigold, china aster, jasmine, crossandra, barleria etc occupy major flower cultivated area of the country as domestic consumption is very high. Tamil Nadu, Karnataka and Andhra Pradesh are leading loose flower producing states. Cut flowers like rose, tuberose, gladiolus, chrysanthemum etc are highly popular and widely cultivated in the country. West Bengal, Karnataka, Andhra Pradesh, Maharashtra and Orissa are the leading cut flower producing states. Important cut flowers exported from India include roses, lilies, carnations and orchids. Major importers of flora products from our country are USA, Netherlands, Germany, UK, Canada and Japan (Singh and Ahlawat, 2014).

Floriculture is an age old farming activity in India having intense potential for generating self-employment among farmers. The production and trade of floriculture have grown consistently over the last 10 years. It has emerged as a profitable agri-business in India and

worldwide. India is on 18th position in the export of floriculture products. In 1996, India exported floriculture products worth 18 million US $ which has increased to 68 million US $ in 2005. The Indian floriculture industry has been growing at a compounded annual growth rate (CAGR) of about 30% and is likely to cross the Rs 8000 crore mark by 2015.It comprises the florist trade, nursery plants, potted plants, bulb and seed production, micro propagation material and extraction of essential oils from flowers (Saha, 2005).

Other most important cut flower plants are Asters, Gladiolus, Tuberose, Gerbera, Carnation, Lilium and orchids. In the recent years dried flowers and foliage have also become an important part of floriculture products. Other floriculture products include dry flowers and essential oils. India is the fifth largest exporter of dried flowers and second largest exporter of dry foliage in the world. The main export markets for cut flowers include USA, Netherlands, UK and Germany. West Bengal accounts for around 70 percent of the dried flower exports from India. India is the second largest exporter of essential oils and is the largest exporter of jasmine oil in the world accounting for over 40 percent of total world exports in jasmine oil (Muthukumaran 2009).

Plant Tissue Culture In Ornamental Plants:

The demand and usage of ornamental plants is increasing day by day. Flowers are the most wanted item in any social occasions for conveying one's status and aesthetic sense. Thus to meet the increasing demands, propagation of ornamental plants via tissue culture has become an accepted commercial practice. The achievements in the field of micro propagation of ornamental plants is attributed to various scientists. Morel is often referred to as the "Father of micro propagation." Morel's successful culture of apical and axillary buds of *Cymbidium* paved the way for rapid development of micro propagation techniques.

In vitro methods are used to speed up propagation. The use of *in vitro* propagation can help to overcome difficulties and can manipulate the phenotypic variation. Natural cultivation or propagation of plants yield heterozygous species only. Thus for slow growing and rare plants, tissue culture provides a cost effective, sustainable and well controlled means for the mass production of disease free plants. The technique can be exploited for the production of

clones particularly in vegetatively propagated plants. The success of the system lies in the development of strict protocols for each species. This involves improving decontamination procedures and determining the effects of various cultural factors on plant growth, both *in vitro* and *ex vitro* to establish optimum growing conditions.

Advantages of Micro propagation:

- Production of virus free plants
- Production of clones of each other
- Formation of somatic hybrids through protoplast culture
- Production of transgenic plants for varietal improvements
- Plant available all round the year
- Produces rooted plantlets ready for growth
- Production of artificial seeds
- Large number of plants can be produced per square meter and propagules can be stored for longer period of time
- Produces more robust plants leading to accelerated growth

Techniques of Micro propagation:

Three basic techniques are used to propagate plants *in vitro*. They are:

1. Multiplication by apical and axillary buds-
    - It is the most common method of micro propagation. This technique ensures genetic stability as the cells of the meristem are uniformly diploid and are least susceptible to genetic changes.
    - Cytokinin rich medium is used for multiple shoot proliferation through apical and axillary buds.

2. *De novo* adventitious shoot formation-

    - New adventitious shoots can be formed either directly from the explants like root, stem, leaf, petiole, flower parts etc. or indirectly from the callus obtained from these explants.

    - Plants obtained through callus may not be true elites because of high incidence of polyploidy and aneuploidy.

3. Somatic or non zygotic embryogenesis-

    - It involves the formation of bipolar embryos which can develop into fully functional plants under appropriate conditions.

Stages of Micro propagation:

Micro propagation involves 4 basic stages.

1. Stage I- Establishment of aseptic cultures:

    - It involves the isolation of explants, surface sterilization and establishment of culture medium. Surface sterilization includes washing of the explants with tap water followed by detergent treatment and then with the antifungal agent.

2. Stage II- Shoot multiplication using definite culture medium:

    It can be achieved through any of the following methods-

    - Multiplication through shoots

    - Multiplication through apical shoots and adventitious buds

    - Multiplication through callus

    - Somatic or non zygotic embryogenesis

3. Stage III- Rooting of regenerated shoots in *in vitro* conditions-

    - This process involves the elongation of shoots prior to rooting.

- Shoots and shoot clusters are transferred to soil for rooting.

4. Stage IV- Hardening and acclimatization of the plantlets-

    - Plants grown under controlled culture conditions are transferred to field conditions. The climatic adaptation of a plant moved to a new environment is known as acclimatization.

    Micro propagated plants are difficult to be transplanted due to two major reasons

    - Heterotrophic mode of nutrition
    - Poor control of water loss

To overcome these problems, plantlets should be transplanted to a well drained soil, sterile medium and maintained initially at high relative humidity (90%) and reduced light for first 10-15 days and should be covered with clear plastic bags. The plants are then transferred to green houses. The transplants are then acclimatized by slowly lowering the humidity. Plants are gradually moved to higher light intensities to promote vigorous growth.

RATIONALE AND OBJECTIVES:

The present investigation was carried out on an important ornamental plant – *Antirrhinum majus* commonly known as snapdragon. Snapdragons are usually grown as annuals. They can be propagated with relative ease through sexual and conventional vegetative multiplication through seeds and cuttings. Natural cultivation through sexual means yields only heterozygous species of plants showing great variations in growth, habit and yield. Vegetative propagation ensures genetic stability or clonal fidelity but the plants are invariably infested with pathogens which definitely affects the progeny. Moreover, it is a slow, cumbersome and labour intensive procedure and only a few propagules are obtained by this method. Snapdragons are invariably affected by pests like rusts, aphids and other fungal diseases which can be a problem, especially in wet seasons. Thus using *in vitro* techniques, these problems can be overcome and disease free plants of high quality can be obtained.

The main objectives of the investigation were:

- To establish a protocol for the rapid and mass scale propagation of plants in shorter duration of time and space.
- To obtain genetically pure elites

MATERIAL AND METHODOLOGY

Experimental Material:

Antirrhinum majus belonging to family Scrophulariaceae was selected as the experimental material. It is known by the name of dog flower in India and snapdragon in other places. *Antirrhinum majus* is important commercially as a cut flower, annual bedding plant, and potted plant. Children love opening the jaw of the flower and watching it snap shot. Dog flowers are the most familiar flowers brightening the cottage gardens.

Habitat:

Old walls, rocks, and dry places

Distribution:

The plant is native originally to North Africa, Spain and along the Mediterranean to Italy. Snapdragons have become naturalized in temperate regions in North America and India (Floridata.com).

Morphology:

Snapdragon is an herbaceous perennial plant, growing to 0.5-1 m tall, rarely up to 2 m. The leaves are spirally arranged, broadly lanceolate, 1-7 cm long and 2-2.5 cm broad. The flowers are produced on a tall spike, each flower is 3.5-4.5 cm long, zygomorphic, with two lips closing the corolla tube (Figure 1). Wild plants have pink to purple flowers, often with yellow lips. The fruit is an ovoid capsule 10-14 mm diameter, containing numerous small seeds. The plants are pollinated by bumblebees and the flowers close over the insects when they enter and deposit pollen on their bodies (Wikipedia, the free Encyclopedia). Antirrhinum is an anthocyanin found in *A. majus*. It is the 3-rutinoside of cyanidin (Scott-Moncrieff, R (1930).

FIGURE 1

***Antirrhinum majus* (Snapdragon/Dog flower)**

Cultivation: *A. majus* cultivated as a biennial or annual plant, particularly in colder areas where it may not survive the winter.

Soil: Snapdragons need a very well drained soil and can grow in heavy clay soils and nutritionally poor soils. It can tolerate acidic, slightly alkaline and basic pH.

Light: Snapdragons do best in full sun. It can grow in semi shade or no shade.

Moisture: Snapdragons need frequent watering for the First Couple of weeks after transplanting (daily watering). Once established, water when the top 1 in (2.5 cm) of soil feels dry to the touch.

Hardiness: USDA Zones 4-11. Snapdragons perform best in cool weather, and most cultivars can tolerate frost and an occasional light freeze. They don't do well in summer heat, and in zones 9-11. Snapdragons are grown in the winter.

Pests and Diseases:

Rust causes brown pustules surrounded by yellowed tissue on the leaves. Plants may bloom prematurely, have small flowers, and die early.

Anthracnose attacks the leaves and stems in late summer. On older stems the spots are sunken, oblong, yellowish green to gray with a narrow brown border. On the leaves, the

spots are yellowish green turning dirty white with a narrow brown border. When the stem is girdled the plant dies.

Gray mold causes flower spikes to wilt and light brown areas form on the lower stem of the flower cluster. Infected plants break over below the flowers. The disease is worst in wet weather.

Stem rot can be detected by the presence of cottony growth on stems of infected plants near the soil line. Infected plants die (Gilman, 2011).

Uses:

Snapdragons are usually planted as bedding annuals, often with petunias or pansies. The short and dwarf snapdragon cultivars are excellent for border edges, raised beds and rock gardens. Snapdragons make great cut flowers, and the tall varieties are often grown in the cutting garden for use in bouquets. The dwarf and trailing cultivars Intermediate varieties are best for bedding and mixed border.

Edible Uses: An oil that is little inferior to olive oil is said to be obtained from the seeds. The report also says that the plant has been cultivated in Russia for this purpose (Grieve, 1931)

Medicinal Importance: The leaves and flowers are antiphlogistic, bitter resolvent and stimulant (Chiej, 1984). They have been employed in poultices on tumours and ulcers (Grieve, 1931). It is effective in the treatment of all kinds of inflammation and is also used on haemorrhoids. The plant is harvested in the summer when in flower and is dried for later use (Chiej, 1984).

Other Uses: A green dye is obtained from the flowers, it does not require a mordant. Dark green and gold can also be obtained if a mordant is used.

Glassware:

The glassware used for culture work comprised of 25X125, 25X150 mm borosil or corning test tubes, 250ml, 500ml and 1000ml borosil or corning flasks. Other glassware includes graduated measuring cylinder, Petri dishes, beakers and a range of pipettes. All the glassware was thoroughly washed and cleaned prior to use. It was brushed with alkaline detergent and then washed in running tap water. It was then treated with hot chromic acid (mixture of $K_2Cr_4O_7 + H_2O$) followed by thorough washing with tap water.

All vessels were then allowed to dry. Copper distilled water (5-10ml) was then poured into every culture vessel which was tightly plugged. Plugs were made out of absorbent surgical cotton wrapped in muslin. Glassware was then steam sterilized in an autoclave at a pressure of 15 lb\in^2 (121^{o}C) for 15 to 20 minutes.

Culture Media:

Murashige and Skoog's (1962) medium was used as basal medium. Stock solutions of generally 8-10 times major elements, 1000 times minor elements and 100 times organic constituents were prepared. These stock solutions were stored at 4^{H}C and were mixed in desired proportions only before use. None of the stock solutions were stored for more than 15 days.

Composition of Murashige and Skoog's medium (1962):

Ingredient	**Amount (mg/l)**
Major elements-	
$(NH_4)NO_3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
$FeS0_4.4H_20^*$	27.8
Na_2EDTA	37.3

*$FeSO_4$ was not used alone but in combination with Na_2EDTA.

Minor elements-	
$MnS0_4.4H_20$	22.3
$ZnS0_4.7H_20$	8.6
H_3BO_3	6.2
KI	8.3

$Na_2Mo0_4.2H_2O$	0.25
$CuS0_4.5H_2O$	0.025
$CoCl_2.6H_20$	0.025

Organic constituents-

Myoinositol	100
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Sucrose	20000
Agar-agar	10000

Weighed quantities of all the constituents except agar were mixed and volume was adjusted using distilled water. The pH of solution was adjusted to 5.8 ± 0.2 using 0.1N NaOH or HCl depending upon high or low.

The basal medium was supplemented with various auxins such as NAA, IBA, IAA, 2,4-D and cytokinins like, BAP, Kn in different concentrations. These hormones were used singly or in combinations according to the growth requirements.

Following are some of the supplements, which were used either singly or in combinations:

1. Basal Medium (BM)
2. BMS + NAA, IAA, IBA or 2,4-D (0.5- 4 mg/l)
3. BMS + BAP or Kinetin (1- 4 mg/l)
4. BMS + BAP or Kn (1- 4 mg/l) + NAA (0.5 mg/l)
5. BMS + NAA (0.5- 4 mg/l) + BAP or Kn (1 mg/l)
6. BMS + 2, 4-D (2- 4 mg/l) + BAP or Kn (1 mg/l)
7. BMS + NAA or 2, 4-D (2- 4 mg/l) + BAP or Kn (1 mg/l)

Inoculation:

All the experimental manipulations were carried under aseptic conditions in an inoculation chamber fitted with a bactericidal ultraviolet tube. The floor of the chamber was thoroughly wiped with cotton dipped in alcohol. The surface of all the vessels and other accessories such as instruments (spatula, forceps, needles and scalpel etc.), spirit lamp, matchbox, 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 ultraviolet rays. Alcohol was then sprayed in the chamber with the help of an atomizer. The chamber was then sterilized with ultraviolet tube kept continuously on for one hour.

Surface sterilization of inoculum:

Plant materials to be inoculated are also required to be surface sterilized before placing over the media. Explants like leaves, stem and nodal explants were taken from plants growing under the *in vivo* conditions. These were placed in different bottles and covered with net and kept under running tap water for 30 minutes to remove all the adhering dust particles and microbes from the surface. The explants were then washed with liquid detergent for another 15 minutes and then again washed properly with water to remove detergent. The explants were then treated with bavistin for another 20-30 minutes to remove fungus and then washed properly to remove fungicide. Under the sterile conditions in laminar air flow chamber, the explants were treated with 0.1% $HgCl_2$ solution for 5-10 minutes depending upon the explants. The explants like stem and nodal explants were treated with 0.1% $HgCl_2$ for 3-4 minutes. Similarly explants like leaves were treated with 0.1% $HgCl_2$ for 1-2 minutes. The explants were then thoroughly washed with sterilized distilled water to remove traces of $HgCl_2$. Fresh cuts were given to the stem explants after sterilization to remove undesirable or dead portions.

Cultural Conditions:

All the cultures were maintained in an air conditioned culture room at a temperature of 25 ± 4^{o}C. Illumination was provided by cool white fluorescent tubes (Philips India Limited, Mumbai) and the intensity of illumination was 50µm m-2s-1 lux at the level of cultures and 16 hour light regime was followed by 8 hours of darkness.

Chapter 4

Results and Observations

RESULTS AND OBSERVATIONS

Different vegetative parts like nodal segments, shoot apices, leaf and stem segments were excised from mature, healthy, field grown plant of *Antirrhinum majus* and used for experimental work.

Nodal explant and shoot tip culture

Nodal explants (5 mm long) holding one dormant lateral bud each and young healthy shoot tips were collected from mature, healthy plant of *Antirrhinum majus.* After sterilization, the damaged internodal tissue on both sides of the nodal segment were cut off and the nodal segments 3-4 mm in size and shoot apices were cultured on MS medium supplemented with different concentrations of BAP (1-4 mg/l) or Kn (1-4 mg/l) either alone or in combination with NAA (0.5–2 mg/l). The axillary shoot proliferation was remarkably influenced by the type and concentration of the cytokinin used.

Multiple shoot proliferation from nodal segments

Out of various growth regulators used, the best results were obtained on MS medium supplemented with BAP (4mg/l). The initial bud break from nodal segment occurred after 10 days of inoculation (Fig.1) and 3-4 shoots were formed after 15 days (Fig.2). These shoots multiplied further leading to the formation of a maximum of 12-14 shoots after 6 weeks of inoculation in about 60% cultures (Fig.3). A change in the concentration level of BAP below the optimum value (4 mg/l) resulted in decline in the number of shoots formed.

Fig 1: Nodal Bud Break on MS+BAP (4 mg/l) after 10 days of inoculation

Fig 2: Formation of few shoots on MS+BAP (4 mg/l) after 15 days

Fig 3: Development of numerous shoots on MS+BAP (4 mg / l) after 6 weeks

Shoot tip culture

Multiple shoot proliferation from the shoot apices was effected on both BAP and Kn supplemented medium. Young and healthy shoot apices were collected from mature field grown plant of *Antirrhinum majus* and cultured on MS medium supplemented with different concentrations of BAP and Kn. Out of various concentrations of BAP (0.5-4 mg/l) tested, maximum sprouting occurred on BAP (4mg/l) supplemented medium where 5-6 shoots were formed after 15 days (Fig.4) leading to further proliferation of shoots forming 12-14 shoots after 6 weeks (Fig.5). Once the clusters of shoots were formed, small clumps of 2-3 shoots were excised and transferred onto fresh multiplication medium where they registered further increase in the number of shoots (Fig.6). A noticeable feature observed was the induction of roots along with shoot multiplication on subculturing on BAP (4mg/l).

Fig 4: Regeneration of shoots from shoot tip on MS+BAP (4 mg/l) after 15 days

Fig 5: Numerous shoots formed on MS+BAP (4 mg/l) after 6 weeks

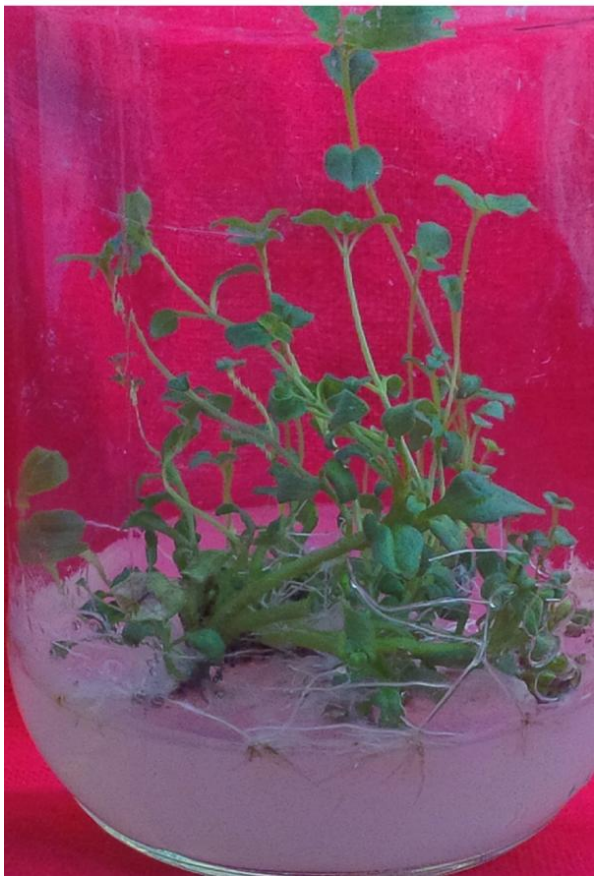

Fig 6: Multiplication of shoots after subculturing on MS + BAP (4mg/l)

Best shoot proliferation, however, occurred on 2mg/l Kn, where 5-6 shoots were formed after 15 days (Fig.7) further leading to the formation of 8-10 shoots after 25 days (Fig.8). The shoots proliferated further forming long, well developed shoots (15-16) with numerous leaves after 6 weeks (Fig.9). The shoots thus formed were further subdivided into smaller clumps having 3 shoots and subcultured onto fresh multiplication medium supplemented with Kn (2mg/l) where the shoots further increased in number along with the formation of roots (Fig.10).

The %age response regarding axillary shoot proliferation from shoot tip on various concentrations of BAP and Kn is depicted in Fig.11 and Table 2. Effects of different concentrations of BAP and Kn on multiple shoot proliferation from the shoot tip is depicted in Fig.12.

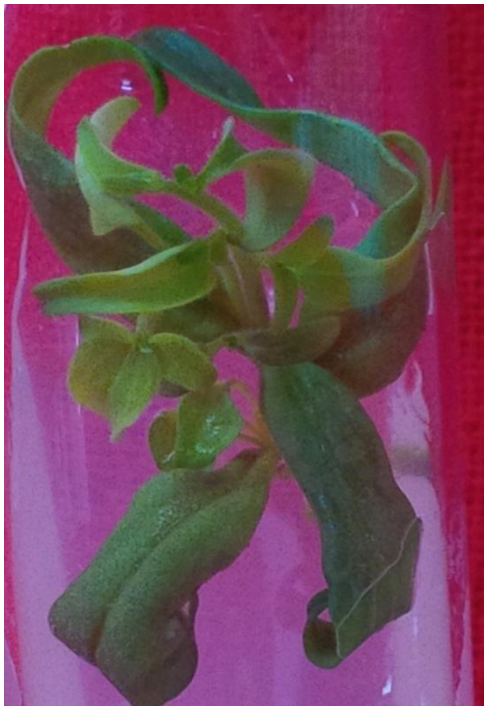

Fig 7: Initiation of multiple shoots on MS+Kn (2 mg/l) after 15 days

Fig 8: Increase in number of shoots on MS+Kn 2 mg/l) after 25 days

Fig 9: Formation of numerous shoots on MS+Kn (2 mg/l) after 6 weeks

Fig 10: Further proliferation after subculturing on MS+Kn (2mg/l)

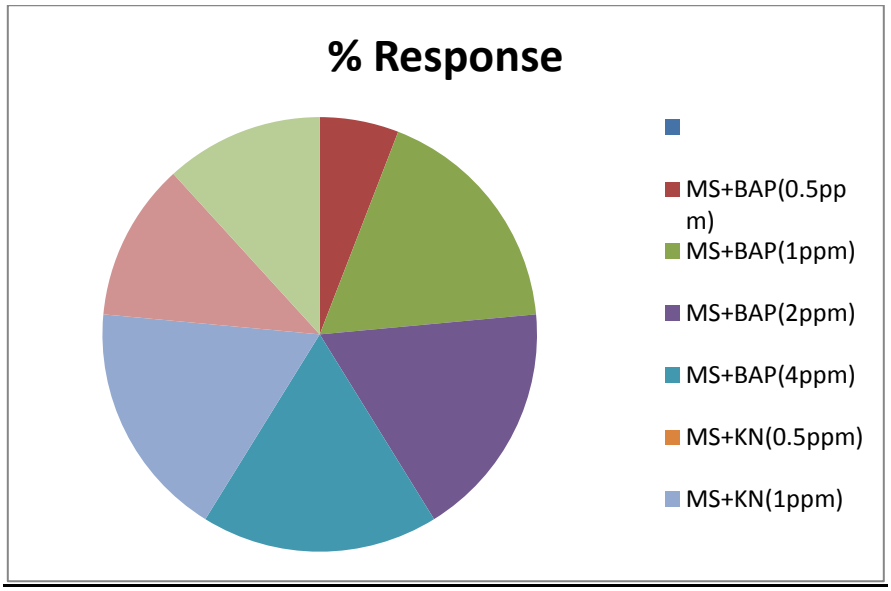

Fig 11: Percentage response regarding shoot proliferation on different media

Table 2: Percentage response and number of shoots formed on different media composition

Hormone Conc.	% Response	No. of shoots formed after 40 days	Mean no. of shoots
MS+BAP(0.5ppm)	20	7 to 8	7.5
MS+BAP(1ppm)	60	8 to 10	9
MS+BAP(2ppm)	60	8 to 10	9
MS+BAP(4ppm)	60	12 to 14	13
MS+KN(0.5ppm)	-	-	-
MS+KN(1ppm)	60	6 to 8	7
MS+KN(2ppm)	40	15 to 16	15.5
MS+KN(4ppm)	40	8 to 10	9

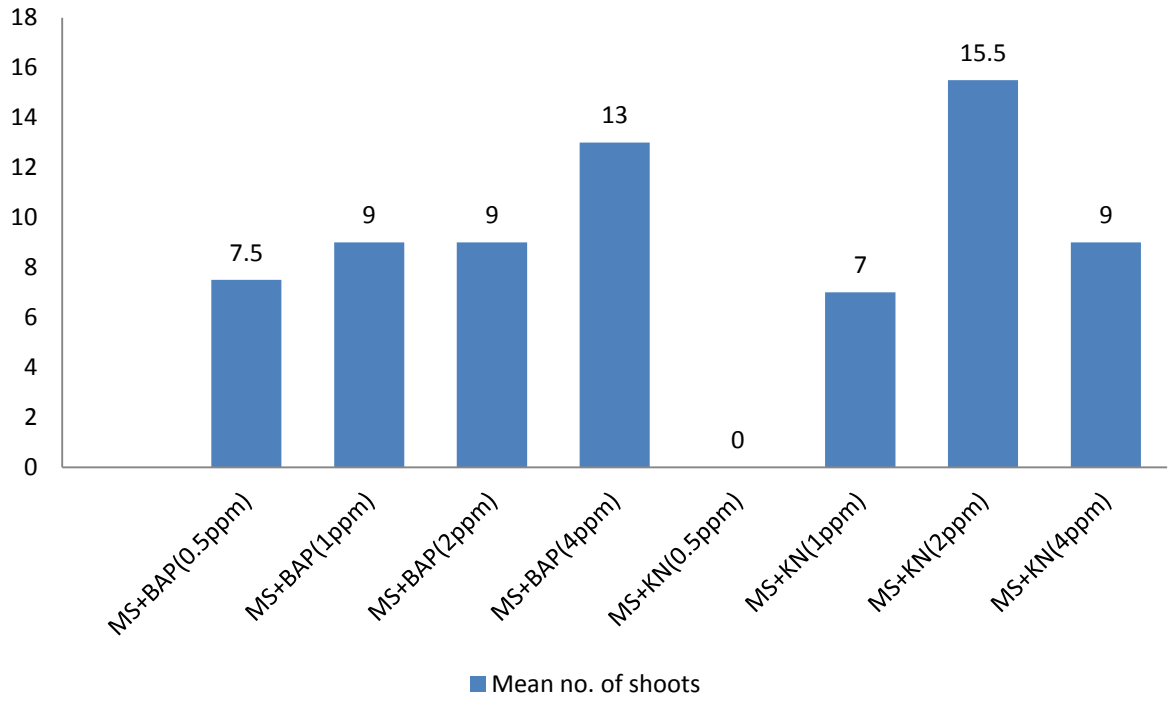

Fig 12: Effects of different concentrations of BAP and Kn on multiple shoot proliferation

Fig 13: Formation of thin, long roots on MS+NAA (2 mg/l) giving rise to complete plantlet

Rooting of microshoots-

Regenerated microshoots were carefully rescued from bottles in laminar air flow on sterile glass plate. These shoots were carefully isolated and inoculated upright in MS medium for root initiation. The shoots were cultured on basal MS medium and MS medium supplemented with different concentrations of NAA (0.5-2 mg/l). Root initiation occurred at the base of the shoot on NAA (2 mg/l) supplemented medium after 2 weeks of culturing forming well developed bunch of roots after 4-5 weeks. The roots were long, white and bore profuse root hairs (Fig.13).

Stem culture:

Direct *de novo* adventitious root formation-

The stem segments 3-4 mm in length were inoculated on basal MS medium supplemented with different concentrations of auxins such as IAA, IBA and NAA for *de novo* adventitious root formation. Out of the various auxins tested, direct rooting from the stem segment was observed only on MS medium supplemented with different concentrations of NAA (0.5-2 mg/l).

Direct rooting was observed on MS medium supplemented with NAA (0.5mg/l), where 2-3 roots were formed after 20 days of culturing (Fig.14). The number increased to 5-6 roots after 30 days (Fig.15). The roots formed were white and bore root hairs.

Fig 14: Root induction from stem segment on MS+NAA (0.5 mg/l) after 20 days

Fig 15: Formation of 5-6 roots on MS+NAA (0.5 mg/l) after 30 days

Maximum rooting was obtained on higher concentrations of NAA i.e. 1-2mg/l. MS medium supplemented with NAA (1mg/l) induced root formation resulting in the formation of 3-4 shoots after 20 days of culturing(Fig.16). The number increased to 6-8 after 30 days (Fig.17) forming 8-10 roots after 40 days of culturing (Fig.18). The roots formed were long, elongated and green or white in color. The roots bore root hair.

Figure 19 depicts the initiation of few thin roots on 2 mg/l NAA which multiplied further covering the entire stem segment after 40 days (Fig.20).

Fig 16: Root induction from stem segment on MS+NAA (1 mg/l) after 20 days

Fig 17: Formation of numerous, long roots on MS+NAA (1 mg/l) after 4 weeks

Fig 18: Increase in number of roots on MS+NAA (1 mg/l) after 6 weeks

Fig 19: Root induction from stem segment on MS+NAA (2 mg/l) after 20 days

Fig 20: Further increase in number of roots on MS+NAA (2 mg/l) after 6 weeks

***De novo* adventitious shoot formation-**

No adventitious shoot formation was observed in cultures of stem segments.

Callusing-

For callus induction, stem segments (4-5 mm) were transferred on MS medium supplemented with different growth regulators. Best callus growth took place on MS medium supplemented with 2,4-D (2 mg/l) and BAP (1mg/l) where callus initiated in 10-12 days, grew further (Fig.21) and within 4 weeks, the entire stem segment was converted into a mass of callus (Fig.22). The callus was whitish green, hard and compact in nature. Likewise, synergistic action of NAA (2-4 mg/l) with BAP or Kn was also found to be effective in inducing callus from the stem explants (Figs. 23 - 26). The callus was compact, non friable and greenish white in color.

Fig 21: Formation of whitish green callus on MS+2,4-D (2mg/l) + BAP (1mg/l) after 20 days

Fig 22: Mass of callus on MS+2,4-D (2mg/l) + BAP (1mg/l) after 4 weeks

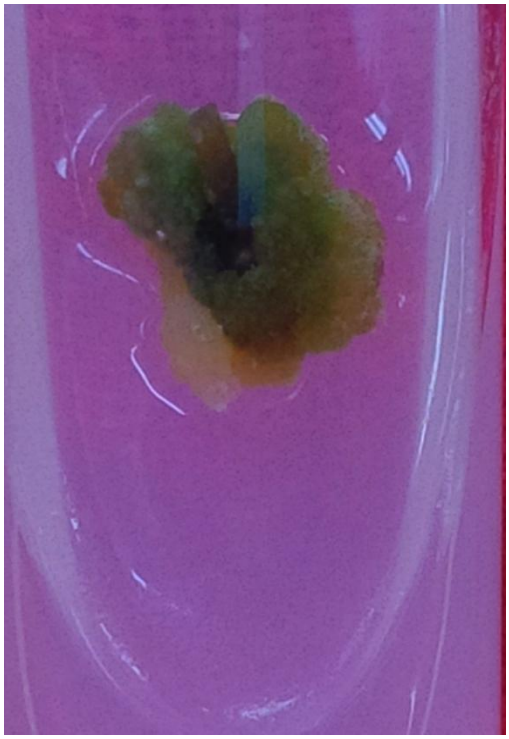

Fig 23: Initiation of callusing son MS+NAA (4mg/l) + Kn (1mg/l) after 10-12 days

Fig 24: Formation of greenish white callus on MS+NAA (4mg/l) + Kn (1mg/l) after 4 weeks

Fig 25: Callus induction on MS+NAA (4mg/l) + BAP (1mg/l) after 10 days

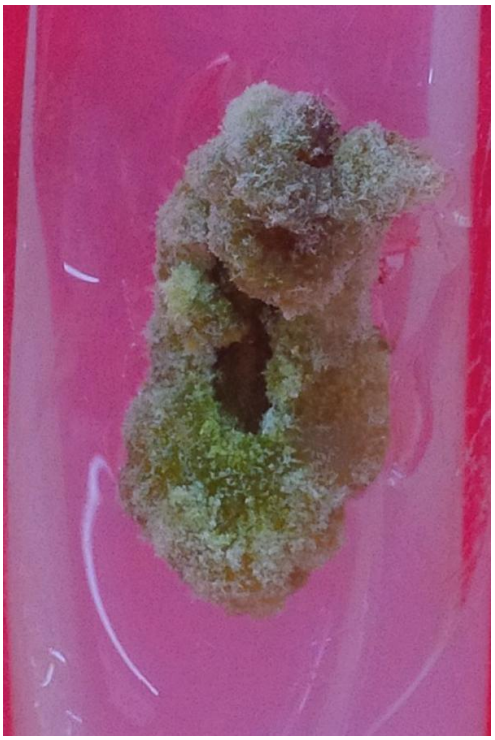

Fig 26: Formation of mass of callus on MS+NAA (4mg/l) + BAP (1mg/l) after 4 weeks

Study of callus-

The callus formed was extremely hard and non friable thus had to be teased properly with needles to study its cell types. The callus was heterogenous in nature comprising of cells of different shapes like ovoid, elongated and spherical. Four week old stem callus revealed differentiation of tracheids which occurred either singly or in groups possessing reticulate thickenings on their walls (Fig. 27).

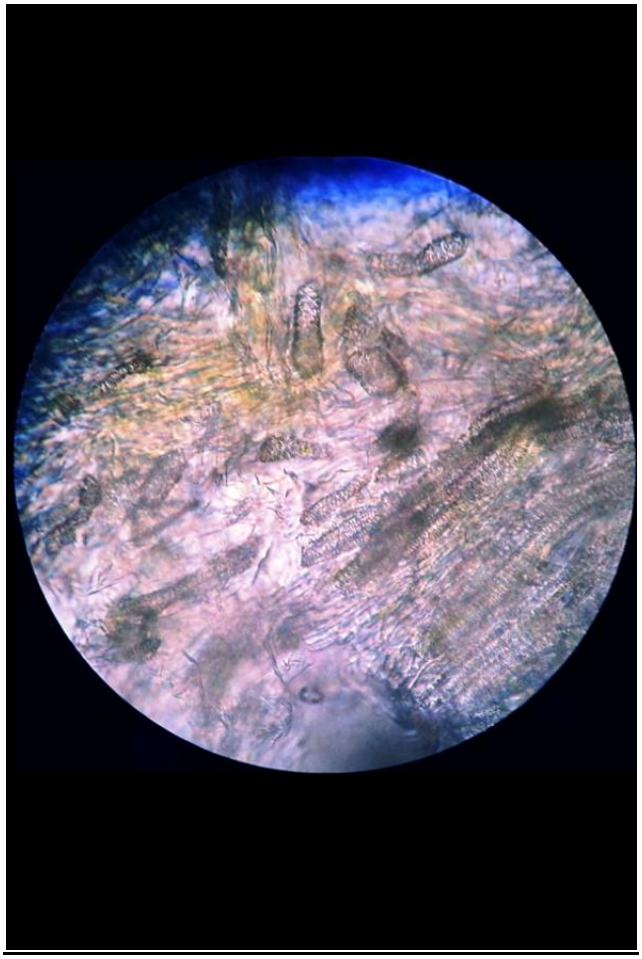

Fig 27: Tracheids with reticulate thickenings on their walls.

Leaf culture

***De novo* adventitious root formation**

Leaf segments 4-5 mm in length were excised and planted on basal medium supplemented with various auxins to promote root induction. Direct rooting from leaf explants was observed only on higher concentration of NAA (4 mg/l). Rooting initiated at one end of the leaf lamina after 20 days of culturing forming 2-3 roots (Fig.28). The number of the roots increased to 8-10 after 4 weeks (Fig.29) and ultimately 15-16 roots were formed after 6 weeks of culturing (Fig.30). The roots formed were long, white and bore profuse root hair.

Fig 28: Root induction on MS+NAA (4 mg/l) after 20 days

Fig 29: Increase in number of roots on MS+NAA (4 mg/l) after 4 weeks

Fig 30: Further proliferation on long, thin profuse roots on MS+NAA (4 mg/l) after 6 weeks

Callusing

Among the various growth regulators tested for establishing callus from the leaf explants, 2,4-D (4 mg/l) in combination with BAP (1mg/l) proved most effective. The callus was hard, compact and whitish green in color (Fig.31). Callus induction from leaf explants also occurred on NAA (2-4mg/l) in combination with BAP or Kn but the growth of callus was slow (Fig.32-34).

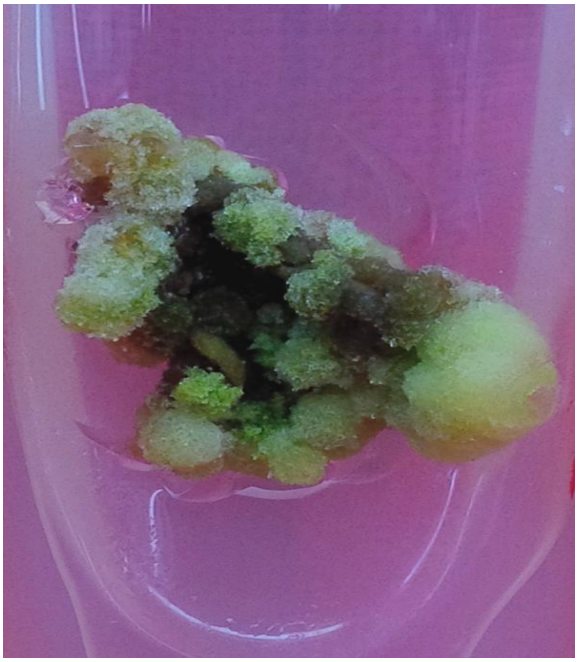

Fig 31: Formation of callus from the cut ends on MS+2,4-D (4mg/l) + BAP (1mg/l) after 4 weeks

Fig 32: Mass of callus formed on MS+NAA (4mg/l) + BAP (1mg/l) after 4 weeks

Fig 33: Formation of whitish green callus on MS+NAA (2mg/l) + Kn (1mg/l) after 2-3 weeks

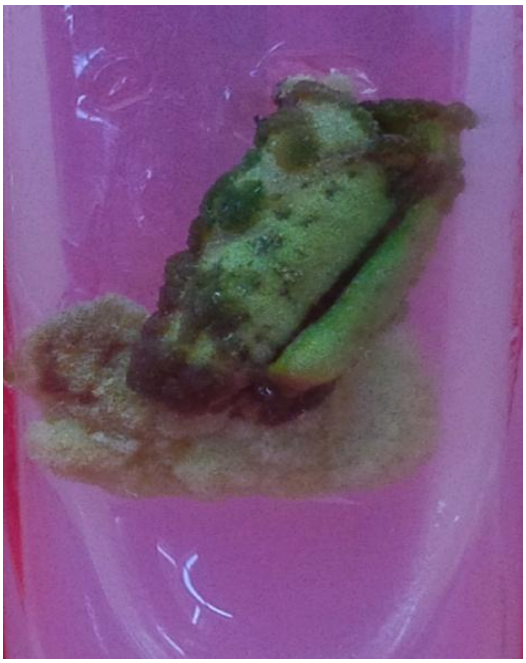

Fig 34: Small mass of callus formed on MS+NAA (4mg/l) + Kn (1mg/l) after 20-25 days

Study of callus

The callus was hard and thus had to be teased with needles to study its cell types. Cell types studied in various calli showed their heterogenous nature with wide variations in size and shapes like ovoid, spherical and elongated. Histogenetic differentiation in the form of tracheids was observed in the calli. Tracheids occurred singly or in groups and possessed reticulate thickenings on their walls (Fig.35).

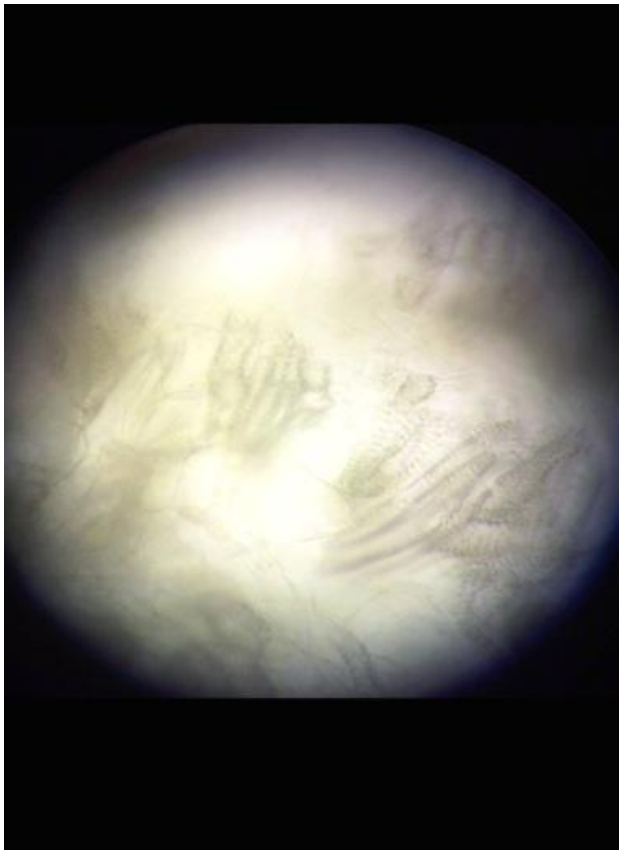

Fig 35: A group of Tracheids showing reticulate thickenings on their walls

No differentiation of root or shoots could be effected from the leaf or stem calli in any of the media tested.

Chapter 5

Discussion

DISCUSSION

The present investigation was carried out on an important ornamental plant *Antirrhinum majus* with an aim to develop an efficient, reliable and reproducible protocol for its clonal propagation under *in vitro* conditions.

Micropropagation through enhanced axillary branching is a reliable technique for clonal propagation as it enables to retain the clonal fidelity and prevents somaclonal variations in the culture unlike the callus tissue. In the present investigation, multiple shoot proliferation was achieved using shoot tip and nodal segment. The axillary shoot proliferation from the cultured explants was remarkably influenced by the type and concentration of the growth regulator used. For shoot proliferation growth regulators especially cytokinins are one of the most important factors affecting the response (Sterk *et.al.,* 2003, Mishra *et.al.,* 2005, George *et.al.,* 2008, Warrier *et.al.,* 2010). A wide range of cytokinins like Kn, BAP, Zeatin and Thidiazuron have been employed in shoot proliferation (Bhojwani and Razdan, 1982 Viji *et.al.,* 2010). However a wide survey of literature suggested that BAP is the most reliable and effective cytokinin for shoot proliferation. A number of plants such as *Dianthus* (Pareek *et.al.,* 2004), *Rosa indica* (Hameed *et.al.,* 2007), *Rosa canina* (Shirdel *et.al.,* 2013), *Gladiolus grandiflorus* (Haula *et.al.,* 2012), *Dianthus caryophyllus* (Brar *et.al.,* 1996; Daniel *et.al.,* 2009) have been successfully multiplied using BAP.

In the present investigation, axillary shoots were induced from the nodal segments and shoot apices on MS medium supplemented with BAP or Kn. For nodal explants best results were obtained on BAP (4 mg/l), where a maximum of 12-14 shoots were formed after 6 weeks of culturing.

Multiple shoot proliferation from shoot tips was achieved on MS medium supplemented with different combinations of BAP (0.5-4 mg/l) or Kn (0.5-4 mg/l). Highest shoot proliferation occurred on BAP (4 mg/l), where a maximum of 12-14 shoots were formed after 6 weeks of culturing and on Kn (2 mg/l), where 15-16 shoots were formed after 6 weeks of culturing. Our results are in agreement with other workers who have reported multiple shoot proliferation from shoot tips and nodal segments in *Antirrhinum majus* using MS medium supplemented with BAP (Atkinson *et.al.,* 1989). Earlier Newbury, 1986 used

nine varieties of *Antirrhinum majus* in a study of *in vitro* multiplication of plants using shoot tip culture. The reported works were done on the shoot tips taken from the seedlings raised from seeds under *in vitro* conditions where as in our case the results are achieved from shoot tips and nodal explants taken from of healthy, mature field grown plants. González-Benito *et.al.* (1996) also reported multiple shoot proliferation from nodal segments of *Antirrhinum majus* on MS medium augmented with BAP (1mg/l) but in combination with NAA (0.5 mg/l).

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. In the present study the *in vitro* regenerated shoots were carefully excised and transferred to basal MS medium alone or supplemented with various auxins for root initiation. Best root formation occurred on 2 mg/l NAA after 15-20 days where a bunch of well developed roots were formed at the base of stem. On the other hand, rooting was achieved in *Antirrhinum majus* when shoots were cultured in the absence of plant growth regulators as reported by Sangwan and Harada, 1975; Pfister Widholm, 1984; González- Benito *et.al.*, 1996.

Attempts are underway to acclimatize the plantlets and transfer them to the field conditions.

De novo adventitious shoot formation through direct regeneration is considered to be a reliable and safe method of micropropagation since in this method genetic uniformity is maintained among the progenies which are less prone to contamination because total number of stages of culturing are reduced significantly. New adventitious roots or shoots can develop directly from the explants like root, stem, petiole, leaf and floral parts. Choice of explants and hormone regime to which the explants are subjected to, are two important factors for their initiation.

The present material exhibited prolific growth of roots directly from leaf and stem segments. Root induction in leaves was observed on MS medium supplemented with NAA (4 mg/l). The roots were thin, long and white in color with profuse root hairs. In case of stem segments the root induction was observed on different concentrations of NAA (0.5-2 mg/l) with 2 mg/l NAA showing the best response where highest number of roots were formed per culture. Similarly Sangwan and Harada (1975) reported the development of abundant roots

from the stem segments of *Antirrhinum majus* seedlings on IAA or NAA supplemented medium. Atkinson *et.al.*(1991) reported *in vitro* adventitious root induction in *Antirrhinum majus* on the shoots formed from seeds. Our is perhaps the first report demonstrating adventitious root induction from the mature explants taken from *ex vitro* plants.

De novo shoot formation directly from leaf or stem segments was not observed on any of the medium tried in the present investigation. However Sangawn and Harada (1975) have reported the regeneration of whole plant from stem explants of seedlings through the interaction of IAA and Kn.

Majority of the plant tissues growing *in vitro* require exogenous hormones in the nutrient medium for dedifferentiation. The reaction of an isolated tissue to an auxin depends upon its endogenous auxin level at the time of its excision and its genetic capacity for its synthesis. In the present investigation, callusing of the leaf and stem segments occurred very rapidly after culturing on optimal media compositions. Best callus formation was observed on 2,4-D in combination with BAP or Kn. Our work is in conformity with the work reported earlier by other authors. Sangwan and Harada, 1975 reported callus formation from stem explants of seedlings in *Antirrhinum majus* on MS media supplemented with 2,4-D where soft friable callus was formed. Rao *et.al.* (1976) also reported callus formation in *Antirrhinum majus* using leaf and stem segments cultured on MS medium supplemented with 2,4-D (1mg/l). Pfister and Widholm (1984) reported the formation of callus from stem internode and seedling root on Sangwan and Harada's medium (1975) augmented with 0.25 mg/l Naphthoxyacetic acid.

The calli formed from stem and leaf segments on the same media were more or less identical in morphology. The calli were heterogenous being composed of ovoid, oblong, semicircular cells with aberrant shapes. According to steward *et.al.* (1963) "even in the most uniform environment, 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 was observed in all the calli. Tracheids occurred singly or in groups and possessed reticulate thickenings on their walls. Tracheids

were seen in the early stages of callus formation. Firstly only a 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.

Poirier-Hamon (1974) reported the formation of embryoids via callus of stem internode of *Antirrhinum majus* on media supplemented with 2,4- D (0.5-2 mg/l). Sangwan and Harada (1975) also reported the formation of embryogenic green callus from internode stem segments of *Antirrhinum majus* on Nitsch *et.al.* (1967) medium supplemented with 0.25-5 mg/l naphthoxyacetic acid or 0.5 mg/l 2,4-D.The shoot differentiation from root callus of *Antirrhinum majus* was reported at low auxin concentration i.e. 0.25 mg/l naphthoxyacetic acid or 0.4 mg/l 2,4-D by Pfister and Widholm, 1984.

In conclusion a reliable and reproducible protocol has been established for the multiplication of *Antirrhinum majus* through forced axillary branching. It has not been possible to induce direct or indirect shoot oragnogenesis from the explants and callus tissue. It is opined that cells in plant are undoubtedly totipotent but some hormonal and nutritional factor or their combination for differentiation could not be discovered by us during the stipulated period of the project.

Chapter 6

Literature Cited

LITERATURE CITED

Altvorst A.C., Koehorst H., Bruinsma T., Custers J., Jong J., Dons J. (1992). Adventitious shoot formation from *in vitro* leaf explants of carnation (*Dianthus caryophyllus*). Scientia horticulturae 51(3): 223-235.

Altvorst V., Koehorst H., Bruinsma T., Dons J. 1994, Improvement of adventitious shoot formation from Carnation leaf explants. Plant cell, Tissue and Organ Culture. 37: 87-90.

Atkinson J., Newbury H. J., Ford-Lloyd B. V. (1991). *In vitro* adventitious root induction in *Antirrhinum majus* L. Plant Cell. Tissue and Organ culture 27: 77-79

Bhojwani S. and Razdan M. (1982). Plant cell Tissue culture theory and practice. Elsevier Sci. Publ. Amsterdam, 1-520.

Brar M. (1996). Effect of Thidiazuron and Benzylaminopurine on *in vitro* shoot proliferation of carnation (*Dianthus caryophyllus* L.). 31(3, Part 2): 61A.

Casas J., Olmos E., Piqueras A. (2010). *In vitro* propagation of Carnation (*Dianthus caryophyllus* L.), Protocols for *in vitro* propagation of ornamental plants 589: 109-116.

Chen T., Tsung J., Chang W. (2004). Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum* orchids. Plant cell, Tissue and Organ Culture 76: 11-15.

Emek Y., Erdag B. (2007). *In vitro* propagation of *Gladiolus anatolicus* (boiss.) stapf, Pak.J. Bot., 39(1): 23-30.

European Commission, General for agriculture and rural development, 2006.

Frey and Janick (1991), Organogenesis in Carnation, J. Amer Soc. Hort. Sci. 116(6): 1108-1112.

Gonzalez-Benito M., Tapia J., Rodriguez N., Iriondo J. M. (1996). Micropropagation of Commercial and Wild Genotypes of Snapdragon (*Antirrhinum* spp.). The Journal of Horticultural Science & Biotechnology 71(1): 11-16.

Haouala and Salhi (2012). Axillary budding and rooting of Gladiolus (*Gladiolus grandiflorus* Hort.) in salt stress conditions, Afr. J. Hort. Sci. 6: 101-110.

Kantia A., Kothari S. (2002). High efficiency adventitious shoot bud formation and plant regeneration from leaf explants of *Dianthus chinensis* L. Scientia Horticulturae 96: 205-212.

Kanwar J.K., Kumar S. (2009). Influence of growth regulators and explants on shoot regeneration in carnation 4:140-146

Karami O., Deljou A., Kordestani G. (2007). Secondary somatic embryogenesis of Carnation (*Dianthus caryophyllus* L.). Plant Cell, Tissue and Organ culture 92: 273-280.

Kharrazi M., Nemati H., Tehranifar., Bagher A., Sharifi A. (2011). *In vitro* culture of Carnation (*Dianthus caryophyllus* L.) focusing on the problem of Vitrification, J. Biol. Environmental Sciences, 5(13): 1-6.

Mahoney (2014). Home Guides by demand media.

Mani T., Senthil K. (2011). Multiplication of *Chrysanthemum* through somatic embryogenesis. Asian J. Pharm. Tech. 1(1): 13-16.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiology 15: 473-497.

Muthukumaram K. (2009). Indian floriculture Industry: Opportunities and Challenges.

Muthukumaran K. (2008). Indian floriculture industry and challenges.

Newbury, H.J. (1986). Multiplication of *Antirrhinum Majus* L. by Shoot-tip Culture. Plant Cell, Tissue and Organ Culture 7: 39-42.

Poirier-Hamon, Rao P. S., Harada H. (1974). Culture of mesophyll protoplasts stem segments of *Antirrhinum majus* (snapdragon): growth and organization of embryoids. Journal of Experimental Botany 25(4): 752-760.

Ruud. L.M., Nico. S.P. (2010). Floriculture World Wide; production, trade and consumption patterns.

Saha A. (2005). Export Growth and Prospect of Floriculture in India.

Sangwan, Harada (1975). Chemical regulation of callus growth, organogenesis, plant regeneration and somatic embryogenesis in *Antirrhinum majus* tissue and cell culture. Environmental Horticulture, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida.

Sharma A., Agrawal V. (2012). Tissue culture aspects of ornamental plants**.** CIBTech Journal of Biotechnology.1: 40-48.

Shirdel M., Azar A., Matloobi M., Nahandi F. (2012). Effect of nodal position and growth regulators on *in vitro* growth of Dog Rose (*Rosa canina*). Journal of Ornamental and horticulture plants 2251-6433.

Singh A., Ahlawat T. (2014). Floriculture as an upcoming industry in India: Present scenario and future prospects. Indian Farmer 1(1): 28-32.

Sudhagar S. (2013). Production and Marketing of Cut flower (Rose and Gerbera) in Hosur Taluk. International Journal of Business and Management Invention 2(5): 15-25.

Tanaka K., Kanoo Y., Kudo S., Suzuki M., (2000). Somatic embyogenesis and plant regeneration in chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura), Plant Cell Reports 19: 946-953.

Udom N., Kanchanapoom K., Kamnoon K., (2009). Micropropagation from cultured nodal explants of rose (*Rosa hybrid*a L.). J. Sci. Technology 31(6): 583-586.

Vantu S. (2006). Organogenesis in *Chrysanthemum morifolium* Ramat (Cultivar "Romica") callus cultures 71-76.

Waseem K., Jilani M.S., Khan M.S.(2009). Rapid plant regeneration of chrysanthemum (*Chrysanthemum morifolium* L.) through shoot tip. African Journal of Biotechnology 8(9): 1871-1877.

Waseem K., Khant M., Jaskant Q., Jilani M., Sohail M. (2009). Effect of different auxins on the regeneration capability of *Chrysanthemum* leaf discs. International Journal of agriculture and biology. 11-4: 468-472.

Xing W., Bao M., Qin H., Ning G. (2010). Micropropagation of *Rosa rugosa* through axillary shoot proliferation, Acta Biologica Cracoviensa Series Botanica 52/2: 69-75.