

***IN VITRO* MUTATION STUDIES IN**
Syngonium podophyllum

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PHK 704

DIVISION OF HORTICULTURE
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***IN VITRO* MUTATION STUDIES IN
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*Affectionately Dedicated To
my Beloved Parents*

*Sister
&
Chairperson*

DIVISION OF HORTICULTURE
UNIVERSITY OF AGRICULTURAL SCIENCES
BENGALURU

CERTIFICATE

*This is to certify that the thesis entitled “**IN VITRO** MUTATION STUDIES IN **Syngonium podophyllum**” submitted in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (HORTICULTURE) in FLORICULTURE AND LANDSCAPE GARDENING** to the University of Agricultural Sciences, Bengaluru, is a record of research work carried out by **Mr. RAJESH, A.M, I.D. No. PHK 704** under my guidance and supervision and no part of the thesis has been submitted for the award of any degree, diploma, associate ship, fellowship or other similar titles.*

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ABSTRACT

An efficient and reliable micropropagation protocol was developed and promising variants were obtained in *Syngonium*, an important foliage plant, by *in vitro* mutagenesis. Shoot proliferation from basal shoot explants were maximum (7.4) in MS media supplemented with 10mg/l of BAP. Length of shoots decreased with increased concentration of BAP. Except for plantlets derived from 20mg/l of BAP, all other plantlets showed 100 per cent establishment on the field condition. The shoots obtained with 20mg/l of BAP, failed to root.

The mutagenic treatments of basal shoot explants of *Syngonium*, showed morphological variations. A general retardation in the shoot and root parameters of the regenerated plantlets was observed with ethyl methane sulphonate treatment. Greater variations were noticed in leaf morphology including reduction in size, length, curling of leaf. Few leaves turned albenic (reduction in chlorophyll) and this was more evident at higher level of EMS. Reduction in chlorophyll content and cultures turning albenic was observed when microshoots were treated with EMS. Lower concentration of EMS (0.5 per cent) did not induce variation in chlorophyll color, but with the increase in EMS concentration (1, 1.5 and 2 per cent) changes in chlorophyll color such as microshoots turning light green, albenic were seen. Stunted growth was observed when microshoots were treated with higher concentration of EMS.

The encouraging results obtained from the present investigation thus emphasize the efficiency of *in vitro* mutation induction for the improvement of this important foliage plant.

Signature of the Student
(Rajesh, A.M.)

Signature of the Major Advisor
(B.N. Sathyanarayana)

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Introduction



I. INTRODUCTION

Foliage plants, in common terminology, are called house plants. Foliage plants, defined literally, would include all plants grown for their attractive leaves rather than for flowers or fruits. In general horticultural term, however, foliage plants are those which are grown mainly for their attractive foliage that are able to survive and grow indoors. Thus, they are used as living plants for interior decoration or interior plantscaping.

Foliage plants from the world's tropical or subtropical regions provide the basis for today's foliage plant industry. From 1990 to 1997, *Syngonium*, *Aglaonema*, *Dieffenbachia*, *Dracaena*, *Epipremnum*, *Ficus*, *Peperomia*, *Philodendron*, *Sansevieria*, and plants from several genera of Palmae (palm) were the major players in the industry. *Philodendron* dominated all other genera accounting for 50 per cent and *Syngonium* 36 per cent of wholesale values in world market in 1990 and 1997, respectively (Smith and Strain 2000). Meanwhile, the wholesale value of foliage plants in the world market increased from \$13 million in 1949 to \$574 million in 2000 (USDA 2001). The use of foliage plants for interior decoration or interior plantscaping has become an integral part of contemporary design, playing an important role in our life (Manaker, 1997).

The genus *Syngonium* includes thirty three species, native to tropical South America (Mexico, Panama, Salvador, Guatemala, Brazil, Honduras) and Africa. *Syngonium podophyllum*. L., commonly known as Arrow Head Plant is one of the most well known and versatile foliage plant. It is grown wildly at South America and African evergreen forests. *Syngonium* is commonly used as hanging baskets. If upright growth is desired, trellis or other support has to be provided. Otherwise the plant can be used as ground cover. The plants can be used in various places

such as offices, hospitals, shops, windows, conference rooms, commercial premises and hotels. *Syngonium* belongs to the family Araceae. It is a perennial, evergreen, herbaceous vine. It is recognized for its juvenile leaves, which are simple and alternatively arranged.

Syngonium propagation is easily done via nodal cuttings, tip cuttings or air layering. The simplest method is to cut sections of the vine into single node or single eye and place it directly in rooting media in mist. But, large-scale propagation of *Syngonium* plants by vegetative mean is time consuming (Henny 1998). Hence, *In vitro* propagation has come as boom for nurserymen. Through tissue culture, large number of plants could be produced in a short duration. Tissue culture could also be employed to induce new variants, which are economically very lucrative.

In the present study an attempt has been made to study proliferation of shoots using auxins, callus induction and the feasibility of induction of variation through mutations. Hence, the present investigation was taken up with the following objectives.

1. Induction of multiple shoots using Cytokinin.
2. Callus induction using different concentrations of Cytokinins in combination with Auxins and Auxins alone.
3. Inducing variation in explants using chemical mutagens.

Review of Literature



II. REVIEW OF LITERATURE

With the tremendous advancements made in the field of biotechnology, the technique of tissue culture has assumed a greater importance, since this technique will be the corner stone of all new biotechnologies that are being developed. The use of tissue culture for large scale production of plants, improvement of crops and conservation of valuable germplasm has been well documented. Micro propagation is the most popular and widely commercialized tissue culture technique for large scale propagation of crops. Many techniques of crop improvement through tissue culture are in vogue.

In the past few years, spectacular developments have been made in plant tissue culture which has come out as an important tool in both basic and applied studies as well as in commercial applications. The technology owes its origin to the ideas of Haberlandt (1902), who conceived and enunciated the concept of totipotency *i.e.*, all living cells containing a normal complement of chromosomes should be capable of regenerating into an entire plant. He visualized growing of plant cells in an artificial media to rejuvenate quiescent and differentiated cells, to trigger them into division and growth, forming tissue and regenerating into a mature plant.

White (1934) demonstrated the idea of Haberlandt for the first time by the successful and indefinite culture of excised roots of tomato. Later, independent studies by Gautheret (1939) and others demonstrated that plant cells in culture could be proliferated continuously and that they also undergo differentiation.

Skoog and Miller (1957) demonstrated that differentiation of shoots and roots (organogenesis) in tobacco pith cultures depend on the relative concentration of Auxins and Cytokinins, thus introducing the concept of

hormonal control of organ formation. Their observations became the guiding principles of *in vitro* organogenesis.

Auxins and Cytokinins established the basis for manipulating organ initiation and provided the principle on which micropropagation depends. Other important developments include regeneration of somatic embryos from callus and cell suspension cultures (Reinert 1959 and Steward *et al.*, 1964), technique of protoplast isolation (Cocking, 1960) and artificial hybridization of plant protoplasts in aseptic culture (Carlson *et al.*, 1972).

Regeneration of plants through organogenesis (Vasil and Hilderbrandt, 1965) and *via* embryogenesis (Steward *et al.*, 1966 and Kohlenbach, 1966) has become an adjunct and indispensable requisite for large scale propagation and genetic manipulation of several plant species. Several genera and species encompassing the gamut of horticulturally important plants are now being micropropagated in millions through these techniques. Of late, plant tissue culture techniques have been increasingly applied as a tool for the improvement of several plants besides resulting in their rapid multiplication. *In vitro* mutation breeding is considered as a valid tool for the improvement of vegetatively propagated plants especially, when one or a few characters of an outstanding cultivar have to be modified (Broertjes and Van Harten, 1988).

2.1. Tissue Culture and Micropropagation

a. Selection of explant

Explant is a piece of tissue or organ, removed from the plant for the purpose of culture. Success in culturing the explants influenced by number of factors inherent to the explant, including the genotype of the explant, the size, its physiological age and the tissue or organ source of

the explant. In general very small explants have low survival rate in both tissue and callus culture. Smaller explants are more likely to form callus, while larger explants maintain greater morphogenetic potential as in gladiolus (Bajaj *et al.*, 1982).

For most micropropagation work, the explant of choice is an apical or axillary bud (Debergh and Read, 1993). It is always more risky to start a micropropagation scheme with the *de novo* formation of buds as the chances of obtaining off-types are more. The use of *de novo* bud formation normally cannot be used for the micropropagation of chimeral plants, since segregation will occur (Debergh and Maene, 1989).

Different explants of *Syngonium podophyllum* Schott var. Imperial White and *Scindapsus aureus* two Araceae species with variegated leaves, showed organogenetic potential on Murashige-Skoog medium containing plant growth factors (PGFs). Initial cultures of *S. podophyllum* were performed with apical and axillary meristems. All buds could produce single or multiple shoots depending on the PGF composition. The behavior in repeated subcultures (up to 4th generation) was similar to that of initial cultures. Initial cultures of *S. aureus* were performed with apical and nodal meristems, leaves and segments. All buds produced single shoots. Leaf explants produced multiple shoots independently of PGF content. In repeated subcultures (up to 4th generation), the behavior was characterized by multiple shoots (Scaramuzzi *et al.*, 1992).

In *Syngonium*, shoot tips, axillary buds or nodes were used as explants and the effects of supplementing MS media with 22.2 μM BA, 23.2 μM kinetin or 24.5 μM 2iP [isopentenyladenine] were investigated. Axillary shoot production per explant was greatest from axillary buds grown on medium containing 2iP. All explant types developed basal tissue in culture from which shoots and roots emerged. Production of this tissue was greatest from axillary buds on medium containing BA (Kozak and Dabski, 1993).

The morphogenetic potential of node, internode and leaf explants of brahmi was investigated by Tiwari *et al.*, (1998 and 2001) to develop reliable protocols for shoot regeneration. They observed that the whole leaf explants gave the largest number of shoot buds followed by node and internode explants.

Stem, leaf and flower bud explants, cultured on MS medium have been reported to form callus, which could give rise to multiple shoots with roots (Tejavathi and Shailaja, 1999). Singh *et al.* (1999) obtained multiple shoots from shoot-tip explants of 1-2 cm derived from field grown plants of *B. monnieri* in MS media.

Srivastava and Rajani (1999) observed adventitious shoot bud induction from leaf and stem explants of brahmi. They reported that the source of explants could influence shoot induction and eventual shoot growth. The best response was obtained in leaf explants taken from *in vitro* grown shoot cultures.

Shoot regeneration response varied with the type of explant. Nodal segments was best compared to shoot tips for multiple shoots and leaf pieces were more responsive to callusing and produced calli of better quality than from the internodal segments in brahmi (Dalia, 2004).

b. Surface sterilization

The contamination of cultures is the most common problem encountered in plant tissue culture techniques. The problem is much prevalent especially, when the inoculum or explant used is not properly disinfected before use. Chemical sterilization aims to eradicate microorganisms. Alcohol, bleach, calcium hypochlorite, sodium hypochlorite, mercuric chloride etc. are the common chemical surface sterilants used. The type of sterilants used, concentration and the time for which treatment is carried out vary with nature of explants and

species. Hence, protocols have to be standardized for each explant and species.

Microbial contamination is considered to be one of the most important reasons for losses during plant propagation. It has been hypothesized that the detrimental effects of contamination may be seen only when the contaminant microorganisms exceed a certain concentration. This was tested by multiplication of contaminated *Syngonium* clusters in an airlift culture vessel containing multiplication medium (Murashige-Skoog medium basal medium with 3 per cent sucrose, 2mg/l kinetin and 1 mg/l Ancymidol) being circulated through a sterilizing filter, at 100 rpm, with a 16 hr photoperiod. After 30 days, the culture in filtered medium produced 19.5 shoot initials/g fresh weight inoculum, compared to 8.7 in unfiltered medium. Clusters grown for 3 subcultures without medium filtration lost their multiplication ability, while clusters grown for 3 subcultures in culture with filtration continued to show 2 to 3 fold increase in fresh weight and shoot production (Leven *et al.*, 1996).

In *Dracaena fragrans*, the results showed that the highest mean value of healthy free of contamination explants was obtained by using 15 per cent (v/v) Clorox and from using Mercuric chloride 0.3g/l. Using Ascorbic acid (100mg/l) inhibited markedly the browning of explants and increased plant length and number of leaves (Badawy *et al.*, 2005).

The explants of *Spathyphyllum* shown better establishment when they were treated with 70 per cent ethanol and then soaked in 10 per cent (v/v) NaOCl solution with few drops of Tween 20 solution for 10 min, followed by three times of sterile distilled water (Meltem *et al.*, 2005).

In *Dieffenbachia*, explants which were treated with 70 per cent alcohol for two minutes and later in 0.1 per cent Mercuric chloride for 20 minutes had shown higher rate of establishment (Taraway *et al.*, 2006).

c. Tissue culture media

The success of plant tissue culture as a means of plant propagation is greatly influenced by the nature of the culture medium used. Plant tissue culture media provides major and minor elements and carbohydrates. Improved results are obtained by providing trace amounts of certain organic compounds, notably, vitamins, amino acids and plant growth regulators (George and Sherrington, 1984).

The composition of macroelements derived by Murashige and Skoog (1962) along with certain microelements and organic constituents for the culture of tobacco callus is found satisfactory for the culture of many plant species. This is true in the case of *Syngonium* also.

d. Direct organogenesis

Internodal segments of *S. podophyllum*, collected from plants grown in greenhouses, were inoculated onto MS medium containing BAP [benzyladenine purines] and kinetin each at 0.5-2.0 mg/litre concentrations. Rooting of microshoot was induced on MS medium containing Auxins, i.e. IBA, NAA and IAA each at 1.0, 2.0 and 3.0 mg/litre. Rooted plantlets were grown in the medium containing equal amounts of coco-peat and sand. After one month of inoculation, small green protuberances developed from the surface of the explants in the media fortified with BAP at 2.0 mg/litre and kinetin at 1.0 mg/litre. Within 35-40 days, these directly developed into shoots, which were subcultured on the same medium for further growth and development. Callus initiation could be noticed from the cut ends in the medium containing BAP at 2.0 mg/litre and kinetin at 0.5 and 2.0 mg/litre.

Subculturing onto the same medium did not promote callus growth. The elongated shoots were transferred to MS medium containing different Auxins. Among the different Auxins, IBA at 1.0-2.0 mg/litre was superior for root induction. Callus formation was observed at the base when NAA was incorporated in the medium. Morphological characters of the roots produced also differed with the Auxins used. The time taken for root initiation was also less in full strength MS medium. Rooted plantlets when transferred from culture tubes to plastic pots containing equal amount of coco-peat and sand recorded maximum survival percentage (70 per cent). Soilrite+sand recorded 65 per cent survival while cow dung+sand and soil+sand recorded 40 and 50 per cent survival (Rajeevan *et al.*, 2002).

Lateral buds of *S. podophyllum* were subjected to primary asepsis in the laboratory followed by a secondary asepsis in a laminar flow bench before culturing in MS medium supplemented with IAA and 6-benzyladenine (BA) during the establishment stage. During the development stage, the explants were subcultured in MS medium supplemented with different BA concentrations. Seedling growth was promoted with the addition of IAA and BA in the MS medium during the establishment stage. The addition of BA during the development of the explants had no significant effects on the number of buds per explant (Schwertner and Zaffari, 2003).

Although all nodal segments of *Syngonium podophyllum* irrespective of their age and different concentrations of benzylaminopurine [benzyladenine] (BAP) showed axillary shoot growth, young nodes cultured on Murashige and Skoog medium (MS) supplemented with 5 μ M BAP gave the best results. Elongated shoots were rooted on half strength MS medium supplemented with 5 μ M 3-indolebutyric acid (IBA). Increase of the frequency of axillary bud

sprouting and decrease in the frequency of root formation with the increase of light intensity were recorded which was accompanied with increase of relative peroxidase activity. Instead of peat, soil consisting of sawdust, sand and clay (1:1:1) was found to be a suitable combination producing valuable plant survival (Hassanein, 2004).

A simple and effective method of regenerating *Syngonium podophyllum* 'Variegatum' via direct somatic embryogenesis has been established. Leaf and petiole explants were cultured on Murashige and Skoog (MS) medium supplemented with N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) or Thidiazuron (TDZ) with either alpha-naphthalene acetic acid (NAA) or 2,4- dichlorophenoxyacetic acid (2,4-D). Somatic embryos directly formed at one or two sides of petiole explants on MS medium supplemented 2.5 mg/l TDZ with 0.5 mg/l NAA or 2.0 mg/l TDZ with 0.2 mg/ l NAA or with 0.2 and 0.5 mg/l 2,4-D, respectively. The frequency of petiole explants with somatic embryos produced was as high as 86% when cultured on medium containing 2.5 mg l(-1) TDZ with 0.5 mg l(-1) NAA. Up to 85 per cent of somatic embryos were able to germinate after transferring onto medium containing 2.0 mg /l 6-benzylaminopurine (BA) and 0.2 mg l(-1) NAA. Approximately 50-150 plantlets were regenerated from a single petiole explant. However, there was no somatic embryo formation from leaf explants regardless of growth regulator combinations used. Regenerated plantlets from petiole explants were stable and grew vigorously after transplanting to a soilless container substrate in a shaded greenhouse (Zhang *et al.*, 2006).

Tissue culture of *Spathiphyllum* sp. Petite and *Syngonium* sp. White Butterfly were compared on agar-solidified culture medium and interfacial membrane rafts floating on liquid medium. Both media were based on Murashige-Skoog medium and all cultures were performed at 22-26 days, under a 16 hr photoperiod with a photon flux density of 60

umol/sq m.sec provided by cool white fluorescent lamps. After 25 days culture, the best shoot multiplication and fresh biomass gain were achieved by plant material grown on rafts. Shoot multiplication of *Spathiphyllum* and *Syngonium* spp. on membrane rafts reached a maximum at day 25, while the fresh biomass increased through the culture period (40 days). The number of shoots of *Spathiphyllum* and *Syngonium* spp. grown on membrane rafts remained constant between 25 and 40 days of culture. The plants grown on membrane rafts also developed more roots (Watad *et al.*, 1997).

The average number and average fresh weight of *Spathiphyllum floribundum* cv. Petite axillary shoots grown on MS medium with three concentrations of BA (1, 2 and 5 mg /l) in the absence of Auxins or in combination with 0.05 mg /l NAA, 0.5 and 1.0 mg /l IAA or 0.005, 0.05 and 0.1 mg /l 2,4-D were determined. In general, higher shoot number and fresh weight values were achieved in all tested levels of BA combined with 0.05 mg /l NAA than in combination with IAA or 2,4-D. The best response in terms of average shoot number (5.8), average fresh weight (116 mg/explant), leaf number/plant (14.7), lateral shoot number/plant (5.3), plant diameter (14.2 cm), and plant height (13.0 cm) was observed in shoots cultured on medium containing 0.05 mg/l NAA+2 mg/l BA. The shoots produced in this treatment grew vigorously when the plants were acclimatized under greenhouse conditions (Rafel *et al.*, 2001).

The *Spathiphyllum* shoots were sub cultured in MS medium supplemented with either of BA or PBA. At a continuous multiplication of *Spathiphyllum* on 1 mg/l of BA media, the multiplication rate began to increase starting from the first subculture and reached its maximum at the fourth subculture with 8.49 shoots per plant (Meltem *et al.*, 2005).

The proliferation responses in *Spathiphyllum cannifolium* were significantly influenced by the Cytokinin type and concentrations.

Supplementation of the medium with benzyladenine (BA; 4.44 to 13.32 μ M) increased the shoot proliferation rate significantly. When Cytokinins were used with Auxin (indole-3-butyric acid, IBA and naphthalene acetic acid, NAA), the number of shoots per explant increased in comparison with treatments with BA alone (Dewir *et al.*, 2006).

In *Dracaena marginata* cv. "Tricolour", shoot cultures were initiated from shoot tip on MS medium supplemented with 0.5 mg/l BA plus 2.0 mg/l IBA. Different types of Cytokinins (kinetin, BA and 2iP) were added to MS medium in concentration of 4.0 mg/l. The result pointed out that shoot development with no root formation was achieved. Largest numbers of shoots/ explant were obtained when stem cuttings were cultured on MS medium supplemented with 4.0 mg/l Of BA compared with the others (2iP and Kinetin). The highest percentage of root formation (80 per cent) was obtained by the addition of NAA (2 mg/l) compared with IBA and IAA (50 per cent and 40 per cent respectively) (Sawy *et al.*, 2000).

In *Dracaena fragrans*, the longest shoots and the greatest number of leaves were obtained by addition of 1.0 mg/l IAA + 1.0 mg/l Kinetin, while the highest number of shoots was recorded by using 0.5 mg/l IAA + 5.0 mg/l Kinetin. Using MS at half strength increased shoot length and number of leaves, while the number of shoots had not been affected by MS strength. The highest shoot length and number of leaves were obtained from addition of 1.0 mg/l IAA + 1.0 mg/l Kinetin to half strength MS medium, while the highest number of shoots was recorded from using 5.0mg/l IAA + 0.5 mg/l Kinetin to MS medium at half MS salt strength. The highest number and length of roots were obtained from addition of 5.0 mg/l IBA and using MS medium free of NAA (control) (Badawy *et al.*, 2005).

In *Dieffenbachia*, shoot proliferation and induction of adventitious shoots were recorded maximum in MS media supplemented with 3 mg/l of BA. Maximum rooting was recorded on half strength MS media supplemented with 0.5 mg/l of NAA (Zhang *et al.*, 1999).

Dieffenbachia explants were cultured on MS medium supplemented with 0.0, 2.0, 4.0, 6.0 and 8.0 mg/l of BA combined with 0.0, 0.1, 0.5 and 1.0 mg/l of NAA. The highest numbers of shoots (21/jar) were obtained with 8 mg/l of BAP. Explants cultured on MS medium supplemented with 0.5 mg/l of NAA gave the tallest shoot (Taraway *et al.*, 2006).

Anthurium explants were cultured on BM medium, or half strength MS medium with 3 to 20 mg/l BAP. MS medium supplemented with 20 mg/l BAP was the best medium for shoot induction, with the half strength MS medium containing 8 mg/l of BAP being most suitable for shoot development (Jambor *et al.*, 1990).

Spadix explants of *Anthurium* had a better capacity for regeneration than leaf segments on the modified Nitsch media used, and the plantlets derived from Spadix segments were less variable. It was unclear whether the variation in the plants raised was epigenetic or genetic, but it was generally less marked than the variation usual in seed propagated material (Prakash and Pierik, 1991).

In *Anthurium andreaum* cv Agnihotri. Result showed that MS basal medium supplemented with BAP (0.8 mg/l), Vit.B₅ (0.5 mg/l), IAA (0.1 mg/l), PVP (200 mg/l) and Coconut water (150 mg/l) was found best for multiple shoot production (4.66 shoots/ explant). The highest rooting percentage (80 per cent) was observed in MS basal medium supplemented with IAA (1.0 mg/l). A survival rate of 60 per cent was obtained when *in vitro* raised plantlets were transferred in *in vitro*

condition in plastic pots containing soilrite- perlite (10:1) mixture (Mahanta and Paswan, 2001).

To establish an efficient regeneration system for *Anthurium andreanum* cv Rubrun, seeds from plant spadixes were germinated on a medium supplemented with 2.2 mM BA. After 2 weeks, 74 per cent of the seeds germinated and four weeks later, micro-cuttings from these plantlets were sub cultured on a medium containing 4.4 mM BA and 0.05 mM NAA. On an average, 3.6 shoots per explant were obtained (Teresa *et al.*, 2004).

In vitro maintained plantlets of Anthurium were cultured on MS medium with 2 mg/l of IBA and 6 mg/l of Kinetin. Among the different levels of kinetin tried, maximum shootlets induction, mean number of shootlets and mean shoot length was observed at 4 mg/l of kinetin. The root development was better on half strength MS without IBA and IBA at 0.25 mg/l. The complete plantlets were obtained with a survival rate of 80 per cent on the sterilized hardening medium (Dhananjaya and Sulladmath, 2006).

In *Phyllodendron oxycardium*, Media supplemented with 2 mg/l BA and 1mg/l IAA significantly increased the number and length of shoots. Meanwhile, the media supplemented with 3 mg/l IAA significantly increased the root number and length in comparison with other treatments (Koriesh and Manie, 2000).

Five species of Araceae plants were successfully cultured via *in vitro* methods. Multiple shoots were induced from four Araceae species, *Alocasia sanderina*, *Colocasia antiquorum*, *Gonatanthus pumilus* and *Syngonium podophyllum.*, when the axillary buds were cultured in Murashige and Skoog medium (MS) supplemented with 2.0 mg/l N⁶-benzyladenine (BA) and 0.5 mg/l indole-3-butyric acid (IBA). Basic MS

medium supplemented with only 2.0 mg/l BA was found to be the most suitable medium for induction of multiple shoots in *Caladium hortulanum* and *Caladium bicolor* 'Florida Crown'. However, the same medium did not induce multiple shoot formation for *Caladium humboldtii*. The *in vitro* shoot microcuttings of these five Araceae species produced roots when cultured in basic MS medium to form complete plantlets. All the rooted *in vitro* plantlets (100 per cent) survived when transferred to the outside environment after acclimatization for two weeks in an incubator at 25°C (Chan *et al.*, 2008).

e. Indirect regeneration

Plantlet regeneration of *Dieffenbachia maculata* cv. Marianna was carried out using stem segments from *in vitro* culture. Different concentration and combination of indole-3-acetic acid (IAA), 1-naphthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2, 4-D), 6-benzyl adenine (BA) and kinetin (Kin) used for callus induction and shoot organogenesis. The frequency of callus formation reached 87 per cent and highest number of shoots per callus was 4.8 per explants cultured on MS medium supplemented with 15 mg/l of BA and 15 mg/l of NAA. Shoots were further grown and rooted on MS free hormone medium. Regenerated plantlets were successfully acclimatized in green house with 100 per cent survival rate (Singh *et al.*, 2007).

A novel protocol for indirect shoot organogenesis of *Dieffenbachia* cv. Camouflage was established using leaf explants excised from *in vitro* shoot cultures. The frequency of callus formation reached 96 per cent for explants cultured on MS basal medium supplemented with 5 µM thidiazuron and µM 2, 4- dichlorophenozy acetic acid. The number of shoots regenerated was high, with up to 7.9 shoots produced per callus cultured on basal medium supplemented with 40 µM N6-(D2-isopentenyl) adenine and 2 µM indole-3-acetic acid. Regenerated shoots

rooted well in a soilless substrate, acclimatized *ex vitro* at 100 per cent, and grew vigorously under shaded greenhouse conditions (Xiuli *et al.*, 2007).

Spathiphyllum inflorescence explants were cultured on MS medium supplemented with 10 mg/l benzyladenine(BA) and 10mg/1 NAA for callus induction and shoot formation. Shoots were transferred to MS medium supplemented with 2 mg/1 of BA and 0.5 mg/1 of NAA for further development. Rooting was obtained on MS medium with or without 0.5 mg/1 of IBA (Vargas and Garcia, 2007).

In *Anthurium andreaenum*, callus was induced using leaf and petiole explants on a 1/2-strength MS medium containing 0.90 $\mu\text{mol L}^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) and 8.88 $\mu\text{mol L}^{-1}$ N6-benzyladenine (BA). The callus exhibited complete hormone autonomy for growth and differentiation of PLBs. This callus proliferated well and was maintained by subculturing on half strength MS medium containing 0.90 $\mu\text{mol L}^{-1}$ 2, 4-D and 4.44 $\mu\text{mol L}^{-1}$ BA. On an average, 8 protocorm-like bodies could be obtained from a piece of 4 mm callus after being transferred to the half strength MS medium with 4.44 $\mu\text{mol L}^{-1}$ BA after 8 week of culture. The regenerated PLBs formed shoots and roots on half strength MS medium. After 24 week of culture on these medium, well-developed plantlets for potting were produced (Ling *et al.*, 2009).

2.2. Induced mutations

Most of the available genetic variation used in breeding programmes has occurred naturally and exists in germplasm collections of new and old cultivars, land races and other genotypes. This variation through crosses is recombined to produce new and desired gene combinations. When existing germplasm fails to provide the desired recombinant, it is necessary to resort to other sources of variation. Since

spontaneous mutations occur with extremely low frequencies, mutation induction techniques provide tools for the rapid creation and increase in variability in crop species. According to Brock (1977), induced genetic variations represent a more efficient source of genetic variability than gene pools conserved by nature.

Although the use of induced mutations in plant breeding is relatively simple in principle, it took about 30 years to develop this technique to a stage where it could be applied effectively and with a reasonable chance of success.

Induced mutations have contributed significantly to crop improvement worldwide and in some cases, have made an outstanding impact on the productivity of particular crops (Broertjes and van Harten, 1988; Konzak, 1984; Micke, 1991 and Rutger, 1992).

Spontaneous mutations are responsible for a large part of the recent variation used to breed vegetatively propagated crops. Induced mutations consequently have a high potential for bringing about further genetic improvements. The main advantage of mutation induction in vegetatively propagated plants is the ability to change one or a few characters of an outstanding cultivar without altering the remaining genotype (Broertjes, 1972). Through vegetative propagation, the mutant individual can form the commercial clone. It is possible to make use of many mutations that could not manifest themselves, in a generation derived from seed propagation. Vegetatively propagated crops are usually heterozygous and a mutagenic treatment may uncover recessive alleles by mutating or deleting corresponding dominant alleles (Broertjes and Van Harten, 1978).

The significance of artificially induced mutations in cultivated plants has been recognized since the last several years by many plant

breeders world over. Today, mutation breeding is one of the accepted methods available to the breeder to improve his crop and this is especially true in the case of vegetatively propagated crops as it alters only one or a few traits, but saves the overall characteristics of an already superior cultivar.

2.3 *In vitro* mutagenesis

It was not until the 1930's that several prior decades of research culminated in the successful propagation of plant organs and tissues in culture. Thereafter, progress in plant tissue culture was rapid. The improvement in the techniques for *in vitro* tissue and cell culture in the last several years, which allows for the recovery of whole plants from many cultures, has opened the possibility of using this experimental system in the production of variant or mutant tissue and cell lines, and subsequently, whole organisms. The development of efficient *in vitro* culture methods has facilitated the use of mutation techniques for the improvement of both seed and vegetatively propagated crops. In many vegetatively propagated crops, mutation induction in combination with *in vitro* culture techniques may be the only effective method for plant improvement (Novak, 1991). Investigative mutation induction experiment to establish treatment parameters utilizing *in vitro* produced tissues have been reported by several researchers (De Guzman *et al.*, 1980; Omar *et al.*, 1989 and Novak *et al.*, 1990).

In vitro produced tissues overcome the problems of material availability, reproducibility and poor uptake of chemical mutagens. In addition, because of the rapid formation of axillary shoots under *in vitro* conditions, periclinal and homohistont structures can be obtained more rapidly than under *in vivo* conditions. Micropropagation by axillary bud stimulation for sufficient cycles following mutagenic treatments could allow the isolation of somatic mutants with higher frequency and sparing

of space and time compared to traditional somatic mutagenesis (Donini and Micke, 1984). Moreover, induction of mutations *in vitro* can also increase the production of isogenic lines from mutated sectors, single cells or even protoplasts (Kameya, 1975).

In vitro techniques are also becoming more and more important for use in mutation breeding to prevent or restrict chimera formation (Broertjes *et al.*, 1976). Plants or shoots originating from adventitious buds are derived from one or a few totipotent cells (Broertjes and van Harten, 1978). Following mutagen applications, some of the new buds become chimeric with mutated and non-mutated sectors. Separation of mutated sector from the non-mutated sector can be achieved through a number of cycles of micropropagation to obtain the mutated sector alone (Maluszynski *et al.*, 1995).

2.3.1 Mutagens

For the induction of mutational events in plant material, the mutation breeder can choose between two groups of mutagenic agents namely, physical (X-rays, Gamma rays etc.) and chemical (Ethyl methane sulphonate, Methyl methane sulphonate, Sodium azide etc.). The former have been used for many decades whereas, the use of chemicals started in 1940's.

As a result of intensive work done during the last six decades, a number of physical and chemical agents that have mutagenic properties have been identified. They bring about changes in the nucleotide structure by altering the hydrogen bonding in the genetic material.

2.3.2. Chemical mutagens

Chemical mutagens induce different kinds of mutations in the genome of the plant. These chemicals react with the bases of the DNA

and causes chromosome breaks and/or point mutations. They have been divided into different groups based on their structure and mode of action. The most used chemical mutagens are the alkylating agents. These mutagens react with DNA by alkylating the phosphate groups and the purine and pyrimidine bases. The most preferred alkylating agent is ethyl methane sulphonate (EMS).

The mutagenic effect of EMS was first demonstrated by Loveless (1958). Freese (1963) classified EMS as a monofunctional alkylating agent with an active ethyl group. EMS produces point mutations with very little damage to the chromosomes (Amano and Smith, 1965).

Chemical mutagens have been successfully employed for *in vitro* mutagenesis in several horticultural crops like garlic (Malpathak and David, 1990), apple (Fu *et al.*, 1995), banana (Omar *et al.*, 1989) carnation (Singh *et al.*, 2000) and brahmi (Dalia, 2004).

However, chemical mutagens are not usually considered for inducing mutations in vegetatively propagated plants. The lack of success is probably a consequence of poor uptake and penetration of the chemical compound (Bowen, 1965; Moes, 1966 and Nybom, 1961).

In general, most mutation breeders prefer ionizing radiations because of its easy applicability, good penetration, reproducibility and high mutation frequency (Broertjes and van Harten, 1978).

2.3.2. Method of treatment

2.3.3. Chemical mutagen treatment

Chemical mutagens when used can be added either to the autoclaved medium in defined amounts to give the final desired concentration or the explants can be agitated in an aqueous solution of the mutagen (Singh *et al.*, 2000). When incorporated into the medium,

the mutagens have to be filter sterilized and added, since their half-life gets reduced at higher temperatures of autoclaving. All the mutagens mentioned may be diluted in water or in a fraction of the culture medium before addition to the autoclaved medium. After the treatment, the medium must be replaced or the explants must be rinsed and cultured in mutagen free medium.

Shoot-tips excised from banana were dipped into aqueous solutions containing different concentrations of filter-sterilized EMS for three hours at a constant temperature (Omar *et al.*, 1989). The shoot tips were then thoroughly washed with sterile water and cultured on a nutrient media. A similar method was followed by Fu *et al.* (1995) in the case of leaf pieces and shoot segments of apple.

Singh *et al.* (2000) subjected axillary bud explants of carnation cv. Espana to mutagenic treatment with EMS through media supplementation and explant agitation. For media supplementation, filter-sterilized, freshly prepared solution of EMS was added to half-strength MS medium and the surface sterilized explants were inoculated on this media. For explant agitation, surface sterilized explants were agitated with different concentrations of EMS and cultured on the same establishment medium devoid of mutagen.

2.3.4 Mutagenic sensitivity

Before the actual mutagen treatment, the tissue is subjected to sensitivity trials to find out an effective dose level. According to Sparrow *et al.* (1963 and 1967), mutagenic sensitivity depends mainly on the nuclear volume (greater the DNA content, the more sensitive), the number of chromosomes (plants with fewer chromosomes, given a certain nuclear volume, are more sensitive than plants with more, smaller chromosomes) and the ploidy level (the higher it is, the less sensitive). At

the same time, the genetic factors, climatic and other environmental conditions before and after treatment of the plant part as well as the stage of development of shoot or root also seem to be of importance.

2.3.4.1 Choice of doses

For chemical mutagens, dose is a combination of the concentration of the chemical and the duration of treatment. The dose of the mutagen to be applied depends on the mutagenic sensitivity of the species in general and that of the plant part and the stage of development in particular. The best practice is to expose the starting material to a range of doses, centered around the optimum one, either that found in the literature or a calculated optimum (Sparrow *et al.*, 1967).

Mutation frequency generally increases with increasing dose, but survival and the capacity to regenerate decrease with increasing dose. The dosage to be used must be selected after preliminary experiments to characterize the responses produced by a given kind of mutagen. Cultures or plant material to be cultured must be subjected to different doses or concentration of mutagens, which will define the lethal and sub-lethal conditions and those, which have no effects on growth rates. The dosage used in general, must allow for a survival of 40 to 60 per cent of the population in comparison to the untreated cultures (Handro, 1981).

2.3.4.2 Time of treatment

Apart from the dose of mutagen, mutagen effect is also dependent on the time of treatment, especially true in the case of chemical mutagens. Fu *et al.* (1995) treated leaf pieces (0.5cm²) of apple cv. Freedom with EMS at different concentrations for 1.5h and 3.0h. The results showed that the survival percentage of the explants decreased with prolongation of the treatment. At a concentration of 0.2 per cent EMS, the survival percentage of the leaves was reduced from 88.9 per

cent to 55.8 per cent when the time prolonged from 40 to 80 minutes. They concluded that the LD₅₀ in this case was 0.2 per cent EMS for 80 minutes.

2.3.5 Mutation frequency

The main objective in any mutation breeding programme is increased mutation frequency and spectrum. According to Walther (1969), a mutation breeding programme should aim to get the greatest number of geneticall changes per 100 treated propagules. Most authors express mutation frequencies for vegetatively propagated plants as the percentage of plants showing one or more mutations for different visible characters.

Chlorophyll mutation frequencies were used as mutagenic parameters by several workers (Reddy and Annadurai, 1991; Reddy, 1992 and Zareen and Devi, 1995).

2.3.6 Mutagen effects

Mutations caused by chemical mutagens may manifest itself in morphological, cytological or biochemical effects.

2.3.6.1 Morphological changes

Morphological changes can often be measured on an organ or consist of a reaction of the whole organism (Sparrow, 1961).

In vitro derived shoots of apple were cultured on MS medium added with filter-sterilized EMS (0.2 per cent) for three hours followed by medium devoid of the mutagen (Fu *et al.*, 1995). They obtained variants with forked leaves and opposite leaves.

2.3.7 Applications of *in vitro* mutagenesis in horticultural crops

Flower colour variants have also been obtained in other crops like begonia (Roest *et al.*, 1981) and chrysanthemum (Broertjes *et al.*, 1976).

Malpathak and David (1990) observed that treatment of callus cultures of garlic with different concentrations of EMS produced a stimulation of flavor formation at lower as well as at higher doses. Following mutagenic treatment, a part of the callus was induced to produce shoots, while the other part was cultured on callusing medium. It was found that in EMS treated calli, at the lowest dose (0.025 per cent), there was a significant rise in allin content by about four fold, while in the differentiated plantlets derived from EMS treated calli, there was an increase in flavour content by 1.5 to 2.0 times over the control at 0.05 per cent EMS concentration.

Singh *et al.* (2000) subjected axillary bud explants of carnation cv. Espana to EMS treatment. Highest doses of the mutagen were found to induce variations in flower colour. A red colour mutant with white stripes along the petal length was induced at 0.075 and 0.10 per cent EMS supplied through media supplementation. Another variant (pink with white stripes) was also isolated from the population treated with the doses 0.75 and 1.0 per cent EMS supplied through explant agitation.

Heat tolerant mutants were obtained in two commercial potato cultivars, 'Kufri Jyothi' and 'Kufri Chandramukhi' through *in vitro* mutagenesis of plantlets propagated *in vitro* (Das *et al.*, 2000).

Mutagenised cotyledon explants resulted in production of herbicide (atrazine) resistant plants in pepper (Venketiah *et al.*, 2005).

Material and Methods



III. MATERIAL AND METHODS

The present study on *In vitro* mutation studies in *Syngonium podophyllum* was conducted at the Plant Tissue Culture Laboratory of the Division of Horticulture, University of Agricultural Sciences, G.K.V.K. Campus, Bengaluru.

The details of the materials used and the methods adopted in the experiments are described here under.

3.1 Plant material

The plant material for the experiments was collected from Plant Tissue Culture Laboratory of the Division of Horticulture, University of Agricultural Sciences, G.K.V.K. Campus, Bengaluru.

3.2 Tissue culture media

A perusal of the available literature on micropropagation of *Syngonium* has shown that Murashige and Skoog (MS, 1962) is the most commonly used tissue culture media for *Syngonium*. Hence, MS medium was used throughout the investigations and the term 'media' used in the experiment refers MS media. The composition of MS medium is presented in Appendix I.

3.2.1 Preparation of stock solutions

3.2.1.1 Nutrient media stocks

Stock solutions of macronutrients, micronutrients and vitamins were prepared separately (Table 1). Stock solutions of macronutrients (MS-A) was prepared at a strength of ten times the final concentration (10×) required to make a liter of the nutrient media. Micronutrients and vitamins (MS-B, MS-C and MS-D) were prepared at strength of 100 times the final concentration (100×) required to make a liter of the nutrient

media. Each chemical was weighed using an electronic fine balance and dissolved separately in small quantities of double distilled water. The components of each stock were mixed and the final volume was made up using double distilled water. All the stock solutions, after preparation, were stored in proper plastic or glass bottles at 4 – 5°C. The stock - C, which was prepared separately, was stored in amber colored bottle as it is photo labile.

Table 1. Composition of stock solutions of Murashige and Skoog's medium (1962)

I. Stock Solution 1: MS-A (10×)

The following chemicals were dissolved in 1L of distilled water:

| | |
|--------------------------------------|--------|
| NH ₄ NO ₃ | 16.50g |
| KNO ₃ | 19.00g |
| CaCl ₂ .2H ₂ O | 4.40g |
| MgSO ₄ .7H ₂ O | 3.70g |
| KH ₂ PO ₄ | 1.70g |

II. Stock Solution 2: MS-B (100×)

The following chemicals were dissolved in 1L of distilled water:

| | |
|-----------------------------------------------------|---------|
| MnSO ₄ .4H ₂ O | 2.23g |
| ZnSO ₄ .4H ₂ O | 0.86g |
| H ₃ BO ₃ | 0.620g |
| KI | 0.083g |
| Na ₂ MoO ₄ .2H ₂ O | 0.0025g |
| CuSO ₄ .5H ₂ O | 0.0025g |
| CoCl ₂ .6H ₂ O | 0.0025g |

III. Stock Solution 3: MS-C (100×)

The following chemicals were added to 100ml of distilled water:

| | |
|----------------------------------------|--------|
| FeSO ₄ .7H ₂ O | 0.278g |
| Na ₂ EDTA.2H ₂ O | 0.373g |

IV. Stock Solution 4: MS-D (100X)

The following vitamins were dissolved in 100ml of distilled water:

| | |
|----------------|--------|
| Glycine | 0.020g |
| Nicotinic acid | 0.005g |
| Pyridoxine HCl | 0.005g |
| Myo-Inositol | 1.0g |

3.2.1.2 Growth regulator stocks

Stock solutions of Naphthalene acetic acid (NAA), Dichloro phenoxyacetic acid (2,4-D) and 6-Benzylaminopurine (BAP), were prepared by dissolving 10mg of the growth regulator in a few drops of absolute alcohol (ethanol) and the volume was made up to 100ml with double distilled water. The prepared stock solutions of growth regulators were stored in the refrigerator at 4 - 5°C.

3.2.2 Carbon source

Market grade sugar was used as the source of carbon (Adiga *et al.*, 1998). Required quantity (3 per cent) of the sugar was weighed and dissolved in double distilled water (about one-fourths of the final volume of the medium) with the aid of a magnetic stirrer.

3.2.3 Gelling agent

Media were solidified with agar (commercial grade) at a concentration of 0.6 per cent for all experiments.

3.2.4 Preparation of culture medium

The required quantity of sugar was dissolved in double distilled water. Appropriate quantities of the various stock solutions were drawn from the stock solutions and added to it. Growth regulators were added as necessary. After all the ingredients have been added and mixed well, with the exception of the gelling agent, double distilled water was added to make up the volume. PH of the medium was adjusted to 5.7 by adding drop-wise, while stirring, 1N NaOH or 1N HCl as appropriate. Required amount of agar (0.6 per cent) was added to the slightly warm media and mixed well by boiling.

The nutrient media was then dispensed into the culture vessels. About 30ml of the medium was dispensed into baby jars of 300ml capacity.

3.2.5 Culture vessels

Baby jars (300ml capacity) of autoclavable glass were used for the experiment. Threaded and screwable tight fitting polypropylene caps (magenta caps) were used for closing the bottles.

3.3 Sterilization procedures

3.3.1 Media

Baby jars containing the media were autoclaved at a temperature of 121°C and a pressure of 1.2kg cm⁻² for twenty minutes.

3.3.2 Instruments

All the instruments used for sterile handling and transfer of cultures were sterilized by autoclaving at 121°C (at 1.2kg cm⁻² pressure) for twenty minutes.

3.3.3 Sterile techniques in laminar flow cabinets

All the sterile transfer work was carried out in a laminar flow cabinet. Before starting any sterile operation, the inner surface of the cabinet was swabbed with 70 per cent ethyl alcohol. The UV lamp provided within the cabinet was then switched on for 15 – 20 minutes before use, followed by the airflow. During the course of transfer, between each transfer of explant to culture bottles or tubes, the instruments were dipped either in absolute alcohol and flame sterilized or put in steri bead.

3.4 Explant preparation and inoculation

The cut ends of the *in vitro* explants were trimmed off and the explants were carefully inoculated on to the prepared media. The culture vessels were immediately closed labeled and shifted to the incubation room.

3.5 Culture incubation conditions

The cultures were incubated in a growth room at a temperature of 25 ± 2°C under a photoperiodic regime of sixteen hours light and eight hours dark cycle.

3.6 Experimental details

3.6.1 Induction of multiple shoots using BA

3.6.1.1 Plant material

Basal shoots of *in vitro* grown plantlets were used for multiple shoot induction.

3.6.1.2 Tissue culture media

Murashige and Skoog (MS) media (Murashige and Skoog, 1962) supplemented with different concentrations of BAP (1, 5, 10 and 20mg/l) were used for proliferation of shoots from the explants.

3.6.1.3 Experimental design

Completely Randomized Design was adopted for laying out the experiments. The treatments were repeated 10 times.

3.6.1.4 Plantlet acclimatization

Well developed plantlets were removed from the culture bottles, roots were washed free of the adhering media and planted in polytrays containing hardening media. The plantlets were immediately transferred to the mist house.

3.6.1.5 Recording of experimental data

Five representative culture bottles were selected randomly from each replication and from each bottle a plantlet was selected to record the various biometrical observations. The following observations were recorded.

a. Shoot characteristics

The shoot characters were recorded at 15 and 30 days after subculture of the ex plants.

i) Number of shoots

The total number of shoots regenerated per explant at 15 and 30 days after subculture were counted and recorded.

ii) Length of shoot

The average length of the shoots regenerated were measured from its base to the tip and expressed in centimeter.

b. Leaf characteristics

The leaf characters were recorded at 15 and 30 days after subculture of the ex plants.

i). Number of leaves

Total numbers of leaves per treatment were measured at 15 and 30 days intervals.

ii) Width of largest leaf

Width of largest leaf in each treatment was measured and expressed in centimeter.

c. Root characteristics

Representative plantlets were removed from the agar medium at 60 days after inoculation. The root system was washed in running water to remove the adhering media. The following observations on root characteristics were recorded.

i) Number of roots

The average number of roots arising from the base of the shoots were counted and recorded.

ii) Length of roots

After separation of the roots from the media, its average length was measured and recorded in centimeter.

3.6.2 Experiment II: Callus induction using different concentrations of Cytokinins in combination with Auxins and Auxin alone

3.6.2.1 Plant material

Leaves and petioles of *in vitro* grown plantlets were used for callus induction.

3.6.2.2 Tissue culture media

Murashige and Skoog (MS) media (Murashige and Skoog, 1962) supplemented with different concentrations of BAP (1.5, 2 and 2.5 mg/l) in combination with NAA (0.1, 0.2 and 0.3 mg/l) and 2, 4-D (0.2, 0.5 and 1 mg/l) and 2, 4-D alone with different concentrations (1, 5, 10 and 20mg/l) were used for callus induction from the explants.

3.6.2.3 Experimental design

Completely Randomized Design was adopted for laying out the experiments. The treatments were repeated 5 times.

3.6.2.4 Recording of experimental data

Two representative culture bottles were selected randomly from each replication and from each bottle a plantlet was selected to record the various biometrical observations. The following observations were recorded.

a) Characteristics of calli induced

The following characteristics of the calli induced from leaves and petiole explants were recorded 60 days after inoculation.

i) Extent of callusing and quality of callus

The extent of callusing was visually recorded as shown in the table below. The quality of the calli was determined visually with respect to the following characters: embryogenic, non- embryogenic, friable, compact and presence or absence of necrotic patches.

Table 2.

| Score | Quality of callus |
|--------------|----------------------------------------------------------------------------------------|
| 1 | Bad callus (Watery in texture, brown in colour or necrotic) |
| 2 | Good callus (Friable in texture with or without small necrotic patches) |
| 3 | Very good callus (Friable in texture, free of necrotic patches) |
| 4 | Excellent callus (Friable to compact in texture, healthy and free of necrotic patches) |

ii) Color of callus:

Based on visual observation, the color of the callus was recorded as greenish, light green, greenish white, cream yellow or glossy or cream yellow.

iii) Type of callus

The type of callus implies whether the callus produced was compact, friable or compact to friable.

iv) Days for callusing

Observation on the number of days taken for explants to initiate callus by different growth regulators at different concentrations was

taken and expressed as days taken for callusing. The cultures were incubated in a growth room at a temperature of $25 \pm 2^{\circ}\text{C}$ under a photoperiodic regime of sixteen hours light and eight hours dark cycle and cultures are also incubated under complete dark condition.

3.6.3 Experiment III: Induction of variation using chemical mutagens

3.6.3.1 Plant material

In vitro derived plantlets induced from micro shoot explants were used for mutagenic treatment.

3.6.3.2 Mutagens

Ethyl methane sulphonate (HiMedia Laboratories Limited, Mumbai) were used for the induction of variation. Details of the mutagens employed in the experiment are given in Table 3.

Table 3. Chemical mutagens used in the study

| Mutagen | Source | Dose/Concentration applied | Nature of Action |
|--------------------------------|--------------------------------------|---------------------------------------------------------------------|------------------|
| Ethyl Methane Sulphonate (EMS) | HiMedia Laboratories Limited, Mumbai | Plantlets: (0.01, 0.05, 0.5, 1.0, 1.5 and 2.0 per cent). for 1 hour | Alkylation |

3.6.3.3 Method of treatment

In vitro derived plantlets were cultured on basal MS media and these cultures were subjected to mutagenic treatments for one hour.

EMS treatment

Freshly prepared EMS solution was used for the treatments. The plantlets were agitated with different concentrations of aqueous solution

of EMS, each for one hour (microshoots) at room temperature ($27 \pm 2^\circ\text{C}$). After the mutagenic treatment, the plant material were thoroughly washed with several changes of sterile distilled water and cultured on MS basal medium.

3.6.3.4 Assessment of LD₅₀

EMS treatment was carried out at concentrations of 0.01, 0.05, 0.5, 1.0, 1.5, and 2.0 per cent for one hour duration for treating the plantlets. The dose of the mutagen that resulted in 50 per cent reduction in growth compared to the control was considered to be the LD₅₀.

3.6.3.5 Treatment details

The concentration of EMS solutions was fixed based on the LD₅₀ values. Accordingly, the concentrations of EMS solution used were 0.01, 0.05, 0.5, 1.0, 1.5 and 2.0 per cent for 1 hour.

3.6.3.6 Experimental Design

Completely Randomized Design was adopted to lay out the experiment and each treatment was repeated five times.

3.6.3.7 Handling of the treated material

The plant materials, soon after mutagen treatment, were transferred to fresh MS media and these materials were designated as C₀ generation. Thirty days after treatment, each treated plant material was subcultured separately on MS media to get the C₁ (First clonal) generation. Subculturing was continued in a similar manner for one more time to get C₂ (second clonal) generations respectively. In each of these generations, the plantlets were treated as individual ones and subcultured accordingly.

3.6.3.8 Recording of experimental data

Representative plantlets were selected from each treatment to record the various biometrical characters. The following observations, like number of shoots and length of shoots, were recorded in the C₁ and C₂ generations, 15 and 30 days after inoculation on media.

a. Characteristics of shoots and roots regenerated from the plantlets

i. Shoot characteristics

Following observations on shoot parameters were recorded at 15th and 30th day.

- i) Number of shoots
- ii) Length of Shoot (cm)

ii. Root characteristics

Observations on the following root characteristics were recorded at 15th and 30th day.

- i) Number of roots
- ii) Length of roots (cm)

b. Other morphological mutations

Other visible mutations, which included changes in leaf (curling, crinkling, and albenic) and plant morphology (decreased and increased growth) were recorded.

Experimental Results



IV. RESULTS

The results of the *in vitro* mutation studies in *Syngonium podophyllum* is presented in this chapter. Experiments were conducted to standardize protocols for shoot proliferation, callus induction and induction of variation using chemical mutagens.

4.1 Experiment I: Induction of multiple shoots using BAP

4.1.1 Effect of different concentration of BAP on the shoot characteristics

The growth regulators used were found to significantly influence all the shoot characteristics of the shoot.

4.1.1.1 Number of shoots per explant

The presence of different concentrations of BAP in the media was observed to result in the production of significantly higher number of shoots per explant (Table 1, Fig. 1 and Plate 1).

All treatments were shown significant differences compared to control. However, a maximum number of shoots (9.5) were obtained in explants cultured on media with 20mg/l BAP, which was followed by explants cultured on media supplemented with 10mg/l BAP (7.4).

Among the treatments BAP at 20mg/l resulted in significantly a higher number of shoots (9.5) as compared with the treatments BAP at 5mg/l (5.0), 1mg/l (4.7) and control (2.0). However the number of shoots produced with BAP at 10mg/l (7.4) was statistically on par with that produced with 20mg/l BAP.

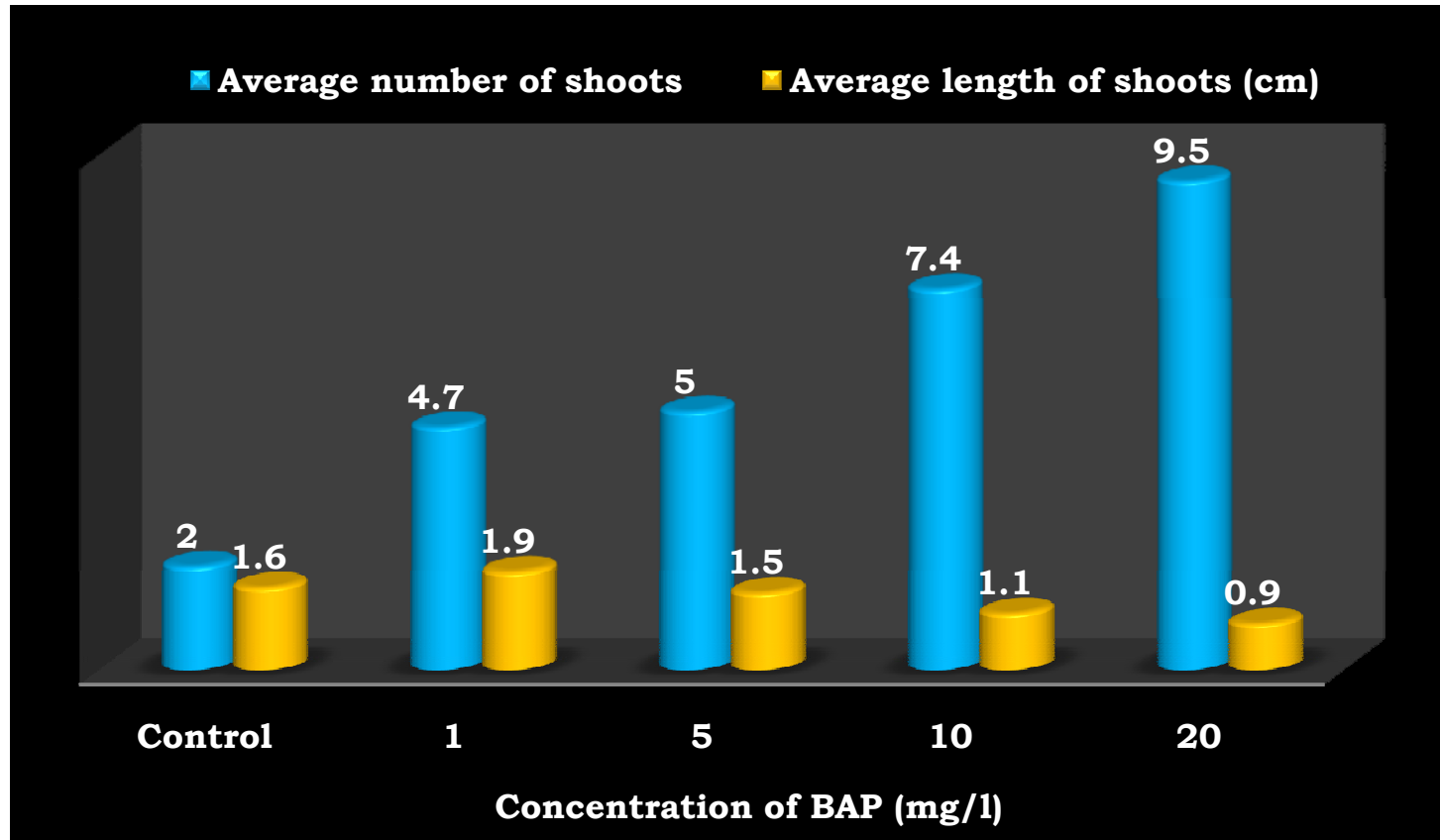


Fig 1: Comparative effect of BAP concentrations on number and length of shoot



Control

1mg/l

5mg/l

10mg/l

20mg/l

Plate 1: Comparative effect of different concentration of BAP on shoot proliferation

Table 1. Effect of BAP on shoot proliferation

| Treatments (mg/l) | Average Number of Shoots | Average Length of Shoots (cm) |
|--------------------------|---------------------------------|--------------------------------------|
| control | 2.0 | 1.6 |
| 1 | 4.7 | 1.9 |
| 5 | 5.0 | 1.5 |
| 10 | 7.4 | 1.1 |
| 20 | 9.5 | 0.9 |
| S. Em ± | 0.74 | 0.05 |
| C.D. at 5% | 2.21 | 0.14 |

4.1.1.2 Length of shoots

Among the treatments, the average lengths of the shoots regenerated from the explants were significantly differing with BAP at different concentrations. Maximum length of shoot (1.9cm) was recorded at lower concentration of 1mg/l BAP followed by control (1.6 cm) (Table 1, Fig. 1 and Plate 1).

With treatment of 1mg/l BAP shoots of significantly increased length (1.9cm) were obtained as compared with the treatments of BAP at 5mg/l (1.5cm), 10mg/l (1.1cm), 20mg/l (0.9cm) and control (1.6cm).

Increase in concentration of BAP resulted in a drastic reduction in shoot length. However, with 1mg/l BAP, where a lesser number of shoots were produced, exhibited maximum length of shoot (1.9cm).

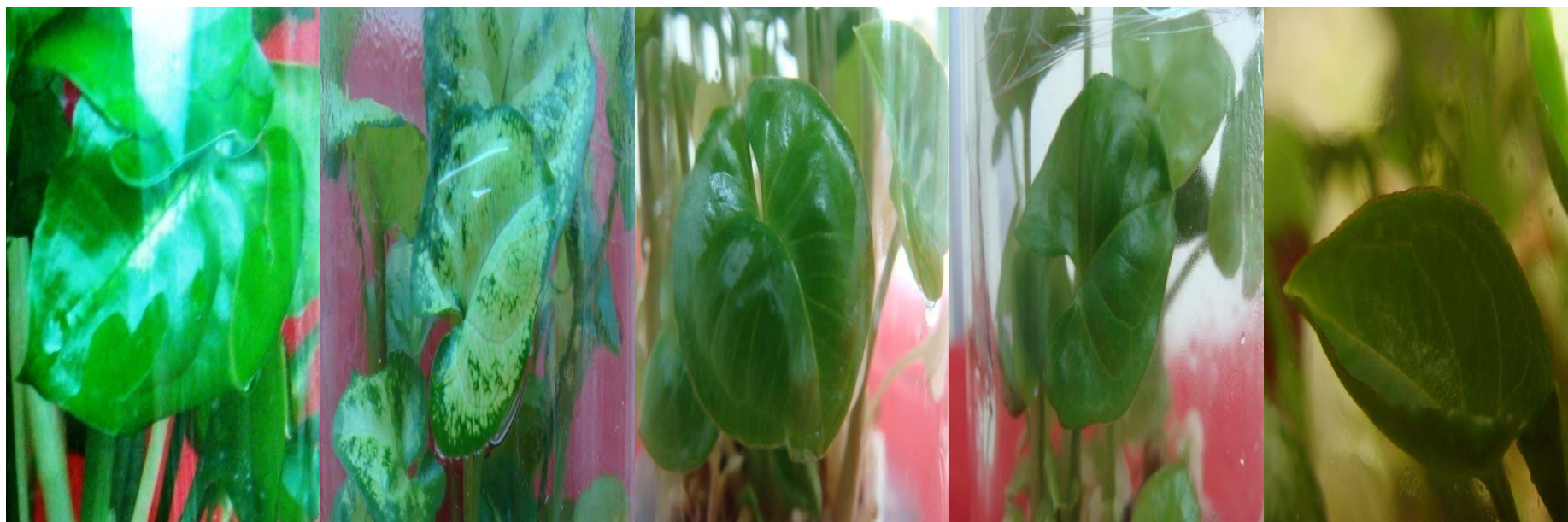
4.1.1.3 Number of Leaves

All the treatments showed no significant differences for the parameter total number of leaves (Table 2 and Fig. 2). However, treatment with 10mg/l BAP recorded maximum number of leaves (3.9cm) which was on par with treatments such as 1mg/l BAP (3.8cm), 5mg/l BAP (3.8cm), 20mg/l BAP (3.6cm) and control (3.1cm).

4.1.1.4 Width of Leaves

The presence of different concentrations of BAP in the media was observed to result in the production of leaves with significant difference in their width (Table 2, Fig. 2 and Plate 2).

Among the treatments, BAP at 1mg/l gave rise to leaves of significantly increased width of leaves (2.4cm) as compared with the treatments of BAP at 5mg/l (1.5cm), 10mg/l (1.1cm), BAP 20mg/l (0.8cm) and control (1.9).



Control

1mg/l

5mg/l

10mg/l

20mg/l

Plate 2: Comparative effect of different concentration of BAP on width of leaves

Table 2. Effect of BAP on total number and average width of leaves

| Treatments (mg/l) | Average Number of Leaves | Average Width of Leaves (cm) |
|--------------------------|---------------------------------|-------------------------------------|
| control | 3.1 | 1.9 |
| 1 | 3.8 | 2.4 |
| 5 | 3.8 | 1.5 |
| 10 | 3.9 | 1.1 |
| 20 | 3.6 | 0.8 |
| S. Em ± | 0.36 | 0.12 |
| C.D. at 5% | 1.09 | 0.37 |

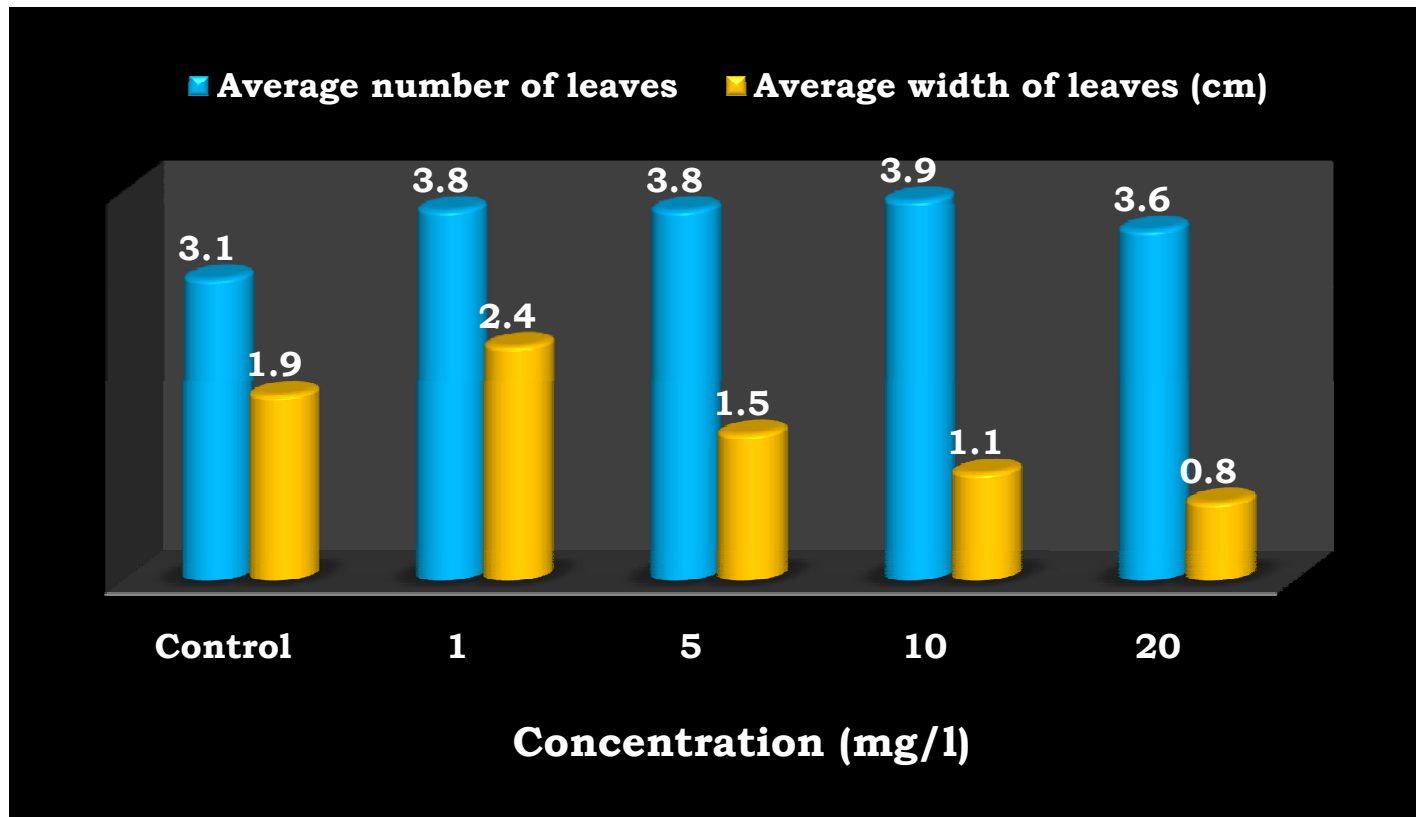


Fig 2: Comparative effect of BAP concentrations on number and width of leaves

Table 3. Effect of BAP on average number and average length of roots

| Treatments (mg/l) | Average Number of roots | Average Length of roots (cm) |
|--------------------------|--------------------------------|-------------------------------------|
| control | 1.7 | 9.7 |
| 1 | 2.5 | 16.6 |
| 5 | 2.6 | 9.6 |
| 10 | 2.8 | 7.9 |
| 20 | 1.8 | 6.5 |
| S. Em ± | 0.31 | 1.12 |
| C.D. at 5% | 0.92 | 3.83 |

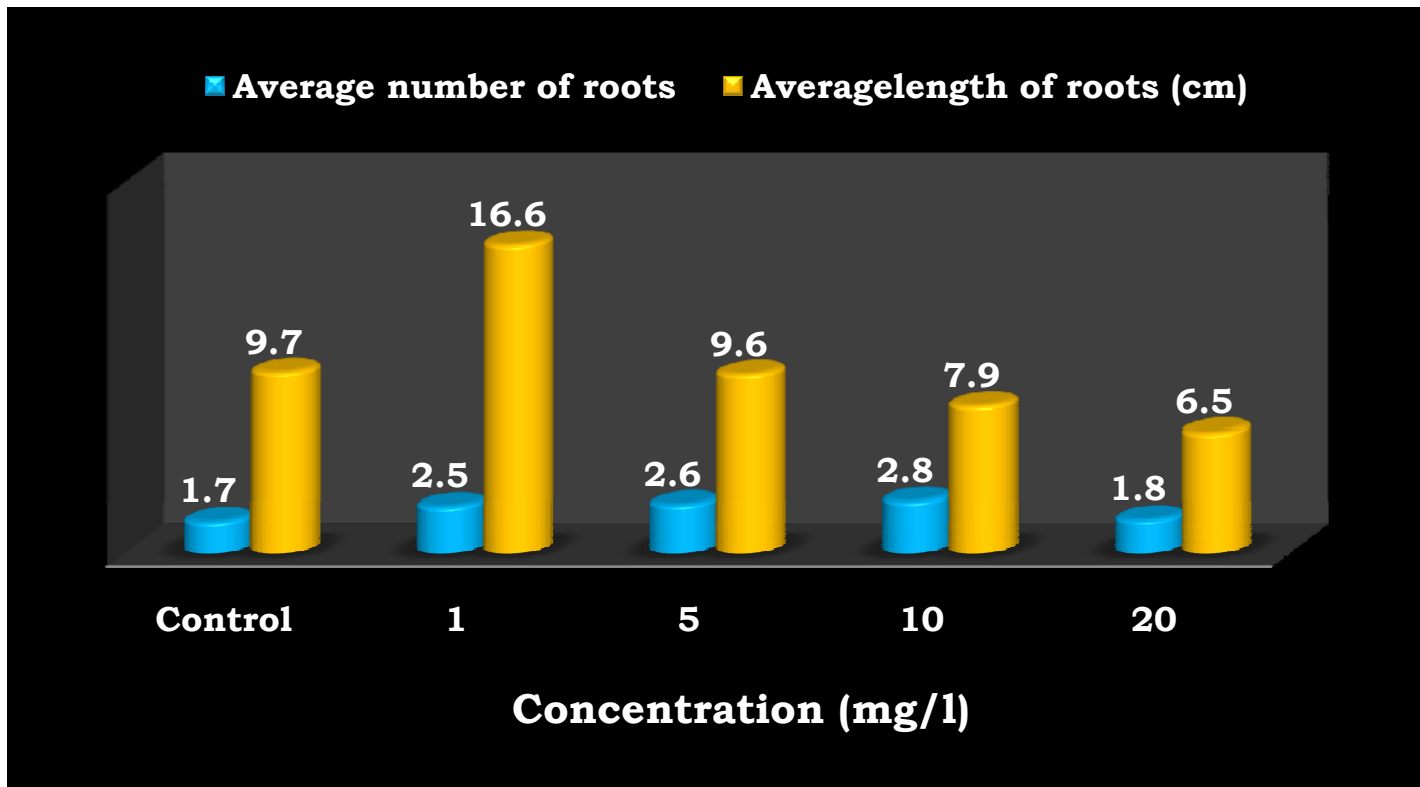


Fig 3: Comparative effect of BAP concentrations on number and length of roots



Control



1mg/l



5mg/l



10mg/l



20mg/l

Plate 3a: Comparative effect of different concentration of BAP on root growth

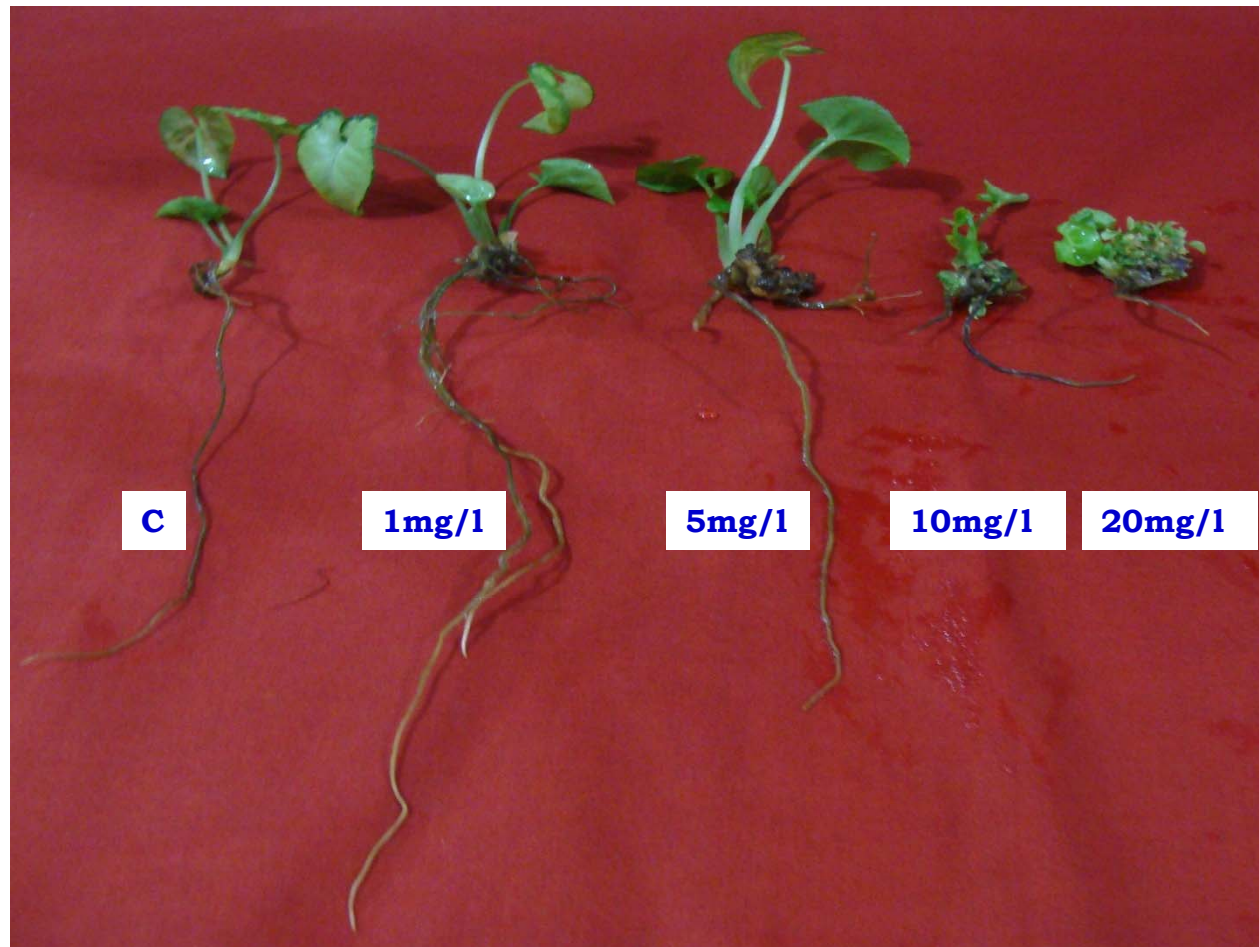


Plate 3b: Comparative effect of different concentration of BAP on root growth

In general, all the treatments were significantly different with control. However, width of leaves produced with treatment of 10mg/l BAP was on par with that produced with 20mg/l BAP.

4.1.2 Effect of different concentration of BAP on root characteristics of microshoots.

The regenerated microshoots found to root better with 1mg/l BAP. In media supplemented with BAP at different concentrations, the root characteristics of regenerated microshoots were found to differ significantly.

4.1.2.1 Number of roots per shoot

Least number of roots (1.7) was produced from shoot explants cultured on media without growth regulator (Table 3, Fig. 3 and Plate 3a).

Among the BAP treatments, maximum numbers of roots per shoot (2.8) were noticed with 10mg/l BAP followed by 5mg/l BAP (2.6) and 1mg/l BAP (2.5) which were found on par but differed significantly from rest of the treatments. Comparatively significant reduction in number of roots was recorded in microshoots treated with 20mg/l BAP, where in only, a mean number of 1.8 roots were observed.

4.1.2.2 Length of the roots

Among the treatments, the average lengths of the roots regenerated from the explants were significantly differing with treatment containing BAP at different concentrations (Table 3, Fig. 3 and Plate 3b). Maximum length of roots (16.6cm) was recorded with 1mg/l BAP which significantly differed from all other treatments.

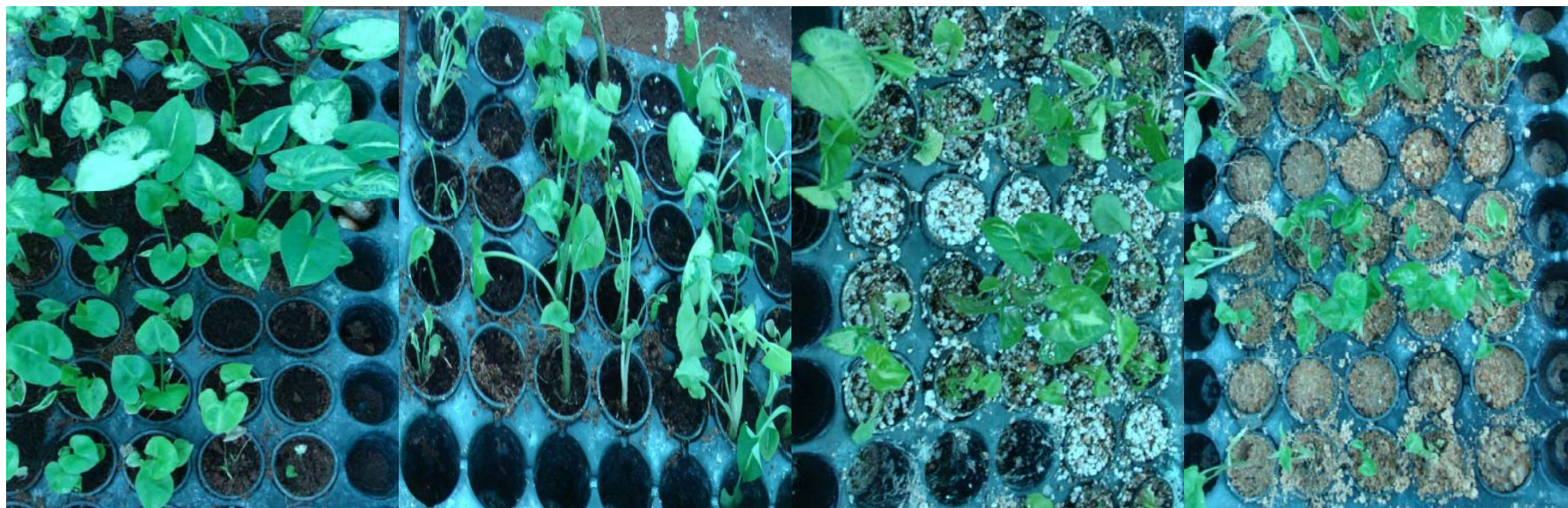
In general, treatment with 5mg/l BAP recorded a mean root length of 9.6cm which was on par with that produced with 10mg/l BAP (7.5cm), 20mg/l BAP (6.5cm) and control (9.7cm).

4.1.3 Acclimatization of microshoots

In vitro microshoots which were rooted on MS media with different concentrations of BAP (1, 5, 10 and 20mg/l) were removed from the media. Roots were washed free of agar and transferred to different hardening media and their survival percentage was recorded at the end of 20th day of transfer. Microshoots transferred in cocopeat medium showed maximum average survival rate of 88 per cent. Other media tried. i.e. Vermicompost, sand and 1:1 mixture of vermiculate + perlite were found to be less suitable for the plant growth and the survival rate was found to be 72, 68 and 68 per cent respectively (Table 4 and Plate 4).

4.2 Experiment II: Callus induction using different concentrations of Cytokinins in combination with Auxins and Auxin alone

Leaves and petiole explants collected from *in vitro* grown plants, when were cultured on various hormones and their different combinations of 2, 4-D alone, BAP + 2, 4-D and BAP + NAA failed to give any callusing response. Both of the explants (leaf and petiole) were completely dried after a period of 5 weeks of inoculation, when cultured on a media containing 2,4- D alone. Inclusion of BAP in the medium in combination with either of the auxin (2,4-D or NAA) resulted in to slight curling of the leaf explants. The explants remained green and surface texture of the explants appeared rough at the end of 6 weeks. But, eventually, after 10 weeks of culture, there was no significant cell growth on the surface.



Vermi compost

Cocopeat

**Vermiculite+Perlite
(1:1)**

Sand

Plate 4: Comparative effect of different hardening media on plant growth

Table 4. *Ex vitro* survival of plantlets as influenced by hardening media at 20th day after transfer

| Hardening media | Per cent Survival | | | | |
|------------------------------|--------------------------|------------------|------------------|-------------------|-------------------|
| | Control | 1mg/1 BAP | 5mg/1 BAP | 10mg/1 BAP | 20mg/1 BAP |
| Vermicompost | 40 | 100 | 100 | 60 | 60 |
| Coco peat | 100 | 100 | 100 | 80 | 60 |
| Sand | 40 | 100 | 80 | 60 | 60 |
| Vermiculite + perlite | 60 | 80 | 80 | 80 | 40 |

Petioles, when used as explants, failed to give any callusing response, irrespective of the growth regulator combination present in the medium. No successful callus was induced from any of the treatments.

4.3 Experiment III: Induction of variation by chemical mutagens

4.3.1 Assessment of LD₅₀ for ethyl methane sulphonate (EMS)

Microshoots (*in vitro* raised basal shoots) were treated with EMS at concentrations ranging from 0.01 per cent to 0.2 per cent, each for duration of one hour (Table 5). Since, the percent survival was more than 90, even at higher concentration of 0.2 per cent EMS, one more experiment was designed to find out LD₅₀ for microshoots. Increase in the concentration of EMS from 0.01 -2 per cent was attempted and the results are presented in Table 5 and Figure 4.

Microshoots showed cent per cent survival at lower concentration of EMS ranging from 0.01-0.05 per cent. Lower concentrations of EMS did not affect the survival of the explants but with the increase in concentration of EMS up to 0.2 per cent progressive reduction to 90 per cent was observed. Since the per cent survival was more than 50 and as such a value of LD₅₀ could not be reached, experiment was redesigned to arrive at LD₅₀ value. Concentration of EMS was increased from 0.01-2 per cent. At 1.0 per cent of EMS nearly 50 per cent mortality of microshoots was observed. Hence, this concentration of EMS was considered as the LD₅₀.

4.3.2 Shoot characteristics of microshoots regenerated from EMS treatments (C₁ generation)

The effect of EMS on the shoot characteristics like the average number of shoots, average shoot length and average width of largest leaf in C₁ clonal generations is given below.

Table 5. Assessment of LD₅₀ of EMS (Microshoots)

| Concentration of EMS (per cent) | Per cent survival |
|----------------------------------------|--------------------------|
| 0.01 | 100 |
| 0.05 | 90 |
| 0.5 | 60.5 |
| 1.0 | 50.2 |
| 1.5 | 45.6 |
| 2.0 | 35.7 |
| Control | 100 |
| S. Em ± | 0.15 |
| C.D. at 5% | 0.50 |

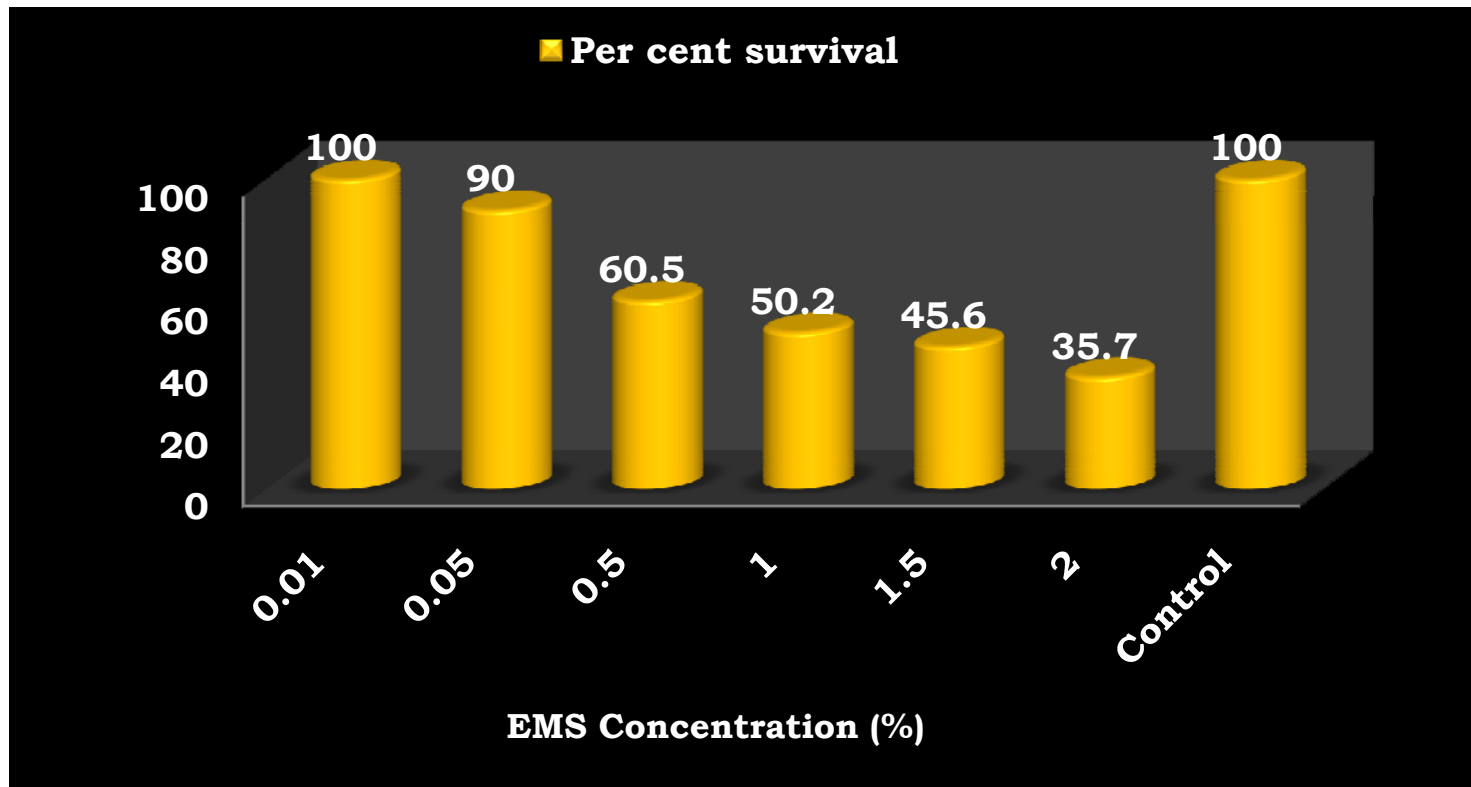


Fig. 4: Effect of different concentration of EMS on survival of explants (LD_{50})

Table 6. Average number and length of shoots regenerated from EMS treated Microshoots (C₁ generation)

| EMS Treatment (%) | Average No. of shoots | Average Length of shoots (cm) |
|--------------------------|------------------------------|--------------------------------------|
| Control | 3.9 | 2.0 |
| 0.5 | 1.0 | 0.9 |
| 1.0 | 1.8 | 0.6 |
| 1.5 | 1.3 | 0.8 |
| 2.0 | 0.9 | 0.7 |
| S. Em ± | 1.14 | 0.07 |
| C.D. at 5% | 2.89 | 0.22 |

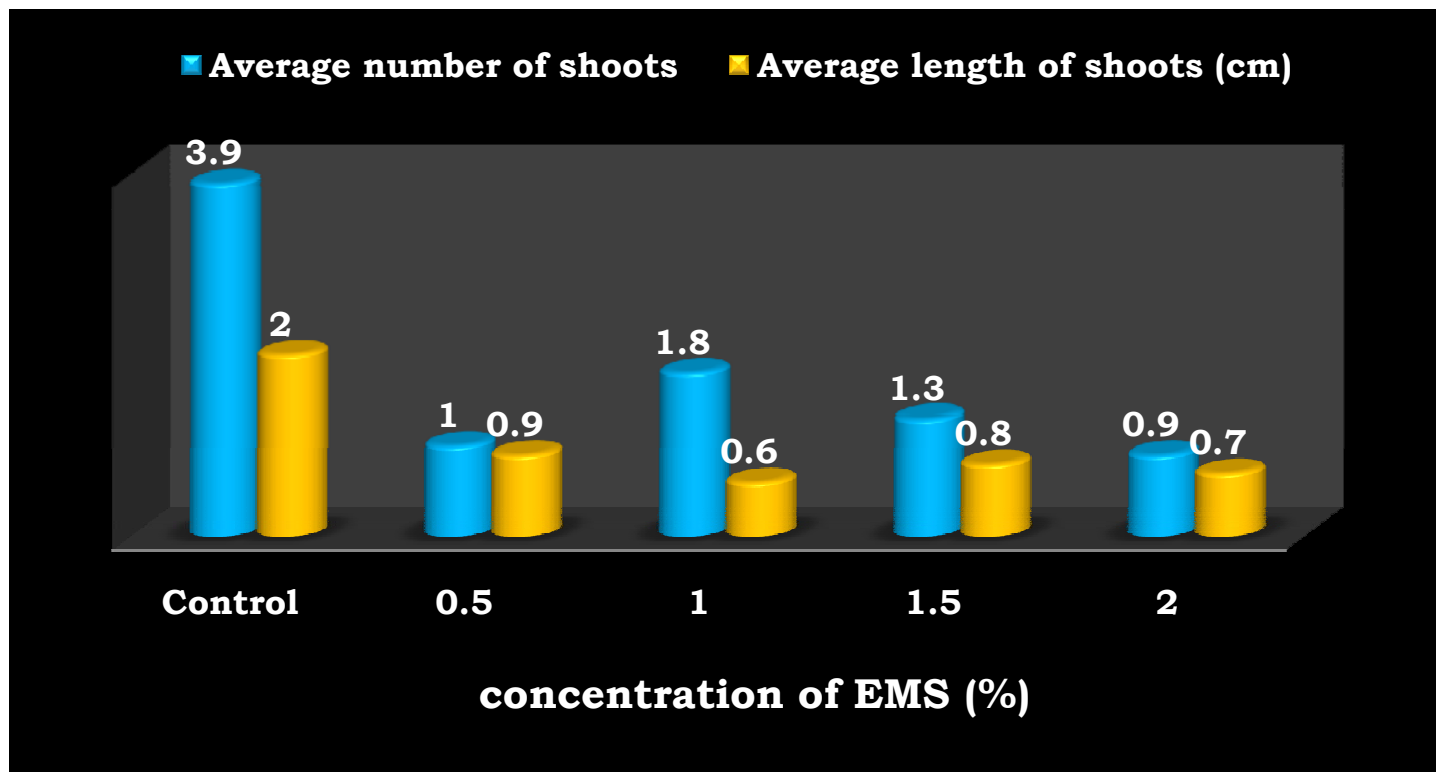


Fig. 5: Comparative effect of EMS concentrations on number and length of shoot (C_1 generation)

EMS treatment resulted in a general reduction in the number of shoots produced per microshoot in C₁ generation. Maximum number of (3.9) shoots per explant were recorded for untreated microshoots. Microshoots treated with EMS at all the concentration displayed poor shoot proliferation. Least number of shoots production (0.9) was observed in case of microshoots treated with 2 per cent EMS (Table 6 and Fig. 5).

4.3.3 Length of the shoots

There was a significant difference in the average length of the shoots produced. The length of the shoots was found to show marked variation with highest shoot length with different treatment value (2cm) for control and was found significantly higher than all other treatments. Least length of shoot (0.7cm) was recorded when microshoots were treated with 2 per cent EMS (Table 6 and Fig. 5).

4.3.4 Width of largest leaf

Significant differences were seen as influenced by different concentrations of EMS on width of largest leaf in C₁ generation (Table 7 and Fig. 6).

Higher width of leaf (1.6cm) was observed for control, whereas, 1 per cent EMS recorded 0.70cm width. Reduced width of leaf (0.40 cm) was noticed when microshoots were exposed to 2 per cent EMS.

All treatments were significantly differing with control. Treatment with 1 per cent EMS recorded significantly smaller leaf width (0.70cm) when compared to 1.5 per cent EMS (0.5cm), 2 per cent EMS (0.40 cm) and control (0.4cm). However, Treatment with 0.5 per cent EMS (0.6cm) was on par with 1 per cent EMS (0.70cm).

Table 7. Average width of largest leaves regenerated from EMS treated Microshoots (C₁ generation)

| EMS Treatment (%) | Average Width of Largest Leaves (cm) |
|--------------------------|---------------------------------------------|
| Control | 1.6 |
| 0.5 | 0.6 |
| 1.0 | 0.7 |
| 1.5 | 0.5 |
| 2.0 | 0.4 |
| S. Em ± | 0.07 |
| C.D. at 5% | 0.23 |

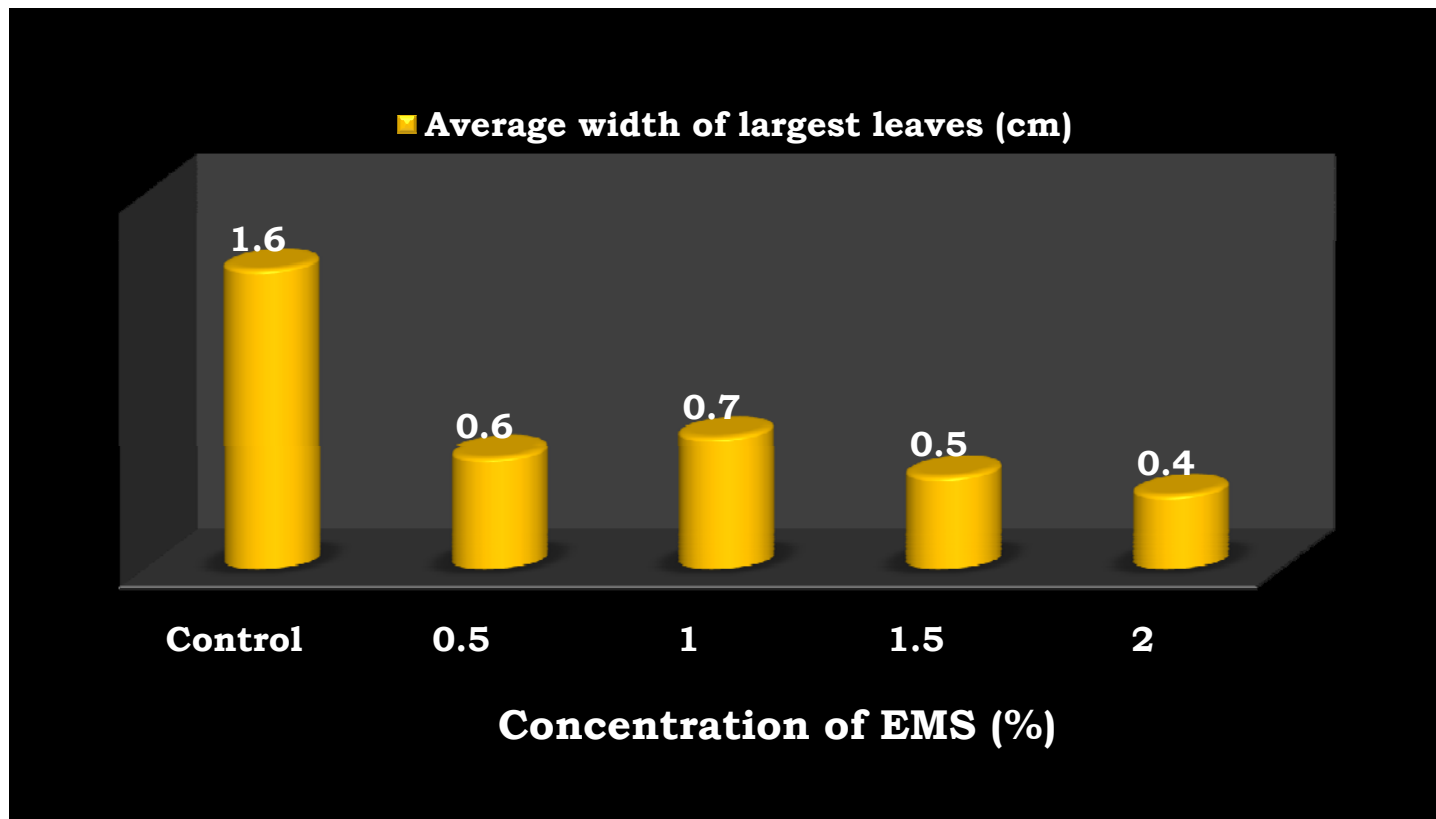


Fig. 6: Comparative effect of EMS concentrations on width of largest leaf (C₁ generation)

4.3.5 Shoot characteristics of microshoots regenerated from EMS treatments (C₃ generation)

The effect of EMS on the shoot characteristics like the average number of shoots, average shoot length, total number of leaves and average width of largest leaf in C₃ clonal generation is presented below.

In contrast to observations made at C₁ generation for the characters such as number of shoots, length of shoots and width of leaf showed marked recovery in which number of shoots, length of shoots and width of leaf were enhanced by the time EMS treated microshoots reached C₃ generation.

4.3.6 Number of shoots

Treatment of microshoots with EMS was found to have a significant influence on average number of shoots (Table 8, Fig. 7 and Plate 5). Maximum number of (5.6) shoots per microshoots were recorded for untreated explant. Microshoots treated with EMS at all concentrations displayed less number of shoots when compared to control. EMS at 2 per cent produced highest number of shoots per microshoots (4.0) and differed significantly from rest of the treatments.

Treatment with 0.5 per cent EMS resulted least number of shoots (1.6) as compared to 2 per cent EMS (4.0). However, treatment with 1 per cent EMS (2.8) was on par with 1.5 per cent EMS (3.8).

4.3.7 Length of shoots

There was a significant difference in average length of shoots produced (Table 8, Fig. 7 and Plate 5). Maximum length of shoot (1.5cm) was observed with control, followed by 1 per cent EMS (1.1cm) and 1.5 per cent EMS (1cm). Shoot length observed in treatments involving EMS

Table 8. Average number and length of shoots regenerated from EMS treated Microshoots (C₃ generation)

| EMS Treatment (%) | Average No. of shoots | Average Length of shoots (cm) |
|--------------------------|------------------------------|--------------------------------------|
| Control | 5.7 | 1.5 |
| 0.5 | 1.6 | 0.9 |
| 1.0 | 2.8 | 1.1 |
| 1.5 | 3.8 | 1.0 |
| 2.0 | 4.0 | 0.9 |
| S. Em ± | 0.55 | 0.12 |
| C.D. at 5% | 1.71 | 0.33 |

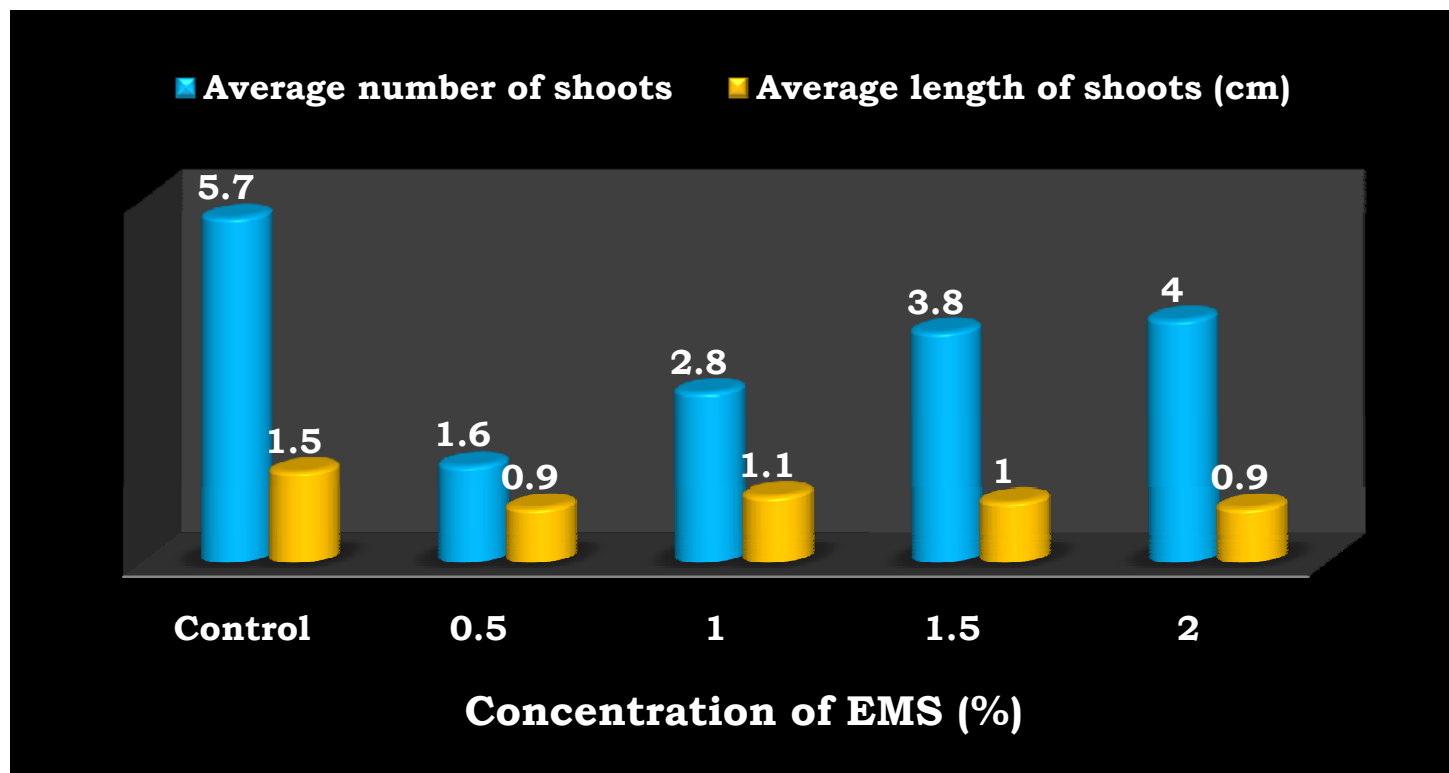


Fig. 7: Comparative effect of EMS concentrations on number and length of shoot (C₃ generation)

Table 9. Average number and width of largest leaves regenerated from EMS treated Microshoots (C₃ generation)

| EMS Treatment (%) | Average Number of Leaves | Average Width of Largest Leaves (cm) |
|--------------------------|---------------------------------|---------------------------------------------|
| Control | 3.1 | 1.9 |
| 0.5 | 7.5 | 1.0 |
| 1.0 | 6.3 | 0.8 |
| 1.5 | 13.7 | 1.4 |
| 2.0 | 10.8 | 1.3 |
| S. Em ± | 1.39 | 0.10 |
| C.D. at 5% | 3.99 | 0.30 |

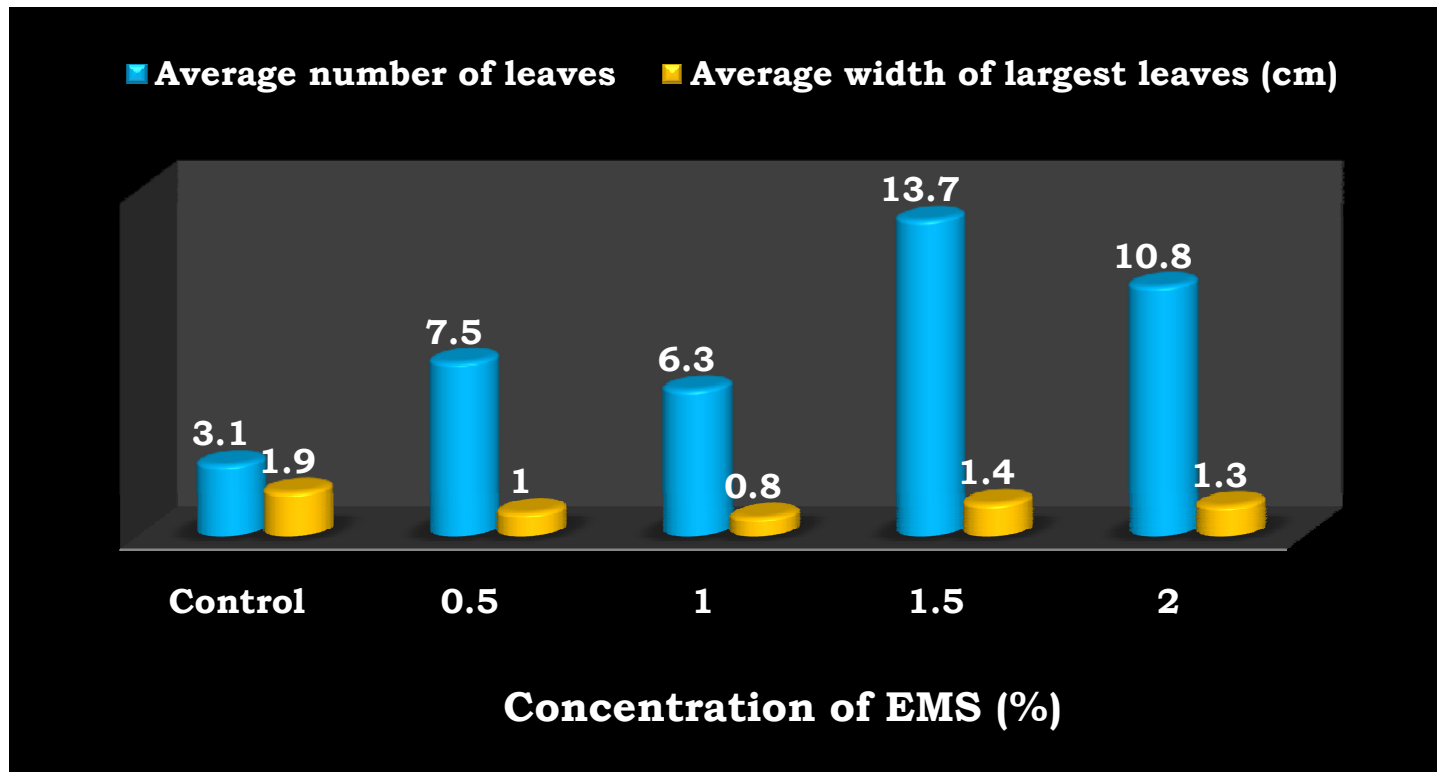


Fig. 8: Comparative effect of EMS concentrations on number and width of largest leaves (C_3 generation)

were all statistically on par. Least shoot length was recorded with 0.5 per cent EMS (0.9cm) and 2 per cent EMS (0.9cm) and was on par.

4.3.8 Total number of leaves

Total number of leaves differed significantly among the treatments as compared with control (Table 9, Fig. 8 and Plate 5). Significantly maximum number of leaves with 1.5 per cent EMS (13.7) followed by 2 per cent EMS (10.8) were recorded. Least number of leaves (3.1) was recorded in control.

4.3.9 Width of largest leaf

Significant differences were seen as influenced by different concentrations of EMS on width of largest leaf in C₃ generation (Table 9, Fig. 8 and Plate 5).

Increased width of leaf (1.9cm) was observed for control and differed significantly from the rest of the treatments. Least width of leaf (0.8cm) was recorded when the microshoots were treated with 1per cent EMS.

In general all the treatments differed significantly. Treatment with 0.5 per cent EMS was recorded (1.0cm) shoot length as compare to 1.5 per cent EMS (1.4cm) and 2 per cent EMS (1.3cm).

4.4 Root characteristics of shoots regenerated from EMS treated microshoots

Roots that had developed from microshoot explants treated with EMS at different concentrations were found to show variation in the average number of roots per microshoot and the average root length.

4.4.1 Number of roots per plant

Significant effects of EMS treatments of explants were observed in the C₃ generation (Table 10 and Fig. 9).

Comparison of the different concentrations of EMS showed production of a significantly higher number of roots per microshoots (4.1) in the case of 1.5 per cent EMS, followed by the concentrations of 2 per cent EMS (3.4) which were statistically on par. Least number of roots (1.5) was recorded in treatment with 0.5 per cent EMS as compared to 1 per cent EMS (3.3) and all other treatments.

4.4.2 Length of roots

The shoots of C₃ generation when cultured for rooting, at higher concentration of EMS of 1.5 per cent gave longest roots (3.9cm), and was found significantly higher compared with roots observed in 0.5 per cent EMS (1.5cm) and 1 per cent EMS (1.6cm). In contrast, roots derived from 0.5 and 1 per cent EMS treatments were short, thin, clasped and appeared more or less at initial stages of growth. The colors of root produced from shoots derived from 0.5 and 1per cent EMS treatments were either light whitish or light green (Table 10 and Fig. 9).

4.5.1. Morphological observations

Reduction in chlorophyll content and cultures turning albenic was observed when microshoots were treated with EMS. Lower concentration of EMS (0.5 per cent) did not induce variation in chlorophyll color, but with the increase in EMS concentration (1, 1.5 and 2 per cent) changes in chlorophyll color such as microshoots turning light green, albenic were seen. Microshoots showing deficiency of chlorophyll was high and they fail to turn normal green in course of time. Stunted growth was observed when microshoots were treated with higher concentration of EMS.

Table 10. Average number and length of roots regenerated from EMS treated Microshoots (C₃ generation)

| EMS Treatment (%) | Average Number of roots | Average length of roots (cm) |
|--------------------------|--------------------------------|-------------------------------------|
| Control | 6.3 | 9.6 |
| 0.5 | 1.5 | 1.5 |
| 1.0 | 3.3 | 1.6 |
| 1.5 | 4.1 | 3.9 |
| 2.0 | 3.4 | 2.4 |
| S. Em ± | 0.68 | 0.61 |
| C.D. at 5% | 2.04 | 1.86 |

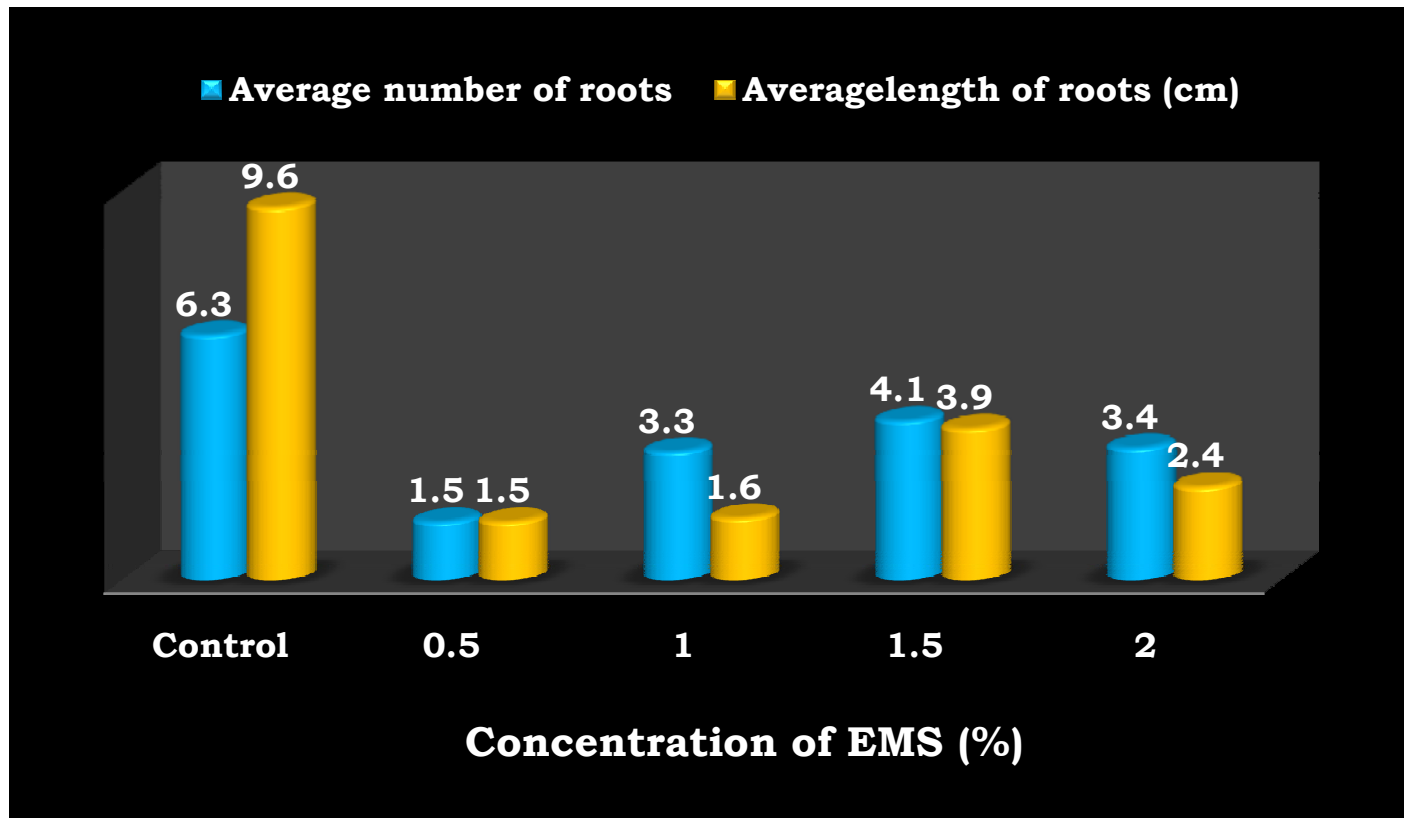


Fig. 9: Comparative effect of EMS concentrations on number and length of roots (C_3 generation)



0.5% EMS control



1.0% EMS control



control 1.5% EMS



2.0% EMS control

Plate 5: Comparative effect of different concentrations of EMS with control (C₃ generation)

4.5.2. Variation in leaf morphology

The effect of EMS treatments on microshoots was manifested in occurrence of varied leaf morphology of the regenerated microshoots. The variation included was crinkling of leaves, twisting and reduced leaf lamina. Crinkling and curling of leaf was more evident, when microshoots were treated with 1.5 per cent and 2 per cent EMS.

Discussion



V. DISCUSSION

The techniques of plant tissue culture have proved to be a practical tool in paving a new avenue for the application of biotechnology in agriculture and horticulture. The past few years have witnessed a dramatic increase in our ability to manipulate and study plant cells and tissues in culture. The fact that a whole plant could be regenerated from a single cell has created an exciting scenario in the field of genetic manipulation and crop improvement. The growing realization of the potentialities of plant cell and tissue culture for plant propagation and breeding has itself provided a substantial impetus for research. The developments in the techniques of plant tissue culture have facilitated the application of mutation breeding techniques for the improvement of both seed and vegetatively propagated crops.

The intricacies involved in the *in vitro* propagation of several horticultural plants have been competently elaborated periodically by several workers, but the use of *in vitro* mutation techniques are few. The present investigation focuses on the standardization of efficient regeneration protocol in *Syngonium podophyllum* and induction of variability by the use of chemical mutagens. The results of the studies are discussed in the foregoing pages in the light of contemporary research works in the field.

5.1 Regeneration of plantlets by direct organogenesis

5.1.1 Shoot organogenesis

The simplest type of *in vitro* plant propagation is the stimulation of axillary bud development. This technique exploits the normal ontogenic route for branch development from axillary meristems (Phillips and Hubstenberger, 1995). The axillary buds are treated with hormones to stimulate the production of multiple shoots. The response of a plant

species to an exogenous growth regulator would depend mainly on the level of that growth regulator (and of other growth regulators as well) in that species (Singh, 1998). In *Syngonium podophyllum*, shoot proliferation was found to occur in growth regulator free medium, but the low multiplication rate of the cultures in MS basal medium was augmented by the addition of growth regulators to the medium.

The role of cytokinins in shoot proliferation is well established (Evans *et al.*, 1983). Although a small quantity of cytokinins may be synthesized by shoots *in vitro* (Kodo and Okazawa, 1980), roots are the principle sites of cytokinin biosynthesis. Both shoot and root synthesize cytokinin (Nordstrom *et al.*, 2004; Taiz and Zieger, 2003) in actively dividing cells through different pathways. Chloroplasts are a prerequisite for cytokinin production in aerial parts (Nordstrom *et al.*, 2004) restricting its production to green plant parts. Exogenous supply of cytokinin is often essential for shoot morphogenesis. In the present investigation also, BAP, a cytokinin, favoured shoot proliferation with improvement in shoot characteristics, when compared to the untreated explants. Similar results in *Syngonium* have also been reported by (Schwertner and Zaffari, 2003 and Hassanein, 2004.)

Presence of BAP in the medium was found to be more effective in improving the shoot proliferation from the explants. A concentration of 1mg/L of BAP was found to be the most effective in shoot proliferation from basal shoot explants with maximum shoot length (1.9cm), while a higher concentration of 20mg/L of BAP was better in enhancing the total number of shoots per explants. Shoot proliferation in terms of the number of shoots produced per explant was found to be quite high (4.7 from basal shoot per explants) compared with the earlier report by Meltem *et al.* (2005), where a maximum of only 2.1 shoots could be induced with BAP supplementation (1mg/L), while at higher

concentrations of 5mg/L to 20mg/L, increased number of shoots per explants were noticed.

Reduced shoot length was observed with the increase in concentration of BAP treatment in comparison with control. This appears to be due to suppression of apical dominance of shoots and the role of BAP in suppression of apical dominance is well documented.

A close examination of the shoot cultures revealed few abnormal plantlets with irregular arrangement of internodes and alternate phyllotaxy. This effect was only transitory. The growth pattern of the abnormal shoots was normal and produced shoots with decussate phyllotaxy. Similar observations have been reported earlier as well (Thakur *et al.*, 1976). These abnormal shoots might be due to growth abnormalities, which would have occurred as a result of overcrowding of shoots. In many cases, such temporary malformations are reported to occur in media where higher concentration of BAP is used as the cytokinin (George and Sherrington, 1984).

5.1.2 Effect of high concentration BAP on shoot proliferation

Explants sub cultured in high concentration BAP resulted in increased of number multiple shoots and number of leaves per shoot and survival percentage after acclimatization. But a significant reduction was noted in internode length and shoot height. This might be due to inhibitory effect of cytokinin on cell elongation. Though cytokinin in high concentrations can stimulate shoot development, they can also have negative effects such as toxicity, reduction of leaf size, internode length and occasionally rooting (Grattapaglia and Machado, 1990). Lateral buds of cytokinin over producing plants grow vigourously developing into shoots that compete with the main shoot. Consequently, cytokinin over producing plants tends to be bushy (Taiz and Zieger, 2003). Applied

cytokinin typically inhibits the process of cell elongation in both stem and roots.

The inhibition of hypocotyl and internode elongation induced by excess cytokinin was due to the production of ethylene (Cary *et al.*, 1995; Vogel *et al.*, 1998). In gas plant (*Dictamnus albus*), it was found that 20 μ M BA reduced the plant height considerably when compared with 5 μ M BA treatment (Jones *et al.*, 1994). Similar results were observed in olive also (Khan *et al.*, 2002).

5.1.3 Rhizogenesis

The shoots in the multiplication media formed roots simultaneously in the same media. The *Syngonium* plant in the field roots easily and creeps along striking roots at nodal points. This easy to root character of the plant was exhibited in the culture too and did not require any special treatment for induction of roots.

5.1.4 Acclimatization

Considerable efforts have been directed to optimize the conditions for *in vitro* stages of micropropagation, but the process of acclimatization of micropropagated plants to the soil environment has not fully been studied. Consequently, the transplantation stage continues to be a major bottleneck in the micropropagation of many plants. Plantlets or shoots that have grown *in vitro* have been continuously exposed to a unique microenvironment that has been selected to provide minimal stress and optimum conditions for plant multiplication.

Plantlets were developed within the culture vessels under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow for heterotrophic growth and in an atmosphere with high level of humidity. These contribute to a culture-induced phenotype

that cannot survive the environmental conditions when directly placed in a greenhouse or field. The physiological and anatomical characteristics of micropropagated plantlets necessitate that they should be gradually acclimatized to the environment of the greenhouse or field.

Inclusion of cocopeat and vermicompost in the hardening media brought a dramatic increase in the survival percentage. The survival of the micro propagated plantlets raise to 88 per cent with the addition of cocopeat and it was found to be 72 per cent with the addition of vermicompost to the hardening media. On an average, sand and mixture of vermiculate + perlite (1:1) as a hardening media, gave only 68 per cent survival. This poor performance is accounted by the fact that *Syngonium* plants were sensitive to drought (Ferriera and Handro, 1988). Hence, the sand with its highly porous nature does not retain any water resulting in death of plantlets.

In the present study, survival percentage of 88 per cent, recorded with addition of cocopeat was spectacular when compared with earlier efforts (Sumana, 1998; Ravindra, 2000).

Cocopeat is available in large quantities as a by-product of the coconut industry. In the last few years, cocopeat has been promoted or considered as a substitute for natural peat in potting media. The particular structure of coconut fibers and their physical and chemical properties, make them suitable for container media purposes (Batra, 1985). In fact the use of coconut fiber in European greenhouse production is well accepted as new technology.

Cocopeat contains equal portions of lignin and cellulose and is rich in potassium and the micronutrients Fe, Mn, Zn, and Cu. Due to the high potassium content of the media; a reduction in potassium fertilization has been shown to produce beneficial results (Savithri *et al.*,

1993). However, some studies have shown that it is necessary to increase the nitrogen fertilization for cocopeat grown plants to compensate for N immobilization of the media. Cocopeat has a low Cation Exchange Capacity (21-30 meq./L). So it does not retain cations or buffer against pH change well (Handreck, 1993). Cocopeat has a high water holding capacity and has been traditionally used to improve the physical and chemical properties of soils (Savithri and Khan, 1993). When applied to agricultural soils, coconut cocopeat can improve moisture retention capacity, and increase available nutrient content, infiltration rate, total porosity, and hydraulic conductivity of that soil (Savithri and Khan, 1993; Abad *et al.*, 1995).

Cocopeat particle size distribution was predominantly within the range (0.25–2.5mm) suggested to be optimal for a growth medium (Abad *et al.*, 1992). Evans *et al.* (1996) reported similar particle size for coir peat between 0.5 and 2.0 mm in diameter. Cocopeat and peat had similar bulk density and particle density, but cocopeat had higher total pore space, water holding capacity, pH, electrical conductance, C:N, and nitrogen drawdown index (NDI₇₅ - a measure of soluble nitrogen immobilization by microorganisms) than peat (Arenas *et al.*, 2002; Martinez *et al.*, 1997; Prasad, 1997; and Wever and Leeuwen, 1994). Cocopeat was less acidic than peat and had higher electrical conductance. Both cocopeat and peat exhibited similar organic matter (>90%) and mineral content (<10%) and were within an acceptable range as cited by Abad *et al.* (1992).

5.2 Callus induction using different concentrations of Cytokinins in combination with Auxins and Auxin alone

In most of plant species different explants behaves in a different manner when cultured on same media or same explant when cultured on

different media. And the factors like physical, chemical and physiological play a vital role in determining the response by a particular species.

Calli of different species may vary in their texture, friability and coloration. Even, callus with different appearances and/or morphogenic capacities can be isolated from the same explant. The various chemical and physical factors for responses of callus cultures have been reviewed by Razdan (1995).

5.3 Induction of variations by chemical mutagens

Most of the available variations used in breeding programmes have occurred naturally and exist in germplasm collections of new and old cultivars, land races and genotypes. When existing germplasm fails to provide the desired recombinant, it becomes necessary to resort to other sources of variation. Since spontaneous mutations occur with extremely low frequency, mutation induction techniques provide tools for the rapid creation and increase in the variability in crop species (Maluszynski, 1990). According to Brock (1977), induced genetic variations represent a more efficient source of genetic variability than genepools conserved by nature. Induced mutations have contributed significantly to plant improvement worldwide. The development of efficient *in vitro* culture methods has facilitated the use of mutation techniques for the improvement of many vegetatively propagated crops, where this may be the only effective method for plant improvement (Novak, 1991). *Syngonium podophyllum*, being a vegetatively propagated crop, is highly suitable for this method of induction of variations.

5.3.1 The choice of doses of mutagens

The mutagen used for induction of variations in the present investigation was ethyl methane sulphonate (chemical mutagen).

The dosage of mutagens to be used in mutation induction experiments must be selected after preliminary experiments to characterize the responses produced by a given kind of mutagen. The *in vitro* cultures of *Syngonium podophyllum* were subjected to concentrations of the mutagen so as to define the lethal and sub lethal doses and those, which have no effect on growth rates. An optimum dosage is one, which allows the survival of 40-60 per cent of the treated population in comparison with the untreated control (Handro, 1981). This could be decided based on the LD₅₀ value for the mutagens. Accordingly, the LD₅₀ value was calculated in the case of microshoots for EMS.

5.3.2 Concentrations of ethyl methane sulphonate (EMS)

Higher concentrations of EMS proved to be best for microshoots. Based on the survival percentage of the EMS treated microshoot even at a higher concentration of 0.5 per cent, a 60 per cent survival was noticed. Hence to standardize the LD₅₀ an experiment was designed with a higher concentration of EMS between 0.5 to 2 per cent. A higher concentration of EMS between 0.5 and 2 per cent was considered as LD₅₀ for microshoots. Hence, the concentration of EMS used for induction of variations were 0.01 per cent, 0.05 per cent, 0.5 per cent, 1 per cent, 1.5 per cent and 2 per cent.

5.3.3 Effect of EMS on morphological characteristics of regenerated shoots

Treatment of the plant material with EMS caused a variation in the response of the shoots with regard to the morphological characteristics. This was more prominent when the EMS concentration was 0.5 and 1.0 per cent. This may be due to the genetic changes caused by the mutagen. EMS, an alkylating agent, reacts with DNA by alkylating the phosphate groups of the purine and pyrimidine bases. They act by adding ethyl

group to guanine, which can thus behave as a base analogue of adenine, leading to pairing errors. EMS often induces point mutations with very little damage to the chromosomes (Amano and Smith, 1965).

5.3.3.1 Characteristics of shoots regenerated from EMS treated microshoots

In general, EMS treatment negatively affected the number of shoots produced and length of shoot, when compared microshoots which were untreated. The damaging effects of EMS on the tissue activities of plants, probably owing to the inactivation of cells consequent to mitotic disturbances or chromosomal aberrations could be the reason for the observations made in the present study (Hentrich and Glawe, 1982).

Microshoots when treated with EMS at 0.5 and 1 per cent displayed a poor root development with respect to the number of roots and length of roots.

5.3.4 Morphological variations noticed

An analysis of the results as a whole, suggested that greater variations were noticed in leaf morphology including reduction in size, length, curling of leaf. Few leaves turned albenic (reduction in chlorophyll) and this was more evident at higher level of EMS. Reduction in chlorophyll content and cultures turning albenic was observed when microshoots were treated with EMS. Lower concentration of EMS (0.5 per cent) did not induce variation in chlorophyll color, but with the increase in EMS concentration (1, 1.5 and 2 per cent) changes in chlorophyll color such as microshoots turning light green, albenic were seen. Microshoots showing deficiency of chlorophyll was high and they fail to turn normal green in course of time. Stunted growth was observed when microshoots were treated with higher concentration of EMS.

The chlorophyll mutations observed in this study occurred at a very less frequency, variations were due to some temporary physiological disturbances (Raju *et al.*, 1980). Chemical mutagens have a higher efficiency and output of mutations if the duration of treatment and concentration are well adjusted (Anon, 1973).

Abnormalities following mutagenic treatments might be due to chromosomal aberration or a change in the route of growth regulator synthesis, disturbance in the metabolism or the accumulation of free amino acids (Gupta *et al.*, 1982). Raju *et al.* (1980) ascribed these variations to physiological disturbances.

The results indicated that EMS was efficient in inducing morphological mutations. This higher efficiency of EMS may be because of the less amount of damage to the tissues caused by it. Gaul *et al.* (1972) reported that high efficiency of mutation is obtained only when the mutagenic effect greatly surpasses the damage to the cells.

The variations such as stunted growth, reduced leaves and slight change in leaf colour were observed in C₃ generation. So probably the variants obtained were solid mutants. Exploitation of these valuable variants may generate new utilities in landscape and indoor gardening as well.

5.4 Future line of work

Based on the results obtained from the present study, it would be worthwhile to pursue further studies on the following lines:

- i. EMS treated *Syngonium* explants were subcultured for three generations and explants showed stable variation. However, there is a need to investigate inheritance of variations for some more generations before exposing to *ex vitro* condition.

- ii. In the present investigation only morphological variations were recorded through visual observations. And there is a need for Genetic level of characterization of the variants using DNA analysis, employing different methods like RFLP, AFLP and RAPD etc.

- iii. In the present investigation *Syngonium* explants were treated only with chemical mutagens. Comparative studies using chemical and physical mutagens may result in some more valuable variants and which can be exploited for commercial purpose.

Summary



VI. SUMMARY

The experiment on *In vitro* mutation studies in *Syngonium podophyllum* were conducted at the Plant Tissue Culture Laboratory of the Division of Horticulture, University of Agricultural Sciences, G.K.V.K. Campus, Bengaluru.

The investigation was carried out for standardizing efficient and reliable micropropagation protocol and induction of variations by subjecting the *in vitro* cultures chemical (ethyl methane sulphonate) mutagens.

Shoot proliferation was enhanced by addition of growth regulators. Maximum number and length of shoot was noticed with BAP compared to control. Maximum number of shoots (9.5) was obtained with 20mg/l BAP. However, with the increase in concentration of BAP reduction in length of shoot was observed.

Maximum shoot length (16.6 cm) was recorded with 1mg/l BAP. However, the same treatment was recorded minimum number of shoots (4.7) per explant compared to all other treatments.

BAP at different concentrations has no influence on total number of leaves. However, treatment with 10mg/l was recorded maximum (3.9) number of leaves.

Widths of leaves were influenced by concentration of BAP. With increase in concentration of BAP gradual decrease in widths of leaves were observed. Treatment with 1mg/l BAP was recorded maximum leaf width (2.4cm) as compare to all other treatments.

Maximum numbers of roots (2.8) were produced on media supplemented with BAP. Roots produced with 20mg/l BAP were very

thin, with reduced root length and were brittle, while, roots produced with 1mg/l BAP were thick and flexible.

Among the treatments, maximum number of roots per shoot (2.8) was observed with BAP at 20mg/l. However, length of roots was recorded maximum (16.6) with 1mg/l BAP, when compared with that obtained with 20mg/l BAP (6.5cm).

Coco peat and vermi compost hardening media resulted in 100% survival of *in vitro* plantlets. The survival percentage of plantlets during hardening in sand and mixture of vermiculite+ perlite (1:1) were 40 to 60 per cent.

The mutagenic treatments generated substantial magnitude of variability for the various morphological characteristics of the plantlets. The optimum concentration of EMS was found to be between 0.5 to 2 per cent for microshoots. Microshoots treated with 1per cent EMS generated lot of variations in leaf morphology.

EMS treated shoots were recorded minimum shoot proliferation as compared to control. However, among the treatments 1 per cent EMS was recorded maximum (1.8) number of shoots and maximum shoot length (0.9cm) at C₁ generation.

Stunted growth and reduced leaf width was noticed among EMS treated explants. Treatment with 2 per cent EMS was recorded very small (0.4cm) leaves compared to all other treatments.

At C₃ generation also EMS treated explants were shown poor shoot proliferation compared to control. However, treatment with 2 per cent EMS was recorded maximum number of shoots (4.0) per explant. Whereas treatment with 1per cent EMS was recorded maximum shoot length (1.1). At all levels of EMS treatment stunted growth was noticed.

Maximum number of leaves (10.8) was recorded with 2 per cent EMS. Width of leaves was recorded maximum with 1.5 per cent EMS.

Total number and length of roots were reduced drastically compare to control. However, among the treatments 1.5 per cent EMS was recorded maximum number of roots (4.1) and maximum length of roots (3.9cm).

The effect of EMS treatments on microshoots also manifested variations in leaf morphology of the regenerated microshoots. The variation included was crinkling of leaves, twisting and reduced leaf lamina. Crinkling and curling of leaf was more evident, when microshoots were treated with 1.5 per cent and 2 per cent EMS.

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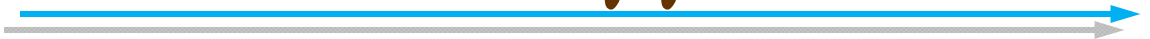
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*Originals not seen

Appendices



APPENDIX

Composition of Murashige and Skoog (MS) basal media (Murashige and Skoog, 1962)

| Components | Concentration (mg L ⁻¹) |
|-----------------------------------------------------|-------------------------------------|
| Macronutrients | |
| KNO ₃ | 1900 |
| NH ₄ NO ₃ | 1650 |
| CaCl ₂ ·2H ₂ O | 440 |
| MgSO ₄ ·7H ₂ O | 370 |
| KH ₂ PO ₄ | 170 |
| Micronutrients | |
| MnSO ₄ ·4H ₂ O | 22.3 |
| ZnSO ₄ ·7H ₂ O | 8.6 |
| H ₃ BO ₃ | 6.2 |
| KI | 0.83 |
| CuSO ₄ ·5H ₂ O | 0.25 |
| Na ₂ MoO ₄ ·2H ₂ O | 0.25 |
| CaCl ₂ ·2H ₂ O | 0.25 |
| FeSO ₄ ·7H ₂ O | 27.8 |
| Na ₂ .EDTA | 37.3 |
| Vitamins | |
| Myo-Inositol | 100 |
| Thiamine HCl | 0.1 |
| Nicotinic acid | 0.5 |
| Pyridoxine HCl | 0.5 |
| Glycine | 2.0 |