IN VITRO TECHNIQUES IN RELATION TO INDUCED MUTATIONS IN GROUNDNUT

BY

K. ARYA, B. Sc. (Ag.)

THESIS

Submitted in partial fulfilment of the requirement
for the degree of
MASTER OF SCIENCE IN AGRICULTURE
Faculty of Agriculture
Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL BOTANY
COLLEGE OF AGRICULTURE
VELLAYANI, TRIVANDRUM

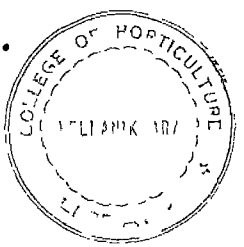
1987

DECLARATION

I hereby declare that this thesis entitled 'In vitro techniques in relation to induced mutations in Groundnut' is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Vellayani,
4-5-1987.

ARYA. K.



CERTIFICATE

Certified that this thesis entitled: 'In vitro techniques in relation to induced mutations in Groundnut' is a record of research work done independently by ARYA. K. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

Dr. N. KRISHNAN NAIR,
Chairman,
Advisory Committee,
Professor of Agricultural Botany,
College of Agriculture.

Vellayani,
4-5-1987.

APPROVED BY:

CHAIRMAN:

Dr. N. KRISHNAN NAIR

MEMBERS:

Dr. Dr.D.Chandramony , Chandramony.D

Dr. S. RAMACHANDRAN NAIR

Dr. S. K. NAIR

EXTERNAL EXAMINER.

Ramachandran

Prof (Dr) K. Ramachandran
Dept. of Botany
Uty of Kerala
Kariavattom, Trivandrum.

ACKNOWLEDGEMENTS

I am extremely indebted to Dr. N.Krishnan Nair, Professor and Head of the Department of Agricultural Botany, College of Agriculture, Vellayani, for the immense interest he has shown in this study. He has been a source of valuable advice, constructive criticism and constant inspiration throughout the period of this study. Words could never adequately express the profound gratitude I have, for his constant help and guidance.

It gives me immense pleasure to acknowledge the valuable services of Dr. P.D. Vijayagopal, Professor of Agricultural Botany, who has helped me considerably during the entire phase of this study. I sincerely thank Dr. S. Ramachandran Nair, Professor of Horticulture and Dr. S.K.Nair, Associate Professor of Plant Pathology, for going through the manuscript and making useful corrections.

My heartfelt thanks are also due to Dr. A.N.Namboothiri, Director of Tropical Botanic Gardens and Research Institute, Trivandrum for permitting me to conduct the study under his supervision making use of the facilities at the Tropical Botanic Gardens and Research Institute. I also wish to thank Dr. P.N.Krishnan, Smt. P.G. Latha and

Miss. C.G.Sudha, Scientists of the TBG & RI, for their keen interest and sustained enthusiasm and help in the project upto its completion.

My sincere thanks are also due to Sri.S.Govindan Potti, Fair Copy Superintendent, Directorate of Health Services and Sri. L.R.K.Potti, Senior Office Superintendent, for their help in the preparation of this manuscript.

Lastly, but not the least, I wish to thank the Kerala Agricultural University and the Dean, College of Agriculture, Vellayani, for the fellowship and for providing all the necessary facilities during the period of the Post-graduate programme.

ARYA K

• • •

C O N T E N T S

.....

LIST OF TABLES

. . . .

LIST OF ILLUSTRATIONS

Plate 10 Growth rate of plantlets as on 40th day of culturing in embryos extracted at time of harvest and treatment after 3 days of culturing.

Plate 11 Growth rate of plantlets asen 40th day of culturing proembryos having 20 days development and treatment after 3 days of culturing.

Plate 12 Growth rate of plantlets as on 40th day of culturing in proembryos having 30 days development and treatment after 4 days of culturing.

Plate 13 Growth rate of plantlets as on 40th day of culturing in embryos extracted at the time of harvest and treatment after 4 days of culturing.

INTRODUCTION

Induced mutations are the ultimate source of variability for having a positive selection response in crop plants. The exploitation of radiations and radiomimitic compounds for inducing mutations/alterations in the base sequence of DNA is one of the most potent lines of contemperory crop improvement programme. It has been proved beyond doubt that green revolutions to a certain extent is a product of induced mutations. It has also been proved that the mutagens whether it is physical or chemical can be beneficially used for tailoring better varieties of crop plants. It is proved both in seed propagated and vegetatively propagated crops that all morphological and physiological characters within the species boundary and even beyond this can be induced by mutations. Whenever the available genetic variation is exhausted in a population further genetic variation can be created only by induced mutation. Whenever the conventional breeding programmes exhaust the natural variability the possibility offered by mutagenic agents for creating variability has got considerable interest. Larger genetic variation means the possibility of greater responses to selection and higher chances of improvement. Gregory (1956) while working on induced

mutations in peanut remarked that radiation is as efficient as hybridisation in supplementing genetic variability for selection and in certain situations induced mutations are the only solution for the problems faced by the breeders. Brock (1971)clearly demonstrated that we can induce any mutation that occured naturally and probably many which have either never occured naturally or have been lost from natural population.

It is a well recognised fact that induced mutations can yield larger variability in crop plants as is evidenced by the reports made by induced mutation breeders. But the large number of created variability is being eliminated by the various factors operated in the sequence of mutational events and screening processes. One of the major factor which plays a decisive role in elimination of mutated sectors is through diplontic selections. Majority of mutation breeding experiments have been initiated with complex multicellular tissues, either with seeds in sexually propagated plants or with buds or cuttings in asexually propagated species. In all cases plants will develop 'chimeras', which will lead to diplontic selection and finally elimination of

mutated sectors as reported by several workers. Swaminathan et al (1970) and Goud et al (1970) reported that plants with drastic induced variations have been eliminated due to a vigorous diplontic selection. Various reports have been made by too many workers that the dormant embryo consists of more than one cell. Swaminathan (1966) infered that the dormant rice seed when exposed to mutagenic treatment had eight to ten initials whibh would give rise to tillers. Kawai (1963a) reported that following treatment with heavy doses of radiation, the same mutations was distributed over almost all the years in certain M_1 plants, indicating that all the panicles originated from a single primordia. In this case the experimental evidences clearly infered that all primordia except one might have been killed by severe radiation injury. These reports and others based on detailed analysis in mutation frequency and segregation ratios clearly showed that sector size plays a major role in induced variability. In general smaller the number of initial cells greater will be the probability for mutation to occur. So many experimental procedures have been adopted to reduce the number of cell involvements during the process of induced mutations to yield solid mutations and to minimise diplontic

selections. Cell culture, meristem culture and pro and embryo culture promises a better scope for getting maximum induced variability in this regard.

Recent advances made in cell/meristem/tissue culture research appear to offer considerable promise in terms of induced mutations for crop improvement programmes. Of all the various factors, the most determinate one is that it is easy to handle a large number of irradiated materials within a small volume. Or in other words induced mutations in relation to tissue culture economise space. In addition, embryo/tissue culture can be carried out at any time of the year, whereas growing plants in field may be restricted to particular season. It is also an advantage that selection for a particular trait may be done at the tissue level. It has been reported that any part of a plant can be induced to form callus, including embryos - mature or immature, root, leaf or stem sections, anthers and pollen grains; though seeds are the easiest material to start with. Embryo culture seems to be the second best easy material for induced mutagenesis. The cell or meristem culture which involves the formation of callus results in the creation of somaclonal variations, thereby the

assessment of induced variability can never be properly carried out. Taking into account of all these factors, in the present investigation to standardise the techniques of induced mutagenesis by using proembryos and embryos having different maturity were taken up. The main objectives of the investigation include:

1. To standardise a suitable culture medium for an early and maximum embryo take and differentiation in groundnut.

2. To standardise a very successful surface sterilisation technique for proembryos and embryos of groundnut.

3. To assess the influence of maturity of the proembryos and embryos for tissue culture techniques in relation to induced mutagenesis.

4. To standardise the best technique of mutagen treatment while adopting embryo culture techniques.

5. To assess the optimum dose requirement for maximum embryo set and differentiation to yield maximum mutations.

REVIEW OF LITERATURE

Tissue culture techniques in Crop Improvement programmes:

The basis for plant tissue culture is the concept of totipotency underlined in the cell theory by Schleiden (1838) and Schwann (1839). Now a days tissue culture has become an important tool for use by the commercial plant propagators (Zimmerman, 1981). When the micropropagation aspects of tissue culture are combined with approproate indexing and explant establishment techniques, then plant tissue culture emerges as a potent research tool in fundamental and applied aspects of Agriculture, Horticulture, Forestry and drug manufacture.

The pioneering experiments of White (1934, 1939), Gautheret (1938), Nobecourt (1939), Reinert (1958), Steward et al (1958) and Morel (1960) are often cited as the landmarks in the developmental phase of plant tissue culture. The first major completelyddfined medium was developed by Murashige and Skoog (1962). To obtain optimal growth and to induce the nutrient media is often supplemented with natural extracts like cocoonut milk, yeast extract, fruit juice etc. (Steward et al 1958; Hilderbrandt, 1963; Vasil and Hildebandt 1966; Butenko, 1968; Street, 1969). Majority of the nutrient

media now being used are chemically defined and can be adopted to various uses by slight modifications (Vasil and Vasil, 1980).

The technique of artificial culturing of embryos has been employed in growing interspecific hybrids, intergeneric hybrids, monoploids and polyploids (Heinz et al, 1977). The procedure is based on the principle of producing an abundant supply of nutrition to the developing embryos so that they are not hindered by selection pressure and competition. Emrgyo culture technique provides a way to produce viable mutants in several crop plants (Ghosh, 1982).

The best commercial application of tissue culture techniques has been in the production of clonal plants at a very rapid rate compared to the conventional methods. Such plants are reported to grow faster and to mature earlier than seed propagated plants (Vasil and Vasil,1980). In vitro clonal propagation procedure have been standardised for a number of important plant species including sugarcane, cotton, maize, rice, tobacco, grasses and several horticultural crops. Excellent reviews on the subject have been made by Murashige (1974, 1978). Vasil and Vasil (1980) Hu and Wang (1983), Styer and Chin (1983)

and Sharp et al (1984). All species in which organogenesis and plant formation can be achieved *in vitro* may not be suitable for large scale clonal propagation (Vasil and Vasil, 1980). Genetic variation may sometimes take place in the clonal population of plants as a result of aneuploidy and polyploidy introduced during cell proliferation *in vitro* (Murashige, 1974).

Morel (1960) could standardise a procedure to render the orchid, (*Cymbidium*) free of virus and thereby *in vitro* clonial multiplication gained momentum. The greatest success using this technique has been achieved in herbaceous species. This success has been partially due to the weak apical dominance and strong root regenerating capacity of many herbaceous plants (Hu and Wang, 1983). The role of cytokinins to release the lateral buds from apical dominance was reported by Wickson and Thimann (1958). This finding was effectively utilised for large scale clonal propagation in strawberry by Boxus (1974).

Yie and Leaw (1977) established papaya seedlings shoot tips *in vitro* and obtained proliferative growth on MS medium containing 0.3 /^{u}M IAA and either 2.3 /^{u} M Kinetin or 2.2 - 4.4 /^{u} M BA. The applicability of the

excised shoot tip culture techniques to a number of banana clones was assessed by de Guzman et al (1976).

Although the first report on enhanced release of axillary buds was made by Custer et al (1980) , the pioneer work on application of in vitro techniques on coffee (Coffea SPP) was reported by Staritsky (1970). Nodal explants of asceptically grown 3 month old coffea arabica plants cultured in MS medium with BA and IAA developed shoots at the average rate of 2.2 per node after two to five weeks.

Meristem culture techniques, as first applied to cassava (Manihot esculenta) cultivars employed the MS medium containing vitamins as in the B5 medium (Gamborg et al, 1908), supplemented with NAA, BA and GA at 1.0, 0.5 and 0.1 μ M, respectively (Kartha et al 1974). GA had a stimulating effect on shoot growth from meristem culture as was reported by (Nair et al, 1979).

Vegetative propagation of various palm species through tissue culture methods was attempted only during the last decade. Techniques have been standardised for oil palm (Jones, 1974; Corley et al, 1976, Rabechault and Martin, 1976), date palm (Tisserat, 1979; 1981) and

coconut (Blake and Eauwens, 1981; Raju et al 1984) Blake and Eauwens (1980) obtained shoot like structures with no intervening callus stage; but these did not develop into plantlets.

Skoog and Miller (1957) reported a high concentration of auxin and a low concentration of cytokinin promoted abundant cell proliferation with callus and low auxin and high cytokinin concentration in the medium promoted shoot morphogenesis. Evans et al (1981) reports that somatic organogenesis is useful in inducing genetic variability or to recover pre existing natural genetic variability.

Khan and Ghosh (1984) developed plantlets in Cicer arietinum through tissue culture, 3-5 shoot buds developed from each cotyledonary node of young seedlings raised in Gamborgs B5 medium supplemented with BAP 1.0 mg/lit and these multiple buds developed profuse roots in B5 medium with NAA 0.5 mg.

A wide range of variation in chromosome numbers was noted in the regenerated plantlet from shoot meristem culture of Vigna sinensis (Ahmed and Ghosh; 1982) Excised shoot tips of Vigna mungo and Vigna radiata cultured on Gamborgs basal medium developed shoots and roots (Goel et al, 1983). Techniques and media for

culture of Soybean, pea, vigna embryos from mature and immature seeds were tested and successfully given by Vagera and Hanackara, (1980).

Callus and root formation from sugar beet anthers was induced on the Linsmaier and Skoog medium containing various growth substances and several saccharose levels. (Rogozinska and Goska, 1975). The regeneration potential of shoot apical meristems of Soybean, cowpea, peanut, chickpea and bean was studied on agar solidified MSmedium supplemented with various concentrations of BA and NAA alone or in combination (Kartha et al., 1981).

A method to culture immature embryos of Populus deltoides in vitro was successfully given by Kouider et al (1984). Multiple shoots were formed when cultured on MS medium.

The possibility of clonal propagation of banana through tissue culture was explored. Shoot tips isolated from the rhizomes were found to be suitable material for plantlet production in vitro. Excised shoot tips with the youngest leaves produced only one plantlet. (Doreswamy et al, 1983).

Tissue culture techniques in Groundnut:

Spielman et al (1979) produced fertile inter-specific hybrids in large numbers despite the ploidy differences between some wild species and the cultivated groundnut. More recent reports of plant regeneration in tissue and organ cultures creates hopes for regeneration from single cells and application of these techniques to some of the refractory problems in groundnut breeding (Sastri et al - 1981 and Mroginski et al 1981).

Mroginski and Fernandez (1979, 1980) succeeded in inducing plantlets in anther cultures. The plantlets have been obtained from anther cultures of Arachis pusilla and Arachis Sp. P.I.No.276233. Pittmann (1981) cultured peanut anthers, cotyledons and leaflets under in vitro techniques. When peanut cotyledons were grown in media with IAA and BAP or NAA, 2,4-D and BA, callus were produced plantlets were regenerated from anther cultures but no plants were recovered.

Meristem culture:

Production of plants from meristem cultures and their survival after freezing for 28 days is utilised for

generation of disease free populations, long term storage and international transfer of germplasm (Sastri et al; 1981, Kartha et al, 1981 and Bajaj, 1979).

Plants of Arachis hypogaea and Cicer arietinum regenerated from the freeze preserved cultures of excised meristems were transferred to soil, grown to maturity and harvested. The seeds showed normal germination and no change in number of chromosome was observed (Bajaj, 1983). Green immature leaflets (2-5 mm in length from shoots of germinated seeds or greenhouse grown plants) from seven species of Arachis were cultured aseptically on a medium composed of Murashige and Skoog salts. Gamborge B5 vitamins, 0.8% Difco agar with 1 mg/lit each of NAA and BA and organogenesis occured in the leaflet cultures and plantlets were recovered,(Pittmann et al ; 1983). Leaflets, epicotyls, hypocotyls, primary and secondary roots and cotyledons excised from seven day old seedlings of four cultivars were cultured on MS medium supplemented with 2, 4-D, IAA and NAA at various concentrations in combination with Kinetin and plantlets were regenerated from callus (Narasimhulu and Reddy, 1983).

Embryo culture:

Embryo culture technique is a tool for securing viable mutants in many legumes (Ghosh et al, 1979).

Culture of embryos in incompatible crosses in Arachis was suggested by Gregory (1946) but was not taken up. Gynophore development and pod initiation and development from these incompatible crosses have been induced by hormone treatments (Sastri and Moss, 1982) and Anonymous 1981). Embryos of Arachis spp was cultured to produce interspecific hybrids (Bajaj et al 1982) Embryos of Arachis hypogaea M 13 (2 n = 4 x = 40), Arachis villosa (2 n = 2x = 20) and their hybrid were cultured in vitro and plants were successfully transferred to soil. The hybrids had 2 n = 3 x = 30 and resembled Arachis hypogaea. In vitro plantlet regeneration of Arachis from embryo axes and cotyledon segment was successfully done (Atroya et al ;1984). In ovule culture of embryos has been useful in a number of plants where the dissection and culture of proembryos is difficult (Raghavan, 1976). Martin (1970) cultured ovules from aerial gynophores of Arachis hypogaea and obtained plants, using MS with Heller's microelements, vitamins, aminoacids,

caseun hydrolysate and kinetin. Sastri et al (1980) although failed to culture ovulues succeeded in in vitro pollination of ovulues.

Ziv and Zamski (1975) were the first to successfully culture the terminal segments of aerial gynophores and to obtain pods. Rangaswamy et al, (1965) reported root formation from callus in pericarp cultures.

Induced mutation in crop improvement:

The idea of induced mutation in quite old. It was proposed first by de Vries as early as 1901 and the term mutation was derived from the latin word 'mutare' to denote change. X-ray was the first radiation to be applied to cells and chromosomes and subsequently the installation of cobalt and cesium sources made gamma rays also available. The discoveries of Muller (1927) in Drosophila and that of Stadler in (1928) in barley and maize opened up a new field of research which has significantly advanced our knowledge in genetics. In addition to several ionizing radiations, a number of chemical mutagens also produce mutations in plants when applied singly or combined with other chemicals and in succession or simultaneously with

with radiation. (Ehrenberg et al., 1971, Konzak et al ; 1965.

Chemical mutagenesis was tried by Shiemann (1912) with some encouraging results. The experiments of Auerbach and Robson (1947) with Drosophila using sulphur and nitrogen mustards brought the usefulness of chemicals for the induction of mutation. Freese (1963) classified chemical mutagens as base analogus substitutes, dyes, acids, metals and alkylating agents. In higher plants the last group, especially EMS has proved to be very effective. The relatively low toxic and high genetic effects of EMS (Gaul, 1961) and its high mutagenic effectiveness as well as efficiency in higher plants (Konzak et al; 1965) demand attention for enhanced practical application.

Irradiation resulted in delayed germination and reduced germination percentage at higher doses as observed by Shirshov and Shain (1966) using gamma radiation in field beans; in pea using gamma rays (Sidorova et al., 1966; Maslov and Stepanova, 1967) and by Alikhan et al (1973) in red gram with EMS and gamma rays. Louis and Kadambavanasundaram (1973) found that

in cowpea, germination was reduced by higher doses of gamma rays and there was complete suppression of germination in 60 Krad and above. In groundnut, Reddy et al (1977) found that germination ranged from 24 to 57 percent.

Reduced germination was noticed with higher doses of chemical mutagens like EMS, MMS, ethyleneimins etc. in Pisum and groundnut (Blixt and Gelin, 1965). In pea Wellensiek (1965) observed that germination decreased rapidly with increase in concentration of EMS.

Several workers have studied the relationship between doses of mutagen and survival percentage. In black gram Jana (1964) observed a direct relationship between doses of neutron and lethality. Teodoradze (1966) noticed a decrease, in Soybean and frenchbean. Constantin et al (1976) observed that in populations of Soybean grown in green house the survival of plant decreased with increase in dose of EMS. The percentages of healthy seedlings and full grown plants decreased rapidly with increasing concentration of EMS in pea (Wellensiek 1965). NMU, DES and NEU decreased the survival rate in

pea (Tarasenkov 1969). In cowpea at 0.25 percent EMS and at 0.025 percent MMS the survival percentage was drastically declined (Narsinghani and Kumar,1976).

Constantin et al (1976) reported that in Soybean, seedling height decreased asthe dose of gamma rays, neutrons, EMS and DES increased. With EMS, MMS and EI in Pisum, there was a considerable reduction in height (Blixt and Gelin, 1965). Blixt et al (1966) reported that intervarietal differences in pea, following varying treatments with EMS, were shown by reduced seedling growth.

Chemical composition in induced mutant groundnut was studied by Gadgil and Mitra (1983). Twenty one cultures were obtained from X-ray and gamma irradiation of Spanish improved either as direct mutants or by selection amongst hybrids from intermutant crosses. Some cultures showed a 2.3% increase in oil content over Spanish improved large seeded cultures contained 5.6 - 8% Sucrose compared to Spanish improved in 3.6%.

Preferential segregation of two allelic mutations for small leaf character in groundnut was studied and reported by Patil and Mouli (1984). Two radiation

induced small leaf mutants in Spanish improved had more than 50% reduction in leaf size associated with a/n increased number of imparipinnate leaves in one mutant and light yellow flower color in the others. Genetic studies demonstrated that the two mutations were allelic and controlled by recessive factors designated S1-imp and S1, respectively. Segregation ratios in the M_4, M_5 and M_6 indicated a lower frequency of mutants than expected which is attributed to preferenial segregation in favour of normal leaf size.

Ethidium bromide and acriflavine were superior to in mutagenic activityto X-rays, EMS and DES in groundnut (Levy, 1976). The frequent mutations induced affected chlorophyll and height.

Radiation induced mutants produced large yield with large kernals and high oil content. This was reported by Patil in (1977).

Mutants DP 1 and DP 2 of Arachis hypogaea which have a compact habit, large kernels and large pods and show early and mid early maturity, respectively were derived from the late maturing variety 41 C, which has a spreading habit and kernels and pods of medium size. In trials over three years these out yielded the predominant variety (Sinha and Rahman, 1979).

A faint orange flower coloured short statured mutant in groundnut was identified by Desale (1985). This mutant variety showed both qualitative and quantitative changes with respect to the cultivated varieties and hence be suggested that pleiotropy is involved and the overall reduction in size may be related to chromosome number or structure. The faint orange colour could be a useful genetic marker for maintaining varietal purity.

CO_2 Mutant 3 released in 1984 was derived from Arachis hypogaea cv POL-1 treated with the mutagen ethylmethane sulphonate. This was reported by Sivaram et al (1985). Due to increased secondary branching and more pods/plant, it has a higher pod yield than POL-1 with a higher shelling outturn.

The performance of gamma ray induced mutants of groundnut in Sri Lanka was studied by Pathirana (1985) and he reported that Mutant 180/21 and 180/22 from the 5 Krad treated cv GN 13 consistently outyielded the control variety 45 during three seasons and mutant 980/30 from the 15 Krad treated cv Vietnam gave a 5% lower yield but higher hyields than 45 in subsequent seasons. All three mutants had better shelling percentages, larger kernels and more 2 seeded pods than the

recommended cultivars.

Seeds of groundnut cv, NC AC 12, NC AC 10, NC AC 2144, M 13, GDM and PI 259747 were irradiated with 5,10,15 and 20 KR gamma rays and grown surrounded by a pollen parent. The effect of irradiation on pollen sterility and other quantitative characters was studied in M_1. Pollen sterility increased linearly with increased dose in each cv. There was negative correlation between inherent pollen sterility and percent increase in sterility. The 15 KR was the most effective dose for inducing a high level of pollen sterility without changing the other characters. The correlation between pollen sterility and cross pollination was positive. This was reported by Dutta et al; (1986). Joshua and Bhatia (1983) isolated two large seed mutants in groundnut. They are TG 1 obtained from Arachis hypogaea cv Spanish Improved by X-irradiation TG 19, selected from the A.hypogaea cross TG 17 x TG 1.

Ramanathan (1984) developed five induced high yielding mutants in groundnut using 30 krad gamma irradiation of 40 MM EMS in TMV 9 and using a combination of 20 krad gamma irradiation and 40mM EMS in Ah. 7911.

Tissue culture techniques in relation to Induced mutagenesis

Among the various techniques now available in tissue culture, the use of tissue culture induced mutagenesis for plant modification is a relatively new and very attractive area of research. Individual plant cells or pollen grains growth in cultures can serve as mutable material which can be grown into entire plants (Yoshida and Ogawa, 1983). Bajaj (1971) studied the direct and indirect effects of gamma irradiation on the seeds, seedlings, callus tissue cultures, excised roots, ovules and embryos and reported that callus tissue cultures are more radio resistant than the intact seedlings. George and Rao (1979) studied the in vitro regeneration of mustard plants on cotyledon explants from non-irradiated, irradiated and mutagen-treated seed. Shot formation in cotyledon explants of mustard was obtained in MS medium with NAA. Complete plants were obtained when shoots were rooted in MS medium. EMS had an inhibitory effect on shoot regeneration and gamma rays in doses above 2 KR suppressed shoot regeneration but stimulated callus growth.

An in vitro propagation of two genotypes of Begonia hiematis was achieved through adventitious shoot

formation on cultured leaf disc explants and subsequent transplantation to soil of explant parts with adventitious shoots. After irradiation of detached leaves with different doses of X-rays and two cycles of adventitious shoot formation on *in vitro* cultured leaf disc explants, plantlets were produced. About 30% of these plants was mutated with respect to the colour, size and form of the leaves and flowers. The great majority of the mutants (98.5%) proved to be non chimeric (Roest *et al.*, 1980).

Skirvin (1977) suggests a novel approach to intraclonal plant improvement which will utilize both the natural and induced variation associated with clonally propagated plants through various *in vitro* and *in vivo* procedures. Many plants obtained are of single cell origin and hence of pure mutant type avoiding the chimerism phenomenon.

Harten *et al* (1980) investigated what potentialities for mutation breeding of potato are offered by using adventitious sprouts that arise *in vitro* from leaf explants after X-irradiation. Mutation frequency and chimerism were studied in subterranean and aerial parts in three vegetative generations. Plants obtained

from irradiated series produced a very high mutation frequency, a wide mutation spectrum and a very low rate of chimerism.

X-irradiation of tuber eye-pieces of the potato cultivar Burmania (B - 173) has resulted in a plant with aberrant leaves which has been designated as " ivy leaf " and subjected to further investigations and found that ivy leaf is dominant without any pleiotropic effects. This was reported by Harten et al (1973).

Large numbers of protoplasts showing reproducible high plating efficiency can be isolated from in vitro propagated, haploid and diploid, plants of Nicotiana sylvestris. Their successful use in the selection of biochemical mutants depends on culture medium, cell density, age of cells at selection etc. (Negrutin and Muller, 1981).

Ghosh (1982) reports that tissue culture can be used as a tool for raising viable mutants, through embryo cultures in Vigna sinensis and Vigna radiata and also according to him chromosome instability in tissue culture of crop plants like Vigna sinensis, Pisum sativum

Triticum aestivum cv Sonalika makes helps in creating variability through induced mutagenesis. The chromosome disturbances which can be created by a mutagen will become easier if instability is available either in the genic or chromosome level.

MATERIALS AND METHODS

The present investigation to standardise the *in vitro* culture techniques for induced mutagenesis in groundnut (Arachis hypogaea L) was carried out at the Plant Tissue Culture Laboratory attached to Tropical Botanic Gardens and Research Institute, Kumarapuram, Trivandrum during 1985-86. The project also envisages to analyse the *in vitro* regeneration of embryos and proembryos and performance of plantlets in culture media. The details of the various procedures adopted are presented hereunder.

MATERIALS

The materials for the present investigation used were proembryos and embryos (ex-plants) of groundnut (Arachis hypogaea L) having four different maturities. They include:

(1) Proembryo having 20 days development,

(2) Proembryo having 30 days development,

(3) Embryo from fully matured nut, immediately after harvest and

(4) Embryos from dry nut, one month after harvest.

The mutagen used was Ethylmethane sulphonate (EMS) at 0.125%, 0.250%, 0.500% and 0.750% concentrations. The media tested were White's medium, Murashige-Skoog medium and Modified Murashige-Skoog medium.

METHODS:

(A) Raising the crop:

The land was well prepared by digging twice and properly ploughed and levelled. Furrows and ridges were taken with provision for good drainage facilities. Uniform seeds were collected from well dried nuts after one month of harvest. The seed nuts were selected out and sown in ridges at 30 x 15 cm spacing. The plants were maintained properly following the package of practices recommended for the crop by Kerala Agricultural University. Special care was taken to provide uniform field condition to the crop till harvest. The crop was raised as an irrigated one by providing irrigation as and when required. All the after cultivation practices including earthing up just prior to flowering was provided uniformly for all the plots. About thousand plants were raised for the extraction of proembryos and embryos at different developmental phases. The date of pegging in all the plants were properly labelled to know the different maturity days of the nuts.

(B) Extraction of proembryos and embryos:

(a) Proembryo having 20 days development:

On the twentyfifth day of pegging the plants were pulled out carefully by giving proper irrigation and washed

thoroughly in running tapwater. The nuts attained twenty days development based on the date of flowering were carefully removed and again washed thoroughly in water. Two hundred and fifty such nuts were collected and used for the study. The plants were pulled out from a single row leaving the border plants as guard rows. The immature pods attained twenty days development were carefully split open by using fine edged scalpel and forceps and the nuts were separated out from the shell. These immature nuts were thoroughly washed in sterile distilled water for 2 - 3 times and sterilised by using 70 percent alcohol. Again the nuts were washed in sterile distilled water and made ready for proembryo extraction.

b) Proembryo having 30 days development:

On the thirtyfifth day of pegging the plants were pulled out carefully by giving proper irrigation and washed thoroughly in running water. The nuts attained thirty days development based on the date of flowering were carefully removed and again washed thoroughly in water. Two hundred and fifty such nuts were collected and used for the study. The plants were pulled out from a single row leaving the border rows as guard rows. The immature pods attained thirty days development were

carefully split open by using fine edged scalpel and forceps and the nuts were separated out from the shell. These immature nuts were thoroughly washed in sterile distilled water for 2 - 3 times and sterilised by using 70 percent alcohol. Again the nuts were washed in sterile distilled water and made ready for proembryo extraction.

(c) Embryo from fully matured nut:

The fully matured plants based on the changing of the colour of the basal leaves from green to yellow were pulled out carefully by giving proper irrigation and washed thoroughly in running tapwater. The nuts attained full maturity were carefully removed and again washed thoroughly in water. Two hundred and fifty such nuts were collected and used for the study. While pulling out the plants the border plants were left behind as guard rows. The fully matured pods were carefully split open by using fine edged scalpel and forceps and the nuts were separated out from the shell. These nuts were thoroughly washed in sterile distilled water for 2-3 times and sterilised by using 70 percent alcohol. Again the nuts were washed in sterile distilled water and made ready for embryo extraction.

(d) Embryo from dry nut:

The nuts from freshly harvested plants were separated and washed thoroughly in running water to remove the soil, They were then kept for drying and stored for one month. After one month the nuts were washed thoroughly in water and two hundred and fifty such nuts were used for the study. The pods were split open by using fine edged scalpel and forceps and the nuts were separated out from the shell. These nuts were thoroughly washed in sterile distilled water for 2-3 times and sterilised by using 70 percent alcohol. Again the nuts were washed in sterile distilled water and made ready for embryo extraction.

(c) Standardisation of surface sterilisation technique:

Surface sterilization:

Embryo from dry nuts were used for this preliminary investigation. The dry nuts presoaked in distilled water for 24 hours by placing them in a wet muslin cloth were split open by using sterile scalpel and forceps. The embryos were extracted by separating them out from the endosperm and the extracted embryos were washed thoroughly in sterile distilled water. They were dipped in 70 percent ethyl alcohol for 10 seconds. The alcohol treated embryos

were taken out and then they were washed thoroughly in sterile distilled water. These surface sterilized explants by using alcohol and sterile distilled water were kept in mercuric chloride solution of different concentration as is shown in Table 1. The mercuric chloride sterilised explants were planted in MS medium in aseptic condition to study the survival percentages.

TABLE 1

SURFACE STERILISATION TREATMENTS

Sterilant.	Concentration (in percentage)	Time (in minutes)	Survival (in percentages)
Mercuric Chloride	0.05	3	28
		5	40
		10	70
	0.1	3	90
		5	70
		10	60
	0.2	3	20
		5	10
		10	Nil

The survival percentage resulted due to various concentrations of mercuric chloride clearly indicated that to get maximum embryo differentiation and survival, treating the surface sterilised embryos in 0.1 percent mercuric chloride for three minutes was the best technique.

(D) Sterilisation of proembryos and embryos:

The surface sterilised nuts by using 70 percent alcohol and sterile distilled water were split open by sterile scalpel and forceps and the proembryos and embryos hidden inside the endosperm were taken out for further studies. Immediately after extraction, the proembryos and embryos were thoroughly rinsed in sterile distilled water, sterilised in 0.1 percent mercuric chloride for 3 minutes and again washed thoroughly in sterile distilled water before treating with the mutagen wherever it was necessitated direct planting of proembryos and embryos were done the surface sterilised proembryos and embryos with mercuric chloride and sterile distilled water were planted in culture tubes having 15 ml of modified solid MS medium in an aseptic condition by using tissue culture hood provided with laminar air flow. Immediately after planting the proembryos and embryo in culture medium they were arranged in racks provided with fluorescent lamps

in an air conditioned chamber.

(E) Standardisation of Culture medium:

The different culture media tested to standardise the suitable medium for groundnut embryo includes:

1) White's medium
2) Murashige-Skoog medium
3) Modified Murashige-Skoog medium.

The details of the ingredients is presented in Table 2.

TABLE 2

CHEMICAL CONSTITUENTS IN CULTURE MEDIA TESTED (1 litre)

Whites medium.		MS medium		Modified MS medium	
Constituents	Quantity (in gm)	Constituents	Quantity (in gm)	Constituents	Quantity (in gm)
Calcium nitrate	0.200	Ammonium nitrate	1.65	Ammonium nitrate	1.65
Potassium nitrate	0.080	Potassium nitrate	1.90	Potassium nitrate	1.90
Sodium dihydrogen phosphate	0.165	Calcium chloride	0.440	Calcium chloride	0.440
Potassium Chloride	0.065	Magnesium sulphate	0.370	Magnesium sulphate	0.370
Sodium sulphate	0.200	Potassium dihydrogen phos.	0.170	Potassium dihydrogen phosphate.	0.170
Magnesium sulphate	0.3600	Sodium EDTA	0.037	Sodium EDTA	0.037
Ferric sulphate	0.0025	Ferric sulphate	0.028	Ferric sulphate	0.028
		Manganese sulph.	0.022	Manganese sulphate	0.022
Manganese sulphate	0.0045	Zinc sulphate	0.0086	Zinc sulphate	0.0086
Zinc sulphate	0.0015	Boric acid	0.0062	Boric acid	0.0062
		Potassium Iodide	0.00083	Potassium Iodide	0.00083

contd..

TABLE 2 (contd)

Whites medium.		MS medium		Modified MS medium	
Constituents	Quantity (in gm)	Constituents	Quantity (in gm)	Constituents	Quantity (in gm)
Boric acid	0.0015	Molybdic acid	0.00025	Molybdic acid	0.00025
Potassium Iodide	0.00075	Copper sulphate	0.00025	Copper sulphate	0.000025
Sucrose	20	Cobalt chloride	0.000024	Cobalt chloride	0.000024
agar	14			myoinositol	100 mg
		Sucrose	30 gm	Sucrose	30 gm
		agar	14 gm	agar	14 gm

All the chemicals used to prepare the different media were of Analytical Grade from British Drug House (BDH), Sisco Research Laboratories (SRL) and Merck (Sigma). Standard procedures Biondi and Thorpe (1981) were adopted for the preparation of the media. The pH of Whites medium was adjusted to 5.6 and that of MS medium, basal and modified, to 5.8 with the help of a p^H meter. The solidification of media was done by using 14 gm/lit agar (Merck).

Corning brand culture tubes and conical flasks were used for the study. Sterilisation of the media was done at 15 psi for 18 minutes and sterilisation of glass wares (culture tubes and conical flasks) at 15 psi for 30 minutes. All aseptic manipulations were carried out in a laminar air flow chamber.

The embryos extracted out from 24 hours presoaked dry nuts were used for this preliminary investigation. The presoaked nuts were thoroughly washed in sterile distilled water followed by 70 percent alcohol. Embryo extraction was carried by using sterile scalpel, forceps and needle. The embryos extracted out in aseptic condition were treated with 0.1 percent mercuric chloride for 3 minutes and thoroughly washed in sterile distilled water

before planting it in different culture media. The different culture media planted with the surface sterilised embryos were incubated at 26 ± 2° C with a 16 hr photoperiod (1000 lux), supplied with cool day light fluorescent tubes. Twentyfive tubes each were provided for each medium.

TABLE 3

EMBRYO DIFFERENTIATION ANALYSIS IN DIFFERENT CULTURE MEDIA

Medium.	Days taken to embryo differentiation.	Percentage differentiation.
Whites medium	12 days	75%
MS medium (Basal)	10 days	80%
MS medium(modified)	6 days	85%

Analysis on the embryo differentiation presented in Table 3 clearly demonstrated that Modified MS medium gives an early and higher percentage differentiation of embryos compared to basal MS medium and Whites medium. Therefore in the present investigation to standardise techniques in vitro culture methods in relation to induced mutagenesis by using ethyl methanesulphonate,

Modified MS media was selected out as the best medium.

(F) Preparation of mutagen solution:

The chemical mutagen - ethylmethanesulphonate (EMS), on alkylating agent was used as the mutagenic agent. The various concentrations ranging from 0.125% to 0.75% at 0.25% interval was prepared by dissolving the mutagen in double glass distilled water, immediately before use. One hundred cc each of the various concentrations were prepared by taking the solution by micropipette and mixing it with 20 cc of distilled water. The standard solution was adjusted to 100 cc by using standard flasks. The p^H of the solution was adjusted to neutral using sodium phosphate dibasic salt as buffer. The mutagen solution of various concentration was sterilised at 15 psi for 17 minutes. The preparation of mutagenic solution was done at room temperature.

(G) Planting of Explants in Culture Media:

The planting of explants were done in culture tubes or conical flasks, sterilised at 15 psi for 30 minutes. Twenty to twentyfive ml. each of the sterilised and solidified media was taken in culture tubes and conical flasks. The extracted proembryos and

embryos having the prescribed maturity growths were planted into the culture media by using a tissue culture hood fitted with laminar air flow. The explant planted media in tubes or flasks were incubated at 26 ±2°C with a 16 hr photoperiod (1000 lux) supplied by cool day light fluorescent lights. Proper sterilisation of the forceps, scalpel and needles were done before they were used for taking the explants for planting in culture media. As the total number of treatments in the experiment was very high and the experiment was envisaged only to have a preliminary investigation on the standardisation of various techniques, no replication was provided for each treatment. Ten tubes/flasks having two explants each were provided for each treatment. Too much variation in survival and other performances of explants were expected due to the involvement of various techniques of treatments. Hence a full-fledged statistical approach became very difficult.

(H) Mutagen Treatments:

The proembryos and embryos of the different maturity groups were subjected to the following methods of treatment.

(i) Treating the material at the time of planting:

The proembryos and embryos were treated with the mutagen in specified concentrations. The materials were

soaked in mutagenic solution for six hours with intermittent shaking at half hourly interval. The treated proembryos and embryos for six hours were taken out from the solution and washed thoroughly in sterile distilled water and kept in running distilled water for half an hour. The mtagen treated and thoroughly washed proembryos and embryos were surface sterilised with mercuric chloride as was standardised for the present investigation. The surface sterilised proembryos and embryos were then washed thoroughly in sterile distilled water to remove the traces of mercuric chloride from the sterilised explants. The thoroughly washed explants were then placed in the folds of a sterile blotting paper to remove the excess quantities of water from the explants. These treated and sterilised explants were planted in the culture medium for further analysis.

(ii) Treating the explants after two days of culturing:

The excised explants of different maturity groups were cultured properly in the standardised medium as presented earlier and allowed for incubation for two days. After two days of culturing, the explants were taken out from culture media and washed thoroughly in sterile distilled water to remove traces of media.

The explants thus taken out from culture medium having two days development were treated with respective concentration of mutagen solution for six hours as in case of direct treatment. The treated explants were washed in sterile distilled water and allowed to be in the running sterile distilled water for half an hour. The mutagen treated and thoroughly washed explants were again surface sterilised with mercuric chloride. The surface sterilised explants were then washed thoroughly in sterile distilled water and planted in the fresh media.

(iii) Treating the explants three days after culturing:

The cultured proembryos and embryos wereallowed to grow in the media for three days as was presented in previous case. After three days of culturing in the media, they were taken out from the growing media and with the mutagenic solution as was done earlier. After proper treatment and thorough washing, the treated explants were replanted in a fresh medium and allowed to grow.

(iv) Treating the explants four days after culturing:

The cultured proembryos and embryos were allowed to grow in the media for four days as was presented in

previous case. After four days of culturing in the media, they were taken out from the growing media and treated with the mutagenic solution as was done earlier. After treatment and thorough washing the treated explants were replanted in fresh medium and allowed to grow as was standardised.

(v) Incorporating the mutagen culture media at different phases of explant growth:

One of the objectives of the present investigation was to treat the explants in culture media treated with the mutagen solution at different developmental phases of explant growth. The technique include treating the explants with the mutagenic solution through the culture media at the time of planting and two, three and four days of growth in the culture medium. The main idea was to feed the embryo with the mutagenic solution along with the nutrient media without. disturbing the explants having the different developmental phases in the media. But unfortunately, wherever the mutagenic solution was added to the culture medium, solidification of the medium was quite impossible and the explants became contaminated within two/three days of culturing and got disintegrated.

(I) Observations:

Detailed observation on the following characters were taken from the cultured explants at different stages of their growth. Special care was taken not to disturb the growing explants under aseptic condition. All the materials used to take observations were sterilised properly before use. The observations taken include:

(a) Days taken for Embryo set.

(b) Percentage setting.

(c) Length of plantlets at fifteen days interval.

(d) Number of leaflets per plantlet at fifteen days interval.

(e) Growth rate analysis based on height and number of leaves.

(a) Days taken for Embryo set:

The number of days taken by different treatments to transform the whitish colour of the explant to light green colour was counted and calculated as the number of days taken for embryo set in the culture media.

(b) Percentage setting:

From the third day of planting of the explants, observations were taken on the transformation of original

colour of the explant to the greenish pigmentation. Based on the number of explants set out of total planted the percentage differentiation for each treatment was calculated.

(c) Length of plantlets at fifteen days interval:

From the fifteenth day of planting of explants, observations on plantlet height was taken by using properly sterilized and marked glass rods. Observation on plantlet height was taken at fifteen days interval till the sixtieth day of explant set.

(d) Number of leaflets per plantlet at fifteen days interval

Proper counts on the number of leaflets per plantlet was taken at fifteen days interval from the day of explant set to the sixtieth day.

(e) Growth rate analysis:

Observations made on plantlet height and leaflet number from the fifteenth day of embryo set to sixtieth day at fifteen days interval was taken into consideration to compute the growth rate analysis due to various treatments.

R E S U L T S

Embryo-take analysis

The days taken for embryo-take as influenced by different embryonic stages, methods of mutagen treatment and the different concentrations of mutagen is presented in Table 4.

(a) Proembryo with twenty days development:

The number of days taken for embryo set in proembryos having twenty days development ranged from four (0.250% in first and second mode of treatment) to nine days of culturing (highest concentrations under third and fourth mode of treatment). Not much difference was observed in control under different modes of treatment except in the case of treating the material four days culturing. The lower concentrations (0.125 and 0.250% does not show any difference among the different mode of treatment. The higher concentrations took the maximum days for tissue or embryo set when compared to the lower concentrations and the control population. The maximum number of days for embryo uptake was observed under the highest concentration of the mutagen (0.75%) in the materials treated three days after culturing and both by 0.50% and 0.75% when embryos were treated four days after culturing. While comparing the different concentrations and modes of treatment it is seen that 0.25% in

the first and second mode of treatment gave an earlier embryo set (4 days). Not much influence was noticed under 0.125% concentration even after the disturbances created four days after culturing.

(b) Pro-embryo with 30 days development:

The number of days taken for embryo set in pro-embryos having 30 days development ranged from four days (0.250% in first, third and fourth mode of treatment) to nine days in the highest concentration in majority of the modes of treatment. As in the case of the first embryonic stage there was not much difference in the days taken for embryo differentiation in the case of control even when it was in the thirtieth day of maturity. The lower concentrations failed to show any difference in embryo set compared to control population. In the case of treating the material four days after culturing, when control population took seven days it was nine days in the case of the highest concentration. All the other concentrations were at par or lower to the control value. The first, second and third mode of treatment did not show much difference in the number of days taken for embryo uptake under control and lower concentrations

of the mutagen. The higher concentrations took a longer period for differentiation. The lower concentrations, 0.125% and 0.25% does not show any difference among the different modes of treatment. The maximum number of days (nine days) taken for embryo set was shown by the highest concentrations in first, third and fourth mode of treatment and in 0.50% (eight days) under second method of treatment. While comparing concentrations and modes of treatment, 0.25% under the first, third and fourth modes of treatment gave an early embryo differentiation. Not much difference was observed under 0.125% even after the disturbances created four days after culturing.

(c) Embryos from fresh nuts:

The period taken for embryo set by fully developed embryos ranged from three days, 0.250% in the first mode of treatment to nine days in the highest concentration in fourth mode of treatment. Comparing to the control population not much difference was observed under different modes of treatment except in the case of treating the material four days after culturing. The higher concentration showed the maximum number of days for embryo differentiation compared to lower concentrations and the

control. The lower concentrations (0.125% and 0.250%) does not show any difference among the different modes of treatment.

The maximum number of days taken (9 days) for embryo set was shown by 0.75% under the last mode of treatment. Embryos from fully matured nuts also showed an earlier embryo uptake when they were treated with 0.250% concentration of the mutagen. Even after the disturbances created after four days of culturing not much difference was noticed under 0.125% concentration. Comparatively a stimulatory effect in embryo uptake was induced by 0.25% (3-5 days) compared to control (5-7 days) in all the four modes of treatment. In general this effect was also noticed in the case of 0.125% where the range was from 5-6 days.

(d) Embryos from dry seed nuts:

The number of days taken for embryo uptake in embryos from dry nut ranged from three days in 0.25% concentration in the first mode of treatment to eight days in the highest concentrations (0.750%), under third and fourth mode of treatment and in 0.500% concentration under the second and fourth mode of treatment. There was not much difference in the days

taken for embryo uptake among the different modes of treatment in the lower concentrations (0.125 and 0.250%). Here also the maximum number of days for embryo uptake was shown by the higher concentrations (0.500% and 0.750%), compared to lower concentrations and the control. Among the different modes of treatment under 0.500% concentration the maximum number of days were taken when the embryos were treated two and four days after culturing. Under 0.750% concentration these values were observed in materials treated after three and four days of culturing. While comparing the different modes of treatment and concentrations of mutagen, the first mode of treatment under 0.25% concentration of mutagen gave an earlier embryo uptake. There was slight difference in the days taken for embryo set under 0.125 concentration after the disturbances created four days after culturing. Here also the lower concentrations gave an earlier embryo uptake in all the four modes of treatment compared to control and higher concentrations.

In general the number of days taken for embryo set was the minimum (3-5 days) in 0.25% concentration compared to control and all other concentrations in all the four modes of treatment. When the days taken for

embryo uptake ranged from 3-5 days in 0.25%, it was 5-7 days in control, 4-6 days under 0.125% and 5-9 days under 0.50% and 0.75% concentrations. Irrespective of mode of treatment and concentrations, the number of days taken for embryo set ranged from 4 - 9, 4 - 9, 3 - 9 and 3 - 8 under first, second, third and fourth stages of embryonic development. Earlier the extraction of embryos longer was the days taken for embryo set. In all the four embryonic stages analysed and the four different modes of treatment adopted shortest period for embryo set was noticed under 0.25% compared to control and all other concentration of EMS tested. Just reverse was the trend in the case of the highest concentrations i.e. irrespective of embryonic stages and mode of treatment the maximum days were taken by the highest (0.75%) concentration.

Embryo Differentiation:

The effect of different stages of embryo development, methods of treatment and the different concentrations of mutagen on embryo differentiation is presented in Table 5. The different techniques and the different concentrations adopted showed much influence on embryo differentiation.

TABLE 4

DAYS TAKEN FOR EMBRYO-TAKE

Embryonic stages.	Mode of treatment.	Concentrations(%)				
		Control	0.125	0.250	0.500	0.750
D_1	T_1	5	6	4	6	7
	T_2	6	5	4	7	7
	T_3	5	6	5	8	9
	T_4	7	6	5	9	9
D_2	T_1	5	5	4	6	9
	T_2	6	5	5	8	6
	T_3	6	6	4	5	9
	T_4	7	6	4	7	9
D_3	T_1	5	5	3	6	6
	T_2	7	5	4	7	8
	T_3	6	6	5	8	7
	T_4	7	5	4	7	9
D_4	T_1	6	4	3	5	5
	T_2	5	5	4	8	7
	T_3	6	6	5	6	8
	T_4	7	6	5	8	8

(a) Proembryos having twenty days development:

The percentage embryo differentiation ranged from zero (in higher concentrations) to 80% (in control population). When the first mode of treatment gave a value of 0 - 80%, it was 0 to 70%, 0 to 60% and 0 to 30% under second, third and fourth modes of treatment respectively in proembryos having twenty days of development. Under the first mode of treatment 0.50% and 0.75% concentrations failed to show any embryo differentiation. Control population gave a higher percentage differentiation (80%), compared to 0.125% and 0.25% concentrations(70%). In second mode of treatment the maximum tissue differentiation was observed in 0.25% concentration (70%) compared to 0.125% (60%) and control (50%). Here 0.50% concentration gave only 20% differentiation. In the third and fourth mode of treatment both 0.50% and 0.75% failed to show any embryo differentiation. In the third mode of treatment 0.25% and control population gave an equal percent differentiation (60%) compared to 0.125 (40%). The same trend was noticed in the case of the last mode of treatment. When 0.125% concentration gave 20% differentiation it was 30% under both control and 0.25% treatments. In general not much difference was observed between 0.25% concentration (30 - 70%) and control population (30-80%),

compared to 0.125% (20-70%). When 0.75% failed to show any embryo differentiation in all the four mode of treatments, 0.50% gave 20% differentiation in the second mode of treatment.

(b) Proembryos having 30 days development:

Embryo differentiation as influenced by thirty days of embryo maturity and various modes of treatment and concentration showed variation depending upon the techniques adopted. When the percentage differentiation ranged from 30% (higher concentration) to 80% (0.125%) in the first mode of treatment, it was 20% (0.75%) to 70% (0.25%) in the second mode of treatment. The highest concentration (0.75%) failed to show any embryo differentiation under the third and fourth mode of treatment. When 0.50% under the third mode of treatment gave 20% differentiation it was zero in case of fourth mode of treatment. In the third mode of treatment the lower concentrations (0.125% and 0.250%) and the control population gave a comparatively uniform value in tissue differentiation. In the fourth mode of treatment the maximum percentage was noted under 0.25% (70%) followed by control (40%) and 0.125% concentration (30%). Even in embryos having thirty days of

maturity, the higher concentration were not able to give a good percent of embryo differentiation. While comparing the effect of two lower concentrations, it can be noted that 0.25% gave an uniform effect (50-70%) compared to 0.125% (30-80%).

(c) Embryos from fully matured nut:

The embryos extracted from the seeds immediately after harvest gave a comparatively higher percentage performance in embryo differentiation compared to proembryos of different maturity. The percentage differentiation ranged from 10-80 in the case of first mode of treatment in the highest concentration the control and the lower concentrations respectively. A reduction in percentage differentiation was noted only in the case of higher concentrations, 60%(0.50%) and 10% (0.75%). An uniform embryo differentiation under the first mode of treatment was noticed under control, 0.125% and 0.25% concentrations. In the second mode of treatment a comparatively better performance was noted in almost all treatments. The values ranged from 20% (0.75%) to 80% (control and 0.25%). In the third and fourth mode of treatments 0.25% gave the maximum percentage performance compared to control and all other

concentrations. In both third and fourth modes of treatment the performance of 0.125% was at par with control. A reduction in percentage differentiation was noted for higher concentrations of EMS in third and fourth modes of treatment. As in the case of second mode of treatment the maximum percentage embryo differentiation was noted under 0.25% (60-80%) compared to control and 0.125% (50-80%), 0.50%(40-60%) and 0.75% (10-20%). Here it was so clear that irrespective of mode of treatment higher the concentration lesser was the percentage differentiation.

(d) Embryo from dry seed nut:

The embryos extracted from dry seed nuts showed a poor performance in embryo differentiation compared to the embryos extracted immediately after harvest and proembryos having thirty days maturity. The fourth mode of treatment failed to show any embryo differentiation in any concentration including control. When the percentage differentiation ranged from 20 to 60% in control it was 20-50%, 30-60%, 20-40% and 0-20% under 0.125%, 0.25%, 0.50% and 0.75% concentrations respectively. In the first mode of treatment the range in values were 20 to 60% (control and 0.25%). When 0.50% concentration gave 40% it was 50% under 0.125% EMS. In the second

mode of treatment, 0.50% and 0.75% failed to show any embryo differentiation. When control population gave 40% differentiation it was 50% and 30% under 0.25% and 0.125% concentrations of EMS respectively. In the third mode of treatment the percentage differentiation were comparatively very poor. It ranged from 20% (0.5%, 0.125% and control) to 30% (0.25%). The maximum percentage differentiation was noticed in 0.25% concentration compared to control and lower and higher concentrations.

In general the embryos having twenty days of maturity and full physiological maturity gave a poor performance compared to embryos having thirty days maturity and those extracted immediately after harvest. When percentage differentiation ranged from 0-80% in proembryos having twenty days development, it was 0 - 60% under embryos extracted from dry seed nuts. The control population (30-80%) and the lower two concentrations (20-70%) gave comparatively a higher percentage differentiation in proembryos having twenty days maturity when compared to the control population and the two lower concentrations (20-60%) in embryos extracted from dry seed nuts. While comparing the performance of embryos having thirty days of maturity and embryo from seeds immediately after harvest, a better performanced was noted in embryos

extracted immediately after harvest. Both control population and lower concentrations gave a comparatively higher percentage differentiation (50-80%) in the embryo extracted from seeds immediately after harvest irrespective of mode of treatment. In this case higher concentration also gave a higher percentage differentiation compared to all other embryos having different maturity.

TABLE 5

EMBRYO DIFFERENTIATION AS INFLUENCED BY DIFFERENT EMBRYONIC STAGES, MODE OF TREATMENT AND MUTAGEN CONCENTRATION

Embryonic stages.	Mode of treatment.	Concentration(%)				
		Control	0.125%	0.250%	0.500%	0.750%
D_1	T_1	80	70	70	-	-
	T_2	50	60	70	20	-
	T_3	60	40	60	-	-
	T_4	30	20	30	-	-
D_2	T_1	70	80	70	30	30
	T_2	60	60	70	30	20
	T_3	50	40	50	20	-
	T_4	40	30	70	-	-
D_3	T_1	80	80	80	60	10
	T_2	80	60	80	50	20
	T_3	50	50	60	40	-
	T_4	60	60	70	50	20
D_4	T_1	60	50	60	40	20
	T_2	40	30	50	-	-
	T_3	20	20	30	20	-
	T_4	-	-	-	-	-

Analysis on growth rate of Plantlets:

(a) Pro-embryos with twenty days development:

The influence of the various concentrations of the mutagen due to the direct treatment in proembryo having twenty days development at different intervals of growth is depicted in Table 5-1.

In the control population the height of the planlet/ length of shoot varied from 3.5 to 15.2 cm from 15th day to 60th day of growth respectively. The growth rate was maximum during 45th to 60th day. Under 0.125% concentration the length of the shoot vvaried from 2.2 to 6.9 cm. The growth rate increased with increase in days of establishment in culture media. The growth rate was comparatively poor compared to control. When 0.50% and 0.75% concentration failed to show any plantlet establishment, 0.25% concentration gave the maximum growth rate compared to 0.125%. Here the height of plantlets ranged from 3.4 to 10.4 under 15th day to 60th day respectively. The growth rate was maximum during the period from 30th day to 45th day of establishment. Compared to control population the growth rate was slow under 0.25% concentration especially in later period of growth.

The number of leaves/plantlet as influenced by the different treatment is also represented in the same table. In the control population the number of leaves per plantlet ranged from 6 - 12 under fifteenth and sixtieth days of establishment respectively. There was a steady increase in leaf production as influenced by the increase in days of maturity. Under 0.125% concentration the number of leaves increased from 3 to 12 under fifteenth day to sixtieth day respectively. Even though the number of leaves per plantlet was lesser in early period of growth, on sixtieth day of development the number of leaves attached the same as in control population. The number of leaves under 0.25% concentration ranged from four (fifteenth day) to fifteen (sixtieth day). Compared to control and lower concentration, 0.25% gave a higher leaf production potentiality especially under later stages of development. On 45th day and 60th day of establishment the number exceeded the valuesin control population. The data clearly show that both under 0.125% and 0.25% concentrations the growth rate was poor in early stages of establishment compared to control population. The higher concentrations (both 0.50% and 0.75%) failed to produce any leaf during the entire period of growth.

Growth rate of plantlets as influenced by different concentration of mutagen in proembryos having twenty days of development and the treatment after two days of culturing is presented in Table 5-2.

The length of shoot in control ranged from 0.5 to 2.5 cm, when it was 1.0 to 4.5 cm, 1.0 to 5.5 cm and 0.0 to 0.7 cm under 0.125%, 0.25% and 0.50% concentrations of EMS respectively. Here the lower concentrations showed a higher value in length of shoot in all the different stages of growth when compared to control. When control plantlets gave a maximum height of 2.5 cm, it was 4.5 cm and 5.5 cm under 0.125% and 0.25% concentrations respectively. Even in the early stages of establishment and development treatment with lower concentration gave a stimulatory effect compared to control. While comparing the lower concentrations it can be seen that growth rate under 0.25% concentration was higher compared to 0.125% eventhough it was the same under the early period of development. The growth rate under 0.50% was quite insignificant when compared to control and other two lower concentrations. The maximum plantlet height attained on sixtieth day of differentiation (0.7 cm) was even lower to the plantlet height on fifteenth day under 0.125% and 0.25 concentrations. The lower concentrations were able to

give a higher value even on the first day of observation than that on the plantlet height on the sixtieth day(0.7 cm) under 0.50%concentration.

The number of leaves per plantlet as influenced by various treatment is also included in the same table. The number of leaves per plantlet varied from 1 to 7 in control, (fifteenth to sixtieth day of development) when it was 3 to 7 and 3 to 11 under 0.125% and 0.25% concentrations respectively. Eventhough the number of leaves per plantlet was low in control population on fifteenth day of establishment when compared to 0.125% concentration the number of leaves per plantlet attained the same in both, control and 0.125% concentration, on sixtieth day of development. From the thirtieth day of development the number of leaves per plantlet under 0.125% and control were at par. The number of leaves per plantlet under 0.25% concentration showed a higher value (3-11) compared to 0.125% (3-7) and control (1-7). There were differences in leaf production potentiality between control and 0.25% concentration even from early period of development. On the sixtieth day of establishment the maximum number of leaves per plantlet was shown under 0.25% concentration compared to 0.125% and control plantlets. The maximum reduction in number of leaves per plantlet was recorded

under 0.50% concentration. The development of leaves in this treatment started only after thirtieth day of tissue differentiation. On the sixtieth day of differentiation the number of leaves per plantlet was only four when the lower concentrations and control gave the same number on thirtieth day of establishment. In general 0.25% concentration gave a stimulatory effect in growth rate of plantlets while 0.75% concentration did not produce any leaves at all.

The influence of different concentrations of the mutagen in proembryos having twenty days development and the treatment after three days of culturing is presented in Table 6-3.

A marked reduction in growth rate, both in height of plantlets and number of leaves per plantlets was noticed when the treatments were done three days after culturing. In the control population theheight of plantlets ranged from 1.8 to 9.1 cm when it was 0.2 to 1.1 cm and 1.6 to 7.5 cm under 0.125% and 0.25% concentration respectively. The higher concentrations (0.50% and 0.75%) failed to show any differentiation of tissues even after sixtieth day of planting. The lowest concentration (0.125%) gave a comparatively very poor performance in increase in length of shoots compared to control population. On the sixtieth day of development it was able to give only 1.1 cm height

when it was 1.8 cm in control population on the fifteenth day of development. The growth rate in early period of development under 0.125% concentration was quite insignificant. While comparing the two later concentrations (0.125% and 0.25%) it can be seen that 0.25% concentration also gave a higher growth rate. It is seen that the treatment of mutagen on the third day of culturing influence the stimulatory effect of 0.25% concentration noted in the previous two modes of treatment. Here the growth rate under 0.25% ranged from 1.6 to 7.5 cm when it was 1.8 to 9.1 cm in control. The difference is mainly noted in the early stages of development.

The number of leaves per plantlet as influenced by different treatment is also represented in the same table. A direct reflection of the plantlet height is noted in case of number of leaves also. In the control population the number of leaves per plantlet ranged from 3- 11 when it was 0 to 5 and 3 to 7 under 0.125% and 0.25% concentration respectively from fifteenth to sixtieth day of establishment . A less influence on the leaf production potentiality is noted under 0.25% concentration compared to control. Marked reduction in the production of leaves were noted under 0.125% compared to 0.25% and control. The maximum number of leaves available on the sixtieth day

of embryo differentiation was 11 leaves per plantlet in control when it was 7 leaves per plantlet under 0.25%, when the plantlets showed the same number of leaves in the early stages of development in both the treatments. (3-6 and 3-5 under control and 0.25% concentration respectively on the fifteen and thirtieth day of establishment).

Table 6-4 represent the growth rate of plantlets due to the effect of different concentrations of EMS in proembryo having twenty days development and treatment after four days of culturing. Tye growth rate based on length of shoot and number of leaves per plantlet showed a marked reduction in all the treatments. Height of plantlets ranged from 0.25 to 0.50 cm in control population when it was 0 to 0.50 cm and 0 to 0.63 cm under 0.125% and 0.25% concentrations respectively from fifteenth to sixtieth day of development. Maximum reduction due to the influence of mutagen was noted in early stages of development compared to control. Plantlet height initiated only on the thirtieth day under both the concentration of mutagen (0.125% and 0.25%), when it was available on the fifteenth day of differentiation in control population. On the thirtieth day of development when control plantlets gave a height of 0.30 cm it was 0.05 and

0.17 cm under 0.125%and 0.25% concentration respectively. On the 45th day of establishment the plantlet height under 0.25% concentration (0.30 cm) was at par with the control plantlets (0.33 cm) but 0.125% concentration showed a marked reduction compared to 0.25% and control population. On the sixtieth day of establishment the plantlet height both under control and 0.125% concentration were at par (0.50 cm each), when it was 0.63 cm under 0.25% concentration. When there was a steady increase in plantlet height in control, under 0.25% concentration a stimulatory effect was noticed in later stages of growth. Both the higher concentrations (0.50% and 0.75%) failed to show any embryo differentiation and growth.

The number of leaves per plantlet included in same table show that there was not much variation between the mutagen treatments and the treated population with the control plantlets. Under 0.125% and 0.25% concentrations leaf production started when before the plantlet increase or shoot length increase was initiated whereas in control population leaf production started only after attaining a particular shoot length. Leaf production in control plantlets started only on thirtieth day of establishment when it was noted on fifteenth day under both

the lower concentration of the mutagen. The number of leaves per plantlet ranged from 1 to 5 in control plantlets, when it was 2 to 5 under the mutagen treatment from fifteenth to sixtieth day of establishment. Leaf production was not at all influenced by the mutagen treatment.

TABLE 6-1

GROWTH RATE OF PLANTLETS AS INFLUENCED BY DIFFERENT CONCENTRATIONS IN PROEMBRYO HAVING TWENTY DAYS DEVELOPMENT UNDER DIRECT TREATMENT

Treatment	Length of shoot (cm)	Number of leaves
Control		
15th day	3.5	6
30th day	5.9	7
45th day	8.7	10
60th day	15.2	12
0.125		
15th day	2.2	3
30th day	3.3	5
45th day	5.0	7
60th day	6.9	12
0.25		
15th day	3.4	4
30th day	5.0	8
45th day	8.1	11
60th day	10.4	15
0.50		
15th day	-	-
30th day	-	-
45th day	-	-
60th day	-	-
0.75		
15th day	-	-
30th day	-	-
45th day	-	-
60th day	-	-

TABLE 6-2

GROWTH RATE OF PLANTLETS AS INFLUENCED BY DIFFERENT CONCENTRATION IN PROEMBRYO HAVING TWENTY DAYS OF DEVELOPMENT AND TREATMENT AFTER TWO DAYS OF CULTURING

Treatment.	Length of shoot (cm)	Number of leaves
Control		
15th day	0.5	1
30th day	0.9	3
45th day	1.8	5
60th day	2.5	7
0.125		
15th day	1.0	3
30th day	1.6	4
45th day	2.7	5
60th day	4.5	7
0.25		
15th day	1.0	3
30th day	2.1	4
45th day	4.1	7
60th day	5.5	11
0.50		
15th day	0.9	0
30th day	0.4	0
45th day	0.6	2
60th day	0.7	4
0.75 15th day	-	-
30th day	-	-
45th day	-	-
60th day	-	-

TABLE 6-3

GROWTH RATE OF PLANTLETS AS INFLUENCED BY DIFFERENT CONCENTRATIONS IN PROEMBRYO HAVING TWENTY DAYS DEVELOPMENT AND TREATMENT AFTER THREE DAYS OF CULTURING

Treatment	Length of shoot (cm)	Number of leaves.
Control		
15th day	1.8	3
30th day	3.8	6
45th day	7.6	8
60th day	9.1	11
0.125		
15th day	0.2	0
30th day	0.3	1
45th day	0.6	3
60th day	1.1	5
0.25		
15th day	1.6	3
30th day	2.3	5
45th day	3.8	6
60th day	7.5	7
0.50		
15th day	-	-
30th day	-	-
45th day	-	-
60th day	-	-
0.75		
15th day	-	-
30th day	-	-
45th day	-	-
60thday	-	-

TABLE 6 - 4

GROWTH RATE OF PLANTLETS AS INFLUENCED BY DIFFERENT CONCENTRATIONS IN PROEMBRYO HAVING 20 DAYS DEVELOPMENT AND TREATMENT AFTER FOUR DAYS OF CULTURING

Treatment	Length of shoot (cm)	Number of leaves
Control		
15th day	0.25	-
30th day	0.30	1
45th day	0.33	3
60th day	0.50	5
0.125		
15th day	0	2
30th day	0.05	3
45th day	0.20	3
60th day	0.50	5
0.25		
15th day	0	2
30th day	0.17	3
45th day	0.30	5
60th day	0.63	5
0.50		
15th day	-	-
30th day	-	-
45th day	-	-
60th day	-	-
0.75		
15th day	-	-
30th day	-	-
45th day	-	-
60th day	-	-

(b) Pro-embryo with 30 days development:

The influence of the various concentrations of the mutagen due to the direct treatment in proembryo having thirty days development at different intervals of growth is depicted in Table 7-1.

In the control population the height of the plantlet varied from 3.3 to 10.2 cm from fifteenth day to sixtieth day of growth respectively. The growth ratewas maximum during the 45th to 60th day . Under 0.125% concentration the length of shoot varied from 1.3 to 8.1 cm and the growth rate was comparatively poor compared to control and 0.25%. The maximum growth rate was noticed under 0.25% concentration (2.4 to 12.1 cm). Eventhough the growth rate during the early days of differentiation was comparatively less in 0.250% concentration than the control, in the later stages of development there was a steady increase in the length of shoot. During the thirtieth day of differentiation the length of shoot under 0.25% concentration (4.5 cm) was almost at par with the control (4.9 cm). The growth rate under 0.50% and 0.75% was quite insignificant when compared to control and the other two lower concentrations. The maximum plantlet height attained on sixtieth day of differentiation, 1.3 cm and 0.8 cm, both under 0.50% and 0.75%

concentrations respectively was even lower to the plantlet height on fifteenth day under 0.25% concentration and control and was at par with 0.125% concentration. In general 0.25% concentration gave a stimulatory effect in growth rate of plantlets.

The number of leaves per plantlet as influenced by various treatment is also included in the same table. The number of leaves per plantlet varied from 3 to 13 in control (fifteenth to sixtieth day of development) when it was 2 to 8, 4 to 14, 1 to 5 and 1 to 5 under 0.125%, 0.25%, 0.50% and 0.75% concentrations respectively. There was steady increase in number of leaves as influenced by the increase in the days of maturity. Compared to 0.25% and control, the leaf production potentiality was poor in 0.125% concentration of the mutagen. The number of leaves per plantlet showed a higher value in 0.25% (4 - 14) compared to 0.125% (2-8), 0.50% (1-5), 0.75% (1-5) and control (3-13). The maximum number of leaves per plantlet (14) on the sixtieth day of development was shownunder 0.25% concentration. The maximum reduction in number of leaves per plantlet was recorded both under 0.50% and 0.75% concentrations. On the sixtieth day of differentiation the number of leaves per plantlet was only five in these concentrations where the lower concentrations

and control gave the same number during the early stages of development. There was a stimulatory effect produced by 0.25% concentration on the growth rate, both in length of shoot and number of leaves per plantlets, of plantlets cultured.

Growth rate of plantlets as influenced by different concentration of mutagen in proembryos having thirty days of development and the treatments two days of culturing is presented in Table 7-2.

The length of shoot in control ranged from 1.4 to 9.7 cm, when it was 2.0 to 12.cm, 4.3 to 17.7 cm, 0.8 to 4.2 cm and 0.3 to 1.7 cm in 0.125%, 0.25%, 0.50% and 0.75% concentration of EMS respectively. Here the lower concentrations (0.125% and 0.25%) showed a higher value in length of shoot in all the different stages of growth when compared to control and the higher concentrations (0.50% and 0.75%). When control plantlets gave a maximum height of 9.7 cm, it was 12.1 cm and 17.7 cm under 0.125% and 0.25% concentrations respectively. Even in the early stages of establishment and development treating with lower concentrations gave a stimulatory effect compared to control and higher concentrations. While comparing the lower concentrations, it can be seen that growth rate under 0.25% concentration was higher compared to 0.125%.

The growth rate under 0.50% and 0.75% was quite insignificant when compared to control and the other two lower concentrations. The maximum plantlet height attained on sixtieth day of differentiation under 0.50% and 0.75% (4.2 cm and 1.7 cm) was even lower to the plantlet height on fifteenth day under 0.25% concentration.

The number of leaves per plantlet as influenced by various treatment is also included in the same table. The number of leaves per plantlet varied from 4 to 14 in control, when it was 5 to 14, 6 to 18, 1 to 4 and 1 to 5 under 0.125%, 0.25%, 0.50% and 0.75% concentrations respectively from fifteenth day to sixtieth day of establishment. Eventhough the number of leaves per plantlet was low in control population on fifteenth day of establishment when compared to 0.125% concentration, the number of leaves per plantlet attained the same in both control and 0.125% concentration on sixtieth day of development. The number of leaves per plantlet under 0.25% showed a higher value (5-18)compared to 0.125%, (5-14) 0.50% (1-4), 0.75% (1-5) and control (4-14). There was difference between control and 0.25% concentration of mutagen in leaf producing potentiality even from early period of development. The maximum number of leaves per plantlet

produced (18) by 0.25% on the sixtieth day of development was higher when compared to control and other concentrations of EMS. The maximum number of leaves attained under 0.50% and 0.75% (four and five respectively) was at par with the number of leaves attained during the fifteenth day (four and five) under control and both under 0.125% and 0.25% concentrations respectively. In general, 0.25% concentration gave a stimulatory effect in growth rate of plantlets even when the treatments were done two days after culturing the embryo.

The influence of different concentrations of the mutagen in proc,bryos having thirty days development and the treatment after three days of culturing is presented in Table 7-3.

The length of shoot varied from 1.5 to 10.0 cm in control (from fifteenth day to sixtieth day) when it was 2.5 to 12.1 cm, 1.2 to 8.3 cm and 0.6 to 1.5 cm under 0.125%, 0.25% and 0.50% concentration of EMS respectively. The highest concentration (0.75%) failed to show any development on the differentiation of tissues even after sixtieth day of development planting. The lowest concentration (0.125%) gave a comparatively better performance in the length of shoot compared to control and other

concentrations. On the sixtieth day of development it was able to give 12.1 cm height. The growth rate increased with increase in days of establishment in culture media. The maximum height attained under 0.50% concentration on sixtieth day of establishment (1.5 cm) was at par or even lower to the height attained on fifteenth day of development under all other concentrations and control. While comparing with control population, 0.125% concentration gave a higher growth rate. It is seen that the treatment of mutagen on the third day of culturing influences the stimulatory effect of 0.25% concentration noted in the previous two mode of treatments. Here the growth rate under 0.25% ranged from 1.2 to 8.3 cms when it was 2.5 to 12.1 and 1.5 to 10.0 in 0.125% and control population respectively.

The number of leaves per plantlet as influenced by different treatment is also presented in the same table. A direct reflection of the plantlet height is noted in case of number of leaves per plantlet also. In the control population the number of leaves per plantlet ranged from 2 to 9 and it was 2 to 8, 2 to 8 and 0 to 5 under 0.125%, 0.25% abd 0.5% concentration from fifteenth to sixtieth day of establishment. Eventhough leaf production initiated

only during the 45th day under 0.50% concentration there was not much difference between the treated population of different concentrations and the control. The highest concentration (0.75%) failed to produce any leaf during the entire period of development.

Table 7-4 represent the growth rate of plantlets due to the different concentrations of EMS in proembryo having thirty days development and treatment after four days of culturing. The growth rate, based on length of shoot and number of leaves per plantlet showed a marked reduction in all the treatments except in 0.25% concentration. Height of plantlets ranged from 1.7 to 8.8 cm in control population when it was 0.9 to 6.7 cm and 2.8 to 14.3 in 0.125% and 0.25% concentrations respectively from fifteenth to sixtieth day of establishment. Maximum reduction in growth rate due to the effect of mutagen was noted in early stages of development. Both the higher concentrations (0.50% and 0.75%) failed to show any differentiation of tissues during the period of development. The maximum height attained by plantlets under 0.125% concentration (6.7 cm) was lower to the maximum height attained by the control (8.8 cm) and 0.25% concentration (14.3 cm). The height of plantlet on 45th day of establishment under 0.25% (8.9 cm) was at par with the maximum height attained by the control

(8.8 cm). In general 0.25% concentration of EMS gave a stimulatory effect on the growth rate of plantlets even when treatments were done after four days of culturing.

The number of leaves per plantlet included in same table show that there was variation among the various concentrations ofthe mutagen and the treated population with the control plantlets. The number of leaves per plantlet ranged from 4 to 12 in control, when it was 3 to 9 and 5 to 19 under 0.125% and 0.25% concentration from fifteenth dayto sixtieth day of establishment. The leaf production potentiality under 0.50% and 0.75% concentration of the mutagen was practically nil while 0.25% concentration of EMS gave the maximum number of leaves from fifteenth day to sixtieth day of establishment. The growth rate was higher during the later stages of development. Eventhough the number of leaves per plantlet was almost the same on fifteenth day of development in 0.125%, 0.25% and control, the increase was at a slower rate in 0.125% while it was steadily increasing in 0.25% and control. The maximum number of leaves attained by 0.125% concentration on sixtieth day of development (9) was at par with the number of leaves per plantlet on 45th day of establishment in control population

and with the number of leaves on thirttieth day in 0.25% concentration of EMS. There was a sudden increase in number of leaves per plantlet from 45th day to 60th day of development under 0.25% concentration. In general, 0.25% concentration produced a stimulatory effect on the growth rate of plantlets even after the disturbances created to the medium after four days of culturing.

TABLE 7-1

GROWTH RATE OF PLANTLETS AS INFLUENCED BY DIFFERENT CONCENTRATIONS IN PROEMBRYO HAVING THIRTY DAYS DEVELOPMENT UNDER DIRECT TREATMENT

Treatment.	Length of shoot(cm)	Number of leaves
Control		
15th day	3.3	3
30th day	4.9	5
45th day	7.9	9
60th day	10.2	13
0.125 15th day	1.3	2
30th day	2.3	3
45th day	4.3	5
60th day	8.1	8
0.25 15th day	2.4	4
30th day	4.5	7
45th day	8.8	12
60th day	12.1	14
0.50 15th day	0.1	1
30th day	0.5	1
45th day	0.8	2
60th day	1.3	5
0.75 15th day	-	1
30th day	0.2	3
45th day	0.5	4
60th day	0.8	5

TABLE 7-2

GROWTH RATE OF PLANTLETS AS INFLUENCED BY DIFFERENT CONCENTRATION IN PROEMBRYO HAVING THIRTY DAYS OF DEVELOPMENT AND TREATMENT AFTER TWO DAYS OF CULTURING

Treatment		Length of shoot(cm)	Number of leaves
Control			
	15th day	1.4	4
	30th day	2.4	6
	45th day	5.3	10
	60th day	9.7	14
0.125			
	15th day	2.0	5
	30th day	3.6	7
	45th day	7.4	11
	60th day	12.1	14
0.25			
	15th day	4.3	5
	30th day	7.2	9
	45th day	11.3	15
	60th day	17.7	18
0.50			
	15th day	0.8	1
	30th day	1.1	1
	45th day	1.9	3
	60th day	4.2	4
0.75			
	15th day	0.3	1
	30th day	0.7	1
	45th day	1.0	4
	60th day	1.7	5

TABLE 7-3

GROWTH RATE OF PLANTLETS AS INFLUENCED BY DIFFERENT CONCENTRATION IN PROEMBRYO HAVING THIRTY DAYS DEVELOPMENT AND TREATMENT AFTER THREE DAYS OF CULTURING

Treatment	Length of shoot(cm)	Number of leaves
Control		
15th day	1.5	2
30th day	2.3	4
45th day	5.0	6
60th day	10.0	9
0.125		
15th day	2.5	2
30th day	4.0	3
45th day	6.9	4
60th day	12.1	8
0.25		
15th day	1.2	2
30th day	2.1	3
45th day	4.5	6
60th day	8.3	8
0.50		
15th day	0.6	-
30th day	1.0	-
45th day	1.1	3
60th day	1.5	5
0.75		
15th day	-	-
30th day	-	-
45th day	-	-
60th day	-	-

TABLE 7:4

GROWTH RATE OF PLANTLETS AS INFLUENCED BY DIFFERENT CONCENTRATIONS IN PROTHBRYO HAVING THIRTY DAYS DEVELOPMENT AND TREATMENT AFTER FOUR DAYS OF CULTURING

Treatment	Length of shoot(cm)	Number of leaves
Control		
15th day	1.7	4
30th day	2.5	6
45th day	4.8	9
60th day	8.8	12
0.125		
15th day	0.9	3
30th day	1.8	4
45th day	2.7	6
60th day	6.7	9
0.25		
15th day	2.8	5
30th day	5.0	8
45th day	8.9	12
60th day	14.3	19
0.50		
15th day	-	-
30th day	-	-
45th day	-	-
60th day	-	-
0.75		
15th day	-	-
30th day	-	-
45th day	-	-
60th day	-	-

(c) Embryo from fully matured nut:

The influence of the various concentrations of the mutagen due to the direct treatment in embryos from fully matured nut at different intervals of growth is depicted in Table 8-1.

In the control population the length of shoot varied from 1.2 to 5.5 cm from fifteenth day to sixtieth day of growth respectively. The growth rates was maximum during 45th to sixtieth day. Under 0.125% concentration the length of shoot varied from 0.8 to 3.4 cm. There was a decrease in the length of shoot in all the concentrations of EMS compared to control population. The length of shoot ranged from 1.1 to 3.1 cm, 0.6 to 1.6 cm and 0 to 0.2 cm under 0.25%, 0.50% and 0.75% concentrations of EMS respectively from fifteenth day to sixtieth day of development. There was a linear decrease in growth rate with increase in the concentration of mutagen. With increase in concentration of mutagen from 0.125%, 0.25%, 0.50% to 0.75% the shoot length decreased from 3.4 cm to 0.2 cm.

The number of leaves per plantlet as influenced by the different treatment is also represented in the same table. In the control population the number of leaves

per plantlet ranged from four to ten under fifteenth day to sixtieth day of establishment respectively. There was a steady increase in number of leaves as influenced by the increase in days of maturity. Both under 0.125% and 0.25% the number of leaves increased from two to eight under fifteenth day to sixtieth day respectively The number of leaves per plantlet ranged from one to seven from fifteenth to sixtieth dayunder 0.50% concentration of EMS. Under 0.75% concentration the leaf production started only from thirtieth day (2) and attained a maximum of four leaves per plantlet on sixtieth day. The maximum number of leaves stained on sixtieth day of development under 0.50% and 0.75% (7 and 4) was even lower or at par with the number of leaves produced by all other concentrations of EMS and control populations. A direct reflection on the effect of mutagen on shoot length is seen in case of number of leaves per plantlet also. With increasing concentration of mutagen there was a gradual reduction in the number of leaves per plantlet.

Growth rate of plantlets as influenced by different concentrations of mutagen in embryos from fully matured nut and the treatment after two days of culturing is presented in Table 8-2.

The length of the shoot in control ranged from 1.6 to 6.0 cm when it was 0.8 to 3.1 cm, 1.10 to 3.5 cm, 0.6 to 2.8 and 0 to 0.3 under 0.125%, 0.25%, 0.50% and 0.75% concentration of EMS respectively. Here the control plantlets gave a higher growth rate compared to the plantlets under different concentrations of mutagen. When control plantlets gave a maximum height of 6.0 cm, it was only 3.1 and 3.5 cm under 0.125% and 0.25% concentrations respectively. The two lower concentrations gave similar kind of growth rate. The maximum height attained by the lower concentrations (3.1 and 3.5 cm) was lower by or at par to the height attained by control plantlets during the thirtieth day of growth (3.3 cm). The growth rate under the higher concentrations was comparatively poor compared to control and both the two lower concentrations. The growth started only after the thirtieth day of establishment under 0.75% and the growth rate was negligible. The maximum height attained by the plantlets under 0.50% concentration was also lower to the height attained by control plantlets on the thirtieth day of development.

The number of leaves per plantlet as influenced by various treatment is also presented in the same table. The number of leaves pef plantlet varied from 5 to 10 in control, when it was 5 to 11, 3 to 9, 2 to 8

and 0 to 5 under 0.125%, 0.25%, 0.50% and 0.75% concentration of EMS from fifteenth day to sixtieth day of establishment. Not much difference was noticed between the treatment and the treated populations with the control except under 0.75% concentration. Under 0.75% concentration the leaf production started only on thirtieth day to sixtieth day of establishment. The highest number of leaves (11) per plantlet was produced under 0.125% concentration on sixtieth day of development. On the fifteenth day and sixtieth day of development, the number of leaves under 0.125% and control were at par. But on the 45th and 60th day there was a slight increase in the number of leaves per plantlet under 0.125%. While comparing the different concentrations of EMS, 0.125% gave a stimulatory effect in the leaf production potentiality than the other 3 concentrations. There was not much difference among 0.25% and 0.50% in leaf production. The maximum number of leaves per plantlet produced under 0.75% (5) on the 60th day was at par with the number of leaves produced by control plantlets and 0.125% concentration on the first day observation (5 each).

The influence of different concentrations of the mutagen in embryos from fully matured nut and the treatment after three day of culturing is presented in Table 8-3.

In control population the length of the shoot varied from 1.7 to 4.4 cm (from fifteenth day to sixtieth day of development) when it was 1.3 to 3.9, 2.1 to 4.8 and 0.3 to 1.2 in 0.125%, 0.25% and 0.50% respectively. The highest concentration (0.75%) failed to show any tissue development even after sixtieth day of planting. The lower concentrations gave a similar growth rate as in control. The maximum height of plantlet attained was in 0.25% (4.8 cm) concentration of EMS. Here from the early stages of growth itself there was a steady increase in the length of shoot. In control population and 0.125% concentration the increase in length of shoot was maximum in the early stages of growth (from fifteenth to thirtieth day). The maximum length of shoot attained under 0.50% concentration (1.2 cm) on 60th day was even lower to the height attained by plantlets in control population, 0.125% and 0.25% concentrations on fifteenth day of development. The growth rate in early period of development under 0.50% concentration was negligible. While comparing the two lower concentrations (0.125% and 0.25%), it can be seen that 0.25% concentration gave a higher growth rate.

The number of leaves per plantlet as influenced by different treatment is also presented in the same table.

In control population the number of leaves ranged from 3 to 7, when it was 3 to 8, 2 to 8 and 1 to 6 in 0.125%, 0.25% and 0.50% concentration of the mutagen respectively. The highest concentration (0.75%) did not produce any leaf. A direct reflection of the effect of 0.75% concentration of mutagen in length of shoot is seen in number of leaves also. The maximum number of leaves per plantlet (8) was produced under 0.125% and 0.25% concentrations. Eventhough the number of leaves per plantlet under control and 0.125% were the same (3) on first day of observation, 0.125% concentration of EMS gave a slightly better performance than control. The maximum number of leaves attained on sixtieth day of establishment under 0.50% (6) was at par with the number of leaves on thirtieth day of development under 0.125% and was even lower to the maximum number of leaves attained on sixtieth day of development under control, 0.125% and 0.25% concentration of EMS.

Table 8 - 4 represent the growth rate of plantlets due to the different concentrations of EMS in embryo from fully matured nut after four days of culturing. Height of plants ranged from 2.6 to 4.3 cm in control and 1.7 to 4.3 cm, 2.0 to 5.1 cm, 0.4 to 1.2 cm and 0 to 0.4 cm

under 0.125%, 0.25%, 0.50% and 0.75% concentration of EMS respectively. Eventhough the growth rate was lesser than the control in 0.125% and 0.25% during the early stages of growth, attained the same length during the later stages of growth. The maximum length was noticed under 0.25% concentration (5.1 cm). The growth rate was quite insignificant and practically nil under 0.50% and 0.75% concentration of EMS. Growth started only by the 45th day under 0.75% and the maximum height attained (0.4 cm) was even lower to the height attained on first day of observation under all other concentrations of EMS and the control plantlets.

The number of leaves per plantlet included in the same table show that there was not much difference among the different concentration of the mutagen and the treated population with the control except in 0.75% concentration. Under the higheet concentration (0.75%) leaf production started even before the shoot length initiation (on thirtieth day). But the maximum number of leaves attained here (3) was at par with the number of leaves on the fifteenth day under 0.125%, 0.25% concentrations of EMS and control. The control plantlets and the plantlets under 0.125% concentration gave equal number of leaves in all the different stages

of growth (3,4,6,8). There was a stimulatory effect under 0.25% concentration and the maximum number of leaves was noticed here (11).

TABLE 8-1

GROWTH RATES OF PLANTLETS AS INFLUENCED BY DIFFERENT CONCENTRATIONS IN EMBRYO FROM FULLY MATURED NUT UNDER DIRECT TREATMENT

Treatment	Length of shoot(cm)	Number of leaves
Control		
15th day	1.2	4
30th day	2.5	6
45th day	3.8	8
60th day	5.5	10
0.125		
15th day	0.8	2
30th day	2.6	5
45th day	3.0	7
60th day	3.4	8
0.25		
15th day	1.1	2
30th day	1.6	4
45th day	2.5	6
60th day	3.1	8
0.50		
15th day	0.6	1
30th day	0.9	3
45th day	1.3	4
60th day	1.6	7
0.75		
15th day	-	-
30th day	-	2
45th day	0.2	2
60th day	0.2	4

TABLE 8-2

GROWTH RATE OF PLANTLETS AS INFLUENCED BY DIFFERENT CONCENTRATIONS IN EMBRYO FROM FULLY MATURED NUT AND TREATMENT AFTER TWO DAYS OF CULTURING

Treatment	Length of shoot(cm)	Number of leaves.
Control		
15th day	1.6	5
30th day	3.3	7
45th day	4.9	8
60th day	6.0	10
0.125		
15th day	0.8	5
30th day	1.7	7
45th day	2.3	9
60th day	3.1	11
0.25		
15th day	1.1	3
30th day	1.9	6
45th day	2.6	8
60th day	3.5	9
0.50		
15th day	0.6	2
30th day	1.2	4
45th day	2.0	5
60th day	2.8	8
0.75		
15th day	-	-
30th day	-	3
45th day	0.2	4
60th day	0.3	5

TABLE 8 - 3

GROWTH RATE OF PLANTLETS AS INFLUENCED BY DIFFERENT CONCENTRATIONS IN EMBRYO FROM FULLY MATURED NUT AND TREATMENT AFTER THREE DAYS OF CULTURING

Treatment	Length of shoot (cm)	Number of leaves
Control		
15th day	1.7	3
30th day	3.1	4
45th day	3.6	5
60th day	4.4	7
0.125		
15th day	1.3	3
30th day	2.7	6
45th day	3.4	7
60th day	3.9	8
0.25		
15th day	2.1	2
30th day	3.4	4
45th day	4.3	6
60th day	4.8	8
0.50		
15th day	0.3	1
30th day	0.6	3
45th day	1.0	5
60th day	1.2	6
0.75		
15th day	-	-
30th day	-	-
45th day	-	-
60th day	-	-

TABLE 8-4

GROWTH RATE OF PLANTLETS AS INFLUENCED BY DIFFERENT CONCENTRATIONS IN EMBRYO FROM FULLY MATURED NUT AND TREATMENT AFTER FOUR DAYS OF CULTURING

Treatment	Length of shoot(cm)	Number of leaves
Control		
15th day	2.6	3
30th day	3.0	4
45th day	3.7	6
60th day	4.2	8
0.125		
15th day	1.7	3
30th day	2.9	4
45th day	3.7	6
60th day	4.3	8
0.25		
15th day	2.0	3
30th day	4.1	7
45th day	4.6	8
60th day	5.1	11
0.50		
15th day	0.4	1
30th day	0.7	3
45th day	1.0	6
60th day	1.2	7
0.75		
15th day	-	-
30th day	-	2
45th day	0.3	3
60th day	0.4	3

TABLE 8-4

GROWTH RATE OF PLANTLETS AS INFLUENCED BY DIFFERENT CONCENTRATIONS IN EMBRYO FROM FULLY MATURED NUT AND TREATMENT AFTER FOUR DAYS OF CULTURING

Treatment	Length of shoot(cm)	Number of leaves
Control		
15th day	2.6	3
30th day	3.0	4
45th day	3.7	6
60th day	4.2	8
0.125		
15th day	1.7	3
30th day	2.9	4
45th day	3.7	6
60th day	4.3	8
0.25		
15th day	2.0	3
30th day	4.1	7
45th day	4.6	8
60th day	5.1	11
0.50		
15th day	0.4	1
30th day	0.7	3
45th day	1.0	6
60th day	1.2	7
0.75		
15th day	-	-
30th day	-	2
45th day	0.3	3
60th day	0.4	3

leaves as influenced by increasing days of maturity. Under 0.125% concentration the number of leaves increased from 2 to 6 from fifteenth to sixtieth day. The growth rate was comparativdly poor than control and 0.25% and 0.50% concentration. The number of leaves ranged from 7 to 21 and 8 to 19 under 0.50% and 0.25% concentration.

TABLE 9

GROWTH RATE OF PLANTLETS AS INFLUENCED BY DIFFERENT CONCENTRATIONS IN EMBRYO FROM DRY NUT UNDER DIRECT TREATMENT

Treatment	Length of shoot (cm)	Number of leaves
Control		
15th day	1.31	5
30th day	2.2	9
45th day	3.9	14
60th day	5.8	20
0.125		
15th day	0.5	2
30th day	1.1	3
45th day	1.7	5
60th day	3.2	6
0.25		
15th day	0.9	7
30th day	1.9	12
45th day	3.4	17
60th day	5.6	21
0.50		
15th day	1.8	8
30th day	2.7	13
45th day	4.8	18
60th day	6.1	19
0.75		
15th day	0.3	-
30th day	0.5	-
45th day	0.8	-
60th day	1.0	-

D I S C U S S I O N

The present investigation to standardise the *in vitro* culture techniques in relation to induced mutagenesis in groundnut using proembryos and embryos of different maturity seems to be first of its kind. The main objectives envisaged include standardisation of embryo culture techniques, techniques of mutagen treatment and the best developmental phase of embryo for mutagen treatment. The various results obtained during the investigation is discussed below.

1) Standardisation of culture media for groundnut embryo:

In the present investigation the three culture media tested include White's medium, M.S. medium and Modified M.S.Medium. The three media tested gave satisfactory embryo take and tissue differentiation. On comparative analysis it was seen that the modified Murashige Skoog medium gave a better embryo take and an early tissue differentiation. Comparing the embryo take in White's media and M.S.basal media, it was seen that MS medium gave an early embryo take and tissue differentiation. Analysis on the modification made in the basal MS medium, myo-inositol gave an early bud take and tissue differentiation compared to basal M.S. Medium. Standardisation of best medium was done using fully matured embryos from

dry nuts. An early embryo take and tissue differentiation as noticed in present investigation using modified Murashige-Skoog medium was reported by various workers in various crop plants including in groundnut by Ziv and Zamski (1975), Mroginski and Fernandez (1980), Bajaj et al (1981), Mroginski et al (1981) , in tomato by Tal et al (1977), in forages by Rangan (1976) and in cocoa by Jalal and Collin (1979). The various results obtained by using modified MS medium and the different treatment techniques as included in project is presented below.

(2) Days taken for embryo set and embryo differentiation :

The effect of embryos in different growth phases, methods of treatment under different concentration of the mutagen presented in Table-4 clearly show that the effect is modified by all the factors analysed. In proembryo having twenty days development, the medium dose level (0.25%) gave an early embryo set compared to control and lower and higher mutagen doses employed. This particular concentration when applied on the fourth day of culturing gave a similar effect as in the case of control population. But in higher concentrations a delay was experienced irrespective of method of treatment.

This clearly demonstrates that the mutagen treatment either direct or after culturing was effective. A delay in seed germination as affected by the mutagen has been reported by various workers in various crops, including field beans by Shirshov and Shain (1966); in pea by Sidorova et al; (1966); Maslov and Stepanova, (1967) in red gram by Alikhan et al (1973) and in cowpea by Louis and Kadambavanasundaram (1973a). The other three different types of embryos tested, embryos having thirty days development, embryos from fresh seed nuts immediately after harvest and embryo from dry seed nuts also gave a similar stimulatory effect in medium and lower concentration of EMS compared to control population. In proembryo having thirty days development and in embryos from nuts immediately after harvest, the effect of the higher concentration (0.50%) was almost identical to that of control. But the highest concentration caused an inordinate delay for embryo set in all the methods of treatment adopted. The effect of the lower and higher concentrations were also at par. The trend of stimulatory effect in the medium and to a certain extent by the lower concentration and a drastic delay in the highest concentration clearly spells out the fact that mutagen treatment by the techniques adopted was effective and can have a detailed

analysis on induced mutagenesis in this crop in the concentration above 0.25%. Analysis on the effect of different methods of treatment and the various concentrations of mutagen tested clearly show that proembryos will be in a position to give a better result compared to matured or physiologically matured embryos. This is evidenced by the fact that mutagenic effect as regards the embryo set under higher concentrations was much delayed in embryos compared to proembryos, where the effect due to medium and lower concentrations were similar, or in other words the matured embryos were more mutagen resistant compared to proembryos, but this was not much supported by the data available for proembryos having both twenty and thirty days of development. The effect due to highest concentration was identical in both these proembryos. It is also evidenced that when we use matured or physiologically matured embryos, the concentration of mutagen solution can be increased. The present investigation also clearly demonstrate that when we use proembryos for induced mutagenesis, the concentration of mutagen solution can be lowered. Higher the maturity of embryo greater will be the mutagen resistance for the embryos of groundnut. Detailed analysis on other factors concerned with the direct effect

of mutagen in M_1 generation is required to have a final conclusion in this regard.

The effect of different stages of development, mode of treatment and the different concentration of mutagen used presented in Table-5 clearly demonstrate that embryo differentiation in culture media is much influenced by the factors analysed in detail.

The data clearly demonstrates that the stage of embryo development determines the mutagen sensitivity. When the proembryos having twenty and thirty days development were not able to give a satisfactory embryo differentiation, the performances of matured embryos were remarkable and it was also interesting to note that regardless of the maturity factor the mutagen concentration played a major role in embryo differentiation in culture medium. On an average the control population gave a satisfactory performance when embryos were grown without any disturbances in the substratum. Higher the disturbances created during the development in culture medium lesser was the percentage embryo differentiation. This factor was reflected in all the embryos of different maturity. But this general trend of drastic reduction in embryo differentiation was not noted in higher concentrations especially when the embryos were extracted immediately

after harvest. But the general trend as is observed in control was noticed in lower concentrations in almost all the proembryos and embryos having different maturity. Though related literature is not available in this particular crop it can be presumed that this embryo differentiation may be due to disturbances created during the early stages of development of embryo in culture medium. In general, a higher percentage reduction in embryo differentiation was observed in mutagen treated materials compared to control. This reduction in embryo differentiation compared untreated materials can be attributed to the bio chemical changes created by the mutagen in early stages of development of proembryos and embryos.

On comparison of the differences in embryo diffdrentiation in proembryos having twenty and thirty days development, it can be observed that embryo differentiation was better in proembryos of thirty days of development compared to proembryos of twenty days development. But when we compare percentage differentiation in embryos extracted immediately after harvest and those from dry seed nuts, it can be noticed that the

maturity of the embryo and embryo differentiation due to various treatments do not have close relationship. This statement is supported by the fact that embryos from seed nuts immediately after harvest gave a very high differentiation compared to embryos having full physiological maturity and also from immature nuts. A general trend of higher embryo differentiation was noticed in embryos extracted from nuts immediately after harvest compared to embryos having full physiological maturity and proembryos of different maturity. It is better to designate it as a higher mutagen reponse rather than to designate it as mutagen resistance by these embryos having full maturity. When proembryos having twenty days development and embryos with full physiological maturity were able to give only a poor performance, a maximum embryo differentiation was noticed in embryos extracted from nuts immediately after harvest followed by proembryos of 30 days development. Based on this result, it can be suggested that best stage for embryo extraction for mutagen treatment is from seeds immediately after harvest.

Comparing the effect due to various concentrations of mutagen adopting various modes of treatment in pro and

and embryos having different maturity it was observed that the middle and lower concentrations of mutagen gave a better performance to higher concentrations in all the different treatment techniques adopted. Of the two lower doses employed, 0.25% gave the maximum response. The effect due to the middle dose was at par to the control material in majority of the treatments, but it varied depending upon the embryonic stage. In general the maximum response was noticed due to 0.25% concentration compared to 0.125%. This response was much evidenced in embryos having the full maturity embryos extracted immediately after harvest of nuts. The deleterious effects noticed by the various concentrations in embryos having full physiological maturity and proembryos with least maturity and a comparatively lesser effect in proembryos having thirty days maturity clearly demonstrate that for maximum embryo differentiation, the correct stage for embryo differentiation is from seeds immediately after harvest.

Growth rate of plantlets as influenced by different doses of the mutagen and different modes of treatment in proembryos having twenty days development showed a remarkable variation - in growth and development. The data clearly demonstrated that it is always better to create only a minimum disturbance to the embryo after planting it in the culture medium. This statement was

made more clear by the fact that even in untreated control, the maximum growth rate of plantlets was observed in media having direct treatment. In control population the growth rate was also maximum in undisturbed media compared to all other modes of treatment, taking into account the length of shoot and number of leaves. A steady increase in growth as was measured based on length of shoot and number of leaves was also observed in concentration of mutagen solution. But this increase in growth rate was at its maximum in the undisturbed media compared to all others. The higher concentrations of mutagen in general failed to show any growth in embryos planted, which may be due to the deleterious effect creatdd by the higher concentration through the non differentiation of proembryos and embryos. The growth rate analysis clearly demonstrate that it is not advisable to recommend concentrations above 0.25% EMS for detailed analysis on mutational events in this particular crop variety. The maximum growth rate irrespective of mode of treatment in proembryos of twenty days development was observed under direct treatment both in the case of length of shoot and number of leaves

per plantlet. Thus it can be inferred that when proembryos having 20 days development is treated with EMS concentration of the medium doses at the time of planting the proembryo can be recommended for *in vitro* techniques in induced mutations.

Growth rate analysis based on shoot length and number of leaves per plantlet in proembryos having thirty days development presented in Table 7 shows a remarkable variation when compared to proembryos having twenty days development. In the case of direct treatment irrespective of the concentrations tissue differentiation and growth rate was satisfactory in all the concentrations both under direct treatment and treatment after two days of culturing. Whereas the highest concentration on the third day of culturing and the higher concentrations (0.50% and 0.75%) on fourth day of culturing failed to show any tissue differentiation and further developments. In control and lower concentrations not much reduction in growth rate was noticed when the proembryos were treated at the time of culturing or two to three days of culturing. But even the lower concentrations showed a reduced growth rate when proembryos were treated four days after culturing. There was a gradual decrease in growth rate in

higher concentrations as the treatment days were enhanced and was nil on the fourth day of culturing. A poor growth rate in higher concentrations on the treatments, the first, second and third, and complete failure of differentiation on fourth day of culturing clearly demonstrates that higher concentrations of mutagen (0.50% and 0.75%) cannot be adopted for *in vitro* techniques of induced mutagenesis in ground nut. The growth rate reduction in the advanced stages of culturing can be attributed to the sensitivity of the cultured embryos for higher concentrations of mutagen and also for their unadjustability with the disturbances created during the developmental process of the proembryos in culture media. Comparing the effects due to the proembryos having twenty and thirty days development it can be inferred that higher the maturity of embryos within the seed greater was the resistance for the higher doses of the mutagens and the disturbances created during the developmental process of the proembryos in the culture medium. This is more clear by the fact that when proembryos having twenty days development gave maximum tissue differentiation and growth rate when the treatments were done at the time of planting, the proembryos having thirty days development gave maximum expressions

when treatments were done two days after culturing and comparatively an insignificant reduction when treatments were done three days after culturing. Thus when proembryos having thirty days development are used for induced mutagenesis adopting *in vitro* techniques there is no harm in treating the materials with the mutagen till the third day of culturing. Comparative analysis on the growth rate was influenced by proembryos having different growth maturity also made it clear that proembryos having thirty days development are better compared to proembryos of twenty days for induced mutagenesis adopting *in vitro* techniques.

Growth rate of plantlets ad influenced by treatment of the mutagen in different days after culturing made it clear that embryos extracted from fully matured nuts immediately after harvest has got a higher resistance to the higher concentrations of the mutagen and was possible to accommodate the disturbances even on the fourth day of culturing. Compared to proembryos having different maturity the embryos from fully matured nuts were able to have tissue differentiation and growth and development even in higher concentrations used and also

in treatments employed four days after culturing. This adds further to the fact that higher the maturity of embryos greater will be the mutagen resistance and accommodability for the disturbances during the early developmental stages of the embryos in culture media. If we compare the performance of proembryos having different maturity with the embryo having full maturity it can also be noticed that for maximum growth rate expressions it is better to use proembryos having thirty days development rather than using proembryos of twenty days development and embryos from full matured nuts extracted immediately after harvest. While comparing the performance of plantlets in culture media from proembryos having twenty days development with that of embryos having full maturity, proembryos having twenty days development performed well to that of embryos of full maturity.

The performance of the embryo from dry nut gave an entirely different picture compared to embryos of all other age groups presented earlier. Eventhough treatments on dry nut embryos were done on second, third and fourth days of culturing the embryo differentiation and further growth and development of plantlets were hindered

by the production of excess quantities of phenolic compounds by the plantlets. This makes to give a general statement that without adopting further techniques it is quite impossible to use the physiologically matured embryos for induced mutagenesis adopting *in vitro* techniques in this particular crop. The frequent production of phenolic compounds requires standardisation of further techniques to remove these growth retarding substances by creating further disturbances of the embryoids during their developmental phases in culture medium. It is doubtful whether these additional processes can give a chance for easy elimination of the matured types. Lesser the disturbances greater will be the expressions of induced variability. The embryos from dry nuts were able to give comparatively a poor performance even when embryos were treated directly at the time of culturing, though the higher concentrations were able to give embryo differentiation and further developments. This statement is further supported by the fact that even the untreated embryos were not able to give a comparable performance with those of embryos extracted from seeds immediately after harvest and proembryos analysed. Thus it can be

concluded that embryos from dry seed nuts cannot be recommended for induced mutagenesis adopting tissue culture techniques in groundnut. The medium or the lower dose levels tested and the embryos extracted from nuts immediately after harvest or proembryos having 30 days development can be recommended for further detailed analysis on induced mutagenic processes in groundnut adopting *in vitro* culture techniques.

S U M M A R Y

The *in vitro* culture techniques in relation to induced mutagenesis in groundnut (Arachis hypogaea .L) were standardised by using the most popular variety, TG-3. The experiment was conducted during 1985-86 at the Plant Tissue Culture Laboratory attached to the Tropical Botanic Gardens and Research Institute. The main objectives of the present investigation were to standardise the embryo culture techniques in groundnut, to standardise surface sterilisation techniques, to standardise the correct age of the embryo for mutagen treatment, to standardise the technique of treatment and also to assess the dose requirement of a chemical mutagen, ethyl methane sulphonate (EMS) to create variability in groundnut. The dose effect was assessed based on various growth indices including days taken for embryo set, percentage setting of embryos, length of shoot at fifteen days interval, number of leaflets per plantlet at fifteen days interval and growth rate analysis. Based on the results emina ted from the investigation the following conclusions are made from the experiment.

1) Of the three culture media tested for groundnut embryo, White's media, Murashige-Skoog media (basal) and Modified Murashige-Skoog media, a modified MS medium was found to be the best.

2) Of the various concentrations of mercuric chloride solution tested and various time intervals adopted for treatment, the treatment 0.1% mercuric chloride treatment for three minutes was found to be the best, based on survival percentage.

3) Treating the explants at the time of culturing was found to be the best technique of treatment of proembryos and embryos by using mutagen solution.

4) Incorporating mutagenic solution with the culture medium was found to be a failure due to non solidification of culture medium and contamination of explants planted.

5) Treatment of embryos extracted from seed nuts collected immediately after harvest followed with proembryos having thirty days development was found to be more suitable for mutagenic treatment compared to proembryo having twenty days development and embryos extracted from seed nuts thirty days after harvest.

6) Fully matured embryos, extracted thirty days after harvest were able to give only a very poor performance compared to all other types of proembryos and embryos tested.

7) Treatment of the embryos and proembryos with mutagenic solutions above 0.50% was found to be lethal.

8) The lower concentration (0.125%) gave a comparable result with that of untreated control.

9) In majority of the factors analysed to assess the mutagenic effectiveness, 0.25% gave a better performance compared to control and lowest concentration tried.

10) The best concentration for the maximum mutational events in groundnut while exploiting <u>in vitro</u> culture techniques seems to lie between 0.25% and 0.50% of EMS.

11) An earlier embryo uptake was noted under 0.250% ethylmethane sulphonate solution, irrespective of the embryonic stage and the mode of treatments tried in the experiment, higher the concentration greater was the delay in embryo take both in proembryos and embryos.

12) The maximum percentage differentiation of embryos was observed in direct treatments compared to other modes of treatments and higher the concentrations, lesser was the percentage embryo differentiation. Treatment of explants with the mutagen at the time of culturing was found to be the best to give maximum tissue differentiation compared to all other modes of treatments.

13) Lesser the maturity of the embryos, greater was the damages created due to delayed treatments with the mutagen.

14) Growth rate analysis based on plantlet height and number of leaves per plantlet clearly demonstrated that maturity of the embryo plays a very important role on the performance of the plantlets. Embryos extracted from seed nuts immediately after harvest gave the maximum expression compared to all other three maturity groups of embryos tested. It was also made clear that embryos extracted from seed nuts thirty days after harvest are not at all suited for induced mutagenesis adopting

in vitro culture techniques.

15) Based on the results eminated during the present investigation it is recommended that for detailed analysis of induced mutagenesis in groundnut adopting *in vitro* culture techniques, modified MS medium, embryos extracted from seed nuts immediately after harvest and a concentration of ethylmethanesulphonate solution in between 0.25% and 0.50% will result in maximum mutational events to give an economic response to induced variability by minimising the diplontic selection.

REFERENCES

1. Ahmed, Rina and Ghosh,P.D.(1982): Morphogenetic studies in two varieties of Vigna radiata. In: Proc. National Symposium on Recent Trends in Botanical Researches (In press) Patna University.

2. Alikhan, W.M, Sivaswamy, N. and Ramaswamy, K.R(1973). Sensitivity of Z red gram (Cajanus Cajan(L) Millsp.) Straine to different mutagens. Madras Agric. J., 60: 406-407

3. Anon,(1981)"Icrisat Annual Report 1979-1980" Icrisat.

4. Athwal, D.S(1963). Some x-ray induced and spontaneous mutations in Cicer. Indian Jr. Genet. 23. 50-57.

5. Atroya, C.D., Rao, J.P. and Subrahmanyam, N.C.(1984). In vitro regeneration of peanut (Arachis hypogaea.L)Plantlets from embry axes and colgledon segments. Plant Sci. Lett. 34(3) 379-383.

6. Auerbach, C. and Robson, J.M.(1947). The production of mutations by chemical substances. Proc. R.Soc. Edinb. (Secct. B1)62:271-283.

7. Bajaj, Y.P.S. and Saettler, A.W. (1970). Gamma irradiation studies on seeds of Phaseolus vulgaris. Radiat. Bot., 10: 35-37.

8. Bajaj, Y.P.S.; Kumar, P.; Singh, M.M. and Labana, K.S. (1982) Interspecific hybridisation in the genus Arachis through embryo culture. Euphytica 31(2):365-370.

9. Bajaj, Y.P.S.(1983). Production of normal seeds from plants regenerated from the meristem of Arachis hypogaea and cicr arietinum cryopreserved for 20 months. Euphylica 32(2): 425-430.

10. Blake, J and Eeuwens C.J.(1980). Inflorescence tissue as source material for vegetative propagation of coconut palm. In: Proc. Int. Cont. Cocoa coconuts, Kulala Lumpur pp549-556.

11. Blake, J. and Euwens, C.J (1981) Culture of coconut palm with a view to vegetative propagation. In: Proc. COSTED Symp.on Tissue culture of Economically Important Plants, Singapore (A.N. Ra. ed)pp.145-148.

12. Bajaj, Y.P.S.(1971). Direct and indirect effects of gamma irradiation on Plant tissue cultures. In: International Symposium on use of Isotopes and Radiation in Agriculture and Animal Husbandry Research. 285-301.

13. Yoshida,S. and Ogawa,M(1983). The application of tissue culture - induced mutagenesis to crop improvement. Tech.Bull. (73).

14. Blixt, S. and Gelin, O(1965). The relationship between leaf spotting (A-sectors) and mutation rate in Pisum. The use of Induced Mutations in Plant Breeding . (Rep.FAO/IAEA. Tech.Meeting, Roma, 1964) Pergamon Press, pp. 251-262.

15. Blixt, S, Ehrenberg. L. and Gelin, O (1966).Studies of induced mutations in peas XVIII. EMS treatment of different duration of the varieties weitor and Witham Wonder. Agri. Hort. Genet. 24: 138-146.

16. Boxus, P.(1974) The production of strawberry plants by in vitro micropropagation. I. Hort. Sci. 49:209-210.

17. Butenko, R.G. (1968) Plant Tissue culture and Plant Morphogenesis. Israel Program for Scientific Translations, Jerusalem.

18. Constantin, M.J, Klobe, W.D. and Skold, L.N(1976) Effect of physical and chemical mutagens on survival, growth and seed yield of soybean. Crop. Sci. 16 (1):49-52.

19. Corley, R.H.V., Barrett, J.H. and Jones, L.H.(1976) Vegetative propagation of oil palm via tissue culture. In: Malayasian Int. Agri. Oil Palm. Conf. Incorporated Society of Planters, Kuala Lumpur pp. 1-7.

20. Custer, J.B.M, Vanee, G. and Buijs, L.C(1980). Clonal propagation of coffea arabica L. by nodal cultures. In: Proceedings IX International Collegium of Coffee, Assoc. scient. Intern. Coffee, London.

21. de Guzman, E.V., Ubalda, E.H. and Del Rosario, A.G. (1976). Banana and coconut in vitro cultures for induced mutation studies. In: Improvement of vegetatively propagated plants and Tree Crops through Induced mutations. pp 33-54, International Atomic Energy Agency, Vienna.

22. Desale, S.C. (1985) A faint orange coloured short stature mutant in groundnut. Curr.Res.Rep. 1(1):92-93.

23. Doreswamy, R., Srinivasa Rao, N.K and Chacko, E.K., (1983). Tissue culture propagation of banana. Scientia Hortic. 18: 247-252.

24. Duuta, M; Bandyopadhyay, A., Arunachalam, V; Kaul, S.L. and Prasad, M.V.R. (1986). Radiation Induced pollen sterility and enhanced outcrossing in groundnut. (Arachis hybogaea L). Envt. Exp.Bot. 26(1): 25-29.

25. Ehrenberg.L., Gustafsson, A and Lundquist, U.(1961) Viable mutant induced in barley by ionizing radiations andchemicals. Herditas, 47: 243-282.

26. Freeze, E.(1963). Molecular mechanism of mutation. Molecular Genetics. Part I Taylor (ed) Academic Press, New York and London, Ch.v. pp 207-289.

27. Evans, D.A., Sharp, W.R. and Flinck, C.E. (1981) Growth and behaviour of cell cultures: Embryogenesis and organogenesis. In: Plant Tissue Culture: Methods and Applications in Agriculture. (T.A. Thorpe, ed) pp.45-114. Academic Press, NewYork, 1st ed.

28. Gadgil, J.D., Mitra, R.(1983). Chemical composition of seeds in Induced groundnuts mutants and their derivatives. Ind. J.Agric. Sci. 53 (5): 295-298-

29. Gamborg, p.L., Miller, R.A. and Ohyama, K.(1968) Nutrient requirements of suspension cultures of Soybean root cells. Exp.cell Res. 50:148-151.

30. Gaul, H (1961). Use of induced mutants in seed propagated species. Mutation in Plant Breeding. NAS-NRC., 891:206-251.

31. Gautheret, R.J. (1938). Sur la repiquage des cultures de tissue cambial de Salix Caprasa l.r. hebd. seanc. Acad. Sci. Paris 206:125-127

32. George, L. and Rao, P.S.(1979). In vitro regeneration of mustard plants (Brassica juncea var. RA.5) on cotyledon explants from non irradiated, irradiated and mutagen-treated seed. Ann.Bot. 46, 107-112.

33. Ghosh, P., Mitra, G.C. and Sharma, A.K (1979). Embryo culture as a tool for securing viable mutants in certain legumes after gamma ray treatment. Ind. J. Hered. 11(1):1-15.

34. Ghosh, P.D.(1982). Induced mutagenesis and chromosome instability in tissue culture of certain crop plants. Sci. Acad. Medals for Young Scientists. Lectures, 24-42.

35. Gladstones, J.S.(1958). Induction of mutation in the West Australian blue lupin (Lupinus digitatus Forks). by X-irradiation. Aust. J. Agri. Res., 9:473-482.

36. Goel, S. Anup, K.M, and Gupta, S.C (1983). Development of plants from in vitro cultured shoot tips of Vigna mungo and Vigna radiata Trop. Plant. Sci. Res. 1(1)31-33.

37. Goud, J.V., Nayar, K.M. and Rao, N.G.(1970). Mutagenosis in sorgnum. Ind. J. Genetics. Pl.Breed. 30 (1):81-90.

38. Gregory, W.C.(1946). Peanut breeding programm underway. Research and Forming, 69th Ann. Rep.N.C. Agric. Exp.Sta. 42-44.

39. Harten, A.M.V, Bouter, H. and Schut,B.(1973). Ivy leaf of potato (Solanum tuberosum) , A radiation-induced dominant mutation for leaf shape. Rad. Bot. 13: pp 287-292.

40. Harten, A.M.V, Bouter, H. and Broertjes, C (1980)In vitro . Adventitious and techniques for vegetative propagation and mutation breeding of potato (Solanum tuberosum L) II. Significance for mutation Breeding. Euphytica. 3 1-8.

41. Heinz, D.J, Krishnamurthi,M., Nickell, L.G. and Maretski, A (1977). Cell, tissue and organ culture in sugarcane improvement. In: Applied and Fundamental Aspects of Plant Cell, Tissue and organ culture(J.Reinert and Y.P.S. Bajaj, eds) pp 3-17. Springer-Verlag, New York, 1st Ed.

42. Hildebrandt, A.C. (1963) Growth of single cell clones of diseased and normal tissue origins. In: Plant Tissue culture and Morphogenesis. pp. 1-22. Scholar's Library, NewYork.

43. Hu, C.Y. and Wang, P.J. (1983) Meristem, Shoot tip and bud cultures. In: Handbook of plant cell culture. Vol.1. Techniques for Propagation and Breeding (D.A. Evans, W.R.Sharp, P.V. Ammirato and Y. Yamada, eds) pp.177-227. Macmillan Publishing Co., New York, 1st.

44. Jalal, M.A.F. and Collin, H.A.(1979). Secondary metabolism in tissue culture of Theobroma Cacao New Phytol. 83:343-349.

45. Jana, M.K (1964). Effects of x-rays and neutron irradiation of seeds of Phaseolus mungo Genet. Agrar. 18$_2$: 617-628

46. Jones, L.H. 1974, Propagation of clonal oil palms by tissue culture. Oil Palm News 17:1-8.

47. Joshua, D.C., Bhatia, C.R. (1983). Increased cotyledonary cell size in induced large seed mutants in three grain legumes. Envt.Exp. Bot. 23(2):175-181.

48. Kartha, K.K, Gamborg, O.L, Constable, F. and Shyluk, J.P. (1974) Regeneration of cassava plants from shoot apical meristems. Plant Sci. Lett. 2:107-113.

49. Kartha, K.K., Pahl, K., Leung, N.L. and Mroginski, L.A (1981). Plant regeneration frommmeristems of grain legumes: Soybean, cowpea, peanut, chickpea and bean. Can. J.Bot. 59 ; 1671- 1679.

50. Kawai, T.(1963a). Mutations in rice induced by radiation and their significance in rice breeding I. Mutation by radiophosphorous. Bull. Natn. Inst. Agric. Sci.(Japan) 10: 1-75

51. Khan, S.K and Ghosh, P.D. (1984). Regeneration of plantlets in Cicer arietinum L through tissue culture. In: Perspectives in Cytology and Genetics (G.K. Manna and U.Sinha, eds) 4: 131-135.

52. Konzak, C.F, Nilan, R.A., Wagner, J. and Foster, R.J. (1969). Efficient chemical mutagenesis. The use of Induced Mutations in Plant Breeding (Rep.FAO/IAEA. Tech.Meeting, Rome, 1964) Pergamon press, pp 49-70.

53. Kouider, M, Skirvin, R.M.,Saladin, K.P, Dawson,J.O., and Jokela, J.J. (1984). A method to culture immature embryos of Populus deltoides in vitro Can. J. Forest Res. 14(6):956-958.

54. Levy, A. (1976) The mutagenic activity of the acridine ethidium bromide in peanuts, Arachis hypogaea. L. From Oleagineux 33(819).

55. Louis, I.M. and Kndambavanasundaram, M, (1973a). Mutation breeding in Cowpea I. An evaluation of Selection methods in M_2 generation. Madras Agric. J. 60:1361-1368.

56. Martin, J.P.(1970), Culture in vitro d' ovules d' ovules d' Arachide, Oleagineaux 25:155-166

57. Maslov, A.B. and Stepanova, N.D. (1967). The effect of different doses of gamma rays and chemical mutagens on wheat, barley and pea. Genetika, 3: 27-34.

58. Mroginski, L.A. and Fernandez, A.(1979) Cultivo in vitro de anteras de especies de Arachis. (Leguminosae), Oleaginoaux 34: 243-248.

59. Mroginski, L.A., Kartha, K.K, and Shyluk, J.P(1981) Regeneration of peanut (Arachis hypogaea) plantlets by in vitro culture of immature leaves, J.Bot. 59:826-830.

60. Morel, G.(1960). Producing virus-free cymbidium. Am. Orchid. Soc. Bull. 29:495-497.

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

62. Murashige, T. (1974) Plant propagation through tissue cultures. Ann. Rev. Plant Physiol. 25:135-166.

63. Murashige, T (1978). Principles of rapid propagation. In : Propagation of Higher Plants through tissue culture, a Bridge between Research and Application. (K.Hughes, R. Henke and M.Constantin, eds) pp.14-24, Tech. Int. Centre, U.S.Dept. of Energy, Oak Ridge.

64. Nair, N.G., Martha, K.K and Gamborg, O.L(1979) Effect of growth regulators on plant regeneration from shot apical meristems of cassava (Manihot esculenta orantz) and on the culture of internodes in vitro . Z. Pflenzenphysiol. 95: 51-56.

65. Narasimhulu, S.B and Reddy, G.H(1983). Plantlet regeneration from different callus cultures of Arachis hypogaea L Plant Sci. Lett. 31(2/3): 157-163.

66. Narsinghani, V.G. and Kumar, S. (1976). Mutation studies in Cowpea. Indian Jr. Agric. Sci. 46(2):61-64.

67. Negrutin, I. and Muller, J.F. (1981). Culture conditions of protoplast derived cells of Nicotiana Sylvestris for Mutant Selection. Plant Cell Reports. (1):14-17.

68. Nobecourt, P. (1939) Sur la parennite et l' augmentation de volume des cultures de tissues vegetaux. C.R. S'eanc. Soc.Biol.130: 1270-1271.

69. Pathirana, R.(1985). Performance of gamma ray induced groundnut mutant in Sri Lanka - Mutation Brec. Newsletter 26, 10-11.

70. Patil, S.J., and Mouli, C.(1984). Preferential segregation of two allelic mutations for small leaf character in groundnut. Theo. Appl.Genet. 67(4):327-332.

71. Pittman, R.N., Banks, D.J., Kirby, J.S., Mitchell, E.D. and Richardson, P.E.(1983) In vitro culture of immature peanut (Arachis spp) Leaves: Morphogenesis and Plantlet Regeneration. Peanut Sci., 10:21-25.

72. Rabechault, H. and Martin, J.P. (1976) Multiplication vegetative du palmier a' huile (Elaeis guineensis Jacq) a' l' aide de cultures de tissues. Foliaries. C.R. Acad. Sci 283:1735-1737.

73. Raghavan, V.(1976) " Experimental Embryogenesis in vascular plants". Academic Press, London.

74. Raju, C.R., Prakash Kumar, P., Mini Chandramohan andIyer, R.D. (1984) Coconut plantlets from leaf tissue cultures. J.Plant. Crops 12(1):75-91.

75. Ramanathan, T.(1984). Induced high yielding mutants in Arachis hypogaea. Madras Agric J. 71(2):85-88

76. Rangan, T.S. (1976). Growth and plantlet regeneration in tissue cultures of some Indian millets - Paspalum scrobiculatum L Eleusine coracana Gaertu and Pennisetum lyphoideum Pers. 2 PFlanzenphiol. 78:208-216.

77. Rangaswamy, N.S; Rangan, T.S. and Rao, P.S.(1965) Morphogenesis of peanut (Arachis hypogaea L pericarp in tissue cultures, Naturwiss .22:1-2

78. Reddy, P.S., Reddy, M.V., Thammi Raju, B. and Mahaboob Ali, S. (1977). Creation of genetic variability by recourse to irradiation in groundnut (Arachis Hypogaea L). Oleagineux, 32(2):59-63.

79. Reinert, J. (1958) Morphogenese and ihre kontrolle an gewebekulturon aus karotten. Naturwissenschaften 45: 344-345.

80. Roest, S., van Berkel, M.A.E., Bokelmann, G.S and Broertjes, C (1980). The use of an in vitro adventitious but technique for mutation breeding of Begonia x Hiemalis. Euphytica 30:381-388.

81. Rogozinsk,a J.H. and Gonka M. (1975) Growth and differentiation of sugarbeet anthers and callus. Bulletin De L' Academie Polonaise Des Sciences. Serie des sciences biologiques Cl.ii. Vol . XXIV, No.1-

82. Sastri, D.C, Nalini, M.S and Moss, J.P. (1980). Arachis ovule and ovary culture in vitro , in: Proc. Symposium on plant tissue culture, somatic hybridization and Genetic manipulation in plant cells, " P. Rao et al; eds., BARC, Bombay.

83. Sastri, D.C; Nalini, M.S. and Moss, J.P(1981), Tissue culture and prospects of crop improvement in Arachis hypogaea and other oil seed crops, in " Proc. of Symposium on Tissue Culture of EconomicallyImportant Crops in Developing countries, " Nat. Univ. Singapore (in press).

84. Sastri,DC. and Moss I.P(1982). Hormonal requirement for incompatible crosses in the genus Arachis (in preparation).

85. Schleiden, M.J.1838, Beitrage zur phytogenesis, Muller, Arch. Anat. Physiol. Wiss. Med. pp. 137-176.

86. Schwann, T.(1839) 'Mikroskopische untersuchungen uber die uboreinstimmung in der struktur und dem Wachstume der Tiere und Pfanzen'. ('Ostwalds Klassiker der exakten Wissenschaften', No.176. Engelmann, Leipzig, 1910).

87. Sharp, W.R., Evans, D.A., Ammirato, P.V. and Yamada,Y. (1984) Handbook of plant cell culture. Vol. 2. Crop. Species. Macmillan Publishing Co., New York, 1st Ed).

88. Shirshov, V.A. and Shain, S.S.(1966). Variation of legumes under the influence of gamma irradiation. Experimental mutagenesis of agricultural plants and its application for plant breeding. Trans., Moscow, Soc.Nat, 23: 159-163.

89. Sidorova, K.K., Kalinina, N.P and Uzhintseva, L.P. (1966) Peculiarities of mutational variability in pea cultivars and forms. Experimental mutagenesis of agricultural plants and its application for plant breeding. Trans. Moscow, Soc. Nat., 23: 141-149.

90. Sinha, P.K., and Rahman, H (1979). Promising mutant varieties of groundnut evolved through gamma irradiation. In symposium on the role of induced mutations in crop improvements.

91. Sivaram, M.R., Rathnasamy, R., Appadurai, R (1985). Details regarding the origin and the characteristics of the groundnut mutants var " CO-2" (Mutant 3) Mutation Breed. Newsletter 26(2):

92. Skirvin, R.M. (1977) Natural and induced variation in tissue culture. Euphytica 27 241-266.

93. Skoog, F. and Miller, C.O. (1957) Chemical regulation of growth and organ formation in plant tissues cultivated in vitro. In: Biological action of growth substances. Symp.Soc. Exp. Biol. 11: 118-131.

94. Spielman, I.V., Burge, A.P and Moss, J.P (1979) Chromosome loss and meiotic behaviour in interspecific hybrids in the genus Arachis L and their implications in breeding for disease resistance , Z. Pflanzenzuchtg. 83:236-250.

95. Staritsky, G.(1970). Embryoid formation in callus tissues of Coffee. Acta. Bot. Nerrl. 19:509-514.

96. Steward, F.C., Mapes, M.O and Mears, K.(1958) Growth and organised development of cultured cells. II. Organisation in cultures grown from freely suspended cells. Am. J.Bot. 46: 705-708

97. Street, H.E. (1969) Knowledge gained by culture of organs and tissue explants. In: Plant Physiology: A treatise. Vol. VB. (F.C.Steward, ed.)pp.3-224. Academic Press, New York, 1st Ed.

98. Styer, D.J. and Chin, C.K (1983). Meristem and shoot tip culture for propagations, pathogen elimination and germ plasm preservation. In: Horticultural Reviews. Vol.5 (J.Janick, ed) pp-221-277. A.V.I. Publishing Co., Connecticut, 1st ed.

99. Swaminathan, M.S.(1966a). Use of induced mutations. Indian Ring. 16:(34-35)125-127.

100. Swaminathan, M.S.,Siddiq , E.A., Singh C.R., and Pai, R.A.(1970) Mutation Breeding in rice in India. Rice Breeding with Induced Mutations. II (Tech. Rep. Series No.102), IAEA 1, Vienna, 25-43.

101. Tal, M, Dehan, K., and Heiken, H (1977). Morphogenetic potential of cultured leaf sections of cultivated and wild species of tomato. Ann. Bot. 41:937-941.

102. Tarasenkov, I.I (1969). Effect of chemical mutagens on pea and tomato plants. Outdoor Hort., Moscow, 233-241.

103. Teodoradze, S.G.(1966). The use of radiomutants of French and Soybean in breeding Experimental mutagenesis of agricultural plants breedings Trans. Moscow, Soc. Nat., 23: 120-125.

104. Tisserat, B. (1979) Propagation of date palm (Phoenix dactylifera L). in vitro J.Exp.Bot. 30:1275-1283.

105. Tisserat, B(1981). Date palm tissue culture: U.S. Agric. Res. Serv., Adv. Agric, Technol. ATT-W-17, 1-50.

106. Vagera, J., and Hanackara, H (1980): Embryo culture technique and possible utilization Rostlinna Vyroba 25 (4)349-360.

107. Vasil, I.K. and Hildebrandt, A.C. (1966) Variations of morphogemtic behaviour in plant tissue cultures. 11. Petroselinum hortense. Am. J.Bot. 53: 869:874.

108. Vasil, I.K. and Vasil, V. 1980. Clonal propagation. Int. Rev. Cytol. Sup. 11A: 145-173.

109. Venkateswarlu, S., Singh, R.N, Singh, R.B and Singh, B.D(1978). Radiosensitivity and frequency of chlorophyll mutations in pigeon pea. Indian J. Genet. Plant.Breed., 38(1):90-94.

110. Wellensiek, S.K(1965). Comparison of the effects of EMS, neutrons, gamma and x-rays on pea. The use of Induced Mutations in Plant Breeding. (Rep FAO/IAEA. Tech. Meeting, Rome, 1964). Pergamon Press pp. 227-235).

111. White, P.R. (1934) Potentially unlimited growth of excised tomato root tips in a liquid medium Plant Physiol. Lancaser 9:585-600.

112. White, P.R. (1939) Potentially unlimited growth of excised plant callus in an artificial medium. Am. J. Bot. 26:59-64.

113. Wickson, M.E. and Thimann, K.V. (1958). The antagonism of auxin and kinetin in apical dominance. Physiol. Plant. 11: 62-74

114. Yie, S. and Liaw, S.I. (1977). Plant regeneration from shoot tips and callus of papaya. In vitro 13: 564-567.

115. Zir, M and Zamski, E (1975). Geotropic responses and pod development in gynophore explants of peanut, Arachis hypogaea cultured in vitro Ann. Bot. 39: 579-583.

.....

Plate 1: Growth rate as on 40th day of culturing in proembryos having 20 days development (D_1).

Plate 2: Growth rate as on 40th day of culturing in proembryos having 30 days development(D_2).

Plate 3: Growth rate as on 40th day of culturing in Embryos extracted at the time of harvest (D_3)

Plate 4: Growth rate as on 40th day of culturing in embryos extracted one month after harvest (D_4).

T_1 = treatment at the time of culturing.

C_0 - Control

C_1 - EMS 0.125%

C_2 - EMS 0.25%

C_3 - EMS 0.50%

C_4 - EMS 0.75%

Plate 2

Plate 3

Plate 5: Growth rate as on 40th day of culturing in Proembryos having twenty days development (D_1)

Plate 6: Growth rate as on 40th day of culturing in proembryos having 30 days development(D_2).

Plate 7: Growth rate as on 40th day of culturing in Embryos extracted at the time of harvest. (D_3).

T_2 - treatment two days after culturing.

C_0 - Control.

C_1 - EMS - 0.125%

C_2 - EMS - 0.25%

C_3 - EMS - 0.50%

C_4 - EMS - 0.75%

Plate 5

Plate 6

Plate 7

Plate 8: Growth rate as on 40th day of culturing in proembryos having twenty days development (D_1)

Plate 9: Growth rate as on 40th day of culturing in Proembryos having 30 days development(D_2)

Plate 10: Growth rate as on 40th day of culturing in embryos extracted at the time of harvest (D_3)

T_3 - treatment after three days of culturing.

C_0 - Control

C_1 - EMS - 0.125%

C_2 - EMS - 0.25%

C_3 - EMS - 0.50%

C_4 - EMS - 0.75%

Plate 1


$D_3T_3C_1$
$D_3T_3C_0$

Plate 11: Growth rate as on 40th day of culturing in proembryos having 20 days development (D_1)

Plate 12: Growth rate as on 40th day of culturing in proembryos having 30 days development (D_2)

Plate 13: Growth rate as on 40th day of culturing in Embryos extracted at the time of harvest (D_3)

T_4 - treatment after four days of culturing.

C_0 - Control.

C_1 - EMS - 0.125%

C_2 - EMS - 0.25%

C_3 - EMS - 0.50%

C_4 - EMS - 0.75%

Plate 13

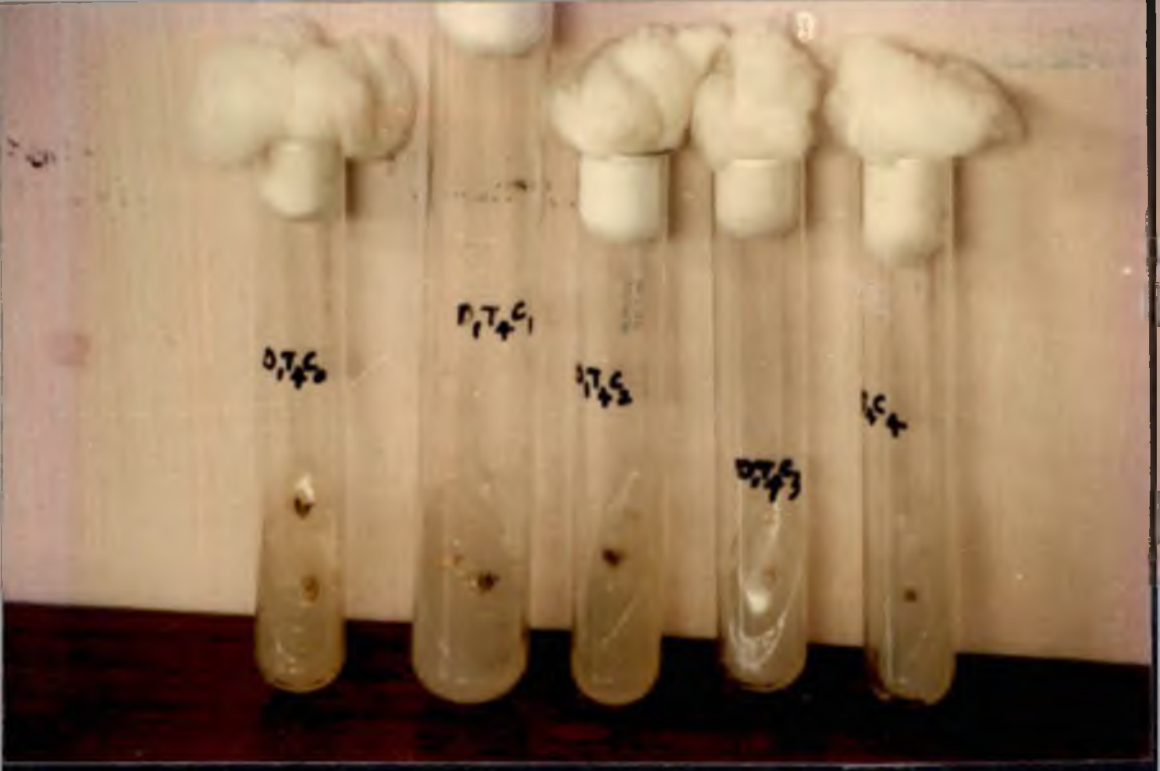


Plate 11

A B S T R A C T

IN VITRO TECHNIQUES IN RELATION TO INDUCED MUTATIONS IN GROUNDNUT

BY

K. ARYA, B. Sc. (Ag.)

ABSTRACT OF A THESIS

Submitted in partial fulfilment of the requirement
for the degree of
MASTER OF SCIENCE IN AGRICULTURE
Faculty of Agriculture
Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL BOTANY
COLLEGE OF AGRICULTURE
VELLAYANI, TRIVANDRUM

1987

The present investigation entitled 'In vitro techniques in relation to induced mutations in Groundnut' was carried out in the Department of Agricultural Botany, College of Agriculture, Vellayani, during 1984-86. All the works related to tissue culture analysis were done at the Plant Tissue Culture Laboratory, attached to Tropical Botanic Gardens and Research Institute, Kumarapuram, Trivandrum. The main objective of the experiment was to standardise the best embryo culture technique in groundnut to standardise a successful mutation breeding programme by using the most potent chemical mutagen, ethylmethane sulphonate. The project was also envisaged to standardise the techniques to assess the correct stage of embryo treatment with the mutagen and to standardise the best mode of treatment of the mutagen solution to the embryoids and embryos.

Three culture media, viz. White's medium, Murashige-Skoog medium (basal) and Modified Murashige-Skoog medium were tested to find out the best culture medium for groundnut embryos. Surface sterilisation of embryos were standardised by using mercuric chloride selecting out three concentrations, 0.05%, 0.1%, and 0.2% and three periods of treatment, 3,5 and 10 minutes.

The correct stage of embryos for the maximum mutagenic effect was done by using embryos having four maturity periods, embryos having twenty and thirty days maturity, embryos from seed nuts immediately after harvest and embryos from dry seed nuts thirty days after harvest. The various concentrations of ethyl methane sulphonate tested include 0.125, 0.25, 0.50 and 0.75%. To standardise the technique of treatment of mutagen the various concentrations tried were treated at different periods, treating the embryo at the time of culturing, locating the embryos two days after culturing, treating the embryos three days after culturing and treating the embryos four days after culturing. Attempts were also made to test whether mutagenic treatment can be carried out through the culture medium. As the main objectives of the experiment were to standardise various techniques of culturing and methods of treatment a fully laid out experiment was not envisaged.

The modified Murashige-Skoog culture medium was found to be the best for groundnut embryos. The best result can be attained with a surface sterilisation of the embryos with mercuric chloride at 0.1% for three minutes. Of the different treatment techniques adopted,

treating the embryos at the time of culturing was found to be the most effective technique. Incorporating the mutagenic solution along with culture medium failed to give any positive result. The lower concentrations (0.125 and 0.25%) gave a better performance based on days taken for embryo set and tissue differentiation. Just reverse was the trend in higher concentrations. Mutagenic solution having a strength of 0.25% to 0.50% is recommended for yielding maximum mutagenic events in groundnut. Analysis on embryoids and embryos having different maturity clearly showed that embryos extracted from seed nuts immediately after harvest gave a better response based on embryo take, percentage differentiation and growth rate analysis compared to all other. Detailed analysis using Modified MS medium, concentrations of ethyl methanesulphonate ranging from 0.25 to 0.50% and treating the embryos extracted from seed nuts immediately after harvest is recommended for detailed analysis on induced mutations in groundnut adopting <u>in vitro</u> culture techniques.

...