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IN VITRO MUTAGENESIS IN RICE
(*Oryza sativa* L.)

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THESIS

Submitted in partial fulfillment of the
requirement for the degree of

Master of Science in Agriculture

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2005

DECLARATION

I hereby declare that the thesis entitled "***In vitro* mutagenesis in rice (*Oryza sativa* L.)**" is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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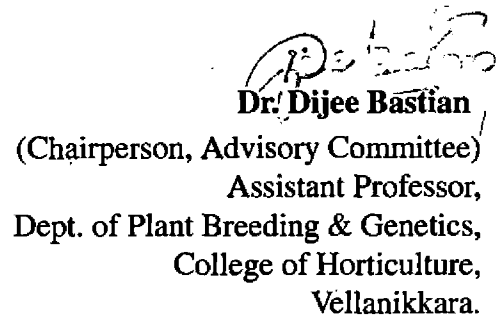

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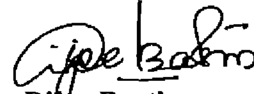

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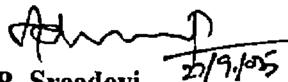

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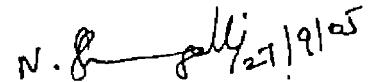

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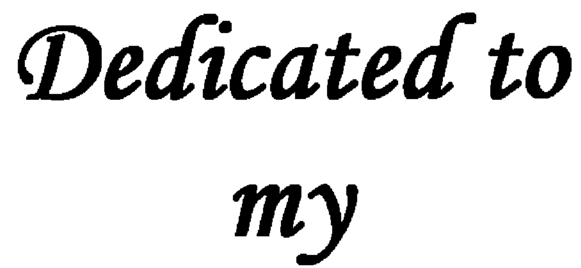

Dedicated to

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LIST OF ABBREVIATIONS

AC	-	Auricle Colour
ApC	-	Apiculus Colour
BA	-	Benzyl Adenine
BLSC	-	Basal Leaf Sheath Colour
BAP	-	6- Benzyl Amino Purine
EMS	-	Ethyl Methane Sulphonate
CC	-	Collar Colour
cc	-	cubic centimetre
CD	-	Critical Difference
CRD	-	Completely Randomized Design
CL	-	Culm Length
cm	-	centimetre
CmA	-	Culm Angle
CmIC	-	Culm Internode Colour
DMRT	-	Duncan's Multiple Range Test
df	-	degrees of freedom
2, 4- D	-	2, 4- Dichlorophenoxyacetic acid
edf	-	error degrees of freedom
FLA	-	Flag Leaf Angle
g	-	gram
GL	-	Grain Length
GL/W	-	Grain Length/Width ratio
GP	-	Grains per Panicle
GW	-	Grain Width
Gy	-	Gray
GY	-	Grain Yield
ha	-	hectare
IAA	-	Indole Acetic Acid
IBA	-	Indole-3- Butyric Acid

kr	-	kilo radon
Kn	-	Kinetin
LA	-	Leaf Angle
LBC	-	Leaf Blade Colour
LBP	-	Leaf Blade Pubescence
LgC	-	Ligule Colour
LgL	-	Ligule Length
LL	-	Leaf Length
LmPC	-	Lemma and Palea Colour
LS	-	Ligule Shape
LW	-	Leaf Width
mg	-	milligram
mm	-	millimeter
mM	-	millimolar
NAA	-	Naphthalene Acetic Acid
No.	-	Number
OD	-	Optimum Dose
PH	-	Plant Height
PL	-	Panicle Length
PnAx	-	Panicle Axis
PnEx	-	Panicle Exsertion
PnT	-	Panicle Type
PP	-	Panicles per Plant
r	-	r adon
SCC	-	Seed Coat Colour
SgC	-	Stigma Colour
TCMG	-	Tissue Culture plants from gamma irradiation
TCME	-	Tissue Culture plants from EMS treatment
TW	-	Test Weight
w.r.t	-	with respect to

INTRODUCTION

1. INTRODUCTION

Rice is an important staple food crop that meets the dietary energy requirements of more than half of humanity. Rice is grown in our country in an area of 43 million hectares with a production of 77.7 million tonnes. It is estimated that the rice demand in 2010 will be 100 million tonnes and in 2025, the demand will be 140 million tonnes (Mishra, 2004). In Kerala, rice occupies an area of 3.9 lakh hectares with an annual production of 7.7 lakh tonnes (FIB, 2002). The country has to produce around 135-140 million tonnes of rice by 2020 to meet the food requirements of its burgeoning population. The target has to be achieved in the backdrop of plateauing yield trends of high yielding varieties and declining resource base of land, labour and water (Khush, 2001). Any research programme carried out to increase the production and productivity of rice will be of great value in the service of mankind and nation.

In India, a number of new high yielding rice varieties were evolved and released through crop improvement programmes, but many of them are unadapted and unaccepted both by the farmers and consumers. The local varieties are still prevailing in major parts of the cultivated areas and such local types constitute an essential basis for further advances in rice breeding and acts as sources of resistant genes for diseases, pests, local adaptation and specialised grain qualities, which are decided by common people (Jyothi, 2002).

There are many traditional bold red rice varieties in Kerala with good consumer preferance but with a few defects like long duration, tall stature, non-responsiveness to fertilizers, photo sensitiveness, low yield, lodging and presence of awns which limits their extensive cultivation. The local cultivars being *indica* types will be more adapted to our region if the defects are rectified and yield improved. Rice variety Varsha [M210X (M210 X Ptb28)] suited to kole lands and resistant to biotic stresses can be quoted as a notable example in this context (Francies 2002).

The exploitation of radiations and radiometric compounds for inducing mutations / alterations in base sequences of DNA of the most potent lines is contemporary crop improvement programme. It is proved both in seed propagated and vegetatively propagated crops that all morphological and physiological characters within species boundary and even beyond can be induced by mutation (Henske *et al.*, 1978).

Mutation breeding is a potential tool for rectifying such few defects in an otherwise good cultivar. Rasmi, a long duration, tall, awnless, saline tolerant mutant from the long awned, saline resistant Oorpandy is a typical example which combines high yield with resistance to biotic and abiotic stresses. It is widely accepted as a donor for multiple resistance breeding.

Totipotency an important quality of plant cells is not exploited in conventional breeding methods. The techniques of cell, tissue and organ culture have made available a new range of hitherto unavailable materials for genetic manipulation (Chandrahasan, 2004). The application of advanced tissue culture techniques like protoplast fusion, gene transfer, induction of somaclonal variation, *in vitro* mutagenesis, cell or callus culture and subsequent plant regeneration, has opened up new avenues in rice improvement. (Nishi *et al.*, 1973).

Induced mutations in relation to recent advances in 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 a particular season. An advantage is that selection for a particular trait may be done at tissue level (Arya, 1987).

Hence the present investigation was envisaged with the following objectives:

i) To study the effect of growth regulators on callus induction and to find out optimum concentration required for better callus induction and subsequent regeneration.

ii) To estimate the LD_{50} values for *in vitro* mutagenesis in rice using gamma irradiation and EMS treatment.

iii) To estimate the optimum doses for *in vitro* mutagenesis in rice using gamma irradiation and EMS treatment.

iv) To identify desirable mutants from the rice cultivar Ptb-26.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Rice is the most important food crop of Kerala. It occupies an area of 3.9 lakh hectares with annual production of 7.7 lakh tonnes (FIB, 2002), which is sufficient to meet only 30 per cent of the requirement. Since the prospect of bringing more area under rice is very remote, increase in production should primarily come through enhanced productivity.

In vitro techniques allow somaclonal variations and regeneration of genetic variants. *In vitro* mutagenesis will amplify the production of somaclonal variants and further exploitation of selected mutants can be carried out in lab conditions under biotic stress free environment. The present study entitled "*In vitro* mutagenesis in rice (*Oryza sativa* L.)" was carried out with a view to produce successful *in vitro* cultures, to fix the LD_{50} values of mutagens, to fix optimum doses of mutagens and identifying desirable *in vitro* mutants. The available literature on *in vitro* culture and *in vitro* mutagenesis in rice are reviewed here.

2.1 ROLE OF LOCAL LAND RACES IN CROP IMPROVEMENT

Eventhough more than sixty high yielding rice varieties were released for various agroecological situations of Kerala, majority of the area (63 per cent) under rice cultivation is still with local cultivars (State Planning Board, 1998).

According to Francies (2002) rice in Kerala finds existence in varied spectrum of agroecological niches, each with specific management and varietal requirements. Among the varieties ruling in the state, traditional cultivars/land races and improved selections from traditional cultivars find a place along with the high yielding varieties. These land races not only offer a pool of resistance genes against pests and diseases which cause considerable damage to the rice crop, but also exhibit excellent adaptability to stress and specific systems of cultivation prevalent in the state.

With regard to cultivated rice (*Oryza sativa* L.), a large number of primitive cultivars, indigenous stocks, landraces and obsolete varieties are available with farmers in addition to the modern cultivars. These "Desi varieties' are distinct, unique and well adapted to the sub-humid tropics due to selection pressures over years. These are dynamic and balanced populations. Farmers have names for them and they are dependable. A few could sustain under zero management condition. Few others hold promise in problem soils such as acid saline, acid sulphate, deep-water soil, etc. Few others possess medicinal properties and superior grain qualities. Still others could be used for genetic improvement especially, in modulating biotic and abiotic stresses (Nizar *et al.*, 2002).

2.1.1 Study Material

Ptb-26 was developed by pureline selection from the local cultivar "Chenkayama" which is characterized by purple pigmentation of the plant especially on the leaf sheath, blade and apiculus. This is an important tall *indica* variety popular in Palakkad district of Kerala. Cultivation of this variety facilitates identification of wild rice and similar weeds, which "mimic" rice, there by making hand weeding more effective. Compared to Thavalakannan, this is less lodging and shorter in duration, maturing in 120 days. It recorded 25% increase in grain yield over the parent cultivar and the average yield is 2500 $kgha^{-1}$. The rice kernel is red in colour, quality good and milling recovery is 78.1 per cent. This variety is also recommended for *Kootumundakan* system of cultivation (Rosamma *et al.*, 2003).

2.2 *IN VITRO* STUDIES

Murashige (1974) noticed that tissue culture cycle involves establishment of a more or less differentiated cell or tissue culture under defined culture conditions, proliferation for a number of cell generations and the subsequent regeneration of

plants. The initiating exploitation of a tissue culture cycle may come from virtually any plant organ or cell type including embryos, microspores, roots, leaves and protoplasts.

Plant cell cultures has often been hailed as one of the most significant potential adjuncts to plant improvement. This is usually seen in terms of ability to apply cellular selection for recovering useful genetic variants. The assembly of genetic variability is vital to any plant breeding enterprise (Skirvin, 1978). Thomas *et al.* (1979) reported that tissue culture *per se* is an expectedly rich and novel source of genetic variability.

Tissue culture techniques have been applied for improvement of many crops. In monocotyledons, especially the Poaceae family, tissue culture is challenging. Research has been conducted in many rice cultivars (Bajaj and Bidani, 1980).

When regenerated plants produced through tissue culture exhibit deviations from parental type, the phenomenon is termed somaclonal variation. Somaclonal variation is a form of genetic variation that may occur during prolonged *in vitro* culture of somatic plant cells and tissue cultures. It is evident that somaclonal variations occur in common wheat and other cereal crops (Larkin and Scowcroft, 1981).

Dunwell (1985) recorded phenotypic variants amongst somaclonal plants regenerated from rice callus. The variants were in traits such as number of tillers, number of panicles per plant, average panicle length, frequency of fertile seed, plant strature and flag leaf sheath.

The nature and causes of somaclonal variations in rice has been discussed by Oono (1987). They attributed some mutations to the genome changes and others to changes in individual genes. Some of the most useful mutations found included those affecting chlorophyll deficiency, plant height and heading date.

Miah *et al.* (1991) used seeds of five semidwarf rice varieties to induce callus. MS medium supplemented with sucrose, yeast extract and 2,4-D was used for callus induction in which the variety BR3 callused best. Vigorously growing callus of BR3 produced multiple shoots on MS medium supplemented with NAA, Kn,

sucrose, yeast extract and casein hydrolysate. Nine regenerants from the callus of a single seed were transferred to pots. Progenies from these plants were tested in experimental plots for various agronomic traits in the R_2 generation and for disease and insect pest reaction in the R_3 generation. Phenotypic variation from the parent variety BR3 was observed for several agronomic characters including plant height, panicle length and grain size.

Choudari *et al.* (1998) revealed minor DNA sequence changes as the reason for somaclonal variants in rice *in vitro* culture. Hendre *et al.* (1998) experimented with the seeds of 46 high-yielding tissue culture derived R_1 plants of cultivar Indrayani. On evaluation, no major changes were observed among progenies for morphological traits, although subtle changes were detected in some traits, including tiller number, panicle length, spikelets per panicle and percentage sterility. Ten variants producing yields 500-2000 kg ha^{-1} higher than Indrayani were identified. This increase in yield was mainly due to an increase in the number of spikelets per panicle.

2.2.1Callus Induction

2.2.1.1 Genotype

Genotypic differences in callusing and plant regeneration ability among the genotypes have been reported (Sticklen *et al.*, 1987). Chen and Zhu (1991) concluded that the genotype of parent is the main factor responsible for the variation in embryo culture parameters.

According to Pandey *et al.* (1994) the mean effects of genotypes, media and genotype X medium interaction for various characters related to callus and plantlet regeneration revealed large genotypic variation.

Kunanuvatchaidach *et al.* (1995) noticed different efficiency in embryogenic response as the genotypes differed. Manipulation of light environment improved callus initiation or embryogenic callus formation.

Interactive effects of genotypes with callus induction and regeneration media combinations on green plantlet regeneration response were studied in the *indica* rice cultivars IR72, IR54 and Karnal Local. Isolated, mature embryos were used to derive scutellar callus. Regeneration percentage as well as shoot bud induction frequency were influenced by genotype, callus induction medium, regeneration medium, interaction between genotype and the two media (callus induction and regeneration), as well as between the callus induction medium and regeneration medium (Khanna and Raina, 1998).

Biswas and Mandal (1999) investigated varietal specificity with respect to *in vitro* culture response involving callus induction and plantlet regeneration in *indica* rice cultivar Annada. Its performance was concomitantly compared with an *in vitro* culture responsive *japonica* cultivar Taipei 309. Plantlet regeneration percentage and number of plantlets obtained from individual seed calli were found to be maximum in Annada.

Genotype X culture media interaction effects on *in vitro* green plantlet regeneration efficiency were examined using mature embryo derived from scutellar calli involving 22 *indica* rice cultivars and five media combinations (Katiyar *et al*., 1999).

Visarada and Sarma (2002) compared *in vitro* response of several elite *indica* rice cultures with that of the most responding *japonica* type, Taipei 309, for enumerating critical tissue culture parameters that subscribe to successful genetic transformation. Callus induced from mature seeds of *indica* rice genotypes was more compact and proliferated slowly. Formation of friable callus, rapid proliferation and sustenance of regeneration capacity for 9-10 weeks, are identified as the key features that favour success of transformation in a variety and recovery of transformant through subsequent selection and regeneration.

2.2.1.2 Explant

Poonsaparaya *et al*. (1989) observed that callus culture growth and development involve a complex relationship between explants used to initiate the

callus, the constituents of the medium and the environmental conditions during culturing period.

Wu *et al.* (1991) found that the efficiency of culture also differed with embryo age. Callus induction rate varied from 3.5% for seven days old embryos to 48.18% for ten days old ones, and that for green shoot differentiation ranged from 25.44% for nine days old embryos to 879.44% for fifteen days old embryos.

Callus induction from mature rice caryopsis commenced within a week of culturing. Callus formation from rice seeds was better in the light than in the dark (Ratisoontorn *et al.*, 1993). Callus induction rate from mature embryos was higher than that from young ones, proliferation was slower during subculture and only some calli differentiated into plantlets (Tan *et al.*, 1999). Vinothini (2004) found mature and dehulled seed to be the best explant for callus induction.

2.2.1.3Culture Media

Immature embryos of IR54 were plated onto callus induction medium consisting of MS. Callus growth and plant regeneration were influenced by addition of organic supplements to the basal media, medium solidifying agents and phytohormone concentration during callus induction (Koetje *et al.*, 1989).

Rapid proliferation of the scutellum and high frequency of callus formation was observed when mature seeds of five *Oryza sativa* cultivars and accessions of *Oryza glaberrima, Oryza perennis* and *Oryza latifolia* were cultured on MS media (Brisibe *et al.*, 1990).

Abbasi *et al.* (2000) investigated callus induction on MS medium supplemented with different combinations of auxins and cytokinins from seed derived callus cultures in three cultivars of rice viz., Basmati 370, Basmati 385 and KS 282.

Deepti *et al.* (2001) studied callus induction and plant regeneration from mature embryo in six *indica* rice cultivars (Pusa Basmati 1, Basmati 370, Type 3, CSR10, Pant Dhan 4 and Pokkali). Maximum callus initiation was observed on MS medium supplemented with 2.0 mg 2,4-D l^{-1} in all the cultivars. Delawar and

Arzani (2001) considered MS and N6 media suitable for *in vitro* culture of rice immature embryos.

Oinam and Kothari (1993) cultured embryos of 15 *indica* cultivars on MS medium supplemented with 3% sucrose and 2.5 mg l^{-1} 2,4-D. Apart from cultivar Himalaya 1; which produced no callus, the genotypes formed either embryogenic or non-embryogenic callus, or both, of which the former was selected for regeneration. According to Vinothini (2004) MS medium is the best one suited for rice tissue culture studies.

2.2.1.3 Growth Regulators

Kucherenko *et al.* (1988) plated mature embryos or embryo parts on MS medium with 2,4-D 2.0 mg l^{-1}. The callus obtained was subcultured on MS medium with 2,4-D 2.0 mg l^{-1}. MS medium containing 2.0 mg l^{-1} 2,4-D combined with 0.5 mg l^{-1} Kn was effective for callus induction (Maheswaran and Rangaswamy, 1989).

Calli seven days after subculture on MS regeneration medium supplemented with 2.0 mg l^{-1} Kn, 2.0 mg l^{-1} BA and 1.0 mg l^{-1} NAA clearly revealed *de novo* somatic embryogenesis occurring during the regeneration process in Nipponbare, Murasakiine and UPLRi5 from callus induced on medium supplemented with 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} Kn. In Sakaikaneko, UPLRi3 and UPLRi4, somatic embryos formed from callus induction on medium supplemented with 0.5 mg l^{-1} 2,4-D, 0.2 mg l^{-1} NAA and 0.5 mg l^{-1} Kn while in IR-8 somatic embryos arose from callus induced on media supplemented with 0.2 mgl^{-1} 2,4-D, 0.2 mgl^{-1} NAA and 1.0-2.0 mg l^{-1} BA (Mendoza and Futsuhara, 1992). Callus initiation in rice cultivar B370 was highest (85%) when mature seeds were cultured on MS medium with 2% 2,4-D (Zafar *et al.*, 1992).

Oinam and Kothari (1993) cultured embryos of 15 *indica* cultivars on MS medium supplemented with 3% sucrose and 2.5 mg l^{-1} 2,4-D. Apart from cultivar Himalaya 1, which produced no callus, the genotypes formed either embryogenic or non-embryogenic callus, or both, of which the former was selected for regeneration.

Modified MS medium with 5.0 mg l^{-1} NAA and 1.0 mg l^{-1} Kn was suitable for inducing callus from rice cultivars Kaodogmali 105, Basmati 370 and RD 15 MS with 4.0 mgl^{-1} NAA and 1.0 mgl^{-1} Kn was suitable for Nangmol S4 and Pradoodaeng, while MS medium with 5.0 mg l^{-1} NAA and 0.5 mg l^{-1} Kn was suitable for Patumthani 60 (Ratisoontorn *et al.*, 1993).

Cunha *et al.* (1994) studied callus development in mature embryos of rice variety BR-IRGA414 grown in MS medium with different concentrations of synthetic auxins, NAA and 2,4-D (0.5, 1.0, 5.0 and 10.0 mgl^{-1}). Each concentration had additional treatments, including the presence and absence of Kn (0.2 mgl^{-1}) and light (cold-white bulb, 4000 lux intensity). The experimental design was completely randomised with three factors and five replications. The addition of auxins improved callus formation.

MS medium supplemented with 2, 4-D gave very good response for callus initiation although this response varied with the change in the levels of 2, 4-D. In general, the MS medium supplemented with 2.0 mg l^{-1} 2,4-D gave best response for all callusing characters studied. A plateau was reached at this level of 2,4-D and thereafter, the response of 2,4-D on callus growth characteristics declined with every unit increase in its level (Pandey *et al.*, 1994).

Kunanuvatchaidach *et al.* (1995) achieved high frequency callus production following the culturing of mature caryopsis on MS medium containing MS vitamins 5 µM 2,4-D, 59 mM sucrose, 3 g l^{-1} casein hydrolysate and 8 g l^{-1} agar.

Singh and Singh (1996) screened *in vitro* development of morphogenic and non-morphogenic cultures in nine cultivars representing four ecotypes of *indica* rice Calli induced by 2.0 mg l^{-1} 2,4-D led to development of morphogenic cultures. Call induced at 8.0 mg l^{-1} 2,4-D reduced the overall morphogenic potential of cultures during regeneration (2.44-48.29%).

Sharma *et al.* (1996) studied callus initiation and plant regeneration from immature embryos of three *indica* rice varieties (Heera, Pankaj and Basmati 370)

and reported that the MS medium supplemented 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA was suitable for callus initiation.

When the MS medium was supplemented with low concentrations of 2,4-D and Kn, the callus resulted in microtillering whereas higher concentrations revealed non embryogenic callus. The callus subcultured on media with lower hormonal composition formed a few shoots and sectors of compact nodular embryogenic callus (Virk *et al.*, 1998).

MS media with varying concentration of 2,4-D(0.5, 1.0, 1.5 and 2.0 mg l^{-1}) and Kn 0.5 mgl^{-1} was used for callus induction while half MS media with BAP (1.0, 2.0 mg l^{-1}), Kn 0.5 mg l^{-1} and NAA 1.0 mg l^{-1} was used as regeneration medium (Kalamani *et al.*, 1999).

Katiyar *et al.* (1999) found callus induction to be greatly influenced by the genotypes and ,4-D concentration in the media. MS media supplemented with 2.0 mg l^{-1} of 2,4-D for callusing was found best in all the 22 genotypes of rice studied.

Gonzalez (2000) grew rice cultivar Amistad82 seeds on MS media containing 0.0, 1.0, 2.0 or 3.0 mg l^{-1} of 2,4-D and BAP each. There was no callus formation in the absence of 2,4-D, and it was reduced in treatments with 1.0 or 3.0 mg l^{-1} each of 2,4-D and BAP. Shoot production was best with 2.0 mg l^{-1} each of 2,4-D and BAP.

The media MS + 2,4-D 2.5 mg l^{-1} + Kn 0.5 mg l^{-1} was found to be best for callus induction. The calli were subcultured for regeneration, MS +BAP 0.5 mg l^{-1} + NAA 0.1 mg l^{-1} and MS + NAA 1.0 mg l^{-1} + GA3 1.0 mg l^{-1} were good for shooting and rooting respectively (Kalamani *et al.*, 2000).

Factors affecting tissue culture were studied in two *indica* rice cultivars, Peiai64S and YanHui 559. The results showed that the optimum concentration of 2,4-D for inducing rice calli was different between cultivars. The optimum concentration of 2,4-D and other exohormones, such as Kn, did not actually improve callus formation, but did improve the quality of calli in terms of growth state of subcultured calli (Wang *et al.*, 2001). MS media with 2,4-D 2.0 mg l^{-1} and Kn 0.5 mgl^{-1} was used for callus induction while basal MS media with BAP 2.0 mg l^{-1}, Kn

1.0 mg l^{-1} and IAA 1.0 mg l^{-1} was used as regeneration medium (Gomez and Kalamani, 2002).

2.2.1.5 Callus Age and Quality

Meifang (1992) observed that the callus emerging in 50 days has good competence for green plantlet regeneration, and those emerging after 70 days are usually larger with poor regeneration capacity. Callus with a diameter 2 mm showed the highest level of green plantlet regeneration. Callus smaller as well as larger than 2 mm will either die or encounter difficulty in regeneration.

The callus quality could be identified by observing callus morphology, callus that is milky-white, compact, moist, smooth and slow growing had excellent ability of plantlet regeneration. On the contrary, callus that was cream coloured or dark yellow; friable, dry and fast growing was poor in plantlet regeneration (Xie *et al.*, 1995).

2.2.2 Regeneration

Plant regeneration from two months old from mature embryos was achieved at 20% and 100% frequency, respectively. The morphogenic potential of embryo derived calli dropped from 100% at the third subculture to 12.5% at the twelfth subculture (Boissot *et al.*, 1990).

Plant regeneration systems were optimized for three commercial rice cultivars (LaGrue, Katy and Alan) and two advanced rice breeding lines. Regeneration was accomplished by callus induction followed by plant regeneration. Genotype, sugar type and the concentrations of Kn, 2,4-D and NAA influenced callus induction and regeneration (Anderson and Al-Khayri, 1996).

Seraj *et al.* (1997) measured the regeneration response of mature and immature rice embryos from fifteen *indica* rice varieties separately in terms of regeneration percentage and total number of regenerated plantlets obtained for a fixed sample size per variety, cultured on standard MS media. The response of the rice varieties was

classified as high, medium or poor, based on the above criteria. The regeneration percentage of genotypes showing high, medium and poor responses were in the range 67-97, 38-63 and 0-36 per cent, respectively. The variation in the regeneration response amongst the defined groups was found to be statistically significant in terms of regeneration percentage and total plantlets for a constant sample size when subjected to one way analysis of variance and Duncan's multiple range tests.

Khanna and Raina (1998) obtained in IR72, the highest regeneration frequency (47.5%) and shoot bud induction frequency (77%) in MS media for both callus induction and regeneration. Although genotype and the composition of the callus induction basal medium were the major determinants of regeneration response, an overall analysis of variation also revealed a significant interaction between the media used for callus induction and plantlet regeneration.

2.2.2.1 Growth Regulators

Maheswaran and Rangaswamy (1989) induced hypocotyl callus from IR50, IR1552 and Co43 induced on medium containing 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} Kn showed higher regeneration capacity than other treatment combinations. Level of regeneration varied with genotype and concentration of growth regulators.

Amirkhanov *et al.* (1991) cultured mature grains of seven *japonica* varieties on MS medium with various supplements. Regenerants were not obtained from all varieties on medium with Kn. The best result in this respect was obtained on medium with BAP (5 mg l^{-1}). Most of the regenerants were green.

Davoyan (1991) obtained the highest frequency of regenerants by subculturing callus on medium with 2,4-D (0.5 mg l^{-1}) and BA (5.0 mg l^{-1}). The medium for organogenesis was most effective when it contained casein hydrolysate (3.0 g l^{-1}) and BA (1.0 mg l^{-1}). Freshly prepared medium surpassed conditioned medium in the frequency of regenerants induced from callus. Callus cultures giving regenerants retained much of their morphogenetic capacity and gave a fairly high regeneration frequency with repeated induction of organogenesis.

Four weeks old calli were maintained on MS medium containing 2.5 mg l^{-1} 2,4-D and after the second subculture were transferred to MS medium (pH 5.8, 0.8% agar) supplemented with various concentrations and combinations of BA (0.5, 1.0 and 2.0 mg l^{-1}) and NAA (0.05, 0.1 and 0.5 mg l^{-1}). At 1.0 mg l^{-1} BA + 0.1 mgl^{-1} NAA, a large number of green plantlets were regenerated from cultivars Chambal, CH1039 and Himdhan. Chambal also produced a high number of albino plantlets (Oinam and Kothari, 1993).

Singh *et al.* (1993) transferred the 35 - 40 days old calli of Basmati 370 and Pusa Basmati1 to regeneration media together with a further series of basal media supplemented with 0.1 mg l^{-1} BA. Regenerated plantlets were transferred to MS basal medium. Regeneration occurred after 30 - 45 days from seed callus with the greatest regeneration frequencies being 44.4 and 45.5%, respectively, in Pusa Basmati and Basmati 370.

Guo and Shen. (1996) used lower concentrations of 2,4-D (0.2, 0.4 or 0.8 mg l^{-1}) to induce rice callus *in vitro.* Addition of 0.2 mg l^{-1} 2,4-D induced a few white, smooth and more compact granular calli under the base of the coleoptile. These calli formed clustered buds and produced fine roots. These clustered buds regenerated into plantlets when cut into pieces and placed in media containing Kn.

Callus proliferation and shoot bud regeneration was achieved on MS medium with 2.0 mg l^{-1} BA and 1.0 mg l^{-1} NAA. For root initiation, differentiated calli were transferred to MS medium containing 1.0 mg l^{-1} Kn and 5.0 mgl^{-1} NAA. Response to *in vitro* culture condition was varietal specific (Sharma *et al.*, 1996).

Katiyar *et al.* (1999) used MS basal medium supplemented with 1.0 mg l^{-1} NAA and 1.0 mg l^{-1} Kn throughout the course of *in vitro* regeneration of rice to assess the genotypic differences to regenerate plants from calli. Calli began differentiating into shoots, which after transferring on to MS basal medium, developed into complete plantlets.

Somatic cell cultures were established from mature seeds of two *indica* (IR-65598-112-2 and PR111) and two *japonica* (Hsinchu-64 and Te-Sen-AI) rice genotypes by culturing on MS medium supplemented with 2,4-D (2 mg l^{-1}) and Kn (0.5 mg l^{-1}). Shoot regeneration was achieved on MS medium supplemented with

NAA (0.5 mg l^{-1}) and Kn (2.0 mg l^{-1}). Among the four genotypes, Hsinchu-64 exhibited better shoot regeneration (70%). Shoots were transferred to half strength MS + NAA (0.5 mg l^{-1}) rooting medium. Tissue culture derived, pot grown plants exhibited normal growth and seed setting (Navraj *et al.*, 1999).

Revathy *et al.* (2000) experimented with rice genotypes IR 50 and Co 43 *in vitro* and reported that MS media with the plant growth regulator treatment, BAP 4.0 mg l^{-1} + IAA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} induced the highest regeneration response in both rice cultivars.

2.2.2.2 Rooting and Hardening of Green Plantlets

Hazarika (1983) reported that rooting and root quality of regenerated rice plantlets was enhanced by lower macronutrients salt concentration in the medium. Most pollen plants regenerated in the test tube have very weak adaptability to the natural environment. Direct transplanting into soil results into their death. Therefore, the plants should be prepared to enable their adaptation to the *in vivo* climate (Chung, 1992). Zhenhua (1992) reported that survival rate can be improved by maintaining the high relative humidity following transplanting of plantlets.

2.3 *IN VITRO* MUTAGENESIS

Idea of induced mutation is quite old. It was proposed first by De Veries in as early as 1901 and the term mutation was derived from 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 Caesium sources made gamma rays also available. The discoveries of Muller in 1927 in Drosophila and that of Stadler in 1928 in Barley and Maize opened up a new field of research, which significantly advanced our knowledge in genetics. Research in the last forty years have elucidated the importance of mutation as a potential tool for modifying plants more or less in the same manner as by conventional breeding methods (Gregory, 1956).

Large number of varieties developed by mutation breeding has been arisen from materials irradiated with ionising radiation (Micke, 1962). In addition to several ionising radiations, a number of chemical mutagens also produce mutation in plants when applied singly or combined with other chemicals and in succession or simultaneously with radiation (Konzak *et al.*, 1965).

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. Bajaj (1983) studied direct and indirect effects of gamma irradiation on the seeds, seedlings, callus tissue cultures, excised shoots, ovules and embryos and reported that callus tissue cultures are more radio resistant than the intact seedlings. EMS had an inhibitory effect on the shoot regeneration but stimulatory callus growth.

According to Vajrabhaya (1977) treatment with chemical or physical mutagens can cause chromosome or gene mutations spontaneously *in vitro*. *In vitro* culture techniques allow selection of the desired variants from large populations of cells.

In vitro technology including anther culture, ploidy manipulation, mutagenesis and a number of other gene manipulation techniques are mainly in their way into the scene, supplementing classical breeding (Sigurbjornsson, 1991). *In vitro* mutagenesis has been proved to be effective method of crop improvement in many vegetatively propagated crops for inducing variability (Novak, 1991). Even though the occurrence of desired mutation is empirical and random, the combination of *in vitro* and mutation techniques can speed up crop improvement (Ahloowalia, 1995).

According to Maluzynski *et al.* (1995) *in vitro* culture in combination with induced mutations can speed up breeding programme from the generation of variability through selection of the desired genotypes.

Plant improvement based on mutation can change one or a few specific traits and thus contribute to crop improvement (Prediere, 2001). Tissue culture increases the efficiency of mutagenic treatments for variation induction, handling of large populations, use of ready selection methods, and rapid cloning of selected variants. Molecular techniques can provide a better understanding of the potential and

limitations of mutation breeding e.g. molecular marker assisted selection, which can lead to the early identification of useful variants. The relatively high number of research reports compared with the low number of cultivars released suggests that mutagenesis in combination with tissue culture is either ineffective or has yet to be exploited.

2.3.1 *In vitro* Mutagenesis using Physical Mutagen

The heading time for rice plant is governed by the basic vegetative growth period, which is influenced by photosensitivity and thermo sensitivity. The former two characters have been altered by *in vitro* mutation with physical agents (Kudo, 1967).

In some mutants dwarfism is combined with earliness and loss in photoperiod sensitivity. Thus it may be possible to grow more than one crop within a year in the respective regions (Hu, 1973). A characteristic feature of many short culm rice mutants is their increased degree of tillering, which often contribute to increased seed production of the plants (Reddy *et al.*, 1975).

Zhu *et al.* (1988) cultured rice calli *in vitro* and treated with ^{60}Co gamma rays. Growth was inhibited by radiation levels of 1000, 2000, 5000 and 10,000 R. There was an increase in the ratio of cultures resistant to *Xanthomonas campestris* pv. *oryzae* on exposure to 1000 or 2000 r. The redifferentiation of plantlets from disease resistant calli was promoted by radiation of 1000 r but inhibited when greater than 1000 r.

Ukai (1988) studied grains of 6 varieties irradiated with 10 to 20 kr gamma rays at a rate of 4 kr per hour. Mature embryos were then cultured on modified B5 media containing 30 μM 2,4-D. Some callus cultures were irradiated 3 days after culturing with 2-6 kr. Increasing exposure resulted in a decrease in callus size and a colour change from white to brown.

The use of induced mutations combined with *in vitro* culture has been utilised in overcoming problems of hybrid seed production by Gao (1989). New

restorer lines were obtained from irradiated progeny and the hybrids with the new restorer lines outyielded the local hybrid, ripened earlier and had better grain quality.

Improvement of anther culture response in recalcitrant but high yielding varieties was studied by Zapata and Aldemita (1989). They opined that to overcome the low response of *indica* varieties to anther culture, radiation treatment can be combined with *in vitro* culture. Minimal stress on the seed to be used as panicle source for anther culture by irradiation could stimulate callus induction and plant regeneration without inducing irreversible genetic changes.

Immature embryos (10-12 days after flowering) from three *indica* restorers were gamma irradiated at 1-3 kr. Young inflorescence segments of 5 mm in length from a maintainer were also irradiated at 0.5-1 kr and mature embryos (mature seeds) from two restorers were irradiated at 2.5-15.0 kr. These were cultured in modified N6 and MS media. Regenerated plantlets were planted in the field and observations were made on variation in chlorophyll content, heading date, tillering ability, plant height, panicle length, fertility and other morphological traits. Results showed that immature embryos had the highest callus induction rate (0.46%) and plantlet regeneration rate (0.90/embryo) at 1 kr. Young panicle had the highest callus induction rate and plantlet regeneration (18.1/panicle) at 0.5 kr and mature embryos had the highest callus induction rate (0.54%) at 0.1 kr and highest plantlet regeneration rate (3.44/explant) at 10.0 kr (Cai *et al.*, 1990).

Mutations were induced with ^{60}Co gamma rays of 15, 30, 45 and 60 Gy to *in vitro* cultured shoots of seven clones of *Musa*. Radiosensitivity was assessed by determining the relative increase of fresh weight of cultures and by the rates of shoot differentiation. *Musa* clones exhibited differences in radiosensitivity and post radiation recovery. Considerable phenotypic variation was observed among plants regenerated from *in vitro* shoot tips after mutagenic treatment (Novak *et al.*, 1990).

Improvement of anther culture response in recalcitrant but high yielding varieties was studied, applying radiation on the seed grown and used as panicle source in anther culture. Seedling lethality was proportional to increasing radiation dosage. Application of radiation improved callus induction and plant regeneration in recalcitrant varieties, but not so in the responsive varieties. Addition of agarose is

beneficial for callus induction and plant regeneration of all genotypes. A total of 1,396 plants were regenerated. Reduction in plant height and per cent fertility was observed with increasing radiation dose (Aldemita and Zapata, 1991).

The effect of gamma rays radiation treatment on somatic cell culture of Basmati 370 selection, an *indica* rice strain was studied. The induction rate of callus reduced with the increase of the radiation dosage on germinating embryo, but the influence of 2.5 kr treatment was not severe. Redifferentiation percentage and/or green plantlets percentage from callus varied with different treatment. Irradiation of *in vitro* cultures reduced the fertility of regenerated plants, but was able to increase their exserted stigma rate and the frequency of early maturing progenies (Min *et al.*, 1991).

Nguyen *et al.* (1991) exposed seeds of the variety 127/d to 100-300 Gy gamma radiations, then cultured on MS medium supplemented with 1-10 mg l^{-1} BAP and finally subcultured to enhance new shoot clump formation. A BAP concentration of 10.0 mg l^{-1} produced the most shoots. Shoots formed the largest number of roots per shoot when cultured on MS medium with NAA. Mean seedling height and number of primary shoots per seed were reduced in M_1 seedlings. The *in vitro* culture of mutagenized seed avoided the problems of chimaeric tiller production.

R_2 generation of somaclones from wheat immature embryo cultures were used as standard material for measuring the frequency of R_2 variant lines for several major characters by Gao *et al.* (1991). M_2R_2 generation from cultures with young embryos irradiated immediately prior to inoculations, and C_2R_2 from calli irradiated with 1 kr gamma rays during the process of culture, were compared to the standard in terms of variant frequencies. The variant frequencies related to M_2R_2 and C_2R_2 significantly surpassed that in R_2 populations. It appeared that irradiation of calli with an optimal dose could benefit the frequency increase and to some extend, spectrum enlargement of the major characters.

Jeyanthi and Rangaswamy (1991) cultured gamma irradiated (0.5-3.0 kr) and non-irradiated anthers of rice cultivar, IR50 *in vitro* and examined them under the

microscope at three days intervals. The anthers irradiated at lower doses formed callus earlier than the non irradiated anther cultures. At the higher doses, the microspores failed to progress beyond the uninucleate stage. Callus induction decreased as the irradiation dose increased.

Rice cultivar Khao Dawk Mali seeds were exposed to different doses of gamma radiation (0, 24, 28, 40, and 44 kr), surface sterilized, and cultured on MS medium supplemented with 25.0 mg l^{-1} BA to induce multiple shoot formation. After five weeks multiple shoot formation was to the tune of 45.9, 83.2, 80.0, 11.4 and 0% respectively. 38 kr reduced the number of multiple shoot-forming seeds by 50% while shoot number per multiple shoot forming seed increased from 5.7 in the control to 21.3 in seeds given 40 kr treatment (Sripichitt *et al.*, 1991).

Ahloowalia (1992) experimented ten weeks old *in vitro* cultured plants of *Chrysanthemum morifolium* cultivars Princess Anne Bright Golden (yellow) and Neptune (white) for gamma irradiation (2000R). Irradiated (M_1V_1) plants were subcultured three times from single node explants to give 10 to 12 times as many plants in the M_1V_4. Twenty new variants were obtained in Neptune, including mutations affecting height, leaf and flower shape, and petal size and curvature. One mutant had light magenta florets. Fifteen of these variants proved stable through three successive conventional propagation cycles.

Gao *et al.* (1992) showed that young inflorescence and mature embryo were satisfactory explants both for *in vitro* culture and for mutagenesis in rice. They noticed higher rate of callusing and plantlet regeneration with both gamma rays treated immature embryos (1 kr) and seed callus cultures treated with different doses of gamma rays (250-500r). A dosage of 0.5-1 kr was recommended as appropriate exposure for young inflorescences to produce culture response and high variability, while 2.5-5.0 kr is the optimal dose range for seed callus from mature embryos.

El-Shoury *et al.* (1994) isolated M_2 plants for earliness, short stature, panicle and grain characteristics after treatment with gamma rays. Shoot tips from banana cultivar Maca were gamma irradiated (20, 40, 60, 80 and 100 Gy) and subcultured *in vitro* for four vegetative generations (M_1V_4). Based on leaf variegation, it was

concluded that the M_1V_4 is the minimum number of generations needed for screening mutants. In the greenhouse, besides leaf variegation, other phenotypic changes (anthocyanin, disturbed plant and leaf architecture) were also quantified. The frequency of variants was highest (13.5%) from the 40 Gy treatment (0.85% in untreated control plants). This dose is recommended for mutation breeding with this cultivar (Domingues *et al.*, 1994).

Seeds of Basmati 370 was cultured on modified MS medium with 2,4-D, NAA and Kn at 1.0, 2.0 and 1.0 mg l^{-1} respectively. Calli from this medium were divided into two groups - three week old subcultures and six weeks old subcultures. The subcultured callus was irradiated with different doses of gamma rays and cultured on medium for regeneration. The three weeks old sub cultures were better than six weeks old calli as regard plantlet regeneration (Pontongkam et *al.*, 1994).

To induce mutations, three weeks old calli from cultured mature seeds were irradiated with 1 to 5 kr gamma rays. The mutated regenerants could be selected from the second generation (Suputtitada *et al.*, 1994).

Ratisoontorn *et al.* (1995) irradiated calli of the cultivar Basmati 370 with 0, 1, 2, 3, 4 and 5 kr. Additional variations for purple stigma, leaf sheath, awn length and other biometrical characters were observed in the M_1R_1 and M_2R_2 plants.

Hammad (1996) exposed dry seeds of *Vicia faba* to 0, 5 and 10 Gy gamma rays. Plantlets regenerated from the cotyledonary node tissue of seedlings. Cotyledonary nodes were germinated on half strength basal MS media. Multiple shoots and buds were developed from the region of the junction between the cotyledon and epicotyl. The cultivars differed in growth and sensitivity to gamma irradiation. 10 Gy caused a slight increase in germination percentage, plant height, fresh and dry weights of all cultivars compared with samples from 5 Gy treatments and the untreated control.

Callus cultures in sugarcane with two cultivars namely Nil, susceptible to rust (*Puccinia melanocephala* and *Puccinia keuhnii*) and KF75-398, susceptible to *Puccinia keuhnii* only, were gamma irradiated. Regenerated plantlets of Nil were screened for resistance under conditions of natural infection. All control plants of both cultivars showed a high incidence of infection while irradiated plants had a

lower incidence. Two resistant mutants, M_1 and M_2 that showed no symptoms of infection were selected from irradiated Ni1 plants. M1 also showed enhanced cane yield (117%) over the wild type (Nagatomi *et al.*, 1996).

Jain *et al.* (1997) showed that protoclonal variation and mutagenesis in *Brassica napus* resulted in variability in seed shape and colour, flowering time and herbicide resistance. Yellow and shrivelled seeds were obtained in the M_1 generation of mutants and protoclones. Flowering time was unstable as a result of epigenetic variation in R_2 and R_3 generations, and seed germination rate was very low. It is suggested that somaclonal variation, in combination with mutagenesis can create stable somaclonal variants and mutants for exploitation by plant breeders in crop improvement.

Kuksova *et al.* (1997) studied somaclonal and *in vitro* mutagen induced variability in grapevine (*Vitis vinifera* cultivar Podarok Magaracha). Plants were regenerated from leaf explants through somatic embryogenesis. Chromosome counts of root tips were used for screening of regenerated plants. Neither chimaeral nor aneuploid plants were observed. Gamma irradiation (50-100 Gy) increased tetraploid plant formation frequency of primary (7%) and embryogenic calli (7.6%), and some aneuploid plants were also found. Variability among regenerated plants was also found after field testing.

The rate of callus formation and green plantlet regeneration varied for different rice types and varieties in anther culture. Irradiation of the anther cultures with 0, 10, 20, 30 and 40 Gy of gamma rays, and incubation for 30 days on induction medium increased the rate of callus formation and green plantlet regeneration. It was found that 10 Gy was the best dosage for callus formation, but 20 Gy was the most favourable for green plantlet regeneration (Lu *et al.*, 1997).

Earliness was induced through EMS and gamma irradiation in Samba Mashuri by Kulkarni and Gangaram (1998). They obtained mutants early by 25-30 days compared to the parent variety.

Ali and Siddiq (1999) reported that physical mutagens induce predominantly mutations affecting glume shape or size while the chemical mutagens appear to induce largely point mutations in rice.

The effect of gamma irradiation on callus induction and regeneration was investigated through M_2 seed culture of rice (Ponni (P) and White Ponni (WP)) on MS medium. Among the doses studied (10, 20 and 40 kr), the lower doses (P10, WP10 and WP20) were best for *in vitro* studies (Punitha, 1999).

Punitha and Kalamani (1999) reported the effects of different doses of gamma irradiation on callus induction and plantlet regeneration in Ponni and White Ponni rice varieties. Gamma irradiated (10, 20 or 40 kr) M_2 generation seeds (P10, WP10, WP20, WP 20 Dwarf, WP40 and WP40 Dwarf) and seeds of their parents were cultured on MS medium containing 2.0-2.5 mg l^{-1} 2,4-D and 0.5-1.0 mg l^{-1} Kn. Induced calli were transferred to regeneration medium (MS medium containing 2.0 mg l^{-1} IAA and 3.0 mg l^{-1} Kn. Regeneration frequency was highest in WP10 (75.88%) and least in WP40 (27.83%). Overall, lower doses of gamma irradiation were best.

Seeds of four basmati rice cultivars were cultured on MS medium. Calli were subcultured, irradiated with 0, 1, 2, 3, 4 and 5 kr gamma rays and subsequently plants were regenerated on MS medium containing 1.0 and 2.0mg l^{-1} BA, 0.5 mg l^{-1} Kn and 1.0 mg l^{-1} NAA. Basmati 370 had the highest regeneration frequency, and 3 kr was determined as the best irradiation dose (Sakila *et al.*, 1999).

Singh *et al.* (1999) studied *in vitro* effects of 5, 10, 20, 30, 40 and 50 Gy gamma rays in Carnation (*Dianthus caryophyllus*) cultivar Espana. Overall, effects on vegetative and floral characters increased with increase in treatment dose. At 5 Gy, gamma rays enhanced various vegetative and floral characters, while doses of 10 Gy and above significantly reduced these parameters. Irradiation with 30 Gy and above induced flower colour variations. Dark pink mutants were observed with 50 Gy treatments at a frequency of 4.44%. Dark pink mutants with red patches were produced by treatment with 30 and 40 Gy doses at frequencies of 1.79 and 3.94%, respectively.

Chu *et al.* (2000) treated tubers of Caladium cultivar Frieda Hemple, an ornamental plant with gamma rays (15Gy). The leaf bits from these treated plants were used for *in vitro* culture. They concluded that combining gamma ray irradiation and *in vitro* culture could increase the rate of mutation in Caladium.

Mittal *et al.* (2000) assessed mutation frequency, mutation effectiveness and efficiency of gamma rays and EMS and reported that gamma rays were more effective while EMS more efficient.

The general methodology of somaclonal breeding in rice involves the induction of embryogenic calli from excised embryos of mature caryopsis and selection of somaclones in the induction or regeneration medium, or even in both the media. However, the genetic variation that arises during somaclonal variation is generally low but could be enhanced through the use of chemical mutagens or irradiation. A protocol for the application of irradiation to enhance the output of stress tolerant somaclones has been perfected for rice (Pathirana *et al.*, 2002).

Lee *et al.* (2003) conducted a study to produce new salt tolerant rice lines through *in vitro* mutagenesis using radiation. Rice cultivar Dongjinbyeo seeds were cultured in MS medium. Callus from the culture were exposed to gamma rays at 0, 30, 50, 70 and 90 Gy for 24 hours, then inoculated in media containing 1.5% NaCl for 72 hours. The callus mass and survival rate of calli irradiated at 30 and 50 Gy were higher than those of the nonirradiated ones in saline media. Salt tolerant regenerants were cultured in auxin free media and grown in the field. The harvested M_1 seeds were used to raise the M_2 and subsequently M_3 generation was raised from selfed seeds of M_2. The M_3 lines were screened for saline tolerance at the seedling stage using 1% NaCl solution. Twelve salt tolerant lines among the 350 M_3s were selected for tolerance in a saline field in Korea Republic. Of these lines, M3-128-12 and M3-130-4 showed higher spikelet numbers, tillering and grain yield than the original cultivar.

A study was conducted *in vitro* using ^{60}Co gamma rays up to 60Gy for irradiating the protocorms of Dendrobium hybrid Sonia 28 x Emma White. The seedlings from irradiated protocorms were smaller than normal ones but the leaves became broader and thicker (Sobhana and Rajeevan, 2003).

In vitro mutagenesis using gamma rays (20 Gy and 30 Gy) was attempted in Jujube (*Ziziphus jujuba*) by Zheng *et al.* (2004) through *in vitro* culture. Calli from leaf explants were cultured on MS medium containing zeatin 1.0 mg l^{-1}, 2,4-D 0.5

mg l^{-1} for shooting. Young adventitious shoots developed were cultured on MS medium containing IBA 1.0 mg l^{-1} and IAA 0.4 mg l^{-1} for rooting.

2.3.2 *In vitro* Mutagenesis using Chemical Mutagen

Chemical mutagenesis was seen to give some encouraging results. Freese (1963) classified chemical mutagens as base analogues, dyes, acids, metals and alkalyting agents. In higher plants the last group, especially EMS has proved to be very effective. The relatively low toxic and genetic effects of EMS (Gaul *et al.*, 1964) and its high mutagenic effectiveness as well as efficiency in higher plants (Konzak *et al.*, 1965) demand attention for enhanced practical application.

Several workers have studied the relationship between doses of mutagen and survival percentage. Constantin *et al.* (1976) observed that in populations of soybean grown in green house the survival percentage of plants decreased with increase in dose of EMS.

Geier (1989) reported that for induced mutations in *in vitro* grown shoots of "Kohleria", an indoor ornamental, by soaking for one hour in a filter sterilized aqueous solutions of N- nitroso-N-methylurea (NMH 500 mgl^{-1}) at 20^0C. After treatment, he obtained a mutant, which is early flowering under low light conditions coupled with significantly shorter internodes as well as smaller and more attractive leaves.

Meiosis and mitosis were observed in two Triticale varieties subjected to gamma irradiation, magnetic fields, EMS and NMU. Most chromosome aberrations were seen in both varieties following gamma irradiation. In the variety DTS962, NMU caused more aberrations than EMS. In DTS1044, EMS caused more aberrations (Farook and Bhalla, 1991).

Checheneva and Larchenko (1997) studied frequencies of callus production and regeneration in the maize lines viz., BC2923, Pioneer 346, Pioneer 502 and Oh43 under various cultural conditions, including application of three chemical mutagens and gamma rays. Optimum concentration of the chemicals, radiation dose

and mode of treatment for the induction of callus and regeneration of plantlets were determined. The rate of callus formation depended on the mutagen used. Regenerants obtained after the use of mutagens were weaker than somaclonal regenerants.

Khan *et al.* (1999) reported that in sugarcane the highest frequency of chlorophyll deficient variants, upto 8.0 per cent, was induced when callus and medium were exposed to 2 mM for 3 hours of MNH.

Shoot apices of *in vitro* grown cultures of banana (*Musa* spp., AAA Group cultivar Highgate) were treated with various concentrations of the mutagens sodium azide, diethyl sulfate, and EMS to evaluate their effectiveness in inducing mutations and also to produce variants tolerant to *Fusarium oxysporum f.sp. cubense*. Based on phenotypic variations in regenerated plants, factors of effectiveness were calculated for each treatment. There were no significant differences between variations induced by the mutagens and between the different mutagen treatment durations. Regenerated plants were screened for tolerance to the fungus under greenhouse conditions. Twelve weeks after inoculation, 4.6, 1.9 and 6.1% of plants regenerated after sodium azide, diethyl sulphate and EMS mutagenesis, respectively, had less than 10% vascular invasion of their corms with no external symptoms of the disease (Bhagwat and Duncan, 1998).

Axillary bud explants of carnation were subjected to mutagenic treatment with EMS under *in vitro* conditions through media supplementation (MS) (0.25, 0.50, 0.75, .10 per cent) and explant agitation (EA) (0.025, 0.050, 0.075, 1.00 per cent). EMS at higher doses of 0.025 and 0.050 per cent in culture medium and 0.25 per cent used for explant agitation showed similar effect on sprouting number of shoots, number of flowers and flower initiation, while higher doses of 0.075 and 0.100 per cent under MS and 0.50, 0.75 and 1.00 per cent employed for EA caused damage to all vegetative and floral characters. Two mutants namely, red with white stripes and pink with white stripes were isolated from the mutagen treated population (Singh *et al.*, 2000).

Alyoshin *et al.* (2001) reported that lines with improved characters were obtained from *in vitro* chemical mutagenesis in rice. Somatic calli of the varieties Kransodarskii 424, Dubovskii 129, Slavyanetz, Liman were treated with a solution of the mutagen NMH (concentration 0.05%; 0.1%; 0.2%+ 0.1% PABA) by 40 minutes at Certormat shaking machine (100 revolutions/ minute). The treated calli were regenerated on MS regeneration media (4.0 mg l^{-1} + 20.0 mg l^{-1} of sucrose) and MS intermediated media (non hormonal + PABA) to obtain regenerants. The best results were obtained from the following treatments namely, 0.05% NMH + 0.01% PABA for varieties Kransodarskii 424, Dubovskii 129, Liman; and 0.01% PABA for variety Slavyanetz.

Mangal and Sharma (2002) observed that black rot resistant plants were selected from regenerated plants derived from calli of Cauliflower (*Brassica oleracea* var. *botrytis*) which were mutagenised with EMS and gamma rays.

In vitro mutagenesis using EMS was carried out in two rice genotypes – Norungan and PMK1. Based on the effect of EMS on callus diameter, callus weight and callus volume the optimum doses were fixed as 4mM and 8mM for creation of variability (Vinothini, 2004).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 MATERIALS

Rice (*Oryza sativa* L.) variety Ptb-26 obtained from the Regional Agricultural Research Station, Pattambi was used in this study. The characteristics of the variety are furnished in Table 1.

Table 1.Morphological description of study material

VARIETY GROUP	*Indica*
LEAF	
Colour	Purple blotch
Length (cm)	52.00
Width (cm)	1.20
Basal leaf sheath colour	Light purple
LIGULE	
Colour	Purple
Shape	Two clefts
Length (cm)	1.70
Collar colour	Purple
Auricle colour	Purple
CULM	
Length (cm)	103.00
Girth (cm)	2.43
Internode colour	Green
Septum colour	White
PANICLE	
Length (cm)	26.0
Exsertion	Well exserted
Number of spikelets/ panicle	158.00

SPIKELET	
Awn	Absent
Apiculus colour	Purple apex
Stigma colour	Purple
Lemma and palea colour	Purple spots on straw
Length (mm)	7.82
Width (mm)	3.21
Thickness (mm)	2.15
100 seed weight (g)	2.49
Classification (FAO Scale)	Medium bold
Seed coat (Bran) colour	Red
Endosperm type	Non waxy
PLANT	
Days to 50% flowering	90-95
Productive tillers/hill	4.00
Height of plant (cm)	129.00

(Rosamma *et al.*, 2003)

3.2 METHODS

The experimental methods consists of following two parts

I. *In vitro* culture

II. *In vitro* mutagenesis

1. Physical mutagenesis

2. Chemical mutagenesis

3.2.1 *In vitro* Culture

3.2.1.1 Callus Induction Studies

The experiment was conducted at the Tissue Culture Laboratory, Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara (Plate 1).

3.2.1.1.1 Selection of Explant

The explants used were mature and dehulled seeds of the Ptb-26.

Plate 1. Protocol for *in vitro* culture

3.2.1.1.2 Media

Media suggested by Murashige and Skoog (1962) was used as the basal medium for the present study.

i) Preparation of Stocks for Basal Media

The stock solutions for the media were prepared in double distilled water and kept in sterile glass stoppered bottles and stored in a refrigerator at 5^0C.The stock formulations and the quantity of stocks used per litre of the respective media are given in Table 2 and Table 3.

ii) Preparation of Stocks for Growth Regulators

Auxin viz., 2,4-D was dissolved in few drops of ethanol, slightly heated and gradually diluted to 100 ml with double distilled water. Cytokinin viz., Kn was dissolved in a few drops of 0.5N NaOH, slightly heated and gradually diluted to 100 ml with double distilled water.

iii) Preparation of Media

All the chemicals of AR grade were used in the preparation of MS medium. Medium was prepared by following the standard procedure adopted by Gamborg and Syluk (1981). Stock solution of major and minor elements were prepared and stored in cleaned amber coloured bottles in refrigerated conditions. Aliquots from all stock solutions were pippetted in proportionate volume into a glass beaker and required quantities of sucrose and inositol were added and dissolved. The desired volume was made by adding double distilled water. The pH of the medium was adjusted in between 5.5 and 5.8 using 0.1N NaOH/HCl. Agar was added and the medium was heated to melt the agar. About 15 ml medium was poured to the test tube (15 X 2.5 cm), and then plugged with non-absorbent cotton.

Table 2.Composition of Tissue Culture Media (MS)

Constituents	MS (mg/l)
Major inorganic nutrients	
NH_4NO_3	1650.0
$(NH_4)_2 SO_4$	-
KNO_3	1900.0
$MgSO_4.7H_2O$	370.0
KH_2PO_4	170.0
$CaCl_2.2H_2O$	440.0
Trace elements	
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Iron source	
Na_2EDTA	37.25
$FeSO_4.7H_2O$	27.85
Organic supplements	
Myo-inositol	100.0
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Glycine	2.0
Carbon source	
Sucrose	30 000
pH	5.8

Table 3. Preparation of stock solutions for MS medium

Sl.no	Ingredients	Quantity (mg)	Volume of stock solution prepared (ml)	Volume of stock solution taken per litre of medium (ml)
1.	**Macro elements**			
	NH_4NO_3	16 500		
	KNO_3	19 000		
	$MgSO_4.7H_2O$	3700	500	50
	KH_2PO^4	4400		
	$CaCl_2.2H_2O$	1700		
2.	**Micro elements**			
	$MnSO_4.4H_2O$	2230		
	$ZnSO_4.7H_2O$	860		
	H_3BO_3	620		
	$Na_2MoO4.2H_2O$	25	500	5
	$CuSO_4.5H_2O$	2.5		
	$CoCl_2.6H_2O$	2.5		
3.	**Iron stock***			
	Na_2EDTA	3725	250	2.5
	$FeSO_4.7H_2O$	2785		
4.	KI	166	200	1
5.	**Organic supplements**			
	Myo-inositol	1000	100	10
	Thiamine HCl	10		
	Nicotinic acid	50	250	2.5
	Pyridoxine HCl	50		
	Glycine	200	100	1

*Iron stock preparation

A quantity of 3.725 g of Na_2 EDTA and 2.785 g of $FeSO_4.7H_2O$ was dissolved separately in 100 ml of double distilled water. Both solutions were warmed up gently. The hot solution of Na_2EDTA was added to the hot solution of $FeSO_4.7H_2O$ and the final volume is made up to 250 ml.

iv) Media Sterilization

The tubes containing the medium were autoclaved at 1.01 kg/cm^2 pressure at 121^{0}C for 20 minutes. The medium was allowed to cool at room temperature and stored at 10°C.

v) Callus Induction Medium

The basal media used was MS medium. The growth regulators used were auxin (2,4-D) and cytokinin (Kn). The media combinations used in this study are given in Table 4.

Table 4. Callus induction medium

Treatment	Basal	2, 4-D (mg l^{-1})	Kn (mg l^{-1})
T_1	MS	1.0	0.5
T_2	MS	1.5	0.5
T_3	MS	2.0	0.5
T_4	MS	2.5	0.5
T_5	MS	3.0	0.5

vi) Disinfectation of Explant

- The mature seeds of paddy were dehusked manually prior to surface sterilisation, as the presence of husk, decreases the effectiveness of sterilization.
- The seeds were washed two to three times with water to remove any impurities.
- Seeds were immersed in a solution containing Teepol (5%) for ten minutes, followed by rinsing with sterile distilled water to remove any traces of soap solution.
- Seeds were transferred to Laminar Air Flow Chamber.
- Seeds were surface sterilized with 70% alcohol for two minutes, followed by rinsing with sterile distilled water for two to three times.

- Seeds were treated in 0.4 per cent mercuric chloride solution for two minutes.
- The seeds were rinsed three to four times thoroughly with sterile distilled water.

vii) Inoculation of Explant

Surface sterilized dehulled seeds were carefully inoculated into the callus induction media at the rate of one seed per tube in each treatment with five replications (20 tubes per replication).

viii) Culture Conditions

Cultures were incubated in a closed room in which temperature was maintained at 26 ± 2^0C and humidity between 60 and 80%. Cultures were examined every alternate day. The contaminations were removed. The callus cultures were maintained in light containing 3000 lux fluorescent light for 16 hours daily.

3.2.1.1.3 Observations

The media combination best suited for seed callus culture was ascertained from the following observations.

i) Number of Days taken for Callus Induction

The number of days required for callus induction was recorded.

ii) Callus Induction Percentage

The callus induction percentage was calculated using the number of calli produced per treatment to the number of explants inoculated per treatment.

$$\text{Callus induction percentage} = \frac{\text{No. of calli produced /treatment}}{\text{No. of explants inoculated /treatment}} \times 100$$

The treatment combination with the highest callus induction percentage was used for further studies.

iii) Callusing Response

The callusing response was classified as given by Katiyar *et al.* (1999).

High Callusing Response - HCR - > 50%

Low Callusing Response - LCR - 0 to 50%

iv) Effect of Growth Regulators

The effect of different growth regulator combinations on callus induction were statistically analysed in CRD (Table 5).

Table 5. Analysis of variance in CRD for 't' treatments

Source	df	SS	MSS	F value
Treatment	t-1			
Error	n-t			
Total	n-1			

SEd = √ 2 X Error Mean Square /r

CD at 5% = SEd x't' value at edf at 5% level of significance

If calculated F value was greater than or equal to table value at 5 per cent level of significance, then the treatments were considered to be significantly different from each other. SEd and CD were calculated to compare the treatment means and choose the best treatment.

3.2.1.2 Callus Proliferation Studies

The best MS medium from the above experiment was used for subculture to effect more calli growth and proliferation. First subculturing was done in 21 days. Thereafter the calli were allowed to proliferate for 21 days and then taken for regeneration. The proliferated calli were studied for the following observations.

3.2.1.2.1 Observations

i) Callus Diameter (cm)

Crosswise breadth of each callus was measured and expressed in centimetre.

ii) Callus Volume (cc)

The radius of the callus was measured in centimetre and the volume was calculated using the formula $4/3\pi r^3$.

iii) Callus Fresh Weight (mg)

Fresh weight of callus was recorded and expressed in milligram.

3.2.1.3 Callus Regeneration Studies

The callus tissues were transferred to regeneration medium when calli attained diameter of 2 cm size (approximately 500 mg weight). The basal medium used for regeneration was MS medium. Two regeneration media with two combinations of growth regulators were used in this study.

1. MS + NAA 2.0 mg l^{-1} + Kn 4.0 mg l^{-1} + BAP 0.5 mg l^{-1} (T_6)
2. MS + NAA 2.0 mg l^{-1} + Kn 4.0 mg l^{-1} + BAP 1.0 mg l^{-1} (T_7)

Green spots appeared in 35 days. Spots turned into shoots, which were allowed to develop for seven days. The shoots obtained were transferred to rooting medium (MS + NAA 2.0 mg l^{-1}+Kn 0.5 mg l^{-1}). The cultures were kept under continuous light (3000 lux intensity) at 26 ± 2^0C for seven days and the following observations were recorded.

3.2.1.3.1 Observations

i) Percentage of Plant Regeneration

The plant regeneration percentage was estimated as the number of green plants produced to the total number of calli plated.

$$\text{Percentage of plant regeneration} = \frac{\text{No. of green plants produced}}{\text{Total number of calli plated}} \times 100$$

The treatment combination with the highest plant regeneration percentage was taken for further regeneration studies of mutated calli.

ii) Regenerating Response

Characterisation of regenerating response of calli was done as given by Katiyar *et al.* (1999).

High Regenerating Response	-	HRR	-	> 70%
Moderate Regenerating Response	-	MRR	-	50 – 70%
Low Regenerating Response	-	LRR	-	< 50%

3.2.1.4 Hardening of Plants

The regenerated plantlets from the above experiment were taken out, washed, dipped in fungicide, again washed and planted in sterilized sand filled in plastic cups. All the plantlets were planted out. Each individual cup with plantlets was covered with a transparent polythene cover to develop sufficient moisture inside to prevent drying of the plantlets. After one week polythene cover was removed. The plantlets were irrigated with sterile water as and when required. When hardened, the plantlets started growing by forming new leaves and roots. A mixture of dried cow dung and topsoil were added around each plantlet, in the cup. After two weeks time the plantlets were transplanted to pots and maintained as pot cultures. The following observation was made.

3.2.1.4.1 Observation

i) Percentage Success of Hardened Plants

Number of plants survived to the total number of plants hardened was calculated and expressed as percentage.

$$\text{Percentage of success of hardening} = \frac{\text{No. plantlets surviving} \times 100}{\text{Total number of plantlets hardened}}$$

3.2.2 *In vitro* Mutagenesis

In vitro mutagenesis is an alternative method to induce variability. Three weeks old subcultured calli were subjected to *in vitro* mutagenesis using physical and chemical mutagens (Plate 2).

3.2.2.1 Physical Mutagenesis

a) Source

Ionising radiation, gamma rays (wavelength 0.1^0A) from ^{60}Co of the Gamma chamber 900 of BARC, Mumbai installed at the Radio Tracer Laboratory of the College of Horticulture, Vellanikkara was used as the source of the physical mutagen. Its mode of action is ionisation.

b) Dose

Mutagenic treatments to fix the LD_{50} dose for gamma irradiation are given in the Table 6. Five replications of ten tubes were irradiated in each dose.

Table 6. Doses of gamma rays used for *in vitro* treatment

Treatment	Gamma dose (Gy)	Duration of treatment
TG_1 (Control)	00	00 minutes, 00 seconds
TG_2	10	03 minutes, 23 seconds
TG_3	20	06 minutes, 46 seconds
TG_4	30	10 minutes, 09 seconds
TG_5	40	13 minutes, 23 seconds
TG_6	50	16 minutes, 55 seconds
TG_7	60	20 minutes, 18 seconds

Three weeks old subcultured calli

Gamma irradiation

EMS treatment

Callus regeneration

Callus regeneration

Rooting media

Rooting media

Hardening in plastic cups

Pot culture

Plate 2. Protocol for *in vitro* mutagenesis

c) Protocol

- Mature, dehulled seeds were inoculated in the medium and the cultures were incubated under diffuse light (3000 lux at 26± 2^0C).
- The calli were subcultured and three weeks old subcultured calli were subjected to different doses of gamma irradiation.
- The irradiated calli were transferred immediately to fresh medium where they were allowed to proliferate. This was to avoid the formation of toxic compounds and enhance radiation efficiency
- After two weeks of proliferation the treated calli along with untreated calli (control) were transferred to regeneration medium.
- The shoots obtained were then cultured in rooting medium.
- The regeneration percentage was used to determine the LD_{50} value based on which two doses lower than LD_{50} value was fixed as optimum dose.
- The above procedure was repeated with the optimum doses.
- The plantlets obtained were hardened and later transferred to pots. Biometrical and morphological observations were recorded on these mature plants at the appropriate stages.

3.2.2.2 Chemical Mutagenesis

a) Source

EMS (Specific gravity 1.204 g/cc; Molecular weight 124.16 g) was used for *in vitro* chemical mutagenesis. The mode of action is alkylation.

b) Dose

The method of explant agitation (EA) was tried. Here the calli were subjected to treatment by soaking in the respective solutions of EMS. Freshly prepared well sterilised EMS solution was used.1mM solution was prepared by dissolving 124.16mg of EMS in 1000ml distilled water. The details of concentrations tried are given in the Table 7. Five replications of ten tubes were treated in each concentration.

Table 7. Concentrations of EMS used for *in vitro* treatment

Treatment	**Concentration (mM)**	**Duration of treatment (Hours)**
TE_1 (Control)	0.00	4
TE_2	2.00	4
TE_3	4.00	4
TE_4	6.00	4
TE_5	8.00	4
TE_6	10.00	4
TE_7	12.00	4

c) Protocol

- Mature, dehulled seeds were inoculated in the medium and the cultures were incubated under diffuse light (3000 lux at 26$\pm$ 2^{0}C).
- The calli were subcultured and three weeks old subcultured calli were subjected to different concentrations of EMS.
- The treated calli were transferred immediately to fresh medium where they were allowed to proliferate. This was to avoid the formation of toxic compounds and enhance treatment efficiency
- After two weeks of proliferation the treated calli along with untreated calli (control) were transferred to regeneration medium.
- The shoots obtained were then cultured in rooting medium.
- The regeneration percentage was used to determine the LD_{50} value based on which two doses lower than LD_{50} value was fixed as optimum dose.
- The above procedure was repeated with the optimum doses.
- The plantlets obtained were hardened and later transferred to pots. Biometrical and morphological observations were recorded on these mature plants at the appropriate stages.

3.2.2.3 Observations

i) Study of Morphology of Mutated Calli

The intensity of colour change was rated as below.

Table 8. Scoring scale

Score	Description
0	Control
1	0 % browning
3	25 % browning
5	50 % browning
7	75 % browning
9	100 % browning

Individual calli were scored and the averages were recorded for each treatment (Plate 3).

ii) Regeneration Percentage for Estimation of LD_{50} Value

LD_{50} is that dose of mutagen, which would kill 50 per cent of the treated calli. In order to fix the optimal dose, the LD_{50} of mutagen was first estimated. The regeneration percentage was calculated to determine LD_{50} value.

iii) Regenerating Response

The regenerating response of mutagen treated calli were characterised as done in the earlier callus regeneration studies.

iv) Regeneration Percentage for Optimum Dose

Regeneration percentage for optimum dose was calculated as done in the earlier callus regeneration studies.

v) Percentage Success of Hardened Plants

The percentage of success of hardened plantlets from regeneration of mutated calli was calculated as mentioned earlier.

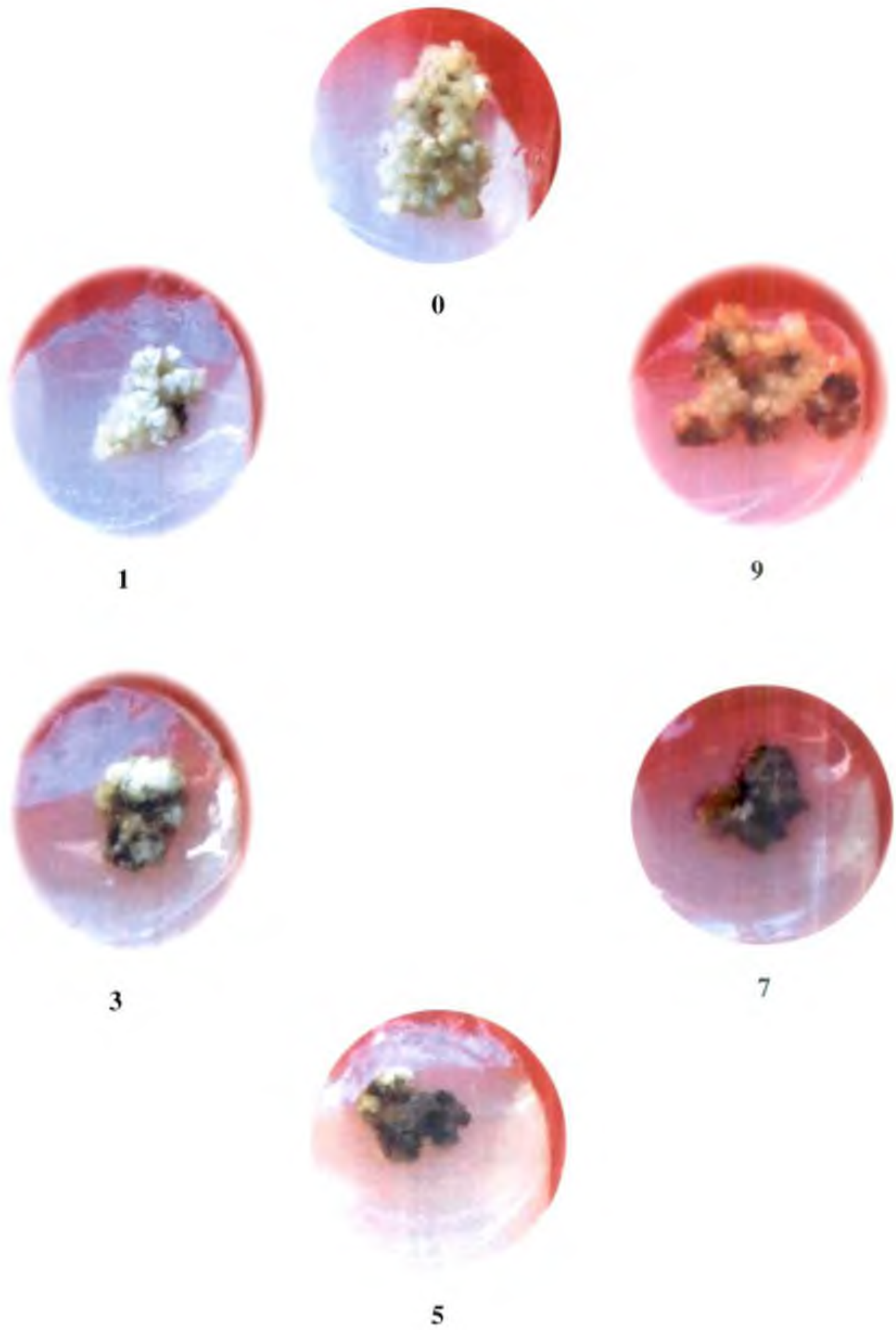


Plate 3. Scoring of mutated calli morphology

3.2.2.4 Study of Plants

The biometrical and morphological characters of the tissue culture plants (gamma irradiation and EMS treatment) were recorded. The rice descriptor in Standard Evaluation System from IRRI (1996) was used for this purpose.

3.2.2.4.1 Observations

a) Biometrical Characters

The following biometrical characters were studied in tissue culture plants (gamma irradiation and EMS treatment).

1. Leaf length (cm)

Leaf length in centimetre was measured on the topmost leaf below the flag leaf.

2.Leaf width (cm)

Leaf width was recorded on the widest portion of the leaf blade on the topmost leaf below the flag leaf in centimetre.

3.Ligule length (mm)

Ligule length was measured in millimetre from the base of the collar to the tip.

4.Culm length (cm)

The length of culm was recorded from soil surface to the panicle base in centimetre.

5.Plant height (cm)

Plant height in centimetre was recorded on the culm at flowering from ground to the tip of the panicle excluding the awns.

6.Panicles per plant

Number of panicles per plant was counted at the time of harvest.

7.Panicle length (cm)

Length of the panicle borne on the main culm was measured from the base of the panicle to the uppermost grain tip in each plant in centimetre.

8. 100 grains weight (g)

Weight of hundred well filled grains were taken at random from plants, dried, weighed and expressed in gram.

9.Grain length (mm)

Grain length was measured as the distance from the base of the lowermost sterile lemma to the tip of the lowermost sterile lemma to the tip of the fertile lemma or palea in five randomly selected grains and expressed in millimetre.

10.Grain width (mm)

Grain width was measured at the widest point on five randomly selected well filled grains and expressed in millimetre.

11.Grain length /width ratio

Length and breadth of grains were measured and its ratio was calculated.

12. Grains per panicle

Total number of grains in the primary panicle was recorded.

13.Grain yield plant^{-1} (g)

All panicles harvested from single plant were threshed, cleaned, dried to 14% moisture level and weighed in gram.

b) Morphological Characters

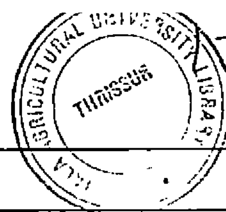
The following biometrical characters were studied in tissue culture plants of gamma irradiation and EMS treatment.

Table 9. Morphological characters

Sl.No.	Characters	Scale	Remarks
1.	Leaf Blade Pubescence	1 glabrous 2 intermediate 3 pubescent	Presence of hairs on the leaf blade surface at vegetative stage
2.	Leaf Blade Colour	1 light green 2 green 3 dark green 4 purple tips 5 purple margins 6 purple blotch 7 purple	At vegetative stage
3.	Basal Leaf Sheath Colour	1 green 2 purple lines 3 light purple 4 purple	On the outer surface of the basal leaf sheath at vegetative stage
4.	Leaf Angle	1 erect 5 horizontal 9 droopy	Angle of openness of the blade tip against the culm on the leaf below the flag leaf at vegetative stage
5.	Flag Leaf Angle	1 erect 3 intermediate 5 horizontal 9 descending	Near the collar as the angle of attachment between the flag leaf blade and the main panicle axis at vegetative stage

6.	Ligule Colour	1 white 2 purple line 3 purple	At vegetative stage
7.	Ligule Shape	1 acute to acuminate 2 two-cleft 3 truncate	At vegetative stage
8.	Collar Colour	1 light green 2 green 3 purple	At vegetative stage
9.	Auricle Colour	1 light green 2 purple	At vegetative stage
10.	Culm Angle	1 erect (<30^0) 3 intermediate (=45^0) 5 open (=60^0) 7 spreading (>60^0) 9 procumbent (rests on ground)	At maturity
11.	Culm Internode Colour	1 green 2 light green 3 purple lines 4 purple	On the outer surface of the internode on the culm at maturity
12.	Panicle Type	1 compact 2 intermediate 3 open	Mode of branching, angle of primary branches and spikelet density at maturity.
13.	Panicle Axis	1 straight 2 droopy	At maturity

14.	Awning	0 absent 1 short and partly awned 5 short and fully awned 7 long awned 9 long and fully awned	At maturity
15.	Panicle Exsertion	1 well exserted 3 moderately exserted 5 just exserted 7 partly exserted 9 enclosed	At maturity
16.	Stigma Colour	1 white 2 light green 3 yellow 4 light purple 5 purple	From blooming spikelets between (9am to 2pm) with the aid of hand lens at maturity.
17.	Apiculus Colour	1 white 2 straw 3 brown (tawny) 4 red 5 red apex 6 purple 7 purple apex	On spikelets at maturity
18.	Lemma and Palea Colour	1 straw 2 golden 3 golden brown 4 brown furrows on straw 5 purple 6 purple furrows on straw 7 brown (tawny) 8 black	After harvest



19.	Seed Coat Colour	1 white 2 light brown 3 speckled brown 4 brown 5 red 6 variable purple 7 purple	After harvest

3.2.2.4.2 Statistical Analysis

a) Biometrical Characters

Analysis of variance was carried out in CRD (unequal number of replications) for the plants for each biometrical character. The biometrical characters that show significant effect due to different treatments of mutagens were identified.

The CD was calculated and DMRT done for comparison among the treatments and then choice of the best optimum dose.

SEd was calculated using the formula

SEd = $\sqrt{2EMS(1/r_1+1/r_2)}$ where,

r_1 and r_2 are the number of replications in the treatments being compared.

CD at 5% = SEd x't' value at edf at 5% level of significanced

b) Morphological Characters

For morphological characters the percentage of variation with respect to control was calculated for comparison among different treatments derived tissue culture plants of gamma irradiation and EMS treatment (Singh *et al.*, 2000).

$$\text{Percentage of Variation} = \frac{\text{No. variants w.r.t. control among tissue culture plants X 100}}{\text{Total number of tissue culture plants}}$$

3.2.2.4.3 Selection of Superior Plants

From the observations recorded on the tissue culture plants the desirable plants were identified for raising the next generation.

RESULTS

4. RESULTS

The results obtained from the various experiments conducted in the study are presented in this chapter.

4.1 *IN VITRO* CULTURE

4.1.1 Callus Induction Studies

Variety Ptb-26 was used in this study. The explant used for research work was mature embryo. The capacity of callus formation was evaluated by mean duration required for callus initiation, percentage of callus induction, callusing response and effect of growth regulators.

4.1.1.1 Observations

i) Number of Days taken for Callus Induction

Callus induction was achieved 14 days after inoculation. Further proliferation of the callus was achieved when they were sub cultured on medium with the same level of growth hormones.

ii) Callus Induction Percentage

The values of callus induction percentage ranged from 0.00 per cent to 98.00 per cent. Maximum callus induction percentage was recorded by T_3 (98.00per cent) followed by T_4 (55.0 per cent), T_2 (32.0 per cent) and the least in T_5 (26.00 per cent) (Table 10).

iii) Callusing Response

The callusing response of calli produced were characterised and furnished in Table 10. It was evident that the callusing response was high in treatments T_3 and T_4. Treatments T_1 and T_5 recorded low callusing response.

iv) Effect of Growth Regulators

The experimental results relating to the effect of growth regulators *viz.*, 2, 4-D and Kn were statistically analysed in CRD. Treatment (T_3) MS + 2,4-D 2.0 mg l^{-1} + Kn 0.5 mg l^{-1}) was found tc be highly significant and the best among all combinations tried. T_1 was the least effective in inducing callus (Table 11).

Table 10. Callus induction percentage and callusing response

Treatment	Treatment combinations	No. of explant inoculated	No. of calli induced	Callus induction percentage	Callusing response
T_1	MS + 2,4-D 1.0mg l^{-1} + Kn 0.5 mg l^{-1}	100	0	00.	LCR
T_2	MS + 2,4-D 1.5mg l^{-1} + Kn 0.5 mg l^{-1}	100	32	32	LCR
T_3	MS + 2,4-D 2.0mg l^{-1} + Kn 0.5 mg l^{-1}	100	98	98.	HCR
T_4	MS + 2,4-D 2.5mg l^{-1} + Kn 0.5 mg l^{-1}	100	55	55.	HCR
T_5	MS + 2,4-D 3.0mg l^{-1} + Kn 0.5 mg l^{-1}	100	26	26	LCR

Table 11. Effect of growth regulators

Treatment	Mean Callus induction percentage	Significance test
T_1	00.00 (00.00)	e
T_2	32.00 (34.45)	c
T_3	98.00 (81.87)	a
T_4	55.00 (47.84)	b
T_5	26.00 (30.66)	cd

Value in parenthesis indicates arc sine transformed values

SEd = 3.715; CD at edf at 5 per cent level of significance= 7.75

4.1.2 Callus Proliferation Studies

The first subculturing was done 21 days after callus induction in the best established medium (T_3) and the morphology of subcultured calli in this medium was studied. All the calli induced were found to be off-white, creamy and hence non-embryogenic.

4.1.2.1 Observations

Table 12 gives the results of the observations recorded.

4.1.3 Callus Regeneration Studies

The callus tissues were transferred to regeneration medium when calli attained a diameter of 2 cm size (approximately 500 mg weight).

4.1.3.1 Observations

i) Percentage of Plant Regeneration

Regeneration percentage for treatments were 75.23 per cent (T_7) and 90.44per cent per cent (T_6). The treatment combination T_6 proved to be the best with the highest regeneration percentage (Table 13).

ii) Regenerating Response

The regenerating responses of calli produced were characterized as given in Table 13. Both the treatments T_6 and T_7 were seen to have high regenerating response.

4.1.4 Hardening of Plants

4.1.4.1 Observation

i) Percentage Success of Hardened Plants

The percentage of success of regenerants to produce rooted hardened plants is presented in Table 14. Both the treatments T_6 and T_7 had 89.47 per cent and 81.01 per cent of success respectively.

Table 12. Observations on the morphology of proliferated calli

Mean callus diameter (cm)	1.04
Mean callus volume (cc)	4.22
Mean callus fresh weight (mg)	326.70

Table 13. Regeneration percentage and response

Treatment	No. of calli plated	No, of green plants produced	Regeneration percentage	Regenerating Response
T_6	105	95	90.48	HRR
T_7	105	79	75.23	HRR

Table 14. Percentage success of hardened plants

Treatment	No. of plants produced	No. of plants surviving	Hardening Success Percentage
T_6	95	85	89.47
T_7	79	64	81.01

4.2 *IN VITRO* MUTAGENESIS

In vitro mutation was experimented using the two mutagens namely, gamma rays and EMS. The following observations were recorded.

4.2.1 Observations

i) Study of Morphology of Mutated Calli

The intensity of colour change in the mutated calli were noted on alternate days. The browning of calli was seen to intensify with the doses of increasing gamma radiation and higher concentration of EMS. Higher score (9) were recorded in TG_7 and TE_7 followed by TG_6 and TE_6 (7). While treatments TG_2, TG_3, TE_2 and TE_3 showed the least score (1). The scoring is given in (Table 15).

ii) Regeneration Percentage for Estimation of LD_{50} Value

The regeneration percentage of mutated calli was used to calculate LD_{50} value of the mutagens under experimental conditions. The data on the effect of different doses of both mutagens on regeneration of calli is furnished in Table 16.

Regarding, gamma irradiation TG_1 recorded the maximum regeneration percentage (100.00 per cent) while TG_6 and TG_7 recorded the minimum regeneration percentage (0.00 per cent). TG_4 (30 Gy) recorded 50.00per cent regeneration percentage maximum survival was observed in control (100.00 per cent). Hence, LD_{50} was fixed as 30 Gy.

Studies on regeneration of the various EMS treated calli revealed that the regeneration percentage ranged 100.00 per cent (TE_1) to 0.00 per cent (TE_7). 50.00 per cent callus regeneration was recorded in TE_4. Hence, LD_{50} was fixed as 6mM.

Table 15. Scoring of morphology of mutated calli

Mutagens	Treatment	Score
Gamma dose (Gy)	TG_1	0
	TG_2	1
	TG_3	1
	TG_4	3
	TG_5	5
	TG_6	7
	TG_7	9
EMS Concentration (mM)	TE_1	0
	TE_2	1
	TE_3	1
	TE_4	3
	TE_5	5
	TE_6	7
	TE_7	9

Table 16. Regeneration percentage and regenerating response for LD_{50} values

Mutagens	Treatment	No. of calli Plated	No. of calli regenerating	Regeneration Percentage	Regenerating response
Gamma dose (Gy)	TG_1	50	50	100.00	HRR
	TG_2	50	48	96.00	HRR
	TG_3	50	44	88.00	HRR
	TG_4	50	25	50.00	MRR
	TG_5	50	8	16.00	LRR
	TG_6	50	0	00.00	LRR
	TG_7	50	0	00.00	LRR
EMS Concentration (mM)	TE_1	50	50	100.00	HRR
	TE_2	50	43	86.00	HRR
	TE_3	50	38	76.00	HRR
	TE_4	50	25	50.00	MRR
	TE_5	50	12	24.00	LRR
	TE_6	50	8	16.00	LRR
	TE_7	50	0	0.00	LRR

iii) Regenerating Response

Regenerating responses of mutated calli were characterized as shown in Table 16 and Table 17. Treatments of lower doses namely, TG_1, TG_2, TG_3, TE_1, TE_2 and TE_3 showed high regenerating response. Treatments of moderate doses TG_4 and TE_4 had moderate regenerating response. Lower regenerating response was seen in treatments of higher doses namely, TG_5, TG_6, TG_7, TE_5, TE_6 and TE_7.

iv) Regeneration Percentage for Optimum Dose

Two doses below 30 Gy namely 10 Gy and 20 Gy were fixed as the optimum dose for gamma irradiation. Similarly two concentrations namely 2 mM and 4 mM of EMS were taken as the optimum doses. The callus regeneration percentage was more than 90 per cent in gamma irradiation and was more than 80 per cent for EMS treatment. Subsequent regeneration and hardening was carried out to recover desirable plants. Regeneration percentages are furnished in Table 17.

v) Percentage Success of Hardened Plants

Percentage of success of regenerants for the estimation of optimum dose in producing rooted hardened plants are presented in Table 18.

For hardened plants from gamma irradiation and from EMS treated ones the percentage of success was the same for control as well as all the treatments. Results show that number of tissue culture plants recovered after hardening was more from gamma irradiated calli when compared to that from EMS treated ones.

Table 17. Regeneration percentage and regenerating response for estimation of optimum dose

Mutagens	Treatment	No. of calli plated	No. of calli regenerating	Regeneration Percentage
Gamma dose (Gy)	TG_1	50	50	100.00
	TG_2	50	50	100.00
	TG_3	50	45	90.00
EMS Concentration (mM)	TE_1	50	50	100.00
	TE_2	50	45	90.00
	TE_3	50	40	80.00

Table 18. Percentage of success of hardening

Mutagens	Treatment	No. of Plants produced	No. of plants surviving	Hardening Success Percentage
Gamma dose (Gy)	TG_1	50	10	20.00
	TG_2	50	10	20.00
	TG_3	45	9	20.00
EMS Concentration (mM)	TE_1	50	10	20.00
	TE_2	45	9	20.00
	TE_3	40	8	20.00

4.2.2 Study of Plants

4.2.2.1 Observations

a)Biometrical Characters

The performance of all the tissue culture plants along with control for all biometrical characters is presented in Table 19 and 20.

i)Gamma Irradiation

Minimal variations were noticed for leaf length and leaf width. Leaf length was 55.30 cm for 10 Gy and 55.40 cm for 20 Gy. While leaf width values were 1.23 cm and 1.25 cm for 10 Gy and 20 Gy respectively. Culm length ranged from 71.30 cm (TCMG12) to 93.00 cm (control). On an average the plants obtained after treatment with 10 Gy had a culm length 92.82 cm and those from 20 Gy was 71.58 cm. Plants TCMG12, TCMG15 recorded minimum values for plant height namely 98.50cm. Both these plants were obtained after treatment with 20 Gy. The control recorded a plant height of 120 cm. Panicles per plant was highest for TCMG12 (6.00) and least in control (3.90). On an average, the panicle length was 27.08 cm for 10 Gy treated plants and 27.12 cm for 20 Gy plants. The grain yield per plant was 15.37 g in control. Maximum grains per panicle were recorded in TCMG12, TCMG13, TCMG15, TCMG16, TCMG17 and TCMG19 (178). Maximum grain yield was recorded in TCMG12 (26.42 g) followed by TCMG15 and TCMG19 (22.02 g). TCMG1 recorded the minimum yield among the plants (16.20 g).

ii) EMS Treatment

Minimum variations were noticed for leaf length and leaf width. Leaf length was 56.88 cm for both 2 mM EMS and 4 mM EMS. While leaf width values were 1.30 cm and 1.38 cm for 2 mM EMS and 4mM EMS respectively. Culm length ranged from 71.00 cm (TCME17) to 92.80 cm (control). On an average the plants obtained after treatment with 2 mM EMS had a culm length 92.60 cm and those from 4 mM EMS was 71.17cm. Plants TCME17 recorded minimum value for plant height namely 98.30 cm. Both these plants were obtained after treatment with 4 mM EMS. The control recorded a plant height of 119.90 cm. Panicles per plant were highest for TCME13 and

Table 19. Performance of gamma irradiation derived tissue culture plants for biometrical characters

Gamma Doses (Gy)	Characters / TC plants	L L (cm)	LW (cm)	LgL (cm)	CL (cm)	PH (cm)	PP	PL (cm)	TW (100 grains)(g)	GL (mm)	GW (mm)	GL/W ratio	GP	GY (g)
0	Control Mean	56.00	1.20	0.16	93.00	120.00	3.90	27.00	2.47	7.82	3.20	2.44	160.00	15.37
10	TCMG1	55.00	1.20	0.16	92.80	119.90	4.00	27.10	2.47	7.83	3.19	2.45	164.00	16.20
	TCMG2	55.00	1.30	0.17	92.80	119.90	4.00	27.10	2.47	7.84	3.20	2.45	164.00	16.23
	TCMG3	55.00	1.20	0.16	92.80	119.80	5.00	27.00	2.47	7.82	3.20	2.44	165.00	20.40
	TCMG4	56.00	1.20	0.16	92.80	119.90	4.00	27.10	2.47	7.83	3.19	2.45	164.00	16.22
	TCMG5	55.00	1.30	0.16	92.80	119.90	4.00	27.10	2.48	7.84	3.21	2.44	165.00	16.36
	TCMG6	56.00	1.20	0.16	92.80	119.90	4.00	27.10	2.47	7.82	3.20	2.44	165.00	16.33
	TCMG7	56.00	1.20	0.16	92.80	119.90	4.00	27.10	2.47	7.84	3.20	2.45	166.00	16.42
	TCMG8	55.00	1.30	0.16	92.90	119.90	4.00	27.00	2.47	7.82	3.20	2.44	165.00	16.43
	TCMG9	55.00	1.20	0.16	92.70	119.80	4.00	27.10	2.47	7.84	3.21	2.44	166.00	16.41
	TCMG10	55.00	1.20	0.16	92.80	119.90	4.00	27.10	2.47	7.82	3.20	2.44	165.00	16.32
	Mean	55.30	1.23	0.16	92.82	119.90	4.10	27.08	2.47	7.83	3.20	2.44	164.90	16.73
20	TCMG11	55.00	1.20	0.16	71.50	98.60	5.00	27.10	2.47	7.84	3.20	2.45	177.00	21.90
	TCMG12	55.00	1.20	0.16	71.30	98.50	6.00	27.20	2.47	7.83	3.19	2.45	178.00	26.42
	TCMG13	56.00	1.20	0.16	71.70	98.80	4.00	27.10	2.48	7.84	3.21	2.44	178.00	17.62
	TCMG14	55.00	1.20	0.16	71.80	98.90	4.00	27.10	2.47	7.82	3.20	2.44	177.00	17.51
	TCMG15	55.00	1.30	0.16	71.40	98.50	5.00	27.10	2.47	7.84	3.20	2.45	178.00	22.02
	TCMG16	56.00	1.30	0.16	71.60	98.70	5.00	27.10	2.47	7.84	3.21	2.44	178.00	22.01
	TCMG17	56.00	1.30	0.16	71.70	98.80	4.00	27.10	2.47	7 82	3.20	2.44	178.00	17.62
	TCMG18	56.00	1.30	0.16	71.50	98.70	5.00	27.20	2.47	7.83	3.19	2.45	177.00	21.88
	TCMG19	55.00	1.20	0.16	71.70	98.80	5.00	27.10	2.47	7.84	3.21	2.44	178.00	22.02
	Mean	55.40	1.25	0.16	71.58	98.70	4.70	27.12	2.47	7.83	3.20	2.44	177.67	21.00

Table 20. Performance of EMS treatment derived tissue culture plants for biometrical characters

MS Concentrations (mM)	Characters / Plants	LL (cm)	LW (cm)	LgL (cm)	CL (cm)	PH (cm)	PP	PL (cm)	TW (100grains) (g)	GL (mm)	GW (mm)	GL/W ratio	GP	GY (g)
0.00	Control Mean	57.00	1.11	0.16	92.80	119.90	4.10	27.10	2.47	7.82	3.18	2.46	165.00	16.72
2.00	TCME1	56.00	1.40	0.16	92.70	119.80	5.00	27.10	2.47	7.81	3.17	2.46	168.00	20.77
	TCME2	57.00	1.40	0.17	92.50	119.70	5.00	27.20	2.48	7.82	3.18	2.46	168.00	20.79
	TCME3	57.00	1.40	0.16	92.60	119.80	4.00	27.20	2.47	7.83	3.18	2.46	169.00	16.72
	TCME4	57.00	1.40	0.16	92.70	119.80	5.00	27.10	2.47	7.81	3.18	2.46	168.00	20.77
	TCME5	57.00	1.30	0.16	92.60	119.80	4.00	27.20	2.48	7.84	3.17	2.45	169.00	16.72
	TCME6	57.00	1.40	0.16	92.50	119.70	5.00	27.20	2.47	7.83	3.20	2.46	169.00	20.91
	TCME7	57.00	1.40	0.16	92.60	119.80	4.00	27.20	2.48	7.83	3.18	2.46	170.00	16.83
	TCME8	57.00	1.40	0.16	92.60	119.80	5.00	27.20	2.47	7.82	3.18	2.46	169.00	20.90
	TCME9	57.00	1.30	0.16	92.60	119.80	5.00	27.20	2.48	7.82	3.18	2.46	170.00	21.04
	Mean	56.88	1.38	0.16	92.60	119.78	4.67	27.18	2.47	7.82	3.18	2.46	168.89	19.50
4.00	TCME11	56.00	1.40	0.16	71.30	98.50	5.00	27.20	2.48	7.83	3.18	2.46	181.00	22.39
	TCME12	57.00	1.40	0.16	71.30	98.50	5.00	27.20	2.48	7.81	3.17	2.46	182.00	22.52
	TCME13	57.00	1.40	0.16	71.70	98.40	6.00	27.30	2.48	7.84	3.20	2.45	182.00	27.04
	TCME14	57.00	1.40	0.16	71.20	98.50	5.00	27.30	2.47	7.82	3.18	2.46	181.00	22.41
	TCME15	57.00	1.40	0.16	71.10	98.40	6.00	27.30	2.48	7.82	3.18	2.46	182.00	27.04
	TCME16	57.00	1.30	0.16	71.20	98.50	5.00	27.30	2.48	7.81	3.17	2.46	182.00	22.52
	TCME17	57.00	1.40	0.16	71.00	98.30	6.00	27.30	2.48	7.83	3.18	2.46	182.00	27.04
	TCME18	57.00	1.30	0.16	71.10	98.40	5.00	27.30	2.48	7.81	3.17	2.46	181.00	23.40
	Mean	56.88	1.38	0.16	71.17	98.44	5.30	27.27	2.48	7.82	3.18	2.46	181.63	24.30

Table 21. Analysis of variance for biometrical characters due to gamma irradiation and EMS treatment

Sl.No.	Characters	Gamma dose (df = 2) MSS	EMS Concentration (df = 2) MSS
1.	Leaf length (cm)	0.2000	0.2800
2.	Leaf width (cm)	0.0010	0.0060
3.	Ligule length (cm)	0.0016	0.0013
4.	Culm length (cm)	1935.1200**	1230.4000**
5.	Plant height (cm)	1400.1200**	1287.0700**
6.	Panicles plant^{-1}	1.9600**	3.6100 **
7.	Panicle length (cm)	0.0350 **	0.0400 **
8.	Test weight (g) (100 grains)	0.0250	0.0040
9.	Grain length (mm)	0.0010	0.0020
10.	Grain width (mm)	0.0005	0.0004
11.	Grain length/ width ratio	0.0010	0.0005
12.	Grains panicle^{-1}	1933.7625**	1388.7500**
13.	Grain yield plant^{-1} (g)	80.5300 **	128.4900 **

** Significant at 5per cent level

MSS – Mean Sum of Squares

Table F value (5per cent)

(2, 26) = 2.908 for gamma irradiation treatments

(2, 24) = 2.919 for EMS treatments

Table 22. Mean performance of both tissue culture plants with respect to characters showing significant effect

Characters / Treatments	Culm Length (cm)	Plant Height (cm)	Panicles $Plant^{-1}$	Panicle Length (cm)	Grains $Panicle^{-1}$	Grain yield $Plant^{-1}$ (g)
Gamma dose (Gy)						
TG_1	93.000 c	120.000 c	3.900 b	27.000 c	160.000 c	15.370 b
TG_2	92.820 b	119.880 b	4.100 b	27.080 b	164.900 b	16.730 b
TG_3	71.580 a	98.700 a	4.700 a	27.120 a	177.670 a	21.000 a
CD* at 5per cent	0.002	0.100	0.590	0.040	3.415	2.530
CD** at 5per cent	0.001	0.230	0.600	0.050	3.508	2.600
EMS Concentration (mM)						
TE_1	92.800 b	119.900 b	4.100 c	27.100 c	165.000 c	16.720 c
TE_2	92.600 b	119.790 b	4.600 b	27.180 b	168.890 b	19.500 b
TE_3	71.170 a	98.440 a	5.300 a	27.270 a	181.630 a	24.300 a
CD* at 5per cent	0.001	0.220	0.590	0.050	2.169	2.540
CD** at 5per cent	0.001	0.240	0.630	0.050	2.294	2.700

* between TG_1 /TE_1 and TG_2 /TE_2

** between TG_2 /TE_2 and TG_3 / TE_3

b) Morphological Characters

Tissue culture plants obtained from gamma irradiation and EMS treatment were studied along with control for several morphological characters as revealed in Table 23 and Table 24.

i) Gamma Irradiation

Variations were observed in leaf blade pubescence, leaf blade colour, basal leaf sheath and lemma and palea colour. Seventeen plants were pubescent, while control and other tissue culture plants had intermediate leaf blade pubescence. Twelve plants derived from 20 Gy had light green leaf blades the rest were green coloured. Light purple colouration was noticed in TCMG4, TCMG8, TCMG9 and all plants derived from 20 Gy whereas control and other tissue culture plants developed purple lines on the basal leaf sheath. Plants TCMG12, TCMG15 and TCMG18 produced brown (tawny) coloured lemma and palea as against the parental colour (purple furrowed lemma and palea).

ii) EMS Treatment

Variations were observed in leaf blade pubescence, leaf blade colour, basal leaf sheath and lemma and palea colour. Six plants were pubescent, while control and other tissue culture plants had intermediate leaf blade pubescence. Five plants derived from 4 mM EMS had light green leaf blades the rest were green coloured. Light purple colouration was noticed in TCME1, TCME3, TCME4, TCME5, TCME6, TCME7, TCME8 and TCME9 and all plants derived from 4 mM whereas control and other tissue culture plants developed purple lines on the basal leaf sheath. Plants TCME13, TCME14, TCME16 and TCMG17 produced brown (tawny) coloured lemma and palea while control and other tissue culture plants produced purple furrowed lemma and palea.

Table 23. Performance scoring of gamma irradiation derived tissue culture plants for morphological characters

Gamma Dose (Gy)	Characters / Plants	LBP	LBC	BLSC	LA	FLA	LgC	LS	CC	AC	CmA	CmIC	PnT	PnAx	Awning	PnEx	SgC	ApC	LmPC	SCC
0	Control Mean	3	2	2	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
10	TCMG1	2	2	2	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
	TCMG2	2	1	2	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
	TCMG3	2	2	2	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
	TCMG4	3	1	3	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
	TCMG5	2	2	2	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
	TCMG6	3	1	2	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
	TCMG7	2	2	2	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
	TCMG8	2	1	3	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
	TCMG9	3	2	3	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
	TCMG10	2	2	2	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
20	TCMG11	2	1	4	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
	TCMG12	2	1	3	1	1	3	2	3	3	1	1	1	1	0	1	5	7	7	5
	TCMG13	2	1	3	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
	TCMG14	2	1	3	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
	TCMG15	2	1	3	1	1	3	2	3	3	1	1	1	1	0	1	5	7	7	5
	TCMG16	2	1	3	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
	TCMG17	2	1	3	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5
	TCMG18	2	1	3	1	1	3	2	3	3	1	1	1	1	0	1	5	7	7	5
	TCMG19	2	1	3	1	1	3	2	3	3	1	1	1	1	0	1	5	7	6	5

Table 24. Performance scoring of EMS treatment derived tissue culture plants for morphological characters

EMS Concentrations (mM)	Characters / Plants	LBP	LBC	BLSC	LA	FLA	LgC	LS	CC	AC	CmA	CmIC	PnT	PnAx	Awning	PnEx	SgC	ApC	LmPC	SCC
0.00	Control Mean	3	2	2	1	1	3	2	3	2	1	1	1	1	0	1	5	7	6	5
2.00	TCME1	2	1	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	6	5
	TCME2	3	2	2	1	1	3	2	3	2	1	1	1	1	0	1	5	7	7	5
	TCME3	2	1	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	6	5
	TCME4	2	2	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	6	5
	TCME5	2	1	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	6	5
	TCME6	3	1	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	6	5
	TCME7	3	2	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	7	5
	TCME8	2	2	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	6	5
	TCME9	2	1	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	6	5
4.00	TCME10	2	1	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	6	5
	TCME11	2	1	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	6	5
	TCME12	2	1	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	6	5
	TCME13	2	1	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	7	5
	TCME14	3	1	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	7	5
	TCME15	2	1	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	6	5
	TCME16	3	1	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	7	5
	TCME17	2	1	3	1	1	3	2	3	2	1	1	1	1	0	1	5	7	7	5

iii) Percentage of Variation

The percentage of variation for morphological characters was worked out with respect to control values for comparison among different treatments (Table 25).

Percentage of variation was higher for tissue culture plants derived from 20 Gy. 100 per cent variation was shown by 20 Gy derived tissue culture plants for leaf blade pubescence, leaf blade colour, basal leaf sheath colour whereas the tissue culture plants derived from 10 Gy showed 70.00 per cent, 60.00 per cent, and 30.00 per cent variation for the above characters respectively. Tissue culture plants derived from 20 Gy gave 33.33 per cent variation in lemma and palea colour, where as no variation was found for this character among tissue culture plants derived from 10 Gy. The variation percentage was seen to be higher for 4 mM EMS derived tissue culture plants. Tissue culture plants derived from 4 mM EMS treatment showed 100per cent variation for leaf blade pubescence, leaf blade colour, basal leaf sheath colour where as 2 mM EMS derived tissue culture plants showed 66.66 per cent, 44.44 per cent and 88.88 per cent of variations for these characters respectively. 4 mM EMS derived tissue culture plants gave 11.11 per cent variation in lemma and palea colour whereas 2 mM derived tissue culture plants showed 50.00 per cent variation.

3.2.2.4.2 Selection of Superior Plants

Six tissue culture variants have been selected based on their superior yield and yield attributing characters from the tissue culture plants of 20 Gy and 4 mM respectively (Tables 26).

Table 25. Percentage of variation in morphological characters due to gamma irradiation and EMS treatment

Sl.No.	Treatments / Characters	Gamma irradiation (Gy)		EMS Treatment (mM)	
		10	**20**	**2.00**	**4.00**
1.	Leaf Blade Pubescence	70.00	100.00	66.66	100.00
2.	Leaf Blade Colour	60.00	100.00	44.44	100.00
3.	Basal Leaf Sheath Colour	30.00	100.00	88.88	100.00
4.	Lemma and Palea Colour	0.00	33.33	11.11	50.00

Table 26. Superior gamma irradiation and EMS treatment derived tissue culture plants

Doses	Characters / Plants	Culm length (cm)	Plant height (cm)	Panicles plant^{-1}	Panicle length (cm)	Grains Panicle^{-1}	Grain yield plant^{-1} (g)
Control		93.00	120.00	3.90	27.00	160.00	15.37
20Gy	TCMG11	71.50	98.60	5.00	27.10	178.00	21.90
	TCMG12	71.30	98.50	6.00	27.20	178.00	26.42
	TCMG18	71.50	98.70	5.00	27.20	177.00	21.88
Control		92.80	119.90	4.10	27.10	165.00	16.72
4mM	TCME13	71.70	98.40	6.00	27.30	182.00	27.04
	TCME15	71.10	98.40	6.00	27.30	182.00	27.04
	TCME17	71.00	98.30	6.00	27.30	182.00	27.04

DISCUSSION

5. DISCUSSION

Rice is one of the most important food crops, since it is the staple food of more than half of the world's population. Much work has been achieved in tissue culture during the last two decades. Rice occupies a pivotal place in India's food and livelihood security system. The country has to produce about 130-135 million tonnes by 2020 to meet its ever increasing food requirements (Vinothini, 2004). It is widely recognized that efforts should now be made to utilize newly emerging techniques of biotechnology combined with conventional plant breeding for rice improvement (IRRI, 1993).

Ceaseless efforts are being made in India in this direction. According to, Sharma and Baksh (2004) plant breeders combine several techniques together to increase efficiency and to make short cuts in each step. In doing so, plant breeders have options to use *in vitro* culture for rapid multiplication, molecular markers to select specific genotypes and mutagenesis to create variability.

In vitro techniques in combination with induced mutations can speed up breeding programmes by creating variability for exploitation. Selection and multiplication of desirable genotypes can be achieved in a shorter time. *In vitro* mutagenesis will amplify the production of somaclonal variants, regeneration of these genetic variants and further exploitation of selected plants can be carried out in lab conditions under biotic stress free environment. The results obtained from various experiments conducted in this study are discussed below in the light of several works done by scientists in rice.

5.1 *In vitro* Culture

The assembly of genetic variability is vital to any plant breeding enterprise. Totipotency of the plant cell varies with plant species and varieties (Murashige, 1982; Rangaswamy, 1993). The totipotency of the cell is also expressed primarily by the interaction between media constituents and endogenous tissues that determine the differentiation of cells. Choudari *et al.* (1998) also opined similar views regarding the harnessing of tissue culture techniques especially in rice.

Genotype used in this study is of *indica* type. The variety Ptb-26 a pureline selection from Chenkayama was used. This variety is popular in Palakkad district of Kerala and is one of the popular varieties for the Koottumundakan type of rice cultivation. Khanna and Raina (1998) and Biswas and Mandal (1999) investigated varietal specificity with regard to *in vitro* culture response involving callus induction in *indica* and *japonica* rice cultivars. Maximum callusing was found in *indica* rice cultivars. On the contrary, Visarada and Sarma (2002) compared *in vitro* response of several elite *indica* rice cultures with that of the most responding *japonica* type, Taipei 309. Callus induced from mature seeds of *indica* rice genotypes was more compact and proliferated slowly.

Somatic cell culture of rice has undergone rapid development. The first report was about the induction of callus from rice stem node (Furuhashi and Yatazawa, 1964).

Mature and dehulled seed was the explant used in this study. Among the various explants used in plant tissue culture such as immature embryo, anther, pollen, ovary and spikelet tried by several rice workers in different genotypes, it was found that seeds excelled in performance for callus induction and subsequent regeneration in the shortest period (Sumathi, 1992; Bastian *et al.*, 1998 and Vinothini, 2004).

MS media was used in this study as several reports confirm the high callus induction frequency of *indica* cultivars in this medium (Bastian *et al.*, 1998; Abbasi *et al.*, 2000; Deepthi *et al.*, 2001). The only other medium with comparable performance was the N_6 medium as reported by Delawar and Arzani (2001) and Vinothini (2004).

5.1.1 Callus Induction Studies

5.1.1.1 Number of Days for Callus Induction

Regarding the callus induction response, number of days taken for callus induction in this study was 10-14 days of inoculation (Plate 4). This is similar to the results of Sharma *et al.* (1996) and Katiyar *et al.* (1999).

5.1.1.2 Effect of Growth Regulators

A combination of auxins and cytokinins were found to be suitable for callus initiation in rice cultivars. The most commonly used growth regulator for callus induction in cereal tissue culture is 2,4-D (Bregitzer *et al.*, 1989). Many cereals show embryogenic competence in the presence of 2,4-D (George and Eapen, 1988).

2,4-D in combination with Kn facilitated callus induction and maintanence. Five levels of 2,4-D (1.0, 1.5, 2.0, 2.5, 3.0 mgl^{-1}) in combination with Kn 0.5mgl^{-1} were used in this study. A critical level of 2,4-D and Kn was found to be essential for optimum level of callus induction.

2,4-D at 2.0 mgl^{-1} with Kn 0.5 mgl^{-1} gave higher responses and hence this combination was selected for callus induction and subculturing. Similar reports were given by Gonzalez (2000), Wang *et al.* (2001) and Gomez and Kalamani (2002).

5.1.1.3 Percentage of Callus Induction

Callus induction percentage ranged from 0.00% to 98.00%. Maximum callus induction percentage was recorded by MS+2,4-D 2.0 mg l^{-1}+Kn 0.5 mg l^{-1} followed by MS + 2,4-D 2.5 mg l^{-1} + Kn 0.5 mg l^{-1}. The percentage of induction varied with different combinations of 2,4-D and Kn (Figure 1). A plateau was reached at this 2.0 mg l^{-1} level of 2,4-D and thereafter, the response of 2,4-D on callus growth characteristics declined with every unit increase in its level as was reported by Pandey *et al.* (1994). However, Singh and Singh (1996) found good callus induction even at a concentration of 2,4-D 8.0 mg l^{-1}.

0th Day

7th Day

14th Day

Plate 4. Stages of callus induction

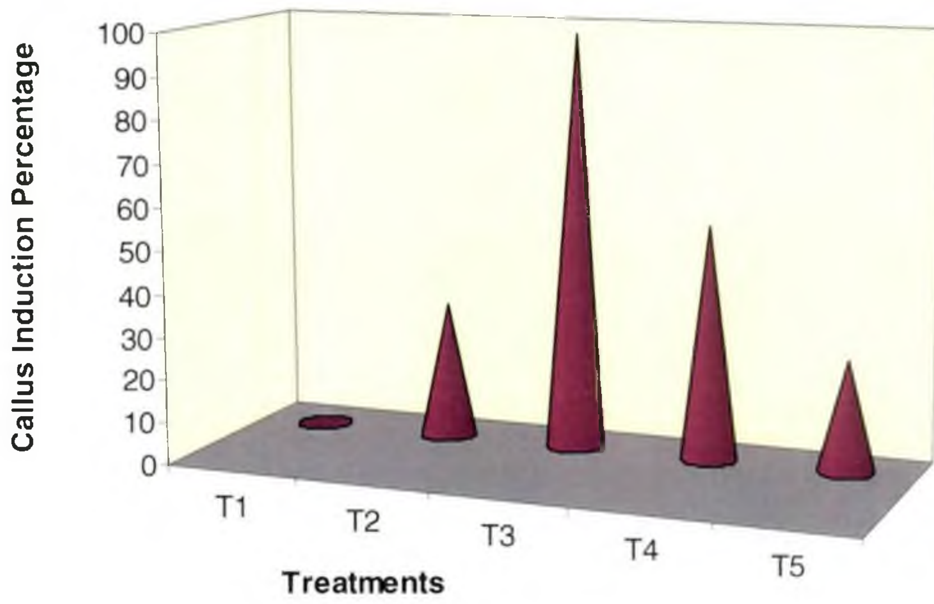


Figure 1. Callus induction

5.1.2 Callus Proliferation Studies

In this study, approximately three weeks old calli were subcultured one time in MS medium with the same hormone level as for induction to effect more callus growth. Subculturing in media with the same hormonal combination produces better callus proliferation and further good regenerants as opined by Sakila *et al.* (1999) and Tan *et al.* (1999). Contrary result was reported by Virk *et al.* (1998) where callus subcultured on media with lower hormonal composition formed shoots.

The callus was allowed to proliferate for three weeks. Reports of Pontongkam *et al.* (1994) reveal that three weeks old subcultures were better than six weeks old subcultures for plant regeneration. Three weeks old subcultured calli were observed for callus diameter, callus volume and callus fresh weight. In the present study on an average the callus diameter was 1.04 cm, callus volume was 4.22 cc and callus fresh weight was 326.70 mg. Callus morphology studies revealed that the all calli obtained in this study were cream coloured and compact. Hence they are classified as non-embryogenic calli. This is similar to the report of Virk *et al.* (1998).

5.1.3 Regeneration Studies

MS medium with NAA 2 mgl^{-1}, Kn 4 mgl^{-1} and BAP 0.5 mgl^{-1} gave the high regeneration response (Plate 5). This is in confirmation with the studies conducted by Anderson and Al-Khayri (1996), Katiyar *et al.* (1999) and Lee *et al.* (2003).

5.1.4 Rooting and Hardening

The rooting media used in this study was MS + NAA 2.0 mg l^{-1} + Kn 0.5 mg l^{-1}. Higher percentage of success using the above rooting media was also reported by Revathy *et al.* (2000). Hardening and acclimatisation is often the critical stage in the overall tissue culture cycle where losses can be high (Jagannathan, 1985).

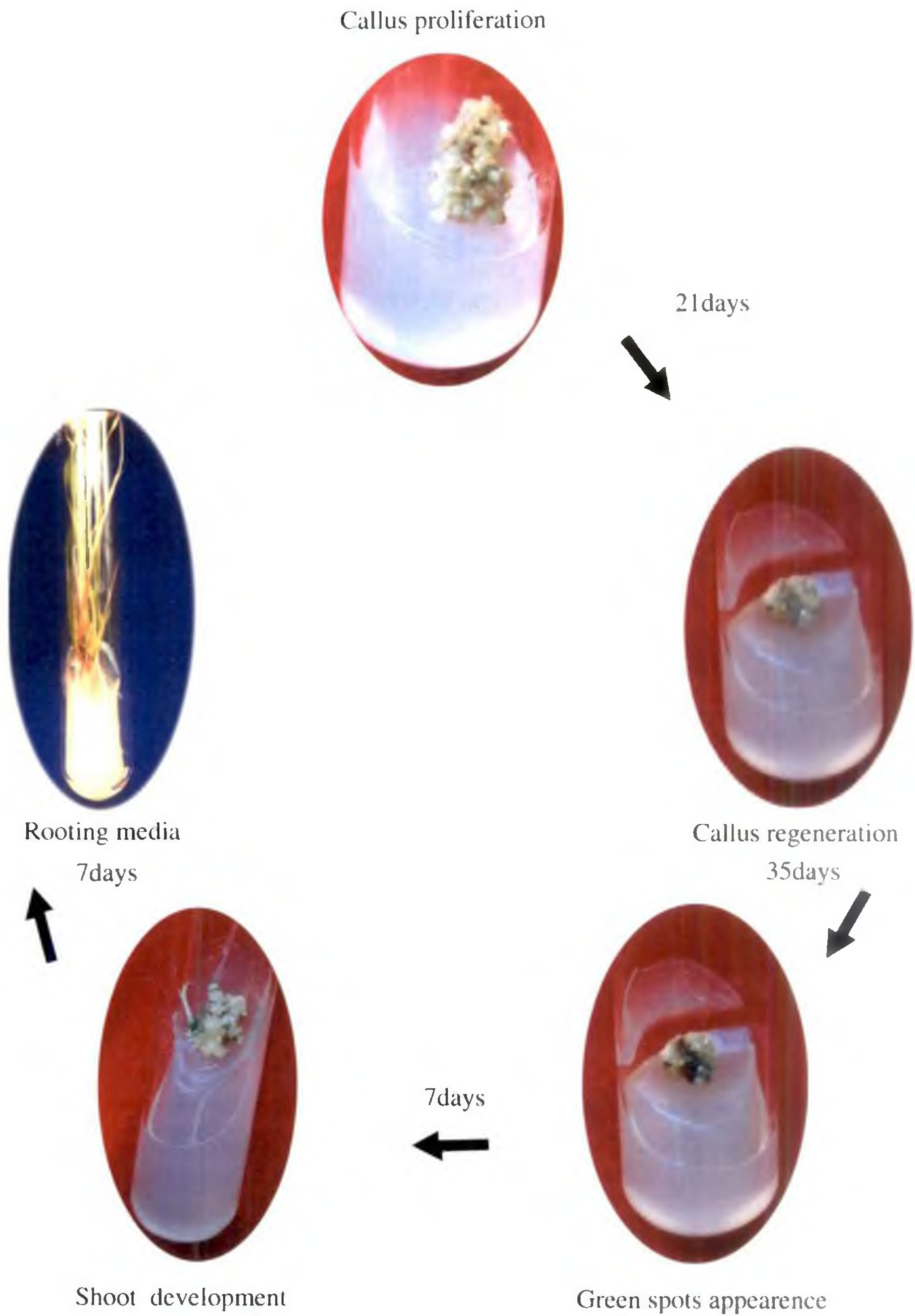


Plate 5. Stages of callus regeneration

5.2 *In vitro* Mutagenesis

Mutations are defined as heritable changes in DNA sequences that are not produced from genetic segregation or recombination. Genetic variation can be induced either by specific treatments with physical and chemical mutagens or by tissue culture (Predieri, 2001). As suggested by Scossiroli (1970) a mutational event will be very important if it has a very small effect on a specific morphological or physiological character because it changes the balance established by natural selection in co-adapted blocks of genes. They have potential for development into new commercial varieties, which can give a stable income to farmers.

Some results point out that the combination of radiation treatment or chemical mutagenesis with rice *in vitro* culture could enhance the redifferentiaiton ability of callus and enhance the rate of variation in progenies (Yin *et al.*, 1983). Therefore, the application of *in vitro* culture combining mutagenesis will be of realistic value in rice improvement programmes.

Moh and Han (1973) opined that the improvement of a cultivar is usually achieved by adding one or two desirable attributes to the initial strain and if these desirable characters are introduced by mutagenesis, it is certainly the simplest means to achieve the breeding objectives. Thus, induced mutations confer specific improvement without significantly altering its otherwise acceptable phenotype (Ojomo *et al.*, 1979).

The present study aims at improving the agronomic characters in *indica* rice cultivar Ptb-26 to obtain desirable plants. This variety has a high consumer preference (Rosamma *et al.*, 2003).

The intensity of colour change in mutated calli was scored. In this study, it was noticed that browning of calli intensified with an increase in the mutagen dosage. This is in confirmation with the results of Ukai (1988).

Moderate doses of gamma rays and lower concentrations of EMS showed stimulatory effect on the growth of calli. It may be due to the increased activity of enzymes involved in biosynthesis of hormones like auxins and cytokinins (Vagera *et al.*, 1976).

Sensitivity to mutagens *in vitro* was assessed by determining by the rates of shoot differentiation. This was explained to be due to death of the embryo immediately after mutagenesis. Fixing of the appropriate dose of the mutagen is done by estimation of LD_{50} values. Based on the percentage of regeneration of mutated calli, LD_{50} was arrived for gamma rays and EMS. The LD_{50} for gamma rays was 30 Gy and for EMS was 6mM (Figure 2). Similar reports were reported by Suputtitada *et al.* (1994), Zhu *et al.* (1988) and Checheneva and Larchenko (1997).

Optimum dose is chosen from the subjected doses of each mutagen below LD_{50} value (Abraham, 2002). Most authors found optimum dose to be around the LD50 value. Optimum dose is one that gives maximum number of desirable mutants with minimum killing. Maximum plant recovery was noticed for two doses of gamma rays *viz.*, 10 and 20Gy and two concentrations of EMS *viz.*, 2mM and 4mM (Figure 3). The mutagen dose for inducing successful *in vitro* mutations in callus cultures was also standardized.

Two optimum doses each for gamma rays (10Gy and 20Gy) and EMS (2mM and 4mM) were fixed in this study. Similar suggestions regarding relationship between dose of mutagen and survival percentage were given by Dunwell (1985), Jeyanthi and Rangasamy (1991), Gao *et al.* (1992), Lu *et al.* (1997), Punitha (1999), Sakila *et al.* (1999) and Singh *et al.* (2000).

5.2.1 Performance of Tissue Culture Plants

Mutations are helpful in rectifying specific defects in an otherwise well adapted commercial variety. Efficient mutagenesis is one, which produce desirable changes free from undesirable linkages (Gupta and Sharma, 1990).

Green plant regeneration percentage from callus varied with different treatments. In this experiment ten tissue culture plants were obtained from 10 Gy treated calli, nine tissue culture plants each from 20 Gy and 2 mM EMS treated calli and eight tissue culture plants from 4 mM EMS treated calli. These tissue culture

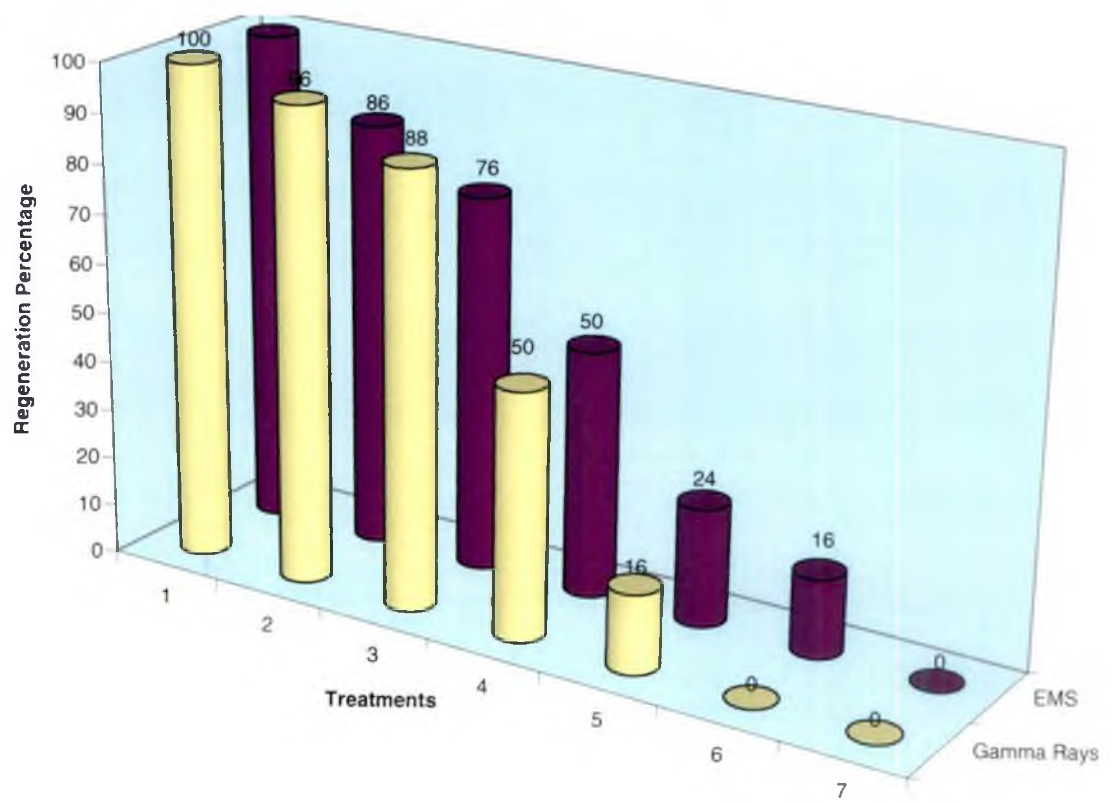


Figure 2. Estimation of LD_{50} values

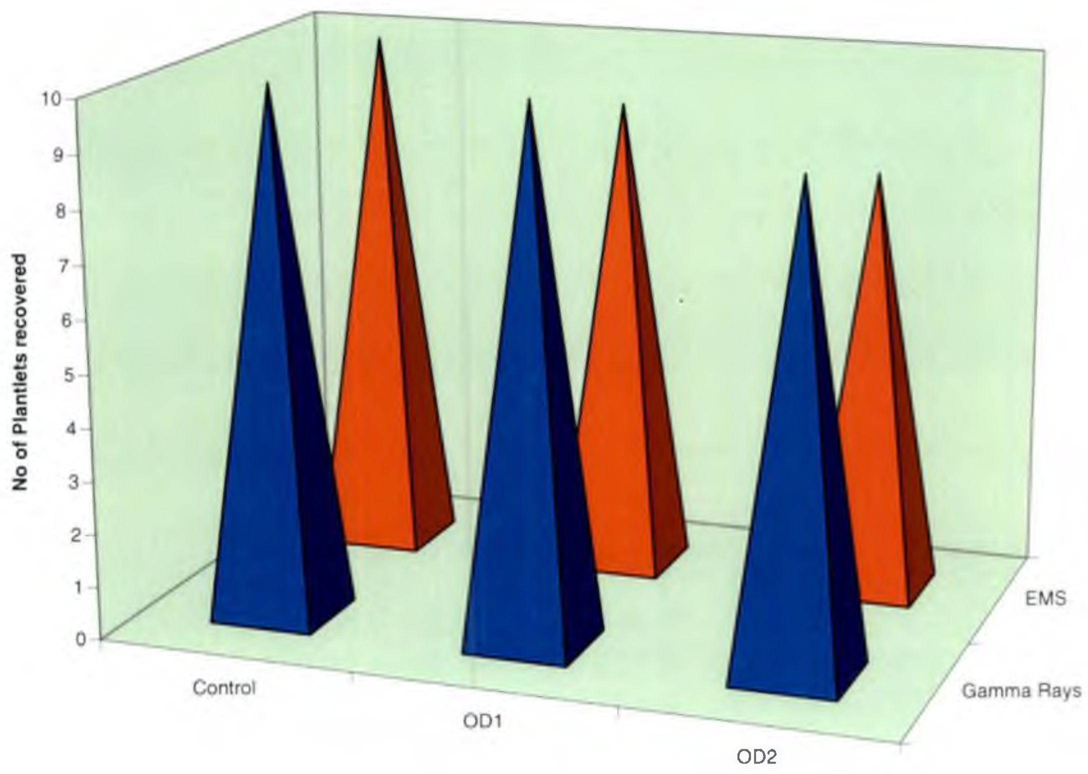


Figure 3. Plantlet recovery

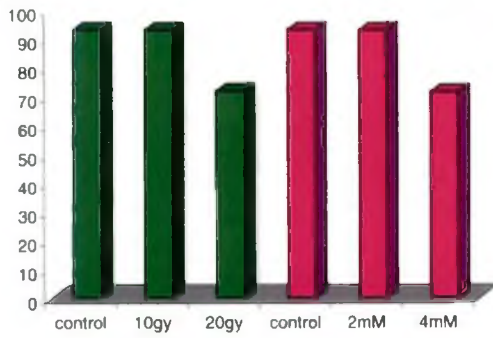
plants along with their respective controls were evaluated for different biometrical and morphological characters for the selection of desirable plants.

Among all the biometrical characters, significant effect due to the different doses and concentrations of gamma rays and EMS respectively was found in five characters namely culm length, plant height, panicles plant^{-1}, panicle length, grains panicle^{-1} and grain yield plant^{-1}.

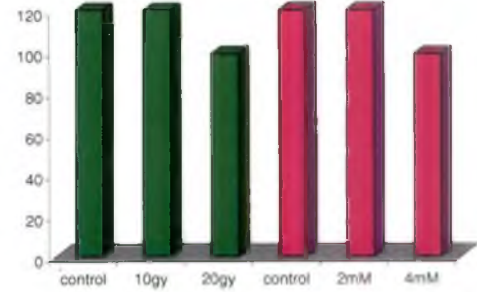
Gamma rays (20Gy) had significant effect on most of the parameters. There was increase in culm length, panicles plant^{-1}, panicle length, grains panicle^{-1} and grain yield plant^{-1} and a decrease in plant height. 20 Gy thus emerges as the best treatment compared to control and 10 Gy (Figure 4). Although the frequency of recovery of plants is less at 20Gy the number of desirable plants was high in this treatment than at the lower doses (control and 10 Gy). Hence, 20 Gy is adjudged as the best optimum dose.

With respect to EMS treatments, EMS concentration of 4mM had stimulatory effect and significant effect on most of the characters. Increase was noted in culm length, panicles plant^{-1}, panicle length, grains panicle^{-1} and grain yield plant^{-1} where as decrease was noted in plant height (Figure 4). Eventhough the recovery of plants was higher in lower concentrations (control and 2mM) the concentration 4mM gave more number of desirable plants. Hence, 4mM is chosen as the best optimum dose.

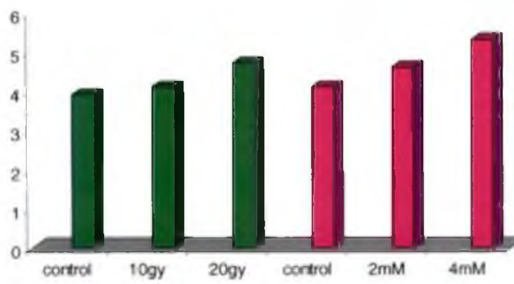
Significant effect of doses of gamma rays and concentrations of EMS on genotype was noticed for leaf blade pubescence, leaf blade colour, basal leaf sheath colour and lemma and palea colour. The percentage of variation with respect to control among the gamma irradiated and EMS treated tissue culture plants was more in 20 Gy and 4mM derived tissue culture plants respectively. Induction of such variations in rice following *in vitro* mutagenesis has been reported earlier also by (Ratisoontorn *et al.*, 1995; Ali and Siddiq, 1999 and Lee *et al.*, 2003).



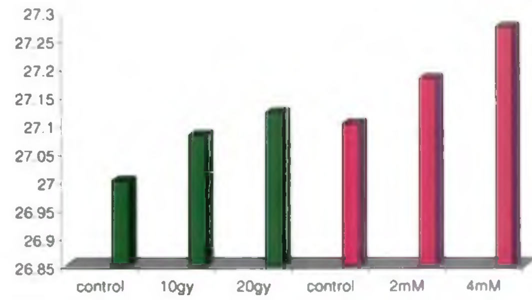
a) Culm length (cm)



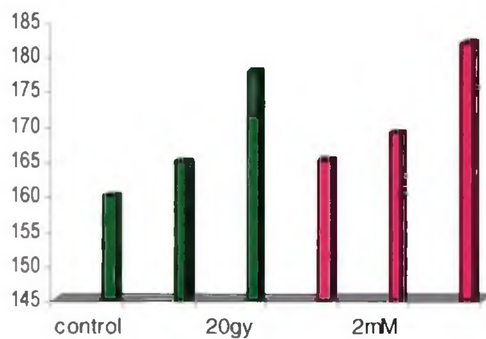
b) Plant height (cm)



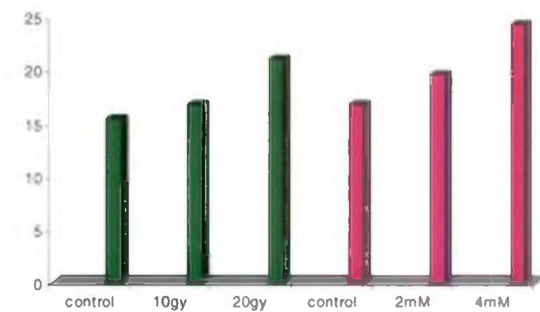
c) Panicles per plant



d) Panicle length (cm)



e) Grains per panicle



f) Grain yield (g)

Figure 4. Variation in biometrical characters

Six desirable variants TCMG11, TCMG12, TCMG18, TCME13, TCME15 and TCME17 (Plate 6 and Plate 7) were found to have increased culm length, panicles plant^{-1}, panicle length, grains panicle^{-1} and grain yield plant^{-1}. The plant height got reduced. This result confirms that the combination of *in vitro* and mutation techniques can speed up crop improvement (Ahloowalia, 1995).

These desirable variants are semidwarf as compared to the parent variety, which is intermediate tall. The reduction in plant height might have led to an increase in the number of panicles per plant and thereby increase in yield. Reddy *et al.* (1975) reports that short duration rice plants have increased degree of tillering which contribute to increased seed production.

5.3 Future Line of Work

Identifying desirable plants especially for agronomic characters governed by polygenes is not quite possible in the immediate generation of treatment. Characters like earliness can be studied only in the next generation for tissue culture derived population. Hence confining the study of these variants to the M_1 generation alone will not throw light on all the potential variants. Segregating generations will be able to ascertain whether the variations are heritable or not.

Raising the segregating generations would lead to increase in homozygosity of the variants. Polygenic traits are micromutations in nature and selection for them is delayed until M_3 or the later generations. As the time frame of this study doesnot permit for such late segregating generations the immediate plan of action will be to evaluate the desirable variants obtained from this study in subsequent segregating generations to see if these variations are indeed inherited and if possible to exploit them commercially.

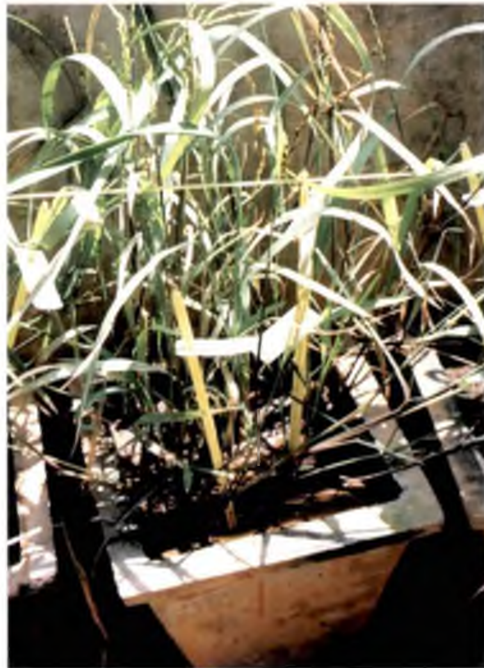
The recovery of plants in this study was small which could be considered as a bottleneck while searching for micromutations. Further refinement in standardization of *in vitro* techniques and mutagenesis would result in higher recovery of better plant types.

TCMG 11

TCMG 12

TCMG 18

Plate 6. Superior tissue culture variants derived from gamma irradiation



TCME 13

TCME 15

TCME 17

Plate 2. Superior tissue culture variants derived from EMS treatment

SUMMARY

6. SUMMARY

The present study entitled "*In vitro* mutagenesis in rice (*Oryza sativa* L.)" was undertaken at the Department of Plant Breeding and Genetics, College of Horticulture, Kerala Agricultural University, Vellanikkara to produce successful *in vitro* cultures of Ptb-26, to fix the LD_{50} values for physical mutagen (gamma rays) and chemical mutagen (EMS), to fix optimum doses of mutagens and identifying desirable *in vitro* plants from it.

1. Genotype used in this study is of *indica* type. The variety Ptb-26 a pureline selection from Chenkayama was used.
2. The explant used for research work was mature embryo.
3. MS medium was chosen as the basal media for this study. Five different levels of 2,4-D (1.0, 1.5, 2.0, 2.5, 3.0 mg l^{-1}) in combination with 0.5 mg l^{-1} of Kn was used in this study.
4. Number of days taken for callus induction in this study was 14 days of inoculation.
5. A critical level of 2,4-D and Kn was found to be essential for optimum level of callus induction. Concentration of MS + 2,4-D 2.0 mg l^{-1} + Kn 0.5 mg l^{-1} (98.00%) was the most effective in promoting callus formation. A plateau was reached at this level of 2,4-D and thereafter, the response of 2,4-D on callus growth characteristics declined with every unit increase in its level.
6. Three weeks old calli were subcultured after 21 days in the same medium with callus induction medium to effect more callus growth.
7. Callus was allowed to proliferate for 21 days and then taken for regeneration.
8. Callus morphology was studied on the proliferated callus. The mean callus diameter, volume and fresh weight were 1.04 cm, 4.22 cc and 326.70 mg respectively.

9. The medium used for regenerating calli were MS + NAA 2.0 mg l^{-1} + Kn 4.0 mg l^{-1} + BAP 0.5 mg l^{-1} and MS + NAA 2.0 mg l^{-1} + Kn 4.0 mg l^{-1} + BAP 1.0 mg l^{-1}.
10. Among the two media used for regeneration the MS + NAA 2.0 mg l^{-1} + Kn 4.0 mg l^{-1} + BAP 0.5 mg l^{-1} was responding better with a regeneration percentage of 90.48%.
11. Regenerated shoots were transferred to rooting medium (MS + NAA 2.0 mg l^{-1} + Kn 0.5 mg l^{-1}) and the percentage of success of hardened plant was 85.24%.
12. The above callus induction and callus proliferation procedure was repeated and three weeks old subcultured calli were exposed to the different mutagens at different levels.
13. 10 Gy, 20 Gy, 30 Gy, 40 Gy, 50 Gy and 60 Gy were the doses fixed for LD_{50} value estimation of gamma rays.
14. 2 mM, 4 mM, 6 mM, 8mM, 10 mM and 12 mM were the doses fixed for LD_{50} value estimation of EMS.
15. The treated calli was subcultured on fresh medium immediately after treatment and callus morphology in terms of browning was studied.
16. Based on the percentage of regeneration of mutated calli, LD_{50} was arrived for gamma rays and EMS. The LD_{50} for gamma rays was 30 Gy and for EMS was 6 mM.
17. The mutagen dose (optimum dose) for inducing successful *in vitro* mutations in callus cultures was standardized. Optimum dose is one that gives maximum number of desirable plants with minimum killing. Optimum doses were fixed as below the LD_{50} value for the respective mutagen.
18. 10 Gy and 20 Gy for gamma rays while 2 mM and 4 mM of EMS were fixed as optimum doses and the procedures from 6-12 were repeated.
19. After successful hardening, ten (TCMG1 to TCMG10) and nine (TGCM11 to TGCM19) tissue culture plants in number were obtained for 10 Gy and 20 Gy respectively. Similarly, nine (TCME1 to TCME9) and eight (TCME10 to

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TCME17) number of tissue culture plants were obtained in 2 mM and 4 mM of concentrations respectively.

20. Among all the biometrical characters, significant effect due to the different doses and concentrations of gamma rays and EMS respectively was found in four characters namely, culm length, plant height, panicles plant^{-1}, panicle length, grains panicle^{-1} and grain yield plant^{-1}.
21. Significant differences were noticed among the tissue culture plants derived from gamma irradiation and EMS treatment for the following morphological characters namely, leaf blade pubescence, leaf blade colour, basal leaf sheath colour and lemma and palea colour.
22. The percentage of variation with respect to control among the gamma irradiated and EMS treated tissue culture plants was more in 20 Gy and 4 mM derived tissue culture plants respectively.
23. 20 Gy thus emerges as the best treatment compared to control and 10 Gy. It is seen to provide most desirable plants though its frequency of recovery of plants is less than at the lower doses. Hence, 20 Gy is the best optimum dose. Similarly the concentration 4 mM of EMS gave more of desirable plants when compared control and 2 mM EMS. Hence, 4mM is chosen as the best optimum dose.
24. Three desirable tissue culture variants each were selected from both gamma irradiated (TCMG11, TCMG12, TCMG18) and EMS treated (TCME13, TCME15, TECM17) tissue culture variants. These variants were found to have increased number of panicles plant^{-1}, panicle length, grains panicle^{-1} and grain yield plant^{-1}. The height of the plant was reduced.
25. Further studies on the segregating generations will throw light on micromutations for polygenic traits.

REFERENCES

REFERENCES

Abbasi, F.M., Hamid, R. and Azra, Q. 2000. Regeneration efficiency and embryogenic callus production of three cultivars of rice. *Pakist. J. agric. Res.* 16: 97-99

Abraham, M. 2002. Assessment and induction of variability for higher yield and photosensitivity in Coleus (*Coleus parviflorus* Benth.). Ph.D. thesis, Kerala Agricultural University, Thrissur, 184 p.

Ahloowalia, B. 1995. *In vitro* mutagenesis for the improvement of vegtatively propagated crops. *Proceedings of Seminar on Induced Mutation and Molecular Techniques for Crop Improvement, June 19 - 23, 1995* (ed. De Garmo, H.). International Atomic Energy Agency, Vienna, Austria, pp. 531-541

Ahloowalia, B.S. 1992. *In vitro* radiation induced mutants in chrysanthemum. *Mutat. Breed. Newsl.* 39: 6

Aldemita, R.R. and Zapata, F.J. 1991. Anther culture of rice: Effects of radiation and media components on callus induction and plant regeneration. *Cereal Res. Commun.* 19: 9-25

Ali, J.A. and Siddiq, E.A. 1999. Induction of mutation affecting floral traits in rice (*Oryza sativa* L.). *Indian. J. Genet.* 59: 23-28

Alyoshin, N. E., Avakyan, E.R. and Alysoshin, E. P. 2001. Obtaining unique large kernel rice using chemical muatgenesis in tissue culture. *Mutat. Breed. Newsl.* 45: 50-51

*Amirkhanov, O.N., Kucherenko, L.A and Dolotova, N.A. 1991. Morphogenesis in the culture of somatic tissues of rice, and the composition of the regeneration medium. *Dopov. Akad. Nauk. A.* (German) 2: 6-8

Anderson, E.J. and Al-Khayri, J.M. 1996. *Effect of genotype and media components on callus induction and plant regeneration of rice*. Research Series No. 453. Arkansas Agricultural Experiment Station, Fayetteville, 315 p.

Arya, K. 1987. *In vitro* techniques in relation to induced mutation in Groundnut (*Arachis hypogaea* L.). M.Sc. thesis, Kerala Agricultural University, Thrissur, 89 p.

Bajaj, Y.P.S. 1983. Production of normal seeds from plants regenerated from the meristem of *Arachis hypogaea* and *Cicer aeritinum* cryopreserved for 20 months. *Euphytica* 32: 425-430

Bajaj, Y.P.S. and Bidani, M. 1980. Differentiation of genetically variable plants from embryo derived callus cultures of rice. *Phytomorphology*. 30: 290-294

Bastian, D., Nadarajan, N.N., Kalamani, A. and Sakila, M.K. 1998. Studies on callus responses in M_3 seeds in rice. National Plant Breeding Congress, 13-16 July 1998, Tamil Nadu Agricultural University, Coimbatore. *Abstract*: 40

Bhagwat, B. and Duncan, E.J. 1998. Mutation breeding of banana cultivar Highgate (*Musa* spp., AAA Group) for tolerance to *Fusarium oxysporum* f.sp. *cubense* using chemical mutagens. *Sci. Hort.* 73: 11-22

Biswas, B. and Mandal, A.B. 1999. Varietal specificity in callus induction and plantlet regeneration in rice (*Oryza sativa* L.). *Crop Improv*. 26: 135-140

Boissot, N., Valdez, M. and Guiderdoni, E. 1990. Plant regeneration from leaf and seed derived calli and suspension cultures of the African perennial wild rice, *Oryza longistaminata*. *Pl. Cell Rep*. 9: 447-450

Bregitzer, P., Somers, D.A. and Rines, H.W. 1989. Development and characterisation of friable callus. *Crop Sci.* 29: 798-803

Brisibe, E. A., Taniguchi, T. and Maeda, E. 1990. *In vitro* plant regeneration from morphogenic callus cultures of cultivars and wild *Oryza* species. *Jpn J. Crop Sci.* 59: 557-565

Cai, Q.H., Gao, M.W. and Liang, Z.Q. 1990. Mutagenesis of *in vitro* culture for improvement of hybrid rice. *Acta Agric. Scand.* (Chinese) 4: 169-174

Chandrahasan, V.T. 2004. Androgenesis in rice (*Oryza sativa* L.). M.Sc. thesis, Kerala Agricultural University, Thrissur, 84 p.

*Checheneva, T.N. and Larchenko, E.A. 1997. Effect of chemical mutagens and gamma irradiation on the *in vitro* culture of inbred maize lines. *Tsitol. I Genet.* (Russian) 31: 65-71

Chen, Z. and Zhu, X.Y. 1991. Inheritance of related characteristics of mature embryos in culture *in vitro* in *Oryza sativa. Chinese J. Rice Sci.* 5: 145-150

Choudari, K.V., Ramakrishna, W., Tamahankar, S.A., Henke, R.R., Gupta, V.S., Sahasrabudhe, N.A. and Ranjerar, P.K. 1998. A minor DNA sequene change in somaclones of rice. *Pl. Cell Rep.* 18: 55-58

Chu, Y., Hu, T., Tsai, Y.C. and Chen, J.J. 2000. Effects of gamma-rays irradiation and leaf blade culture on mutations in Caladium. *J. Chinese Soc. hort. Sci.* 46: 381-388

Chung, G. 1992. Anther culture for rice improvement in Korea. *Anther Culture for Rice Breeders* (eds. Kangle, Z. and Murashige, T.). Zhejiang Academy of Agricultural Sciences, Hangzhou, China, pp. 8-37

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

*Cunha, M.B., Paranhos, J.T., Dorneles, L.T., Franco, E.T.H. and Da, C. 1994. Effect of interaction of auxins and crop system *in vitro* on the induction of callus from embryos of rice. *Cienc. Rural* (Spanish) 24: 87-90

*Davoyan, E. I. 1991. Optimization of conditions for induction of morphogenesis in callus culture of rice. *Dopov. Akad. Nauk. A.* (German) 5: 67-70

Deepti, S., Shankhdhar, S.C., Pant, R.C. and Shankhdhar, D. 2001. Genotypic variation of callus induction and plant regeneration in rice (*Oryza sativa* L.). *Indian J. Pl. Physiol.* 6: 261-264

Delawar, N. and Arzani, A. 2001. Study of callus induction and plant regeneration from immature embryo culture in rice cultivars. *J. Sci. Technol. Agric. Natural Resour.* 4: 57-72

*Domingues, E.T., Tulmann, N.A., Mendes, B.M.J. and Ando, A. 1994. Effects of gamma ray doses on shoot apex of Banana (*Musa* sp.) plants developed *in vitro* aiming at mutation induction. *Pesqui. Agropecu. Brasil.* (Portuguese) 29: 1091-1098

Dunwell, J.M. 1985. Anther and ovary culture. *Cereal Tissue and Cell Culture* (eds. Bright, S.W.J and Jones, M.J.K.). Martinus Nighoff, Dordrecht, pp.1- 24

El-Shoury, K.A., Mohamed, A.A. and Azar, S.A. 1994. Induced variation in M_2 generation of two rice varieties by gamma radiation. *Ann. agric. Sci.* 32: 817-825

Farook, T.T. and Bhalla, J.K. 1991. Effect of different mutagens on induction of chromosomal abnormalities in triticale. *Adv. Pl. Sci.* 4: 110-121

FIB. 2002. *Farm Guide*. Farm Information Bureau, Government of Kerala, Thiruvananthapuram, 49 p.

Francies, R.M. 2002. Breeding for abiotic stress tolerance. *Seventy Five Years of Research* (eds. Nair, R.R., Jyothi, M.L and Balachandran, P.V.). Kerala Agricultural University, Thrissur, pp. 29-33

Freese, E. 1963. Molecular mechanism of mutation. *Molecular Genetics, Part I* (ed. Taylor). Academic Press, New York, pp. 207-289

*Furuhashi, K. and Yatazawa, M. 1964. Indefinite culture of rice stem node callus. *Kagaku Kogaku Ronbun.* (Philippino) 34: 623

Gamborg, O.C. and Shyluk, J.P. 1981. Nutrition media and characteristics of plant cell and tissue cultures. *Plant Tissue Culture – Methods and Applications in Agriculture* (ed. Thorpe, T.A.). Academic Press, New York, pp. 21-24

Gao, M., Cheng, Z. and Liang, Z. 1991. Effect of *in vitro* mutagenesis on the frequency of somaclonal variation in wheat. *Cereal Res. Commun.* 19: 77-88

Gao, M.W. 1989. A summary of the current status of hybrid rice improvement by using induced mutations and *in vitro* techniques. *Advances in Agricultural Biotechnology* (ed. Maluszynski, M.). Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 63-70

Gao, M.W., Cai, Q.H. and Liang, Z.Q. 1992. *In vitro* culture of hybrid *indica* rice combined with mutagenesis. *Pl. Breed.* 108: 104-110

Gaul, H., Grunewaldt, J. and Hesemann, C.U. 1964. Variation of character expression of barley mutants in a changed genetic background. *Mutations in Plant Breeding II.* International Atomic Energy Agency, Vienna, 105 p.

Geier, T. 1989. An improved type of "Kohleria" obtained through *in vitro* chemical mutagenesis. *Mutat. Breed. Newsl.* 34: 7-8

George, L. and Eapen, S. 1988. Plant regeneration by somatic embryogenesis from immature inflorescence cultures of *Sorghum aimum. Ann. Bot.* 61: 589-591

Gomez , S.M. and Kalamani, A. 2002. Variability analysis of traits related to callus growth and plant regeneration in drought resistant local land cultivars of rice (*Oryza sativa* L.). *Asian J. Pl. Sci.* 1: 583-584

Gonzalez, M.C. 2000. Effects of different growth regulators on *in vitro* culture of rice cv. Amistad-82. *Cultivos trop.* (Russian) 21: 27-28

Gregory, W.C. 1956. The comparative effect of radiation and hybridizaion in plant breeding. *Q. Rev. Biol.* 31: 48-51

Guo, Q.S. and Shen, Y. 1996. Study of lower concentration of 2,4-D on enhancement of rice green plantlets *in vitro. Chinese J. Rice Sci.* 10: 103-109

Gupta, S.C. and Sharma, K.D. 1990. Mutagenic effetiveness and efficiency of ethyl methane sulphonate and gamma rays in rice (*Oryza sativa* L.). *Oryza* 27: 217- 219

Hammad, A.H.A. 1996. Regeneration of some bean cultivars via *in vitro* organogenesis after irradiation by gamma rays. *Ann. agric. Sci.* 34: 1117-1122

Hazarika, B.N. 1983. Acclimatization of tissue cultured plants. *Curr. Sci.* 85: 1704 - 1712

Hemalatha, R.G. 1995. Studies on *in vitro* screening for sheath rot (*Sarocladium oryzae*) disease resistance in rice (*Oryza sativa* L.). M.Sc. thesis, Tamil Nadu Agricultural University, Coimbatore, 134 p.

Hendre, R.R., Sahasrabudhe, N.A., Katare, N.B and Ranjekar, P.K. 1998. Agronomic trait evaluation of somaclonal variants in *indica* rice. *Rice Biotech. Q.* 34: 22-23

Henske, R.R., Mansur, M.R. and Constantin, M.J. 1978. Organogenesis and plantlet formation from organ and seedling derived calli of rice (*Oryza sativa*). *Physiol. Pl.* 44: 11-14

Hu, C.H. 1973. Evaluation of breeding semi dwarf rice by induced mutations and hybridisation. *Euphytica* 22: 562-574

IRRI. 1993. *Biotechnology a Common Legacy.* International Rice Research Institute, Manila, Philippines, 85 p.

IRRI. 1996. *Standard Evaluation System for Rice.* International Rice Research Institute, Manila, Philippines, 44 p.

Jagannathan, V. 1985. Factors influencing the survival of tissue culture plantlets in the field. *Biotechnology in Agriculture* (eds. Natesh, S., Chopra, V. L and Ramachandran, S.). Oxford and IBH Publishing Co. Ltd., New Delhi, pp. 185-192

Jain, S.M., Altman, A. and Ziv, M. 1997. Creation of variability by mutation and tissue culture for improving plants. *Acta Hort.* (Chinese) 447: 69-77

Jeyanthi, R. and Rangaswamy, S.R.S. 1991. Effect of gamma irradiation on *in vitro* androgenesis in rice (*Oryza sativa* L.). *Oryza* 28: 305-308

Jyothi, R. 2002. Stability analysis of Kunjukunju rice cultivar (*Oryza sativa* L.). M.Sc. thesis, Kerala Agricultural University, Thrissur, 134 p.

Kalamani, A., Sakila, M., Bastian, D., Narayanan, S. L. and Ashok, S. 1999. *In vitro* studies in rice. National Symposium on Role of Biochemistry and Biotechnology in Twenty first century, 4-6 March 1999. Society for Plant Biochemistry and Biotechnology and University of Agricultural Sciences, Bangalore. *Abstract*: 3

Kalamani, A., Sakila, M. and Bastian, D. 2000. Studies on enhancing *in vitro* response in rice. National Seminar on Recent Advances in Plant Breeding, 3-4 February, 2000. Society for Plant Physiology, Central Plantation Crops Research Institute, Kasargod. *Abstract*: 17

Katiyar, S.K., Chandel, P.S. and Pratibha, R. 1999. Genetic variation and effects of 2,4-D on *in vitro* plant regeneration in *indica* rice cultivars. *Oryza* 36: 254 - 256

Khan, D., Gaj, M.D. and Maluszynski. 1999. *In vitro* mutagenesis in sugarcane callus culture. *Mutat. Breed. Newsl.* 44: 19-21

Khanna, H.K. and Raina, S.K. 1998. Genotype X culture media interaction effects on regeneration response of three *indica* rice cultivars. *Pl. Cell Tissue Organ Cult.* 52: 145-153

Khush, G.S. 2001. Grain quality of hybrid rice. *Hybrid Rice*. International Rice Research Institute, Manila, Philippines, 95 p.

Koetje, D.S., Grimes, H.D., Wang, Y.C. and Hodges, T.K. 1989. Regeneration of *indica* rice (*Oryza sativa* L.) from primary callus derived from immature embryos. *J. Pl. Physiol.* 35: 184-190

Konzak, C.F., Nilan, R.A., Wagner, J. and Foster, R.J. 1965. Efficient chemical mutagenesis. *The Use of Induced Mutation in Plant Breeding*. International Atomic Energy Agency, Vienna, 70 p.

*Kucherenko, L.A., Vlasov, V.G. and Butenko, R.G. 1988. Induction of callus from individual parts of the embryo in rice and the regenerative ability of the callus obtained. *Biol. Zbl.* (German) 116: 234-237

Kudo, M. 1967. GC analysis and photoperiodic response of some early maturity mutants induced from Norin B in rice. *Jpn. J. Breed.* 17: 36-37

Kuksova, V.B., Piven, N.M. and Gleba, Y.Y. 1997. Somaclonal variation and *in vitro* induced mutagenesis in grapevine. *Pl. Cell Tissue Organ Cult.* 49: 17-27

Kulkarni, N. and Gangaram, A. 1998. Genetic changes and phenotypic stability in mutants of rice variety Samba Mahsuri. *Oryza* 35: 322-324

Kunanuvatchaidach, R., Godwin, I.D. and Adkins, S.W. 1995. High efficiency plant regeneration from callus induced on mature *indica* rice caryopsis. *Aust. J. Bot.* 43: 337-348

Larkin, P.J and Scowcroft, W.R. 1981. Somaclonal variation – A novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* 60: 197 – 214

Lee, I.S., Kim, D.S. and Lim, Y. 2003. Selection of salt tolerant variants by radiation and *in vitro* culture in rice. *Korean J. Breed.* 35: 13-17

*Lu, Y.M., Wang, C.L., Shen, M. and Chen, Q. F. 1997. Effect of gamma-irradiation on the formation of calli and regeneration of green plants in rice anther culture. *Acta Agric. Scand.* (Chinese) 9: 123-126

Maheswaran, M. and Rangaswamy, S.R.S. 1989. Effect of 2,4-D and kinetin on callus induction and plant regeneration from somatic cell cultures of rice. *Oryza* 26: 302-305

Maluzynski, M., Ahloowalia, B.S., Sigurbjornsson, B., Cassels, A.C and Jones, P.W. 1995. Application of *in vivo* and *in vitro* mutation techniques for crop improvement. *Euphytica* 85: 303 – 315

Mangal, M. and Sharma, D.R. 2002. *In vitro* mutagenesis for induction of back rot resistance in Cauliflower. *J. Hort. Sci.* 77: 268-272

Meifang, L.I. 1992. Anther culture breeding of rice at the CAAS. *Anther Culture for Rice Breeders* (eds. Kangle, Z. and Murashige, T.). Zhejiang Academy of Agricultural Sciences, Hangzhou, China, pp.75-86

Mendoza, A.B. and Futsuhara, Y. 1992. Histological observations on plant regeneration in rice (*Oryza sativa* L.) calli. *Jpn J. Breed.* 42: 33-41

Miah, A.J., Azam, M.A., Hakin, L., Mansur, M.A., Jalaluddin, M. and Begum, N. 1991. Use of induced mutations in connection with haploids and heterosis in cereals. *Cereal Res. Commun.* 19: 195-199

*Micke, A. 1962. Mutations in plant breeding. *Z. Pflanzenzucht* (French) 49: 33 -38.

Min, S., Xiong, X., Qi, X. and Zhao, C. 1991. Effects of gamma ray irradiation treatment on somatic cell cultures in rice (*Oryza sativa* L.). *Cereal Res. Commun.* 19: 201-208

Mishra, B. 2004. Exploring new opportunities. *Survey of Indian Agriculture 2004* (ed. Ravi, N.). National Press, Chennai, pp. 29-32

Mittal, R. K., Gautam, A.S., Parwar, K.A. and Jungpo, B. 2000. Synergistic effects of combined treatments of gamma irradiation and Ethyl Methane Sulphonate in buckwheat (*Fagopyrum tataricum*). *Indian. J. agric. Res.* 34: 191-193

Moh, C.C. and Han, J.J. 1973. Methods of inducing muations in Cassava and possible uses of mutant. *Induced Mutation in Vegetatively Propagated Plants.* International Atomic Energy Agency, Vienna, 89 p.

Murashige, T. 1974. Plant propagation through tissue culture. *Ann. Rev. Plant Physiol.* 25:135-166

Murashige, T. 1982. Rgeneration of Plants. *California Agric.* 36: 19-20

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Pl.* 15: 473-497

Nagatomi, S., Ujihara, K., Sugimoto, A. and Maeda, H. 1996. Selection of mutants resistant to rust disease in sugarcane induced through gamma irradiation on *in vitro* culture. *Tech. News Inst. Radiat. Breed.* 2: 54 - 57

Navraj, K., Gill, M.S., Raman, G., Bharaj, T.S., Gosal, S.S. and Kaur, N. 1999. Factors enhancing somatic embryogenesis and high frequency plant regeneration in rice. *Crop Improv.* 26: 23-27

Nguyen, T.T., Santangelo, E., Donini, B. and Remotti, P.C. 1991. Avoidance of chimerism from irradiating rice seeds (*Oryza glaberrima*) through *in vitro* culture. *Mutat. Breed. Newsl.* 37:15-16

Nishi, T., Yamada, Y. and Takahashi, E. 1973. The role of auxin in differentiation of rice tissue cultured *in vitro*. *Bot. Mag.* 86: 183-188

Nizar, A.M., Muralitharan, V.K., Joseph, J.K and Karuppaiyan, A. 2002. Collecting indigenous rice germplasm from Kerala. National Symposium on Priorities and Strategies for Rice Research in High Rainfall Tropics, 11-12 October 2002. Regional Agricultural Research Station, Pattambi, Kerala. *Abstract*: 41

Novak, F.I., Afza, R., Duren, M. and Omar, M.S. 1990. Mutation induction by gamma irradiation of *in vitro* cultured shoot tips of banana and plantain (*Musa* sp.). *Trop. Agric.* 67: 21-28

Novak, F.I. 1991. *In vitro* mutation system for crop improvement. *Plant Mutation Breeding for Crop Improvement*. International Atomic Energy Agency, Vienna, 365 p.

Oinam, G.S. and Kothari, S.L. 1993.Genotypic differences in embryogenic callus formation and plant regeneration in *indica* rice. *Int. Rice Res. Notes* 18: 9-10

Ojomo, O.A., Omue, O., Raji, J.A. and Omueti, O. 1979. Studies in induced mutations in Cowpea. The variation in protein content following ionizing radiation. *Nigerian J. Agric. Sci.* 13: 225-23

Oono, K. 1987. Somatic mutation in rice tissue culture. *Proceedings of a Seminar on Cell and Tissue Culture in Field Crop Improvement, October 4-9, 1987* (eds. Bay and Petersen, J.). National Institute of Agrobiological Resources, Tsukuba, Japan, pp. 108-112

Pandey, S.K., Ramesh, B. and Gupta, P.K. 1994. Study on effect of genotype and culture medium on callus formation and plant regeneration in rice (*Oryza sativa* L.). *Indian J. Genet.* 54: 293 - 299

Pathirana, R., Wijithawarna, W.A., Jagoda, K. and Ranawaka, A.L 2002. Selection of rice for iron toxicity tolerance through irradiated caryopsis. *Pl. Cell Tissue Organ Cult.* 70: 83 - 90

Prediere, S. 2001. Mutation induction and tissue culture in improving fruits. *Pl. Cell Tissue Organ Cult.* 64:185 - 210

Poonsaparaya, P., Murray, W., Kerri, W. and Vajrabhaya, M. 1989. *Pl. Cell Tissue Organ Cult.* 16:175 -186

Pontongkam, P., Ratisoontorn, P., Suputitada, S., Peyachoknagul, S., Ngernsiri, L., Silayoi, B., Rodrangboon, P., Klakhaeng, K.J. and Shenghaphan, R. 1994. Induced mutation in rice by tissue culture. *Kasetsart J. Natural Sci.* 28: 381 - 389

Punitha, M. 1999. Effect of mutation on *in vitro* studies of rice. *J. Ecotoxicol. Environ. Monitoring* 9: 273 - 275

Punitha, M. and Kalamani, A. 1999. *In vitro* studies of gamma irradiated rice. *Rice Biotech. Q.* 40: 20 - 25

Rangaswamy, S.R.S. 1993. Comparison of fertility between the F_1, F_2 and anther derived lines in the crosses of *indica/japonica* and *japonica/ indica* in *Oryza sativa* L. *Euphytica* 66: 19 - 25

Ratisoontorn, P., Pongtongkam, P., Suputtitada, S., Peyachoknakul, S. and Ngernsiri, L.1993. Tissue culture in rice. *Kasetsart J. Natural Sci.* 27: 278 - 285

Ratisoontorn, P., Pongtongkam, P., Suputtitada, S., Peyachoknagul, S., Ngernsiri, L., Apisittivanich, S., Silayoi, B., Rodrangboon, P., Klakhaeng, K. and Senghaphan, R. 1995. Induced mutation in rice by tissue culture. *Kasetsart J. Natural Sci.* 29: 229-298

Reddy, T.P., Padma, A. and Reddy, G.M. 1975. Short culm mutations induced in rice. *Indian. J. Genet.* 35: 31 - 37

Revathy, N., Muthusamy, M., Seetharaman, K., Yesuraja, I., Rajeswari, E. and Ramanathan, A. 2000. Effect of BAP on regeneration of IR50 and Co 43 genotypes. *J. Ecol.* 3: 79 - 81

Rosamma, C.A., Elsy, C.R., Balachandran, P.V. and Francies, R.M. 2003. *Pattambi Rice Varieties*. Kerala Agricultural University, Thrissur, 69 p.

Sakila, M., Ibrahim, S.M. and Kalamani, A. 1999. *In vitro* mutagenesis in scented rice cultivars. *Rice Biotech. Q.* 38: 30 - 34

Scossiroli, R. E. 1970. Selection experiments in a population of *Triticum durum* and *Triticum vulgare*. Effects of ionization of seeds. *Radiat. Bot.* 5: 443 - 450

Seraj, Z.I., Islam, Z., Faruque, M.O., Devi, T and Ahmed, S. 1997. Identification of the regeneration potential of embryo derived calluses from various *indica* rice varieties. *Pl. Cell Tissue Organ Cult.* 48: 9 - 13

Sharma, B.K. and Baksh, P. 2004. Induced mutations – New dimensions in gene technology. *Agrobios* 3: 5 - 7

Sharma, J.P., Mukherjee, B.B. and Gupta, S. 1996. Callus induction and plant regeneration from immature embryos of *indica* rice varieties. *Oryza* 36: 32-34

Sigurbjornsson, B. 1991. Use of induced mutations in connection with haploids and heterosis in cereals. *Cereal Res. Commun.* 19: 7 - 8

Singh, D., Ravi, B. and Minocha, J.L. 1993. Efficiency of different explants and media for callus induction and plant regeneration in basmati rice. *Crop Improv.* 20: 139 - 142

Singh, K.P., Singh, B., Raghava, S.P.S. and Kalia, C.S. 2000. Induced flower colour mutations in carnation through *in vitro* application of chemical mutagen. *Indian J. Genet.* 60: 535 - 539

Singh, K.P., Singh, B., Raghava, S.P.S., Misra, R.L. and Kalia, C.S. 1999. *In vitro* induction of mutation in carnation through gamma irradiation. *J. Ornamental Hort.* 2: 107 - 110

*Singh, S. and Singh, H.P. 1996. Somatic cell culture of rice: II. Dose effect of 2,4-D and *in vitro* organ forming potential in different ecotypes of *indica* rice (*Oryza sativa* L.). *Biol. Zbl.* (German) 115: 300 - 306

Skirvin, N. 1978. Natural and induced variation. *Euphytica* 27:241- 266

Sobhana, A. and Rajeevan, P.K. 2003. Effect of ^{60}Co gamma irradiation on seedling development of dendrobium. *J. Ornamental hort.* 6: 39 - 41

Sripichitt, P., Tangman, P. and Saengkaewsook, W. 1991. Effects of gamma radiation on *in vitro* multiple shoot formation of aromatic rice seeds (*Oryza sativa* L.). *Kasetsart J. Natural Sci.* 25: 146-152

Sticklen, M.B., Pumpho, M.G. and Kennedy, R.A. 1987. Somatic embryogenesis in rice seedlings. *Physiol. Pl.* 30: 75-80

State Planning Board. 1998. *Economic Review*. State Planning Board, Thiruvananthapuram, 267 p.

Sumathi, R. 1992. Studies on callus organisation and *in vitro* organogenesis in rice (*Oryza sativa* L.). M.Sc. thesis, Tamil Nadu Agricultural University, Coimbatore, 145 p.

Suputtitada, S., Adachi, T., Pongtongkam, P., Ratisoontorn, P., Peyachoknagul, S., Apisitwanich, S., Klakhaeng, K., Rodrangboon, P., Lertvichai, R. and Kreft, I. 1994. Rice improvement through tissue culture. *Proceedings of the International Colloquium on Impact of Plant Biotechnology on Agriculture, December 5-7, 1994* (eds. Javornik, B. and Bohanec, B.). Centre for Plant Biotechnology and Breeding, Rogla, Slovenia, pp. 73-84

Tan, G.X., Wu, S.G. and Shu, L.H. 1999. *In vitro* culture of different explants in wild rice. *Pl. Physiol. Commun.* 35: 96-100

*Thomas, E., King, P.J. and Potrykus, I. 1979. Improvement of crop plants via single cells *in vitro* – An assessment. *Z. Pflanzenzucht* (French) 82: 1 - 30

Ukai, Y. 1988. Effect of gamma-ray irradiation on growth of calli derived from mature embryos or seeds in barley. *Jpn. J. Breed.* 38: 292 - 300

Vagera, P., Novak, F. J. and Vysko, B. 1976. Anther cultures of *Nicotiana tabacum* L. mutants. *Theor. Appl. Genet.* 47: 109 - 114

Vajrabhaya, T. 1977. Variations in clonal propagation. *Orchid Biology-Reviews and Perspectives-1* (ed. Arditti, J.). Cornell University Press, Ithaca, London, pp. 180 - 193

Vinothini, S. 2004. Enhancement of variability in drought tolerant varieties of rice (*Oryza sativa* L.). through *in vitro* mutagenesis. M.Sc. thesis, Tamil Nadu Agricultural University, Coimbatore, 155 p.

Virk, G.S., Kaur, R., Grover, I.S. and Thukral, A. K .1998. *In vitro* plantlet regeneration from immature embryo and cotyledon derived cultures of *Oryza sativa* L. var. Pakistani Basmati. *J. Environ. Biol.* 15: 275-281

Visarada, K.B.R.S. and Sarma, N.P. 2002. Qualitative assessment of tissue culture parameters useful in transformation of *indica* rice. *Curr. Sci.* 82: 343-347

Wang, Z.B., Pan, X.B., Tang, K.X. and Sun, Z.X. 2001. Studies on tissue culture of *indica* rice varieties. *J. Yangzhou Univ. Natural Sci.* 4: 37-41

*Wu, L.Y., Li, S.H. and Han, G.M. 1991. *In vitro* culture of young embryos of rice. *Nippon. Nogeik. Kaishi.* (Japanese) 6: 13-14

Xie, J., Gao, M., Cai, Q., Cheng, X., Shen, Y. and Liang, Z. 1995. Improved isolated microspore culture efficiency in medium with maltose and optimized growth regulator combination in *japonica* rice (*Oryza sativa* L.). *Pl. Cell Tissue Organ Cult.* 42: 245-250

Yin, Z., Zhao, K., Zheng, X., Qi, Z. and Fu, Y. 1983. Somaclonal genetics of rice *Oryza sativa* L. *Theor. Appl. Genet.* 67: 67-73

Zafar, Y., Wajid, A., Malik, K.A. and Gamborg, O.L. 1992. Establishment of regenerating calli and cell suspension line of basmati rice (*Oryza sativa* L). cv. B.370). *Pakist. J. Bot.* 24: 64 - 71

Zapata, F.J. and Aldemita, R. R. 1989. Induction of salt tolerance in high yielding rice, varieties through mutagenesis and anther culture. *Advances in Agricultural Biotechnology* (ed. Maluszynski, M.). Kluwer Academic Publishers, Dordrecht, pp. 193-202

Zheng, H.R., Ye, Z.W., Su, M.S. and Jin, Y.F. 2004. Induction of mutants in jujube *in vitro* culture by ^{60}Co gamma ray irradiation. *Acta Agric. Scand.* (Chinese) 20: 13-18

Zhenhua, Z. 1992. Anther culture for rice Breeding at SAAS. *Anther Culture for Rice Breeders* (eds. Kangle, Z. and Murashige, T.). Zhejiang Academy of Agricultural Sciences, Hangzhou, China, pp. 38-74

Zhu, Z.W., Sun, L.H. and Tang, B.G. 1988. Effect of gamma rays on resistance of rice calli to bacterial leaf blight and their differentiation. *J. agric. Sci.* 4: 46-51

* Originals not seen

IN VITRO MUTAGENESIS IN RICE
(*Oryza sativa* L.)

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ABSTRACT OF THE THESIS

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ABSTRACT

An investigation entitled "*In vitro* mutagenesis in rice (*Oryza sativa* L.)" was undertaken at the Department of Plant Breeding and Genetics, College of Horticulture, Kerala Agricultural University, Vellanikkara for estimating LD_{50} values and optimum doses for gamma rays and EMS and identifying desirable plants.

Mature, dehulled seeds of variety Ptb-26 were raised in MS medium with different combinations of 2,4-D and Kn. Callus induction studies revealed that MS + 2,4-D 2.0 mgl^{-1} + Kn 0.5 mgl^{-1} was the best to induce callus in rice. The same media was used for callus proliferation. Three weeks old subcultured calli was transferred to regeneration MS + NAA 2 mgl^{-1} + Kn 4 mgl^{-1} + BAP 0.5 mgl^{-1} and the shoots obtained were transferred to the rooting medium MS + NAA 2mgl^{-1} + Kn 0.5 mgl^{-1}. The percentage of success of hardening obtained was 85.20%.

Different doses (10Gy, 20Gy, 30Gy, 40Gy, 50Gy and 60Gy) of gamma rays were given to three weeks old subcultured calli and the LD_{50} value was fixed based on the regeneration percentage. 30Gy was fixed as the LD_{50} value. Six concentrations of EMS (2mM, 4mM, 6mM, 8mM, 10mM and 12mM) were tried to ascertain the LD_{50} value based on the regeneration percentage. 4mM was the LD_{50} value obtained for EMS.

Two optimum doses each for the two mutagens below the LD_{50} value was given to obtain the desirable plants. Ten tissue culture plants for 10Gy, nine for 20Gy, nine for 2mM and eight for 4mM were obtained from this study. They were analysed for biometrical and morphological characters. Based on which the best optimum doses obtained were 20Gy and 4mM for the two mutagens respectively. Significant effect of treatments of gamma rays and concentrations of EMS on genotype was noticed for culm length, plant height, panicles $plant^{-1}$, panicle length, grains $panicle^{-1}$ and grain yield $plant^{-1}$. Significant differences were noticed for leaf blade pubescence, leaf blade colour, basal leaf sheath colour and lemma and palea colour. Six tissue culture variants were identified as desirable based on higher panicles $plant^{-1}$, panicle length, grains $panicle^{-1}$ and grain yield $plant^{-1}$.