“ANTHER CULTURE STUDIES IN INDICA RICE (*Oryza sativa* L.)”

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ABSTRACT

The present study, undertaken at Plant Tissue Culture Laboratory, Department of Agricultural Biotechnology, B.A. College of Agriculture, Anand Agricultural University, Anand during 2005-2008, describes anther culture studies using anthers of two parental lines and their heterotic hybrid *viz*. IR-58025 (B-line), Gujarat- 7 (GR-7) and IR-58025 A x GR-7 (F_1) of rice (*Oryza sativa* L.), an important cereal crop to develop a consistent regeneration system for the production of haploid/doubled haploid plantlets.

The characters studied were percentage contamination, days to callus initiation, percentage callusing, texture and colour of callus, days to plantlet/shoot primordia initiation, per cent calli differentiation, percentage green plantlets regeneration, percentage albino plantlets regeneration, percentage rooting, percentage haploid plantlets, percentage survival of anther culture raised plantlets during hardening and morphological comparison between haploid and diploid/doubled haploid plants.

The results showed that high proportions of microspores at uninucleate stage were observed when the distance between the base

of the flag leaf and the auricle of the penultimate leaf was 5-9 cm. Panicle of main tiller with anther development/blooming in basipetal fashion reached 1/2 - 1/3 of spikelets length was found as morphological indicator for selection of panicle for anther culture studies.

Among the various treatments for surface sterilization, 70 % Ethyl alcohol for 10 seconds to panicle + 0.1 % $HgCl_2$ for 5 minutes to spikelets was found best with significantly reduced the contamination.

Twelve days cold pretreatment at 7 ± 1 °C was found effective to alter gametophytic pathway of anther to sporophytic pathway for better callus induction.

Among the two basal media studied *viz.,* N_6 and MS-1, N_6 (semisolid) medium was found significantly effective for callus induction in all cultivars. However, medium MS (1962) was found superior for calli differentiation and plantlet regeneration.

The N_6 medium used as basal media for optimization of cultural environment with a total of 12525 anthers from two parents and their heterotic hybrid. Auxin: cytokinin and carbon source concentration, genotype specific media sequence found important to obtain highest number of anther derived haploid plants.

Effect of auxin: cytokinin (2,4-D/NAA, Kn) and carbon source (Sucrose) at different concentrations showed that the higher concentration of 2,4-D (3.0 mg l^{-1}) with lower concentration of Kn (0.5 mg l^{-1}) combined with higher sucrose concentration (7.0 %) was a major determinant of response for callus induction. However, the rate of increase was genotype dependent. Per cent callusing was significantly increased in the F_1 (IR-58025 A x GR-7) as compared to its parents.

The plant regenerating capabilities of the calli increased slightly between 50-60 days of incubation (i.e. 60-80 days old calli) of the anthers, and thereafter decreased gradually. The calli differentiation was 35.41, 25.0

per cent for cv. IR-58025 and GR-7 respectively as compared to their hybrid (F_1) (27.08 %) for 50-60 days of incubation.

Among the eight media, response for green plantlet regeneration was remarkably higher when calli were sub cultured/transferred on to MS medium supplemented with 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar. However, differences in green plantlet regeneration were found genotype dependent. Per cent green plantlet regeneration was 38.53 and 15.27 % for the cv. IR-58025 and GR-7 respectively as compared to their hybrid (F_1) (18.05 %).

Among the eight media studied, MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar was found superior for percentage of haploid plantlet regeneration as compared to other combinations of growth regulator and sucrose. The average per cent haploid plants produced using different regeneration media were 29.06 and 22.21 % for the genotype IR-58025, GR-7 respectively as compared to their hybrid (F_1) (30.20 %)

Among the various callus induction – shoot regeneration media sequences studied, N_6 + 3.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 7 % sucrose + 0.9 % agar as callus induction medium followed by transfer on MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA +1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar as shoot regeneration medium was found better for production of maximum number of green haploid plantlets among all genotype.

Maximum percentage rooting (100.0 %) was observed on medium White's + 2.0 % sucrose + 0.9 % agar in all three cultivars.

Acclimatization of anther culture raised plantlets showed the superiority of liquid nutrient followed by soil: soil rite (3:1) as a potting mixture.

Morphological study of haploid plants revealed lowest stomatal area (188.56 μm^2) while diploid showed the highest one (390.02 μm^2).

The stomatal density for haploid plants was 40.90 and diploid showed 30.09 stomata per field of view (124929 μm^2) at 40 x. The anther derived haploid plants showed morphological differences in their plant height(43 cm), flag leaf area (3.90 mm^2), panicle length (28 cm), spikelets length (7.58 mm), spikelets width (2.07 mm) as compared to anther derived diploid/doubled haploid plants.

Overall, cultural modifications can greatly improve anther culture response but improvements hinge on the responsiveness of the genotypes. Where the germplasm is clearly responsive, anther culture has great potential. The implications of these results are considered in the context of the application of anther culture technique to rice research and breeding.

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CONTENTS

CHAPTER	PARTICULARS	PAGE NO.
I	**INTRODUCTION**	1
II	**REVIEW OF LITERATURE**	
	2.1 EFFECT OF PHYSIOLOGICAL STATE OF THE DONOR PLANTS	8
	2.2 EFFECT OF DEVELOPMENTAL STAGE OF THE MICROSPORES	10
	2.3 EFFECT OF PRETREATMENT	11
	2.3.1 COLD TREATMENT	11
	2.3.2 HEAT TREATMENT	12
	2.4 SURFACE STERILIZATION	13
	2.5 EFFECT OF GENOTYPES ON INDUCTION OF CALLUS AND REGENERATION OF PLANTLETS	15
	2.6 INFLUENCE OF POSITION OF ANTHERS AT PLANTING ON CALLUSING AND PLANT REGENERATION	17
	2.7 EFFECT OF CULTURE MEDIUM ON CALLUS INDUCTION AND PLANT REGENERATION	18
	2.8 EFFECT OF CARBON SOURCE ON CALLUS INDUCTION AND PLANT REGENERATION	20
	2.9 EFFECT OF GROWTH REGULATORS ON CALLUS INDUCTION AND PLANT REGENERATION	21
	2.10 EFFECT OF ORGANIC SUBSTANCES	25
	2.11 EFFECT OF PHYSICAL STATE OF MEDIUM	26
	2.12 EFFECT OF CULTURE CONDITIONS	26
	2.13 DIFFERENTIATION OF CALLUS	27
	2.14 ALBINISM	27
	2.15 *IN VITRO* ROOTING	30
	2.16 PLOIDY LEVEL OF ANTHER DERIVED PLANTS	30
	2.17 ACCLIMATIZATION (HARDENING)	34

III	**MATERIALS AND METHODS**	
	3.1 EXPERIMENTAL MATERIAL	43
	3.2 CULTURE MEDIA	44
	3.2.1 CHEMICALS	44
	3.2.2 CULTURE VESSELS	44
	3.3 PREPARATION OF MEDIA	44
	3.4 STERILIZATION OF MEDIA AND CULTURE VESSELS	47
	3.5 PREPARATIONS FOR INOCULATION	47
	3.6 PREPARATION OF EXPLANT	47
	3.7 EXPERIMENTS	49
	3.7.1 PRE-TREATMENT OF ANTHERS	49
	3.7.2 STANDARDIZATION OF SURFACE STERILIZATION TECHNIQUE	49
	3.7.3 MORPHOGENETIC STUDY	49
	3.7.3.1 INDUCTION OF CALLUS	49
	3.7.4 OPTIMIZATION OF CULTURAL ENVIRONMENT	50
	3.8 *IN VITRO* ROOTING	59
	3.9 PLOIDY CONFIRMATION	60
	3.9.1 CYTOLOGICAL METHODS	60
	3.9.2 PLOIDY ANALYZER	61
	3.9.3 ANATOMICAL METHOD	62
	3.10 ACCLIMATIZATION	63
	3.11 OBSERVATIONS RECORDED DURING INVESTIGATION	64
	3.12 STATISTICAL ANALYSIS	66
IV	**RESULTS AND DISCUSSION**	
	4.1 APPROPRIATE STAGE (CYTOLOGICAL AND MORPHOLOGICAL) OF ANTHER FOR *IN VITRO* STUDIES	69
	4.2 SURFACE STERILIZATION OF PANICLES AND SPIKELETS	70
	4.3 COLD PRE-TREATMENT	72
	4.4 MEDIA RESPONSE FOR *IN VITRO* CALLUSING	75
	4.5 OPTIMIZATION OF CULTURAL ENVIRONMENT	99
	4.6 *IN VITRO* ROOTING	136
	4.7 CYTOLOGICAL AND ANATOMICAL STUDY OF ANTHER DERIVED PLANTLETS	138
	4.8 ACCLIMATIZATION	153
V	**SUMMARY AND CONCLUSION**	159 -171
	BIBLIOGRAPHY	i-xxxv

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
3.3.1	Composition of plant tissue culture media used as basal medium.....	46
3.12.1(A)	Structure of ANOVA for individual equal CRD.....	66
3.12.1(B)	Structure of ANOVA for individual unequal CRD.....	66
3.12.2	Structure of ANOVA for 2 factor CRD.....	66
3.12.3	Structure of ANOVA for 3 factor CRD.....	67
4.2.1	Analysis of variance for effect of genotypes (G) and surface sterilization treatments (S) on per cent contamination.....	70
4.2.2	Effect of genotypes (G) and surface sterilization treatments (S) on per cent contamination.....	71
4.3.1	Analysis of variance for effect of genotypes (G) and cold pre-treatments (C) on per cent callusing.....	72
4.3.2	Effect of genotypes (G) and cold pre-treatments (C) on per cent callusing.....	73
4.3.3	Effect of genotypes (G) and cold pre-treatments (C) on days to callus initiation.....	74
4.4.1	Analysis of variance for effect of genotype (G), different basal media (M), Treatments (T) and their interaction *viz.*, G x M, G x T, M x T and G x M x T on per cent callusing.....	76
4.4.2	Effect of genotype (G), different basal media (M), Treatments (T) and their interaction *viz.*, G x M, G x T, M x T and G x M x T on per cent callusing.....	76-78

4.4.3	Effect of genotype (G), different basal media (M), Treatments (T) on days to callus initiation.....	80
4.4.4	Morphogenic effect of various concentration of auxin (2,4-D) and Sucrose added in N_6 and MS-1 medium on anthers of rice cv. IR-58025, GR-7.....	82
4.4.5	Analysis of variance for effect of genotype on per cent callusing and days to callus initiation.....	83
4.4.5.1	Effect of genotypes on per cent callusing and days to callus initiation.....	83
4.4.6	Effect of various basal media and genotypes on calli Differentiation.....	85
4.4.7	Effect of genotype and basal media on per cent plantlet (green and albino) regeneration and days to shoot primordia or plantlet initiation (green and albino).....	89
4.4.8	Effect of genotype, shoot induction basal media and rooting media treatments on percentage rooting.....	93
4.4.9	Effects of genotypes and basal media (Shoot induction) on haploid plantlet regeneration in rice anther culture cv. IR-58025 and GR-7.....	96
4.4.10	Effect of genotype and different potting mixtures (T) on per cent survival of anther culture raised plantlets in rice cv.IR-58025(B-line) and GR-7.....	98
4.4.11	Per cent survival of the anther derived green plantlets of rice cv. IR-58025 (B-line) and GR-7.....	98
4.5.1	Analysis of variance for effect of genotypes (G) and callus induction media (CI) on per cent callusing.....	99
4.5.2	Effect of genotype and callus induction media on per cent callusing from anthers of rice cv. IR-58025, GR-7 and their hybrid (F_1 - GRH 1)	101
4.5.3	Effect of genotype and callus induction media on per cent callusing of three indica rice genotypes	105

	(Duncan's New Multiple Range Test based on an analysis of all three genotypes over all media treatments at 5 % level of significance cv. IR-58025, Hybrid (F_1- GRH-1), GR-7 and average for all three genotypes)	
4.5.4	Analysis of variance for effect of callus induction media (CI_1-CI_{15}) on days to callus initiation for anthers of rice cv. IR-58025, GR-7 and their hybrid (F_1)	106
4.5.5	Effect of callus induction media treatment (CI_1-CI_{15}) on days to callus initiation for anthers of rice cv. IR-58025, GR-7 and their Hybrid (F_1)	107
4.5.6	Effect of different callus induction media on callus morphology in rice cv. IR-58025, GR-7 and their Hybrid (F_1)..	110
4.5.7	Effect of genotype and shoot/plantlet regeneration media *viz*., MS with combination of growth regulators and sucrose on per cent green and albino plantlet regeneration and days to green and albino plantlet regeneration.....	127
4.5.8	Analysis of variance for effect of shoot/plantlet regeneration media treatments (R_1-R_8) on days to green plantlet regeneration of rice cv. IR-58025, GR-7 and their hybrid (F_1).	128
4.5.9	Effect of shoot/plantlet regeneration media treatments (R_1-R_8) on days to green plantlet regeneration of rice cv. IR-58025, GR-7 and their hybrid (F_1)	129
4.6.1	Effect of genotype and shoot induction media treatments on percentage rooting.....	137
4.7.1	Effect of genotypes and shoot/plantlet regeneration media *viz*. MS fortified with growth regulators and sucrose on per cent haploid and diploid/doubled haploid green plantlets in rice cv. IR-58025, GR-7 and their Hybrid (F_1).....	139
4.8.1	Effect of genotypes and different potting mixtures (T) on per cent survival of anther culture raised plantlets in rice cv. IR-58025(B-line), GR-7 and their Hybrid (F_1)	156

	.....	
4.8.2	Per cent survival of the anther derived green plantlets of rice cv. IR-58025, GR-7 and their Hybrid (F_1)	156
4.9.1	Morphological differences between anther culture derived haploid and diploid/doubled haploid plants.....	157

LIST OF PLATES

PLATE NO.	TITLE	AFTER PAGE
I	A. Healthy, green rice plants grown in green house B. Appropriate stage of panicle for boots collection and spikelets for anther inoculation C. Mid-uninucleate stage of pollen/microspore development D. Bi-nucleate microspore E. Anthers (7 days after inoculation) F. Anthers colour change from pale yellowish to brownish after 15 days of inoculation (Stereomicroscopic view)	48
II	A. Emergence of callus B. Proliferation of callus C. Stereomicroscopic view of emerged callus D. A responding anther showing callus emergence through a split anther lobe, 35 days after plating on medium N_6 (1978) (Stereomicroscopic view) E. Green spots first appeared on the callus surface; transferred on to regeneration medium MS (1962) F. Close up of a portion of tube, showing green shoot primordia G. Green shoot formation (Close up view) H. Rhizogenesis in cv. GR-7 callus	87
III	A. Tubes showing high frequency regeneration of green plantlets, 35 days after transfer of calli from medium N_6 to MS B. Variation in height among the regenerated green plantlets C-D. Regenerated green and albino shoots from anther derived calli; subcultured on MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 3 % Sucrose (w/v) + 0.9 % Agar E. *In vitro* rooting on white's medium (1963) + 2 % Sucrose + 0.9 % Agar F. Plantlets under hardening in a 3:1 (Soil:Soilrite) potting mixture	93

	G. Plantlets under hardening in a liquid nutrient medium (White's medium excluding vitamins)	
IV	A. The spikes of haploid and diploid/doubled haploid plant regenerated from anther culture B. The spikelet of haploid and diploid/doubled haploid plant regenerated from anther culture, showing variation in size C-D. Chromosomes of haploid (n=12) and diploid/doubled haploid (2n= 24) derived from anther culture (x 100) E-F. Stomata of haploid and diploid/doubled haploid plant showing variation in stomatal area (x 40)	146
V	A. Anther culture derived plants at hardening B. Close up view of photograph A; showing flowering initiation C. Hardened diploid/doubled haploid plant derived from anther culture D. Hardened haploid and diploid/doubled haploid plant; showing variation in height	158

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE NO.
1	Influence of genotypes (G) and basal media (M) on calli differentiation ability in rice……..	86
2	Effect of callus age on differentiation…….	111
3	Graph showing effect of callus induction media on callus induction and calli differentiation in the rice cv. IR-58025 (B-line)……	113
4	Graph showing effect of callus induction media on callus induction and calli differentiation in the rice cv. IR-58025 A x GR-7 (F_1) ……..	113
5	Graph showing effect of callus induction media on callus induction and calli differentiation in the rice cv. GR-7……	114
6	Graph showing cumulative effect of callus induction media and genotypes on callus induction and calli differentiation (Bar represents per cent callus induction and line represents per cent calli differentiation) ……	115
7	Graph showing average effect of callus induction media on callus induction and calli differentiation among all genotypes……	115
8	Graph showing average effect of genotype on callus induction and calli differentiation among all callus induction media……	116
9	Graph showing effect of shoot regeneration media on calli differentiation and green plantlet regeneration in the rice cv. IR-58025 (B-line) ……	121
10	Graph showing effect of shoot regeneration media on calli differentiation and green plantlet regeneration in the rice cv. IR-58025 A x GR-7 (F_1) ……..	121
11	Graph showing effect of shoot regeneration media on calli differentiation and green plantlet regeneration in	122

	the rice cv. GR-7……..	
12	Graph showing cumulative effect of genotypes and shoot regeneration media on calli differentiation and green plantlet regeneration (Bar represents per cent calli differentiation and line represents per cent green plantlet regeneration) …….	123
13	Graph showing average effect of shoot regeneration media on calli differentiation and green plantlet regeneration among all genotypes……..	123
14	Graph showing average effect of genotypes on calli differentiation and green plantlet regeneration among all shoot regeneration media……..	124
15	Graph showing effect of shoot regeneration media on calli differentiation and albino plantlet regeneration in the rice cv. IR-58025 (B-line) ……..	131
16	Graph showing effect of shoot regeneration media on calli differentiation and albino plantlet regeneration in the rice cv. IR-58025 A x GR-7(F_1) ……..	131
17	Graph showing effect of shoot regeneration media on calli differentiation and albino plantlet regeneration in the rice cv. GR-7……..	132
18	Graph showing cumulative effect of genotypes and shoot regeneration media on calli differentiation and albino plantlet regeneration (Bar represents per cent calli differentiation and line represents per cent albino plantlet regeneration) …….	133
19	Graph showing average effect of shoot regeneration media on calli differentiation and albino plantlet regeneration among all genotypes……..	133
20	Graph showing average effect of genotypes on calli differentiation and albino plantlet regeneration among all shoot regeneration media……..	134
21	Histogram of calibration peaks using diploid mother plant as standard showing single peak at channel 400 in ploidy analyzer……..	143
22	Histogram showing two peaks confirming haploid	144

	nature of the tested sample in comparison to diploid standard in ploidy analyzer.....	
23	Histogram showing single peak at channel 400 confirming the same ploidy level of the tested sample and the standard diploid mother in ploidy analyzer.....	145
24	Effect of callus induction (CI) - shoot regeneration (R) media sequence on regeneration of green haploid plantlet per regenerating callus in rice anther culture cv. IR-58025 (B-line)	148
25	Effect of callus induction (CI) - shoot regeneration (R) media sequence on regeneration of green haploid plantlet per regenerating callus in rice anther culture cv. GR-7.....	149
26	Effect of callus induction (CI) - shoot regeneration (R) media sequence on regeneration of green haploid plantlet per regenerating callus in rice anther culture cv. IR-58025 A x GR-7 (F_1-Hybrid)	149

LIST OF ABBREVIATIONS

ABA	Abscisic Acid
BA or BAP	6-benzyladenine or 6-benzyl aminopurine
Anon.	Anonymous
CD	Critical difference
CH	Casein hydrolysate
CI	Callus Induction
cm	Centimetre
CMS	Cytoplasmic male sterile
cv.	Cultivar
C.V.	Coefficient of variation
CW	Coconut water
°C	Degree Celsius
2,4-D	2,4-Dichloro phenoxy acetic acid
et al.	and others
etc.	Et cetera
F_1	Hybrid
g	Gram
GA_3	Gibberellic acid
GR-3	Gujarat-3
GR-7	Gujarat- 7
GRH-1	Gujarat rice hybrid-1
hr./h.	Hour
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid
i.e.	That is
kg	Kilogram
Kg/ha	Kilogram per hectare
Kn	Kinetin
Lbs	Pound (Pressure unit)
LH	Lactalbumin hydrolysate
LS	Linsmaier and Skoog
MET	Multi effect trizole
mg	Milligram
mgl^{-1}	Milligram per litre
mm	Milli meter
mM	Milli molar
μM	Micro molar
μ	Micron
MS	Murashige and Skoog (1962) medium
MS-1	Modified Murashige and Skoog (1962) medium
MS-2	Modified Murashige and Skoog (1962) medium
NAA	α-Naphthalene acetic acid
2iP	6-(γ, γ – dimethylallylamino) Purine
No.	Number

N & N	Nitsch & Nitsch (1969) medium
NS	Non significant
%	Per cent
#	Number
N_6 medium	Chu *et al*. (1978) medium
PA-II	Ploidy analyzer –II
ppm	Parts per million
RN1	Region 1
S.Em.±	Standard error of mean
UV	Ultra violet
Var.	Variety
viz.,	Namely
v/v	Volume/volume
White’s (WH)	White’s (1962) medium
w/v	Weight/volume
YE	Yeast extract
Zea	Zeatin

I. INTRODUCTION

Rice (*Oryza sativa* L.) is a worldwide crop for staple food as well as a model for cereal biology (Ronald and Leung, 2002; Shimamoto and Kyozuka, 2002).

Rice is the second largest cereal crop after wheat. It belongs to the genus *Oryza*, subtribe *Oryzineae* of the family *Poaceae (Grameneae).*

The genus *Oryza* includes 24 species of which 22 are wild and two, *viz.*, *O. sativa* L., the Asian rice and *O. glaberrima* Steaud., the African rice, are cultivated. Both of these species are diploid (2n=2x=24) in chromosome number. *Oryza sativa* L. has three ecogeographical races *viz*., *indica, japonica* and *javanica* (bulu). (Gosal *et al*., 1997)

Different genotypes of rice are adapted to a wide range of environmental conditions from tropical flooding to temperate dry land, making it an excellent example for real-life, adaptive response.

Rice contributing 55 per cent to the global cereal production is the staple food for more than 65 per cent of our population and source of livelihood for 100 million rural households. About 90 per cent of the world's rice is produced and consumed in Asia, where 60 per cent of the population and about two-third of the worlds poor live. It produces more calories and carbohydrates per hectare than any other cereal (Lu and Chang, 1980).

Rice is the most important crop, grown all over the country, in different ecological niches with a wide range of cultivars. It occupies

first rank in area and second rank in production and consumption next to China.

In India, it accounts for about 65.0 % of the total cropped area under food grains. It is grown in an area of 44.97 million hectare with a total production of 128.44 million tones and productivity being over 2.0 t/ha.

(Rai, 2006).

In Gujarat, rice occupies about 5.0 % of gross cropped area of the state and about 14.0 % of total food grain production. It is grown on 0.66 million hectares of land with total production of about 1.03 million tones,

the productivity being 1549 kg/ha. (Anon., 2006).

Rice is one of the most intensively investigated crop plants. The accumulated knowledge base has been of considerable value in elevating rice productivity and, in turn, in the betterment of millions of people who depend on it as a source of food and income.

Significant progress by breeders has been made in the past with wheat, rice and other cereals species towards higher yield, improved quality, greater disease resistance and other improved characteristics of agricultural importance. But now, and even more in the future, further efforts will be needed to provide the food necessary for a steadily growing world population. The goals of plant breeding in the less developed countries are still yield improvement and stability. (Lorz *et al*., 1988)

As soon as the three lines (A or sterile, B or maintainer and R or restorer line) had been successfully developed through breeding, hybrid

rice was used for production in China. (Deyao and Xigan, 1990). Development and use of hybrid rice technology is a potential alternative approach for enhancing the yield level further to feed the mouth of growing world population and breaking the yield plateau. The successful exploitation of hybrid rice technology on a commercial scale has been demonstrated in China with rice hybrid covering nearly 60% of the total rice area. Hybrid rice has helped China to produce additional 250 million tones of rice during the last two decades. India also has made spectacular progress in development and adoption of hybrid rice technology on commercial scale. In India, about 17 rice hybrids have been released for commercial cultivation and rice hybrids suitable for cultivation in almost every part of the country are now available.

Hybrid breeding includes two major steps. Breeding parental lines and selection of the best hybrids from the cross combination of the parental lines developed. These steps involve a large amount of work in test crossing and field evaluation of experimental hybrids. Breeder continually have to decide which experimental single cross to test, which advanced hybrids to recommend for further testing or commercialization and which inbred parents to cross to form new base population for inbred/population development. Conventional methods employed by plant breeders for their production are cumbersome, laborious and not very efficient. (Lee *et al*., 2004).

Biotechnology offers great potential for creation, conservation, characterization and utilization of germplasm for rice breeding programs.

Non conventional procedures of plant breeding will be of special importance to achieve these aims. *In vitro* culture and recombinant DNA technology open up possibilities to combine new characteristics more precisely, to select for rare genotypes within a large population of individuals, and to transfer a defined character directly as an isolated gene to an already well bred crop line.

A number of *in vitro* techniques are useful tools in plant breeding, utilized both for creating new variability and for increasing selection efficiency.

Tissue culture of rice has been started long back from organ cultures of excised roots (Fujiwara and Ojima, 1955), or from young hybrid embryos (Amemiya *et al*., 1956; Nakajima and Morishima, 1958). Successful callus cultures of rice were achieved using the nodes of young seedlings (Furuhashi and Yatazawa, 1964). Regeneration of plants was done from seed callus (Tamura, 1968; Nishi *et al*., 1968; Maeda, 1968), root callus (Kawata and Ishihara, 1968) and pollen callus (Niizeki and Oono, 1968). (Oono, K. (1984)) The significance of haploids in the fields of genetics and plant breeding has been realized for a long time. Haploid plants are a tremendous asset in plant breeding and genetic studies.

Indica rice has many important genes including resistance to disease and insects, sensitivity to photoperiod and temperature, tolerance to abiotic stresses. Traditionally, the backcross method is being used to transfer traits such as disease resistance controlled by one or a few genetic loci. But it is difficult to transfer these traits from indica rice into japonica rice by traditional methods, because most

useful traits are co-inherited with non desirable traits in their progenies, or often disappeared in the process for selection of progenies. When useful traits are transferred from indica rice in to japonica rice by backcross method, the utilization of an anther culture technique is desirable for producing homozygous lines and selecting target traits.

In vitro haploid production by anther culture was first developed at the Delhi University, Delhi by Guha and Maheshwari (1964, 1966) in Datura. Production of haploid/doubled haploid rice has since been extended to a diverse array of rice genotypes. Production of doubled haploids through anther culture is a rapid approach to achieve homozygosity that shortens the time required for the development of new rice cultivars compared to conventional methods which require at least 6-7 generations. Haploids are also valuable to detect and fix desirable recessive traits introduced through mutation or hybridization. Moreover, anther and microspore culture of F_1 hybrids lead to fixation of gene combinations which otherwise may not be possible to isolate from a segregating population through conventional means. An ideal situation would be the rapid fixation of heterotic gene combinations from an F_1 generation to develop homozygous lines as good as heterotic F_1 hybrids.

Anther culture has also been useful for overcoming F_1 sterility, utilizing heterosis and mapping QTLs with doubled haploids in rice (Zhang *et al*., 1995; Liu *et al*., 1997; Raina, 1997; Yang, 1997; Chen *et al*., 1998; Zhuang *et al*., 2002). (Oono, K. (1984))

Numerous studies have demonstrated that the percentage of green plants from anther culture in cereal crops is under the control of a

complex genetic system involving both additive and non-additive nuclear gene action as well as cytoplasmic factors (Ekiz and Konzak, 1991a, b, c; Larsen *et al*., 1991; Sagi and Barnabas, 1989; Tuvesson *et al*., 1989). Expression of these genes is influenced by environmental factors, such as the growth conditions of donor plants (Bjornstad *et al*., 1989), cold pretreatment of anthers (Konzak and Zhou, 1991; Powell, 1988), presence or absence of tapetum (Mejza *et al*., 1993), ammonium nitrate levels in culture media (Olsen, 1987), the concentration and combination of plant growth regulators (Ball *et al*., 1993; Ziauddin *et al*., 1992), physical and chemical conditions of callus induction media (Hoekstra *et al*., 1993; Orshinsky *et al*., 1990; Zhou *et al*., 1991, 1992), temperature during anther culture (Huang, 1987), and the quality and intensity of light during plant regeneration (Ziegler *et al*., 1990).

Anther culture of rice had been reported to be influenced by the genotypes of the explant (Niizeki and Oono, 1968; Shen *et al.,* 1982), the growth condition of the donor plants (Chen, 1988), the developmental stage of the microspores (Chen, 1977; Genovesi and Magill, 1979), Pre-treatment (Qu and Chen, 1983), the culture methods (Yang and Zhou, 1979; Chen, 1988), the media (Chen, 1988; Sun *et al*., 1990) and the culture conditions (Wang *et al.,* 1977; Qu and Chen, 1983). Among these influencing factors, the genotype of the donor plants has been reported to be the most important factor in anther culture (Chen, 1988; Henry *et al.,* 1994).

Considerable variation in anther culture among rice genotypes has been identified (Niizeki and Oono, 1968; Mukherjee, 1973; Oono,

1975;

Shen *et al*., 1982; Chen, 1988; Li, 1991) and a general trend has been reported as follows: japonica/waxy > japonica/japonica > japonica > indica/japonica > indica/indica > indica (Shen *et al*., 1982). (Yan *et al*., 1996)

Over a dozen varieties and more than hundred inbred lines have been developed especially in China through anther culture predominantly in japonica rice indicating that japonicas are easier to culture *in vitro* than indica cultivars. Although the advantages of anther culture technique attracted the attention of breeders to exploit the tool in rice improvement, the formidable problems especially with indica rice, like genotype dependent response to callus induction, low regeneration of green plants restricted its wider application routinely.

Some of the hybrids studied at Main Rice Research Station, Anand Agricultural University, Nawagam through conventional breeding programme have shown heterosis for yield, varied resistant reaction to diseases etc.

In the present study it is planned to develop efficient protocol for anther callus induction and regeneration of plants from a promising heterotic hybrid of indica rice and confirmation of the ploidy status of the regenerant plants through cytological methods.

The present investigation was therefore, planned with the following objectives:

1. To determine developmental stages of microspores for anther inoculation.
2. To study cold Pre-treatment.

3. Standardization of surface sterilization technique.
4. To study the efficient media for *in vitro* callusing
5. To study the response of different phytohormones for induction of callus and regeneration of plantlets through organogenesis.
6. To study the genotypic response for *in vitro* plantlet regeneration.
7. To study *in vitro* rooting and acclimatization of *in vitro* raised plantlets.
8. To study ploidy status of regenerated plantlets using root tips/flower buds of the anther derived plantlets by using cytological staining methods *viz.*, Acetocarmine squash preparation/Feulgen method or using leaves of the anther derived plantlets through ploidy analyzer.

II. REVIEW OF LITERATURE

The available literature pertaining to developmental stages of the pollen/microspores, Pre-treatments *viz*., cold and heat shock pretreatment, surface sterilization for establishment of aseptic cultures, factors affecting anther culture of rice, induction of callus, plantlet regeneration, *in vitro* rooting and cytological study of regenerated plantlets for ploidy status, hardening of anther culture derived plantlets is reviewed and presented in this chapter. Survey of tissue culture literature in Grameneae family revealed that rice is one of the most intensively and extensively investigated crop plants. In view of this, literature on this crop has been reviewed in the context of the present work.

2.1 EFFECT OF PHYSIOLOGICAL STATE OF THE DONOR PLANTS

The physiological state of the donor plants has a considerable impact on the response of *in vitro* anther/microspore cultures.

Chen and Lin (1976) observed that anthers collected and cultured at the beginning of the flowering period were more productive than those harvested at the end of this period.

The frequency of callus formation was significantly lower in anthers collected from tertiary tillers than in those in main culms and primary and secondary tillers. (

Tsay *et al*., 1988

)

The age and growth conditions of donor plants and nutrients available to them are important factors affecting androgenesis. (Dunwell, 1985; Sunderland, 1974; Foroughi-Wehr *et al.,* 1982 and Tsay *et al*., 1982).

Marked differences in anther response have been noticed among plants grown during different months of the year (Tsai and Lin, 1977; Hu *et al*., 1978; Chaleff and Stolarz, 1981). These seasonal variations in anther response may be attributed to differences in the physiological status of the donor plants which are, in turn, caused by environmental factors such as temperature, photoperiod, light intensity, etc.

But as a whole, anthers should be collected from plants supplied with proper nutrients and maintained in a vigorous and healthy state.

Hu *et al.* (1981) obtained best anther culture response when plants were grown under bright sun at 18-20 ºC.

Huang *et al*. (1983) reported that exposure of donor plants to high temperature had an adverse effect on green plant regeneration.

Raina *et al.* (1987) suggested that Indica cultivars have a specific need of high temperature and gave best anther culture response when plants were grown at 23.2 - 34.2 ºC compared to 16.4 - 29.1 ºC.

Anther culture response of indica rice cultivars was affected by the photoperiod and thermo period under which the plants were grown (Sun *et al*., 1992).

Early flowering plants with numerous flowers generally produce viable microspores that are more responsive to *in vitro* culture. Many factors that affect plant growth, *viz.* plant age, photoperiod, light

intensity, temperature, season, growing conditions and nutritional status also affect the anther culture response. (Gosal *et al*., 1997)

2.2 EFFECT OF DEVELOPMENTAL STAGE OF THE MICROSPORES

The microspore development stage is the most critical factor that affects the frequency of pollen embryo/callus formation.

The relationship between pollen stage and pollen plant production in rice has been critically studied by Chen C.C. (1976, 1977), who cultured five anthers of each spikelet in a single vessel for observation of callus formation, and fixed the remaining anther of the spikelet for cytological determination of pollen stage. This work clearly demonstrated that maximum response occured at the mid-uninucleate microspore stage; before or after this stage response declines sharply.

Maximum response occured at the late-uninucleate microspore stage (Wang *et al*., 1974; Hu *et al*., 1978).

The mid-uninucleate stage appeared also to be the optimal responsive stage for anther culture of other cereals (Quyang *et al*., 1973; Sunderland, 1974).

Cytological examination of the microspores, as has been done by Chen C. C. (1976, 1977), is the most reliable method for the identification of anther stage. But this procedure is too tedious and time consuming for large scale culturing for breeding purposes. Other criteria that can be used are:

(1) the distance between the legules of the flag leaf and the

penultimate leaf (Oono, 1975; Chen and Chen, 1979; Chaleff and Stolarz, 1981), (2) the color and size of the spikelets and anthers (Chen and Chen, 1979), and (3) the texture of the spikelets. These methods, when combined together and after some practice, could be fairly accurate.

In rice, the maximum response shifts from the mid-uninucleate to an early-uninucleate stage when sucrose concentration of the culture medium was increased from 6 % to 9 % (Chen, 1978).

Several researchers have successfully cultured anthers containing microspores at mid-late uninucleate to early-mid binucleate stages. Anthers in mid-late uninucleate responded very highly to callus induction while those in the early-mid binucleate stage had a very lower efficiency reported by Mercy and Zapata (1986).

Rout and Sarma (1986, 1991) reported panicles containing maximum number of florets at mid-late uninucleate state while florets were enclosed within the boot leaf.

2.3 EFFECT OF PRETREATMENT

The normal gametophytic pathway of pollen can be altered to sporophytic pathway by subjecting anthers or buds to various stresses, especially cold or heat shock treatments.

2.3.1 Cold treatment:

Cold-pretreatment of panicles before removing anthers is necessary for better response to callus formation and green plantlet production. The highest increase (four fold) in anther response was obtained by Genovesi and Magill (1979) who pretreated the detached

panicles with mild temperature shock (10 ºC or 13 ºC) for 10 to 14 days.

Longer durations (8 to 15 days) of treatment appeared to be better than shorter ones of 2 to 4 days. However, prolonged cold treatment (beyond 15 days) caused the resultant callus to be more difficult to differentiate (Chen *et al*., 1982) and to form more albino plantlets (Genovesi and Magill, 1979; Zapata *et al*., 1982).

Cold treatment also delayed anther senescence thus allowing sufficient time for anther wall tissue to nurture developing microspores (Zhang and Chu, 1986).

A mild temperature shock of up to 11 days enhanced the frequency of callusing from 12.5 to 32.8 % reported by Gupta and Borthakur (1987).

High callus induction frequencies were obtained in the range of 17 to 27 days of pretreatment, among which 27 days was the optimum. The plating efficiencies of 17, 22 and 27 days cold-pretreatment were 1.17, 1.20 and 1.72 per cent calli per anther and green plantlet regeneration frequencies were 1.8 %, 1.9 % and 3.4 %, respectively. (Xie *et al*., 1997)

Trejo-Tapia *et al*. (2002) studied the effect of cold pretreatment on the induction of callus for both, parental lines and hybrids, Callus induction was inhibited when the cold pretreatment lasted 21 days. A cold pretreatment of 8 days stimulated only callus induction from anthers of F_1 hybrids, whereas the parental lines did not induce callus in this condition. A

pretreatment of 14 days promoted callus for the parental lines and two F_1 hybrids (K x MA92 and MA92 x K). Morelos A-92 and F_1 hybrid K x MA92 presented the highest callus induction values. MA-92 x K and MA-92 x S were the worst responding genotypes with callus induction value lower than 1.3 %.

2.3.2 Heat treatment:

In addition to cold shock, other pretreatments such as high temperatures (Keller and Armstrong, 1977), placing the detached tillers in water at room temperature (Wilson *et al*., 1978) and putting the excised anthers in a water saturated atmosphere (Dunwell, 1985) have also been found beneficial to embryo and plant yields. These discoveries have led to the formulation of a concept that the induction phase was of great importance in androgenesis (Sunderland, 1974), and this phase was probably nutrient independent (Dunwell, 1985).

A short heat treatment has been beneficial for callus induction from anthers.

Reddy *et al*. (1985) induced callus from rice anthers by pre-treating them at 35 ºC for 5 minutes followed by cold treatment at 10 ºC for seven days.

Zapata and Torrizo (1986) reported an increase in callus induction frequency from anthers treated at 35 ºC for 15 minutes prior to cold treatment at 10 ºC for 7 days.

Sathish *et al*. (1995) found that a heat pretreatment of immersing the panicles in a water bath at 32 ºC for 2.5 or 5 hr. enhanced the ability of anthers to initiate callus.

2.4 SURFACE STERILIZATION

Proper surface sterilization of the panicles and spikelets before inoculation is essential to obtain axenic culture free from external contaminants with improved callusing and quality of callus.

Chen *et al*. (1982) used 0.5 % sodium hypochlorite for sterilizing inflorescence.

Balachandran *et al*. (1990) reported 0.1 % mercuric chloride for 15 minutes followed by liberal washing in sterile distilled water at least for six times for spikelets surface sterilization.

Krishna Raj and Shree Rangasamy (1993) surface sterilized panicles with 70 % ethanol for 30 seconds followed by 0.1 % $HgCl_2$ for 10 minutes and were washed three times with double distilled sterile water.

The spikelets were surface sterilized with 0.1 % mercuric chloride for 5 minutes and rinsed thrice with sterile distilled water. (Narasimman *et al*., 1994)

Yan *et al*. (1996) used 10 % (v/v) calcium hypochlorite for 17-20 minutes for surface sterilization of spikes and rinsed 3-4 times in sterile distilled water.

Zhuo *et al*. (1996) reported boots cleaned with 75 % ethanol and sprayed with Amphyl ™ (Sterling Drug Inc., National Lab., Montvale, USA). Florets were disinfected by dipping in 75 % ethanol, immersing in 1 % sodium hypochlorite for 15 minutes and 3 % hydrogen peroxide for 10 minutes and finally rinsing 3-4 times with autoclaved distilled water.

Faruque *et al*. (1998) disinfected spikes with 20 % (v/v) commercial Clorox solution containing 5.25 % (w/v) sodium hypochlorite

for 10 minutes outside the clean bench, followed by 20 minutes in the clean bench after a change in the sterilization solution.

Panicles were surface sterilized by rinsing in 70 % (v/v) ethanol for 40 seconds. Spikelets from the center of the panicles were excised and surface sterilized with 0.1 % (w/v) aqueous $HgCl_2$ solution for 8 minutes and rinsed three times with sterilized distilled water as reported by Bishnoi *et al*., 2000.

The spikes with the leaf sheaths removed were surface sterilized with 70 % (v/v) ethanol for 30 seconds and then surface dried in a laminar flow.

(Lee *et al*., 2004)

2.5 EFFECT OF GENOTYPES ON INDUCTION OF CALLUS AND REGENERATION OF PLANTLETS

In general, Japonica rice cultivars respond better than Indica cultivars (Chen and Lin, 1976; Hu, 1981; Rush and Shao, 1982; Chu, 1982; Woo and Chen, 1982; Zapata *et al*., 1986b; Quimino and Zapata, 1990) and sometimes F_1 hybrids performed better than their inbred parents (Chen, 1986 a, b).

Iyer and Raina (1972) demonstrated that, of 15 cultivars studied, only five showed androgenic response and only one regenerated roots and shoots.

Guha-Mukherjee (1973) found that some Indica and Japonica cultivars did not respond to anther culture, but cv. Assam 5 formed callus in 26 % of cultured anthers.

Oono (1975) reported variation in callus formation capability among the lines tested from 0.5 to 8.0 %, and regeneration of albino and green plants was also genotype dependent.

Chaleff (1980) reported that Indica cultivars regenerated fewer green plants (only 5 %) than Japonica type (up to 90 %).

Hu (1985) regenerated green plants from 10 % of cultured anthers of Japonica cultivars compared to 1% for Indica rice.

Among 11 Indica cultivars examined by Aruna and Reddy (1988), the frequency of green plants regenerated varied from 0.0 to 47.5 %.

Callus forming ability from rice anther culture and time required for callus induction was genotype dependent (Abe, 1992; Reddy *et al*., 1985)

Quimino and Zapata (1990) at Philippines studied plant regeneration from a diallel cross involving two japonica cultivars, Taipei 309 and Taipei 177; and two indica cultivars, IR 36 and Basmati 370. The effect of genotype on green-plant regeneration was found to be significant ($P<0.01$), contributing most to the overall variation in the experiment. Genotypic effects on albino plant regeneration were non significant ($P>0.05$), indicated that this character was not affected by genotype. Per cent plant regeneration (green and albino) was highest in Taipei 177 and lowest in Basmati 370. Varieties Taipei 177 and Taipei 309 generally had high regeneration rates while IR 36 and Basmati 370 had very low per cent regeneration. This supported that the japonicas were more responsive to culture than the indicas. Crosses with IR 36 as female and Taipei 177 or Taipei 309 as male greatly increased green-plant regeneration relative to IR 36. Similar to that in total plant

production, Taipei 177 gave the highest percentage of calli producing green plants followed by crosses with Taipei 309. Green-plant regeneration in these japonica cultivars was considerably reduced in crosses involving Basmati 370 as male or female.

Kim *et al.* (1991) reported the best response from Japonica x Japonica hybrids followed by Indica x Japonica and then Indica x Indica crosses.

Dhaliwal *et al*. (1997) attempted anther culture from six crosses of high yielding indica and basmati varieties. Cultured anthers from all crosses exhibited callusing with varying frequency (1.9 - 10.7 %). Highest callusing (10.7 %) was observed in the cross Pusa Basmati/PAU 1198.

Lentini *et al*. (1997) conducted diallel and backcross inheritance analysis using crosses between true indica (non responsive to anther culture, with long, slender grains) and japonica (high responsive to anther culture, with short, thick grains) genotypes. They examined F_1, (and their corresponding reciprocal), F_2 and backcross (BC) populations with one BC to each parent. The diallel study indicated that the low anther culture (AC) response shows incomplete dominance with respect to high response. Thus, the AC response in the F_1 generation is below that of the most responsive parent. Possible maternal effects-although not statistically different were only noted in the crosses when C8707 was used as the non responsive parent.

Raina and Zapata (1997) at Philippines studied anther culture efficiency of certain indica x javanica and indica x japonica hybrids. However, green plant regeneration frequency was very low (only about

8 %). Differences in anther response and green plant regeneration, among the four hybrids, were not significant. The anther calli that regenerated albino plants (20 %) were about twice those that regenerated green plants (8 %).

Rohilla *et al*. (1997) studied 12 indica rice varieties, six Basmati rice cultivars/breeding lines and 31 heterotic F_1/F_2 hybrids obtained from different indica/Basmati crosses. Regeneration of green plantlets was very low. They obtained high ratio of albino/green plantlets. This suggested that indica rice were low responding to anther culture.

Rout and Sarma (1990, 1991) at CRRI, Cuttack studied regeneration frequencies of different rice genotypes for anther callus culture. They reported that on an average Japonica cultivars responded better than Indica, followed by inter varietal hybrids of *O. sativa* and indica x japonica;
O. sativa x *O. longistaminata* responded least.

2.6 INFLUENCE OF POSITION OF ANTHERS AT PLANTING ON CALLUSING AND PLANT REGENERATION

Mercy and Zapata (1987) studied the effect of anther orientation at plating on callusing response and plant regeneration in japonica rice variety Taipei 309. Most of the anthers plated flat callused on both lobes, but callusing on one lobe was also observed. Most anthers plated on edge produced callus on the upper lobe. Only rarely anthers plated on edge produced callus from both lobes or from the lower lobe only. No special anther inoculation technique is needed to get satisfactory callusing response in rice except to see that anthers are not buried in the medium.

2.7 EFFECT OF CULTURE MEDIUM ON CALLUS INDUCTION AND PLANT REGENERATION

Culture medium is another important component for producing embryos, callus from cultured anthers/microspores. Nutrient requirements may differ for the induction of androgenesis and growth of developing embryos (Wang *et al*., 1974).

Chu *et al*. (1975) developed N_6 medium with reduced $(NH_4)_2SO_4$ and increased KNO_3 specifically for anther culture of rice; this medium has subsequently been demonstrated to be the most suitable (Chu, 1982; Genovesi and Magill, 1979; Chen *et al*., 1982; Tsay *et al*., 1982). Both B_5 (Gamborg *et al*., 1968) and modified LS (Linsmaier and Skoog, 1965) medium which also have high concentrations of nitrate nitrogen and reduced concentrations of ammonium nitrogen have also been found to give good results (Chaleff and Stolarz, 1981; Zapata *et al*., 1982).

N_6 medium was found superior to other media, *viz.,* MS (Murashige and Skoog, 1962), Potato extract, Blayeds (1966) SK-8 and Chaleff's R-2 (Chu, 1982; Lenka and Reddy, 1994).

Huang *et al.* (1978) modified N_6 medium and developed He-2 medium amended with half strength ammonium, double strength KH_2PO_4 and one fiftieth the concentration of $MgSO_4$.

Yang *et al*. (1980) used modified N_6 medium by replacing $(NH_4)_2SO_4$ and KH_2PO_4 with NH_4HPO_4 for Indica cultivars.

The ammonium nitrogen level in the medium was a crucial factor (Chu

et al., 1975; Chu, 1982; Gosal *et al*., 1991, 1993; Sandhu *et al*., 1993a) and its requirement by Indica cultivars was different from that of Japonica.

The N_6 medium devised for Japonica cultivars was unsuitable for Indica varieties. For Japonica and Indica cultivars, the optimal NH_4 content was 7.0 me l^{-1} and 3.0 me l^{-1} respectively, but 4.8 me l^{-1} for Japonica x Indica hybrids (Chen, 1983).

Chen *et al*. (1986) observed a significant increase in anthers forming callus when 0.1 mM Na_2 Fe EDTA was added to the induction medium; however, further increase in the amount of iron (3.2 mM) was deleterious.

He-2, He-5, Potato-2, E-10, MSN modified media have been suitable for Indica rice anther culture (Raina *et al*., 1989; Rout *et al*., 1989; Reddy *et al*., 1985; Zapata *et al*., 1990).

Draz *et al*. (1991) studied the interaction of three different derivatives to B_5, N_6 and MS/B_5 basal media supplemented with different growth regulators and reported that there were significant differences among media as well as a significant interaction between cross and media.

Callus induction medium also plays a role in inducing plant regeneration; for a particular callus induction medium, a specific regeneration medium may give a better response.

Raina and Zapata (1997) at Philippines studied effect of genotype and media interaction on callus induction efficiency and green plant regeneration of different indica x japonica, indica x javanica derivatives

and two of the parents. Data on microspore callus induction showed medium M-III as the most effective, whereas calli derived from medium M-II have shown, overall, better regeneration frequency than the other two media.

In IR-9884 X Moroberekan, higher anther response (22.19 %) obtained in M-III was followed by higher (41.2 %) plant regeneration results. Conversely, the highest response of 33.2 % recorded in M-III was followed by the lowest regeneration results. The results highlight the extent of variation in anther response and subsequent green-plant regeneration, as a consequence of medium x genotype interaction.

An overall regeneration efficiency of 24 % was obtained. Although calli derived from M-II, having 1.75 mM of $(NH_4)_2SO_4$ compared with 3.5 mM in M-III, showed higher regeneration efficiency (32.1 %) even the calli from M-III showed as much as 22 % plant regeneration.

2.8 EFFECT OF CARBON SOURCE ON CALLUS INDUCTION AND PLANT REGENERATION

Sucrose is essential for androgenic plant development and it functions both as a nutrient and an osmotic factor. It influences callus induction and green plant regeneration.

Chen (1978) reported that the rate of callus formation and subsequent organogenesis increased as the sucrose concentration increased from 3 to 9 %, but that calli initiated on a medium with 9 % sucrose regenerated more albino plantlets. A combination of 6 % sucrose in the callus induction medium and

3 % in the plant regeneration medium gave the highest frequency of regenerated green plants.

Optimal sucrose concentration for callus induction was 3 % (Kavi Kishore *et al*., 1989), 4-5 % (Huang *et al*., 1978; Chu, 1982; Chaleff and Stolarz, 1981), and 5 % (Reddy *et al*., 1985; Chen, 1978), or 6 % (Chaleff, 1980).

High sucrose concentration altered the osmotic potential of the medium without providing any additional nutritional requirement such that mannitol (50 mM) can be substituted for additional sucrose (Chaleff and Stolarz, 1981).

Krishna Raj and Sree Rangasamy (1993) studied effect of sugar source on callus induction, maintenance and plantlet regeneration of rice anther cultures. Frequency of callus induction on different combinations of sugars indicated Sucrose 2 % + Sorbitol 3 % (29.6 %) and Sucrose 3 % (23.1 %) to be effective. Combinations Sucrose 4 %, Sucrose 2 % + Sorbitol 2 %, Sucrose 2 % + Sorbitol 4 % and Sucrose 3 % + Sorbitol 3 % also showed reasonably high levels of callus induction. Media containing either no sugar or sorbitol (2 %) alone showed no callus initiation. Plantlet regeneration after 4 or 5 subcultures showed high frequency of plantlet regeneration (56.3 %) when callus was initiated and maintained on sucrose (2 %) + sorbitol (3 %) supplemented N_6 and MS medium, respectively.

2.9 EFFECT OF GROWTH REGULATORS ON CALLUS INDUCTION AND PLANT REGENERATION

A combination of auxins [2,4-D (2,4-dichlorophenoxy acetic acid), NAA (Naphthalene acetic acid), IAA (Indole acetic acid)] cytokinins [BA (Benzyl adenine), Kn (Kinetin), Zea (Zeatin)], and organic substances [Casein hydrolysate (CH), Coconut water (CW), Yeast extract (YE)] has been widely used in rice anther culture media (Niizeki and Oono, 1968; Harn, 1969; Guha *et al*., 1970; Iyer and Raina, 1972).

NAA (2.0 mg l^{-1}) induced a higher frequency of callus than 2,4-D in Japonica varieties and hybrids; however, plant regeneration was poorer for NAA induced calli (Hu *et al*., 1981).

The different effects of 2,4-D and NAA on callus growth might be genotype dependent (Zhang, 1982).

Reddy *et al*. (1985) reported that NAA was better to induce callus, but 2,4-D was better for plantlet regeneration of Indica cultivars. However, high amounts of either NAA or 2,4-D enhanced callus formation but reduced plant regeneration.

A modified medium with 2,4-D (9.0 µM), Picloram (0.3 µM), and Zea (0.15 µM) was most suitable for callus induction and green plant regeneration.

Rout *et al*. (1989) found that NAA improved both callus induction and plantlet regeneration.

Efficient production of microspore callus with high morphogenic potential requires both auxin and cytokinin in the medium (Huang *et al*., 1986; Kavi Kishore *et al*., 1989).

Kavi Kishore *et al*. (1989) regenerated plants at a higher frequency on R2 medium containing 2.0 mg l^{-1} each Kn and IAA or LS medium with 1.0 mg l^{-1} IAA and 4.0 mg l^{-1} Kn.

A combination of 1.0 mg l^{-1} 2,4-D, 2.0 mg l^{-1} NAA and 1.0 mg l^{-1} Kn resulted both in the highest frequency of callus induction and green plant regeneration for interspecific hybrids between *O.sativa* cv. Savitri and *O.rufipogon*, a presumed progenitor of *O.sativa* (Rout and Sarma, 1991).

Draz *et al*. (1991) observed plant regeneration on media with 5.0 mg l^{-1} Kn and 1.0 mg l^{-1} IAA or 2.0 mg l^{-1} Kn and 1.0 mg l^{-1} IAA.

Anther derived callus could be placed into long term maintenance for *in vitro* selection schemes by incubation on MS medium with 2 % sucrose,
3 % sorbitol, and 4.5 µM 2,4-D (Krishna Raj and Sree Rangasamy, 1993).

Krishna Raj and Sree Rangasamy (1993) studied callus induction frequencies in rice cultivar ‘IR-50’ anther cultures under different combinations of 2,4-D and kinetin. Among the ten treatments, anthers inoculated in N_6 + 9.1 µM 2,4-D + 2.3 µM kinetin showed a high percentage of callus induction (11.2 %) followed by N_6 + 9.1 µM 2,4-D + 4.7 µM kinetin (8.2 %) and N_6 + 4.5 µM 2,4-D + 2.3 µM kinetin (7.2 %).

Marassi *et al*. (1993) studied effect of culture medium on callus induction and plant regeneration of rice anthers, variety Colonia Mascias 5 MAG. The best response from the four callus induction media

tested occurred on N_6 medium supplemented with 2.0 mg l^{-1} NAA and 0.5 mg l^{-1} Kn. The high NAA level in this medium in combination with Kn promoted the proliferation of embryogenic callus. In contrast, 2,4-D alone or in combination with NAA, Kn or BA did not promote embryogenic callus.

They further studied interaction between callus induction and shoot regeneration medium on anther-derived callus of rice, *var*. Colonia Mascias 5 MAG. after 35 days in culture. Thirty days old calli subcultured to MS medium supplemented with different combinations and concentrations of NAA, BA and Kn produced shoots. The best response 50 % of calli producing green shoots was achieved with MS + 0.1 mg l^{-1} NAA + 1.0 mg l^{-1} BA. The other growth regulator combinations tried by them were less effective. Callus obtained from N_6 medium plus 2.0 mg l^{-1} NAA and 0.5 mg l^{-1} Kn and subcultured to MS medium plus 0.1 mg l^{-1} NAA and 1.0 mg l^{-1} BA gave the best response compared with the other callus induction media.

Same group of scientists also studied callus induction and shoot regeneration from anther culture. Anthers from seven rice varieties were cultured on N_6 basal medium supplemented with 2.0 mg l^{-1} NAA and 0.5 mg l^{-1} Kn in single step method, calli were transferred from dark to light at 30 days without subcultures. In two step method, calli was subcultured to MS plus 0.1 mg l^{-1} NAA and 1.0 mg l^{-1} BA, prior to transfer to the light. From 30 to 70 % of the anthers from the indica rice varieties, Basmati-370 and Lemmont produce green plants. Many japonica cultivars showed a high capacity for callus production and plant regeneration when exposed to

low concentration of growth regulators. However, indica varieties have displayed a low capacity for both responses, even with high hormone concentrations. This variation might by endogenous factors.

Trejo-Tapia *et al*. (2002) studied plant regeneration from the different genotypes as function of the growth regulators. Differentiation of the calluses was observed 2 weeks after transferring callus to MS medium containing Kn and IAA. No differentiation was accomplished on plant growth regulator free MS medium. The results indicated that regeneration of green haploid plants from anthers of the parental lines Morelos A-92 and Koshihikari could be achieved using IAA and kinetin. However, no plants of the parental line Sasanishiki and the F_1 hybrids, K x MA92 and MA92 x K were obtained, suggesting that various genotypes could have different requirements with regard to plant growth regulators.

Same group of scientists studied the effects of NAA, IAA and carbon source on the induction of callus from anthers of the hybrid Morelos A-92 x Koshihikari. The anthers of the F_1 hybrid, MA92 x K developed callus when NAA or IAA were added to the medium and there was no response with 2,4-D in contrast to that observed for the reciprocal hybrid, K x MA92. The induction of callus was achieved using maltose and ≥ 8.06 µM NAA. The highest CI value was with 10.74 µM NAA and 30 g l^{-1} maltose. This response was four times higher ($p \leq 0.05$) than the obtained with the other levels of NAA tested. For IAA, the induction of callus of the hybrid MA92 x K was accomplished using at least 5.71 µM of this auxin. The highest callus induction value was reached using this concentration of IAA and sucrose. The same level of

IAA in combination with maltose promoted an induction of callus three-fold lower than the achieved using sucrose.

2.10 EFFECT OF ORGANIC SUBSTANCES

Addition of organic substances, *viz*., Coconut water (CW), Yeast extract (YE), potato extract, Casein hydrolysate (CH), Lactalbumin hydrolysate(LH), etc. in the medium has promoted callus formation from rice anthers (Guha *et al*., 1970; Guha-Mukherjee, 1973; Wang *et al.,* 1974; Oono, 1975). By contrast, several studies have shown equally high frequency of callus induction and plant regeneration on media devoid of these substances (Chen, 1977; Chaleff and Stolarz, 1981; Lee and Chen, 1987; Chen *et al*., 1982; Tsay *et al*., 1982).

Anther browning was reduced, suggesting a drop in ethylene promoted senescence. Normally, anther browning develops as a result of quinone production leading to degeneration of anther wall tissue. This can be disadvantageous, as anther wall tissue helps to supply nutrients to the developing microspores. To overcome this problem, ginseng powder at 500 - 1000 mg l^{-1} can be added to the medium to delay anther browning, resulting in increased callus induction frequency (Chen and Tsay, 1986)

Hirabayashi *et al*. (1992) observed that the addition of aspartic acid, glutamine, glutamic acid, tryptophan and CH increased the efficiency of callus formation and plant regeneration.

2.11 EFFECT OF PHYSICAL STATE OF MEDIUM

Physical state of medium i.e. liquid/semi-solid had also affect on callus induction as well as green plant regeneration.

Chowdhury and Mandal (2001) studied microspore embryogenesis and fertile plantlet regeneration in indica rice hybrid and its parents. On an average callus forming ability in liquid culture (5.49 %) was better than in semi-solid medium (3.75 %) across all genotypes. Liquid culture offered the advantage of better gaseous exchange and consistent nutrient availability to the microspore, which are restricted to some extent in agar fortified semi-solid medium. It also provides greater scope to reduce the effects of competitive inhibition among the potential embryonic pollen grains. Furthermore, inhibitors or toxic compounds, produced by the degenerating anther wall diffuse into liquid medium rather than being localized around the anthers as happens in the agar supplemented medium.

2.12 EFFECT OF CULTURE CONDITIONS

The light conditions used for rice anther culture have varied from complete darkness (Niizeki and Oono, 1968; Harn, 1969; Woo and Tung, 1972; Guha-Mukherjee, 1973) to continuous illumination (Nishi and Mitsuoka, 1969; Niizeki and Oono, 1968).

Wang *et al*. (1977) concluded that the formation of green or albino plants was mainly affected by temperature at the initial phase of microspore development and not at the phase of callus differentiation. The temperatures used for rice anther culture generally fall between 25 ºC and 30 ºC.

Light and temperature played important role in androgenesis (Maheshwari *et al*., 1980). Darkness was slightly favorable for the initiation of callus (Cornejo-Martin and Primo-Millo, 1981).

2.13 DIFFERENTIATION OF CALLUS

Pollen callus of rice loses its morphogenetic potential very rapidly during culture and early transfer to the plant regeneration medium has been suggested (Oono, 1975; Chen, C.C., 1977).

The most suitable time for callus transfer has been established to be approximately 10 days after its emergence; transfer before this usually results in death of the callus (Wang, *et al*., 1974). Another advantage of early transfer is that the chances for change in ploidy level during callus proliferation (Chen and Chen, 1979) could be minimized, so that more haploid and few polyploidy and aneuploid plants could be recovered

2.14 ALBINISM

One of the major limitations with rice anther culture is the occurrence of albino plants in large numbers. (Raina, 1989)

Various studies on rice in this aspect have been carried out in China, including the factors related to the albino plant formation, the effect of the components of medium and the mechanism of albino plant formation, etc.

Genetic factor:

The rate of albino pollen plants in rice usually ranged from 5-90 % (Chu, 1982) or even more in some varieties and Hsien/Keng hybrids, which showed that their frequency depended on the varieties or hybrids used.

When the anthers were taken from the diploid pollen plants and cultured, a great number of albino plants were regenerated, which indicated that the occurrence of albinism was not the expression of the recessive albino gene(s) of the parent in pollen plants (Wang *et al*., 1978). When tetraploid rice plants (Keng varieties and Hsien/Keng hybrids) were used as anther donor, the segregation of green and albino plants was not observed in H_2 population (2n). This result further proved that albinism in rice anther culture is not caused by the mutation of recessive nuclear gene (He and Ouyang, 1983); otherwise the rate of albino plant should have been lower in the culture of anthers taken from the tetraploid rice.

The effect of temperature:

It has been observed that among the physical factors studied, temperature was the most critical one in albino plant regeneration. The frequency of albino plants increased with the rise in temperature, especially at the early stage of culture of rice anther. In this period, the longer the anthers were cultured at the higher temperature (35 °C), the higher the rate of albino plant which occurred. The pretreatment of rice panicles at the higher temperature (35 °C) for 3 or 5 days also increased the albino plants. If rice panicles were pretreated at the room temperature (26 °C), all the regenerated plantlets were albinos; however, the pretreatment at lower temperature (10 °C) increased the rate of green plants (Qu and Chen, 1983).

Hu *et al*. (1978) have also noticed that if the anther donor plants were subjected to higher temperature at the stage of earing, it not only

greatly decreased the frequencies of callus formation and plant regeneration, but also increased the percentage of albino plants.

Effect of culture medium and radiation:

High levels of 2,4-D (20 mgl^{-1}, Wang *et al*., 1978) or sucrose (9 %, Chen, 1978) in the callus induction medium enhanced the formation of albino plantlets after the pollen calli were transferred on the differentiation medium.

Biochemical and Physiological changes of albino pollen plants

Rice albino plantlets contained very low content of chlorophylls: on average, chlorophyll a and b were only 1.6 and 2.3 % respectively to those of the normal green plantlets. Electron microscopic observations also demonstrated that protoplastids though exist in the leaf cells of albino plants, but their normal development was disturbed. There was no grana formation in stroma, and no ribosomes were observed in the albino plastids, though DNA fibrils were radially found in them (Sun *et al*., 1974). Some ultrastructural changes were also observed in the cells of callus which would differentiate albino plants (Liang *et al*., 1978). The electrophoretic patterns of soluble proteins from albino pollen plantlets showed that they lost two bands in comparison with that of green plantlets. One of them was identified as Fraction I protein. It was also found that 16S and 23S rRNA was missing, whereas 18S and 25S rRNA was normal (Wang *et al*., 1978). All these results obtained indicated that DNA damage might be responsible for the albino plant formation (Chu, 1982 a)

Wang *et al.* (1978) further reported that emergence of albino plantlets was due to the functional genes which regulate the synthesis

of RNA, was somehow blocked, consequently the necessary proteins (enzyme) for the development of lamellae systems was cut off and finally no chlorophyll would be formed.

2.15 *IN VITRO* ROOTING

Balachandran and Sarma (1990) transferred regenerated plantlets to half strength MS medium and allowed three weeks of growth for hardening before transfer from tube to soil.

Use of MET(multi effect triazole) at 2.5 mg l^{-1} in the rooting medium (MSR-3) reduced plant height, increased plantlet rooting, and made leaves thicker and greener (Kangle and Murashige, 1992)

Green plants regenerant from SK-II were transferred to ¼ strength MS medium in long (20 cm) culture tubes so to allow better root and shoot growth reported by Raina and Zapata (1997).

Bishnoi *et al.* (2000) reported *in vitro* rooting in a phytagel-solidified half strength MS medium supplemented with 2 % (w/v) sucrose, 0.25 mg l^{-1} NAA and 2.5 mg l^{-1} MET.

2.16 PLOIDY LEVEL OF ANTHER DERIVED PLANTS

Regenerants from rice anther calli consist of haploids, diploids, polyploids and mixoploids. Most of the plants (more than 50 %), however, are diploids (Iyer and Raina, 1972; Oono, 1975; Yin *et al*., 1976; Chen and Li, 1978; Huang *et al*., 1978; Shen *et al*., 1983; Zapata, 1985; Loo and Xu, 1986).

Huang *et al*. (1978) examined a total of 2496 rice anther derived plants. The frequencies of haploids, diploids, polyploids and mixoploids were found to be 35.3, 53.4, 5.2 and 6.0 % respectively.

Anther derived rice plants comprised a mixed population with ploidy ranging from 1*x* to 5*x*, although most are haploid (30-35 %) or diploid (50-65 %). (Chen *et al*., 1983; Mercy and Zapata, 1986; Rout and Sarma, 1990). Many factors, such as developmental stage of microspores at the time of culture, nature of pretreatment, genotype of the donor plants, composition of the culture medium and age of the cultivars, affect the ploidy of the regenerated plants. Both endoreduplication and nuclear fusion have been demonstrated to occur during rice anther culture and were presumed to result in diploid regenerants. (Chen and Wu, 1983).

Reiffers and Adelson Freire (1990) studied survival rates and ploidy levels of androgenetic plants. Spontaneous chromosome doubling, frequently observed in rice haploids, is expressed by the formation of fertile panicles, while haploid plants remain entirely sterile. The chromosome number of haploid plants was doubled by colchicines' treatment. The spontaneous chromosome doubling of the androgenetic plants, frequent in rice, might be due to endomitosis or cellular fusion. Plants obtained from the same callus had the same ploidy, whereas different calli from the same cross gave plantlets with either ploidy. The average frequency of spontaneous chromosome doubling in the crosses tested was 59 %, but some crosses it reaches 100 %.

Polyploid-type plants, mainly tetraploids, which represented approximately 5 % of the regenerated plants and semi-sterile diploid plants, were found in the 4 anther culture-derived populations. The frequency of spontaneous doubling averaged 46 % and is similar to

values obtained in anther culturing tropical japonicas (Reffiers and Adelson Freire, 1990).

Guiderdoni *et al*. (1992) studied frequency of spontaneous recovery of haploid and diploid-type anther culture plants. Frequencies of recovery of diploid-type anther culture plants for the 4 hybrids which over yielded 100 plants.

Narasimman and Sree Rangasamy (1993) had also studied frequency of green haploids and spontaneous doubled haploid androgenic plants among the regenerants of indica-japonica and japonica-indica hybrids. Among the anther culture regenerants, the frequency of haploids ranged from 57.7 to 72.9 per cent and doubled haploids ranged from 27.1 to 42.3 per cent. The mean percentage of haploids and doubled haploids were 64.4 per cent and 35.6 per cent respectively over the different crosses.

Haploid and diploid rice exhibits distinct morphological differences. Haploids are characterized by the absence of ligules and auricles, approximately twice as many tillers and 60-70 % of plant height, panicle length and leaf length compared to diploids. However, glumes length has been demonstrated to be the most diagnostic character for distinguishing the ploidy of anther-derived rice (Nakamura *et al*., 1994).

Jones *et al*. (1997) found a 5:4:1 ratio for haploids, doubled haploids and polyploids among the regenerated plants regardless of the crosses' genetic origin. The spontaneous doubled haploid lines frequently displayed partial fertility, particularly when derived from *O. sativa/O. glaberrima* crosses and to a lesser extent, from japonica/indica

crosses. High sterility levels might have been due to aneuploidy of the regenerated plants or fixation of sterility genes.

Speedy ploidy level determination:

In plants, ploidy estimation is usually carried out by chromosome counting on microscopic slides prepared from actively growing root tips. The main limitations to the use of conventional chromosome counting in practical breeding and taxonomic studies include: (1) very low frequency of dividing cells (mitotic index in root tips may be 1 % or less); (2) rare occurrence of well spread metaphase plates suitable for chromosome counting; (3) the time needed to screen sufficient number of samples (Novak, 1992).

Chromosome counting in mitotic cells of root tips is an accurate procedure to determine the ploidy, but it is time consuming and requires much experience. Therefore, indirect methods for ploidy determination were found helpful.

Gilissen *et al*. (1994) found diploids, tetraploids and mixoploid plants in tobacco, using flow cytometric analysis.

Dolezel *et al*. (1997) screened ploidy levels in *Musa* plants regenerated after polyploidization *in vitro* using flow cytometric analysis.

The area of stomata has been used to differentiate diploid and tetraploid regenerants of orchids (Watrous and Wimber, 1988), rye grasses (Speckmann *et al*., 1965), daylilies (Arisumi, 1972; Chen and Goeden-kallemeyn, 1979), barley (Borrino and Powell, 1988) and banana (Hamill *et al*., 1992; Vandenhout *et al.*, 1995; Van duren *et al*., 1996) and differences in stomata density (Vandenhout *et al.,* 1995; Van

duren *et al*., 1996) have also been used as criteria in the distinction between diploid and tetraploid plants. (Silva *et al*., 2000)

The suitability of ploidy determination using stomatal measurements, *viz.*, area and density of stomata was evaluated by Silva *et al*. (2000). Diploid plants presented the lowest stomatal area (1269) while tetraploid showed the highest one (1916), mixoploid presented intermediate values (1441) and stomatal density was 8.0 for diploids, 4.7 for tetraploids and mixoploid stomatal density was 6.6.

2.17 ACCLIMATIZATION (HARDENING)

The most of pollen plants regenerated in test tubes have very weak adaptability to natural environment. Direct transplanting into soil caused to the death of pollen plants.

After enough hardening *in vitro*, the young plants eliminated medium were placed in nutrition water (Yoshida *et al*., 1986) supplemented with
50 ppm SiO_2 at the green house. The plants were grown in the water solution for 3 to 4 days under 50% shading condition, and then cultured for 15 to 20 days under natural condition. When the young plants formed new root system, they were transplanted into soil. These artificial treatments sharply increased the survival ratio of pollen plants. (Raina and Zapata, 1997)

Chung and Sohn (1995) had done some treatments for acclimatization in test tube and green house or growth room by making a hole on the cap (aluminum foil) of test tube.

Tubes with plants about 10 cm height, with well developed roots, were unplugged a couple of days prior to transfer to trays of Yoshida *et al.*

(1986) liquid culture medium for root hardening (~10 days), in a phytotron glass chamber (29 ºC day and 21 ºC night) before transfer to the field reported by Raina and Zapata (1997).

Bishnoi *et al.* (2000) reported plants acclimatized in a plant growth chamber for 7 days under controlled humidity (90 %), temperature (25+1 ºC) and light conditions (35 µmol $m^{-2}s^{-1}$, 16 h photoperiod) before final transfer to the open glasshouse.

Plant material	Explant/stage	Medium	Remarks	Reference
O. sativa L.	Anthers	Blaydes (1966) + NAA (9.4 µM) + Kin (10 µM)	0.57 % regeneration after 4-8 weeks in culture	Niizeki and Oono (1968)
20 Indica cv.	Anthers with Uninucleate pollen	Medium supplemented with IAA, 2,4-D,YE and CW	Variation between cv. For androgenic response. Highest callus was in 'Assam 5'	Guha *et al*. (1970)
Keng, Keng x Shien F_1	Anthers	N_6 or SK-8 liquid medium	Callus induction from unreleased pollen in both media	Chen and Chen (1979)
O.sativa	Anthers with bi-nucleate	Miller's macro + micro + Morel's vit. + NAA (3 mgl^{-1}) + YE (2000 mgl^{-1}) + Sucrose (6 %)	Green haploid and mixoploid plants	de Beauville (1980)
O. sativa L.	Anthers with uninucleate to bi-nucleate	N_6 + MCPA (0.125 mgl^{-1}) + Sucrose (3 %) N_6 or MS + IAA (0.5 mgl^{-1}) + Kin (1-2 mgl^{-1}) + NAA (0.25 mgl^{-1}) + Sucrose (3 %)	Green as well as albino haploids were obtained	Zhou and Yang (1981[a,b,c])
O. sativa L.	Anthers with late Uninucleate to early bi nucleate stage	N_6 + 2,4-D (2 mgl^{-1}) + CH (500 mgl^{-1}) + Sucrose (4 %) N_6 + CH (500 mgl^{-1}) + Kin (2 mgl^{-1}) + NAA (0.05 mgl^{-1}) + Sucrose (3%)	Green haploid, diploid and some albino plants obtained	Kuo (1981)

Minehikari (Japonica)	Anthers with late Uninucleate to early bi nucleate stage	R-3 (modification of LS) + sucrose (4 %) + various combinations of NAA, IAA and 2,4-D + Agarose (0.2 % or 0.4 %)	Sucrose concentration of 4 % was found beneficial, addition of 50 µM mannitol increased the callus induction	Chaleff and Stolarz (1981)
Calrose-76, Labelle, Mars, Nortai, Starbonnet, Ma	Anthers with mid to late Uninucleate stage	Blayde's salts +YE (1 gl^{-1}) + CM (150 ml l^{-1}) + IAA (2 mgl^{-1}) + sucrose (3 %) Blayde's salts + CM (500 mgl^{-1}) + YE (250 mgl^{-1}) + 2,4-D (1 mgl^{-1}) + NAA (0.5 mgl^{-1}) + Kin (1 mgl^{-1}) + sucrose(3 %) MS + glutamine (10-3 M) + Inositol (100 mgl^{-1}) + IAA (1 mgl^{-1}) + Kin (1 mgl^{-1}) + sucrose (3 %)	Obtained plants in 'Calrose 76' and observed variability in the regenerated plants for height, seeds, flowering time, awn length, seed weight, etc.	Schaeffer (1982)
Giza 170, Taipei 309	Anthers with mid uninucleate to early binucleate	E_{10} + sucrose (2 %) + glucose (0.5 %) + BA (0.5 mgl^{-1}) + 2,4-D (1 mgl^{-1}) MS + NAA (1 mgl^{-1}) + Kin (1mgl^{-1})	Used conditioned medium 1:8 dilution and obtained 1.6 times higher callus than control for 'Taipei 309' while conditioned medium reduced the callus and green plantlet induction/regeneration in 'Giza 170'	Zapata *et al*.(1985)
Indica cv. ARC 1557, Tetep, Mohini, Pankaj, Govindobhoa, Radhunipagal small fruits, Meghna	Anthers with Uninucleate microspores	He-2 + 2,4-D(9 µM) or He-5 + sucrose(5 %) or N_6 or R_3 He-2 + different conc. and combination of 2,4-D, NAA, Picloram, Zeatin and Kin + glucose (5 %) He-2 + 2,4-D (9 µM) + sucrose (3,5,7,9 or 11 %) MS + NAA (1.5 mgl^{-1}) + Kin (9.5 9 µM)	Different cultivars responded differently to different media. He-2 and He-5 media induced callus at a higher frequency than R_3 and N_6. Medium with 2,4-D (9 µM), picloram (0.3 µM) and zeatin (0.5 µM) was most effective for callus induction.	Reddy *et al*.(1985)
3 Indica, Indica x Japonica 4 F_2 lines	Anthers	N_6, modified N_6, H_5, modified H_5 and SK8	The response of different cultivars was different in 5 media indicating existence of genotypic variability	Shahjahan *et al*.(1985)
Indica cultivar	Anthers with Mid	B_5 + 2,4-D (1 mgl^{-1}) + BA (0.5 mgl^{-1}) + IAA	Heat treated at 35 °C for 15	Zapata and

IR-42	uninucleate to early bi nucleate microspores	(0.5 mgl^{-1}) or B_5 + Zeatin (1mgl^{-1}) + 2,4-D (2mgl^{-1}) + Picloram (0.07 mgl^{-1})	min increased significantly the callus induction in both media	Torrizo (1986)
Basmati 370	Anthers with uninucleate to early bi nucleate microspores	B_5 + 2,4-D (1 mgl^{-1}) + NAA (0.5 mgl^{-1}) + BA (0.5 mgl^{-1}) + sucrose (2 %) (liquid medium) MS + kinetin (1mgl^{-1}) + NAA (1 mgl^{-1}) + ABA (20 mgl^{-1}) for 4 weeks (semi solid medium) ABA free medium	20 K rad treatment of seeds of donor plants reduced the callus induction and albino plant regeneration No green plants were regenerated in control	Zapata *et al*.(1986b)

Taipei 309, IR-5, Basmati 370, Pankaj	Anthers	E_{10} + 2,4-D (1 mgl^{-1}) + BA (0.5 mgl^{-1}) + IAA (0.5 mgl^{-1}) + sucrose (2%) + glucose (5gl^{-1}) MS + NAA (1 mgl^{-1}) + Kin (1 mgl^{-1}) + sucrose (3%)	60 % of the callus forming anthers was plated on edge and 40% were flat. Most of the anther plated flat callused on both lobes. Most of the anthers plated on edge produced callus on the upper lobe	Mercy and Zapata (1987)
Taipei 309	Uninucleate microspores	E_{10}, E_{10} + alanine (1mM), E_{10} + glutamine (1mM), E_{10} + proline (1mM)	Glutamine and proline had stimulatory effects on pollen culture	Cho and Zapata(1988)
PR 106 x Jaya *O.sativa* x *O. rufipogon* *O.sativa x O. longistaminata*	Anthers	Potato-2 or N_6 + NAA or 2, 4-D (2 mgl^{-1}) + Kn (1 mgl^{-1}) + sucrose (6 %) + agar (0.75 %)	Potato-2 medium was superior to N_6 Early callusing when NAA was present instead of 2,4-D *O.sativa* x *O. longistaminata* gave no response	Rout *et al*.(1989)
17 Japonica, 31 Indica, 2 Indica x Japonica	Anthers	N_6 + NAA (1 mgl^{-1}) + agar (0.8 %) MS + Kin (3 mgl^{-1}) + NAA (0.5 mgl^{-1})	Japonica cultivars were more responsive than	Reiffers and Freire (1990);

F_1 heterotic hybrids WU 1OB	Anthers	N_6 + 2,4-D (2 mgl^{-1}) + sucrose (6 %) MSN or SK-1 + 2,4-D (0.7 mgl^{-1}) + NAA (2.5 mgl^{-1}) + Kin (0.5 mgl^{-1}) + sucrose (4 %)	Indicas; 27 % of the plants obtained were albinos	Hadi and Raina (1989)	Raina *et al.*(1989)
Taipei-309	Anthers with mid Uninucleate to early binucleate microspores	R_2, N_6 or MS with different combinations of 2,4-D and NAA (2.5 mgl^{-1}) + BA (0.5 mgl^{-1}) + sucrose (2 %)	Callus response was highest from main tiller microspores and anthers from primary tillers showed poor response	Karim and Reddy (1994)	
Indica cultivars CR666-34-3, SR 26-B, Basmati Sd-10, Pb 33	Anthers with mid Uninucleate to early binucleate microspores	N_6 + 2,4-D (2 mgl^{-1}) + NAA (0.5 mgl^{-1}) + Kin (0.75 mgl^{-1}) + sucrose (6 %)	Callusing frequency was higher in cultivars raised on N_6 medium than Basmati 370 and Basmati 2	Zapata (1990)	
Indica and Japonica Rasi, Crossa, Pokkali, Tellahemsa,Yerregaluvadhu, Mahsuri	Anthers with early to mid Uninucleate microspores	N_6 + 2,4-D (2 mgl^{-1}) + NAA (0.5 mgl^{-1}) + Kin (0.5 mgl^{-1}) + sucrose (6 %)	Addition of ground green plant tissue to the medium increased the frequency of callus induction	Kavi Kishor and Chengmei (1989)	
The F_1 hybrids of Indica x Japonica; TCA-2 x YVSD, Basmati D5 x Basmati, Mahsuri x Bala, TN-1 x Carreon	Anthers	R_2 + NAA (2 mgl^{-1}) + Kin (2 mgl^{-1}) N6 medium with hormones in 25 %) disposable Petri dishes or in glass jars LS + NAA (1 mgl^{-1}) + Kin (4 mgl^{-1}) + IAA (0.5 mgl^{-1}) MS+ Kin (2 mgl^{-1}) + NAA (0.5 mgl^{-1}) + sucrose (4%)	Regeneration was found to be suitable for callus induction and subculture 5 % sucrose gave better results and increased plant regeneration With an increase in time in culture, the ratio of green/albino plantlets did not change; however, the overall plant regeneration frequency was affected with the time in culture	Zhenghua (1990)	Courtois (1992)
Japonica x Japonica Indica x Indica Japonica-indica cv. Miara	Anthers with Uninucleate microspores Anthers with Uninucleate microspores	N_6M or N_6AK supplemented with MCPA, Kin, NAA with sucrose (4-5 %) or 5 %) N_6AK + NAA (2 mgl^{-1}) + Kin (0.5 mgl^{-1}) For Indica R_5 + Kin (2 mgl^{-1}) + NAA (0.5 mgl^{-1}) + sucrose (6 %) or for japonica R_6 + Kin (1 mgl^{-1}) + NAA (0.1 mgl^{-1}) + sucrose (4 %)	Plating of anthers on different media (250-300 mg/l) combinations (250-300 mg/l) combinations Regeneration of green plants was better in jam jars as against glass tubes Indica and japonica rice varieties showed differential response Maximum 2.9-3.5 fold increase in doubled haploid frequency was recorded in japonica cultivars	Abdullah and Taha (1990) Guiderdoni *et al.* (1992) Guiderdoni (1994)	Abdullah and Taha (1990) Guiderdoni *et al.* (1992) Guiderdoni (1994)
Japonica x Japonica Indica rice cultivars; F1 and F2 resulting from high yielding Indica rice x Basmati cultivars	Anthers Anthers with Uninucleate microspores	N_6 + NAA (1 mgl^{-1}) + sucrose (6 %) N_6 + 2,4-D (2 mgl^{-1}) + sucrose (3 %)	Varied green plants Addition of tryptophan (25 mgl^{-1}), use of mannitol (1%) along with sucrose (3%), cold pretreated (8 °C for 7 days) and culturing anthers from primary tillers enhanced anther callusing	Reiffers and Gosal (1991); Sandhu *et al.* (1993a,b)	Reiffers and Gosal (1991); Sandhu *et al.* (1993a,b)
Japonica x Japonica Jaya, Bangladesh cultivars BR-3, BR-7, BR-10, BR-14, BRRI-Dhan-29 and Hagbaengbyeo (Japonica) Somgaughbyeo (Tongil types)	Anthers with Uninucleate microspores Anthers	N_6 + 2,4-D (2 mgl^{-1}) + sucrose (3 %) Embedded in MS+ Kin (3 mgl^{-1}) supplemented with 2,4-D (0.5 mgl^{-1}) + NAA (2.5 mgl^{-1}) + Kin (0.5 mgl^{-1}) MS + Kin (0.5 mgl^{-1}) + BA (2 mgl^{-1}) + NAA (1 mgl^{-1}) + Kin (4 mgl^{-1}) + agar (1.2 %) Two step method N_6 Y-1 + NAA (2 mgl^{-1}) + Kin (1 mgl^{-1}) + agar (0.8 %) N_6 Y-1 + NAA (0.2 mgl^{-1}) + Kin (1 mgl^{-1}) + agar (0.8 %)	Haploid plants were obtained in the response of different cultivars Single step method was found superior for callus induction and plant regeneration Anther culture is a simple, quick and inexpensive method for obtaining haploids directly from anthers	Bhatt and Wani (1994); Chung *et al.*(1991)	Bhatt and Wani (1994); Chung *et al.*(1991)

Commercial hybrid IR-69690 developed by BRRI	Anthers with late-uninucleate microspores	MS + NAA (0.5 mgl^{-1}) + Kin (3 mgl^{-1}) + sucrose (50 gl^{-1}) + agar (8 gl^{-1})	Higher callus induction was found in a medium supplemented with 2,4-D (1mgl^{-1}) + NAA (2 mgl^{-1}) + Kin (1 mgl^{-1})	Islam *et al*.(2004)
3 Indica parents, two hybrids and One Japonica rice *viz*., IR 58025 A x IR 66 R and IR 58025 x C20 R, two Assam cultures , ARC 15759, ARC 18023 and one Japonica Taipei 309 and one best local variety ASD16	Anthers with Uninucleate microspores	Modified N_6 + 2,4-D (1.5 mgl^{-1}) + CW (5 %) + glucose (5 %) + maltose (50 %) MS + BA + CH + CW	The hybrid IR 58025 A x IR 66 R was found best for anther culture studies when media supplemented with 2,4-D (2.5 mgl^{-1}) + Kin (3 mgl^{-1})	Ramakrishnan *et al*.(2005)

III. MATERIALS AND METHODS

The present study entitled “Anther Culture Studies in Indica Rice (*Oryza sativa* L.)” was conducted at Plant Tissue Culture Laboratory, Department of Agricultural Biotechnology, Anand Agricultural University, Anand, India during 2005-2008.

The general procedure for initiation and maintenance of cultures and different analytical methods in this context are embodied in this chapter. Moreover, a brief description of materials *viz*., plant material as well as chemicals and glassware is also provided.

3.1 EXPERIMENTAL MATERIAL

Two rice cultivars, IR-58025 (CMS B-line) as parent P_1 and GR-7 as parent P_2 and their single cross hybrid (F_1, IR-58025 CMS A-line X GR-7(GRH-1)) used in this study were provided by Research Scientist (Rice), Main Rice Research Station (MRRS), Nawagam, A.A.U., Gujarat, India.

The plants were grown in pots with regular supply of water and fertilizer, under natural light conditions and a day and night temperatures of 35 ºC and 20 ºC, respectively and 70-80 % relative humidity during June – September, 2005 and June – September, 2006 in green house.

The salient features of these cultivars are as follows.

i) Gujarat 7 (GR-7) was released in 2001(Pedigree: GR-3 x Basmati-370), breeding method - hybridization followed by selection. It is an erect, non lodging type variety with strong culm, well exerted and compact panicles with tip awned spikelet and medium slender

kernels. Panicles are compact & well exerted, mild aroma, medium slender grain. It is an early maturing variety with 89 days for 50 % flowering having panicle length of 25 cm, Panicle height-112 cm, Effective tillers - 7, 1000 grain weight- 22 gm and Grain yield - 4500-6000 Kg/ha. It comprises fertility restorer gene and resistance against the major diseases *viz*., Bacterial leaf blight and Blast.

ii) IR-58025 is a cytoplasmic male sterile line (CMS).

Preliminary studies on various aspects were conducted using GR-7 and

IR-58025 (B-line). Where as, both these parents and their heterotic hybrid

(F_1: IR-58025 A x GR-7) was used for optimization. Normally healthy grown plants of the aforesaid cultivars/genotypes served as the source of anthers used throughout the study. (Plate-I, A)

3.2 CULTURE MEDIA

3.2.1 Chemicals

The chemicals used were of analytical grade (AR) and were obtained from Qualigens, Loba, SRL (Sisco Research Laboratory), E. Merck, Hi-Media, Partec (Germany) and Sigma chemical company (USA).

3.2.2 Culture vessels

According to the type of culture, “Borosil” make test tubes (150 x 25 mm, 200 x 25 mm) and “Yera” make small round bottles (size: 150 ml) were used. Before use, all vessels were cleaned with 40 per cent chromic acid (Potassium dichromate in concentrated sulphuric acid). The traces of acid were removed by thorough rinsing of glassware

under running tap water. Finally, all the glass containers were rinsed with single distilled water and dried in an oven at 60 °C for 3 hours.

3.3 PREPARATION OF MEDIA

In the present investigation several media *viz*. Chu *et al.,* 1975 (N_6 medium), Murashige and Skoog, 1962 (MS) and its modifications *viz*., Murashige and Skoog (1962) based callus induction medium designated as MS-1 and regeneration medium designated as MS-2, Nitsch and Nitsch, 1969 (N & N), White's, 1963 (WH) were tried as basal medium during different studies. Detailed composition of all these media is given in Table 3.1.

Separate stock solutions were prepared according to the composition of medium and were stored at 5 ºC. The compounds which precipitates in the stock solution were added fresh in the calculated quantity. Culture medium was prepared from concentrated stock solutions prepared in double Quartz distilled water and stored at 5 ºC. All the supplements were added in to the medium prior to the final adjustment of volume.

The pH of the medium was adjusted to 5.7 ± 0.1 on pH meter (Systronics, Model-μ pH system 361) using sodium hydroxide and hydrochloric acid of different concentrations as the case might be.

Table: 3.1 Composition of plant tissue culture media used as basal medium

(mg l^{-1})

	Constituents	N_6	MS	MS-1	MS-2	White's	N & N
1	**MACRO ELEMENTS**						
	Ammonium nitrate	-	1650	-	1650	-	720.0
	Potassium nitrate	2830	1900	3150	1900	80.0	950.0
	Calcium chloride	166.0	440	150	440	-	219.9
	Magnesium sulphate		370	185	370	750.0	185.0
	Pot. Dihydrogen-phosphate*	185.0	170	540	170	-	68.0
	Potassium chloride	400.0	-	-	-	65.0	-
	Sodium sulphate		-	-	-	200.0	-
	Sodium dihydrogen -orthophosphate	-	-	-	-	19.0	-
	Potassium sulphate	-	-	-	-	-	-
	Calcium nitrate tetra -hydrate	-	-	-	-	300.0	-
	Ammonium sulphate	-	-	-	-	-	-
		-					
		463.0					
2	**MICRO ELEMENTS**						
	Boric acid	1.6	6.20	6.0	6.2	1.5	10.0
	Potassium iodide	0.8	0.83	1.0	0.83	0.75	-
	Sodium molybdate	-	0.25	0.25	0.25	-	0.25
	Cobalt chloride	-	0.025	-	-	-	-
	Manganese sulphate	4.4	22.3	22.3	22.3	5.0	25.0
	Zinc sulphate	1.5	8.6	1.5	8.6	3.0	10.0
	Copper sulphate	-	0.025	0.02	0.02	0.01	0.025
	Molybdic acid	-	0.25	5	5	0.001	-
	Ferrous sulphate**	27.85	-	-	-	2.5***	27.85
	Na_2 EDTA	37.2	27.85	27.85	27.85	-	37.25

		5	5 37.25	37.25	37.25		
3	**VITAMINS**						
	Nicotinic acid	0.5	0.5	2.5	0.5	0.05	5.0
	Pyridoxine hydrochloric acid	0.5	0.5	2.5	0.1	0.01	0.5
	Thiamine hydrochloric acid	1.0	0.1	2.5	1.0	0.01	0.5
	Glycine	2.0	2.0	2.0	2.0	3.0	2.0
	Myo inositol*	-	100.0	100.0	100.0	-	100.0
	Biotin	-				-	0.05
	Folic acid	-	-	-	-	-	0.5
			-	-	-		

* Fresh addendum, ** The $FeSO_4.7H_2O$ is dissolved in approx. 50 ml distilled water. The Na_2.EDTA is dissolved in approx. 50 ml distilled water separately and heated and mixed (under continuous stirring) with $FeSO_4.7H_2O$ solution. After cooling the volume is adjusted to 250 ml. Heating and stirring result in a more stable Fe-EDTA complex

*** In White's (1963): Iron was supplied as ferric sulphate instead of ferrous sulphate.

3.4 STERILIZATION OF MEDIA AND CULTURE VESSELS

Fixed volumes of boiled medium after thorough dissolving of agar (0.9 %) were dispensed into culture vessels using auto dispensor (AES Laboratories, Model 71518-00). After closing with autoclavable plastic lids or polypropylene caps or cellulose bunks and aluminum foils, the vessels containing media were sterilized by autoclaving at a pressure of 15 lbs at 121 ºC temperature for 20 minutes. Petri plates and other tools *viz.* scalpels, spatula, forceps, scissors, metal needles were sterilized at same conditions for 30 minutes.

3.5 PREPARATIONS FOR INOCULATION

Aseptic conditions

All the operations related to tissue culture *viz*., surface sterilization, inoculation, sub-culturing etc. were carried out on a sterile laminar airflow hood (Klenzaids, India). The aseptic conditions in the clean zone (i.e. inoculation and culture room) were maintained with the use of air pressurizing module and ozonization of both the rooms using ozonizer at weekly interval. The vessels were exposed to ultra-violet (UV) light under laminar flow for 20 minutes to get the vessels surface sterilized. The hand tools used for inoculation and sub-culturing (scalpels, forceps, spatula, needles, scissors etc.) were frequently flame sterilized and subsequently sterilized using glass bead sterilizer (dent-eq Model no. BS-2000) during different operations.

3.6 PREPARATION OF EXPLANT

3.6.1 Anthers

i) Developmental Stage:

To determine the developmental stages of pollen/microspores for anther inoculation, Spikelets of rice cv. GR-7 and IR-58025 were collected from the green house when the distance between the base of the flag leaf and the auricle of the penultimate leaf was 5-9 cm (Plate-I, B). Spikelets were fixed in a carnoy's fluid (Ethyl alcohol: Glacial acetic acid (3:1)) mixture for 24 hrs., the anthers were cut open at the two ends and pollen were teased out of the anthers in a drop of 2 % aceto-carmine placed on a clean glass slide. A cover slip was dropped in to place and sealed with parafilm wax.

The collected boots were wrapped in plastic bags and kept in the refrigerator for cold pre-treatment at 7-8 ºC for 4, 12 and 22 days and were selected for the experimental work. Panicles/Boots were surface sterilized with the surface sterilent followed by 3 - 4 washes of sterile double distilled water.

ii) Inoculation of anthers

Panicles were surface sterilized and carefully isolated by opening the flag leaf with the help of sterilized needles. The spikelets in the top third and bottom third section of the panicle were rejected and only the spikelets in the middle section of the panicle having pollen at uni-nucleate stage were used for the study.

Each floret was cut from the base so that the filament of the anther was also cut. Thus carefully dissected excised out anthers using pointed needles under stereomicroscope (attached with fibre optic light) were placed on the medium.

Twenty to twenty five anthers per bottle were inoculated. All cultures were incubated at 25 ± 1 °C temperature and 60 - 80 per cent relative humidity under darkness using clean corrugated card board boxes for callus induction.

3.7 EXPERIMENTS

3.7.1 Pre-treatment of anthers:

To study the effect of cold pre-treatment, the collected main culms/primary types panicles were wrapped in plastic bags/muslin cloth

and kept in the refrigerator for cold pre-treatment at 7-8 ºC for 4, 12 and 22 days and were used for the experimental work. Effect of cold treatment on callus induction was studied using anthers of healthy grown plants of cv. GR-7 & IR-58025 (B-line).

3.7.2 Standardization of surface sterilization technique

Boots/Panicles of two genotypes (GR-7 and IR-58025) were subjected to surface sterilization treatments as follows:

S_1 – 0.1 % $HgCl_2$ for 3 minutes to spikelets

S_2 – 70 % Ethyl alcohol for 10 seconds to panicle + 0.1 % $HgCl_2$ for 3 minutes to spikelets

S_3 – 70 % Ethyl alcohol for 10 seconds to panicle + 0.1 % $HgCl_2$ for 5 minutes to spikelets

Observation for per cent contamination was recorded for each treatment.

3.7.3 Morphogenetic study:

3.7.3.1 Induction of callus

Effect of basal media, phytohormones and carbon source on callus induction was studied using anthers of healthy grown plants of cv. GR-7 and IR-58025(B-line).

Twenty anthers per treatment placed in a glass bottle were replicated
6 times on different callus induction media.

Auxin (2, 4-D) were incorporated in N_6 (Chu *et al.,* 1975) and MS-1 (MS - modified) medium along with 2 levels of sucrose (3 & 6 %) and 0.9 per cent agar. Three levels of 2, 4-D (1.0 mg l^{-1}, 1.5 mg l^{-1}, 2.0 mg l^{-1}) were studied. The details of treatments are as under:

Where, T_1 : 1.0 mg l^{-1} 2,4-D + 3 % Sucrose + 0.9 % Agar
T_2 : 1.5 mg l^{-1} 2,4-D + 3 % Sucrose + 0.9 % Agar
T_3 : 2.0 mg l^{-1} 2,4-D + 3 % Sucrose + 0.9 % Agar
T_4 : 1.0 mg l^{-1} 2,4-D + 6 % Sucrose + 0.9 % Agar
T_5 : 1.5 mg l^{-1} 2,4-D + 6 % Sucrose + 0.9 % Agar
T_6 : 2.0 mg l^{-1} 2,4-D + 6 % Sucrose + 0.9 % Agar

Observations *viz*., days to callus initiation, per cent callusing and callus color & callus texture was recorded.

3.7.3.2 Plantlet/Shoot regeneration:

Effect of basal media and various phytohormones on shoot induction was studied using 3-4 mm calli induced on N_6 + 2.0 mg l^{-1} 2,4-D + 6 % sucrose + 0.9 % agar from anthers of healthy grown plants of cv. GR-7 and IR-58025 (B-line).

Auxin (NAA - 1.0 mg l^{-1}) in combination with cytokinin (BA - 1.0 mg l^{-1}, Kn - 0.5 mg l^{-1}) were incorporated in MS and MS-2 (MS - modified) medium along with 3 per cent sucrose and 0.9 per cent agar. Observations *viz*., days to plantlet/shoot primordia initiation, per cent regeneration/calli differentiation, per cent green and albino plantlets regeneration, per cent rooting, per cent haploid and diploid plantlets and per cent survival were recorded.

3.7.4 OPTIMIZATION OF CULTURAL ENVIRONMENT

Effect of genotype, auxin:cytokinin and sucrose combination on callus induction and plantlet regeneration was carried out using all the three

cv. GR-7, IR-58025(B-line) and their hybrid GRH-1 as follows:

Code		Media
CI_1	-	N_6 + 6 % Sucrose + 0.9 % agar (Control)
CI_2	-	N_6 + 1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 5 % sucrose + 0.9 % agar
CI_3	-	N_6 + 1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 7 % sucrose + 0.9 % agar
CI_4	-	N_6 + 1.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 5 % sucrose + 0.9 % agar
CI_5	-	N_6 + 1.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 7 % sucrose + 0.9 % agar
CI_6	-	N_6 + 2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 5 % sucrose + 0.9 % agar
CI_7	-	N_6 + 2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 7 % sucrose + 0.9 % agar
CI_8	-	N_6 + 2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 5 % sucrose + 0.9 % agar
CI_9	-	N_6 + 2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 7 % sucrose + 0.9 % agar
CI_{10}	-	N_6 + 3.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 5 % sucrose + 0.9 % agar
CI_{11}		N_6 + 3.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 7 % sucrose + 0.9 % agar
CI_{12}		N_6 + 3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 5 % sucrose + 0.9 % agar
CI_{13}		N_6 + 3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 7 % sucrose + 0.9 % agar
CI_{14}		N_6 + 2.0 mg l^{-1} 2,4-D + 1.5 mg l^{-1} NAA + 0.5 mg l^{-1} Kn + 6% sucrose + 0.9 % agar
CI_{15}		N_6 + 2.0 mg l^{-1} 2,4-D + 2.5 mg l^{-1} NAA + 0.5 mg l^{-1} Kn + 6 % sucrose + 0.9 % agar

Twenty five anthers were inoculated per treatment in a glass bottle replicated 9 times on above callus induction media. Observations

viz., days to callus initiation, per cent callusing, callus color and callus texture were recorded.

Calli (3-4 mm) were placed on regeneration medium for complete plantlet regeneration. Callus mass from each media combination (CI_2 to CI_{15}) was inoculated on different auxin-cytokinin combinations used to study regeneration of shoot as under:

Code		Media
R_1	-	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
R_2	-	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
R_3	-	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
R_4	-	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
R_5	-	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
R_6	-	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
R_7	-	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
R_8	-	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar

The details about the sequential transfer are as under:

Callus induction		Shoot Regeneration	
Code	Callus Induction Media	Code	Shoot Regeneration Media
Cl_3	N_6 + 1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 7 % sucrose + 0.9 % agar	R_1	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_2	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_3	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_4	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_5	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_6	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_7	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_8	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
Cl_5	N_6 + 1.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 7 % sucrose + 0.9 % agar	R_1	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_2	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_3	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_4	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_5	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_6	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_7	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar

		R_8	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar

Cl_6	N_6 + 2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 5 % sucrose + 0.9 % agar	R_1	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_2	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_3	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_4	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_5	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_6	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_7	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_8	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
Cl_7	N_6 + 2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 7 % sucrose + 0.9 % agar	R_1	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_2	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_3	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_4	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_5	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_6	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_7	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar

		R_8	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar

Cl_8	N_6 + 2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 5 % sucrose + 0.9 % agar	R_1	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_2	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_3	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_4	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_5	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_6	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_7	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_8	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
Cl_9	N_6 + 2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 7 % sucrose + 0.9 % agar	R_1	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_2	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_3	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_4	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_5	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_6	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_7	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar

		R_8	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar

CI_{10}	N_6 + 3.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 5 % sucrose + 0.9 % agar	R_1	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_2	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_3	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_4	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_5	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_6	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_7	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_8	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
CI_{11}	N_6 + 3.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 7 % sucrose + 0.9 % agar	R_1	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_2	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_3	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_4	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_5	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_6	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_7	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar

		R_8	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar

Cl_{12}	N_6 + 3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 5 % sucrose + 0.9 % agar	R_1	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_2	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_3	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_4	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_5	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_6	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_7	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_8	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
Cl_{13}	N_6 + 3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 7 % sucrose + 0.9 % agar	R_1	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_2	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_3	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_4	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_5	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_6	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_7	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar

		R_8	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar

CI_{14}	N_6 + 2.0 mg l^{-1} 2,4-D + 1.5 mg l^{-1} NAA + 0.5 mg l^{-1} Kn + 6 % sucrose + 0.9 % agar	R_1	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_2	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_3	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_4	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_5	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_6	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_7	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_8	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
CI_{15}	N_6 + 2.0 mg l^{-1} 2,4-D + 2.5 mg l^{-1} NAA + 0.5 mg l^{-1} Kn + 6 % sucrose + 0.9% agar	R_1	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_2	MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_3	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_4	MS + 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_5	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_6	MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar
		R_7	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar

		R_8	MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar

1. The callus of GRH-1 (IR-58025 A x GR-7) induced on CI_1, CI_2, CI_4 and CI_{15} were too small showing necrosis and thus could not be transferred to regeneration medium.
2. The callus of IR-58025 (B-line) induced on CI_1, CI_2, CI_3, CI_4, CI_5, CI_{14} and CI_{15} could not be taken for sequential transfer due to less number of calli induced.
3. The callus of GR-7 induced on CI_1, CI_2, CI_3, CI_4, CI_5, CI_{10} and CI_{12} media could not be taken for sequential transfer due to less number of calli induced and were too small showing necrosis and thus could not be transferred to regeneration medium.

Observations on days to plantlet/shoot primordia initiation, per cent regeneration/calli differentiation, per cent green and albino plantlets regeneration and per cent haploid plantlets were recorded.

3.8 *IN VITRO* ROOTING

Shoots regenerated on all different regeneration medium were subjected to various combinations of three basal media (½ MS, N & N and White's) for induction of rooting. The treatment combinations are as under:

Code		Media
P_1	-	½ MS + 0.9% agar
P_2	-	N & N + 1% sucrose + 0.9% agar
P_3	-	White's + 2% sucrose + 0.9% agar

Observations on percentage rooting were recorded for each treatment.

3.9 PLOIDY CONFIRMATION:

3.9.1 Cytological methods:-

To determine/check the ploidy status of anther culture derived plantlets; actively growing roots were collected from in *vitro* rooted plantlets at an appropriate timing and pre-treated with 0.1 % (w/v) colchicine for 1 hour & saturated Para dichlorobenzene for 3 hours by addition of a small pinch of esculin in it (Incubated at 4 ºC for 10 min. then shifted to 7-8 ºC for 1 or 3 h. respectively). Roots were washed thoroughly with distilled water after pre-treatment and fixed in carnoy's fluid (Ethyl alcohol: Glacial acetic acid (3:1)) mixture for 24 h, washed the roots once in distilled water, and hydrolysis of root tips was carried out by incubating them in 1 N HCl at 60 ºC for 10-15 min. These hydrolyzed roots were placed on different clean glass slides and root cap was removed with a razor blade. Less than 1 mm of root tip (milky white region only) was placed in a drop of aceto carmine stain (2 %) with ferric acetate and slides were prepared by making a squash preparation.

3.9.1.2 Photomicrography:-

Sony made digital camera (7.5 Mega pixels) was used to take photographs of the culture vessels showing growth, morphogenesis and differentiation.

Axioskop 2 mot (Zeiss) microscope plus with fluorescent attachment with CCD camera (Cohu make) fitted with x10 or x15 projection eyepiece and 10/0.3, 45/0.65 or 100/ (oil emersion) objectives, Karyotype software (Biocis) and Computer system (IBM)

were used to analyze the preparations and microphotographs were taken with the help of camera mounted on the microscope.

3.9.2 Ploidy analyzer:

Plant material:

Young leaves from anther culture raised plantlets were used for this

study.

Sample preparation:

To check the DNA ploidy status of the anther culture derived plantlets, approximately 0.5 cm^2 leaf tissues were chopped with sharp razor blade in partec (Germany) make extraction buffer (400 µl) and nuclei suspension was filtered through a cell-Trics® disposable filter directly in to the sample tube, these suspension was incubated 0.5 to 1.0 min. and later partec make staining buffer (1600 µl) was added in to the sample tube and were finally analyzed with UV excitation in Partec Ploidy analyzer (PA-II, model no. 02-2-2000, Partec GmbH, Munster, Germany).

DNA ploidy analysis:

Relative fluorescence intensity of stained nuclei was analysed using a ploidy analyzer (PA-II). Traditionally, relative DNA content is given in C units. The 1C value is DNA content of haploid set of chromosomes (n). The distribution of fluorescence intensities (relative DNA content) obtained after flow cytometric analysis is usually in arbitrary units (channel numbers). For ploidy screening, this scale must be calibrated with a reference. In this study, a sample prepared from

rice (2n=2x=24) as a diploid reference and ploidy analyzer was adjusted so that the peak representing its G_1 nuclei appeared at channel 400. This setting was kept constant and other samples were characterized by the relative position of their G_1 peak.

Here, the unwanted noise signal was cut off by increasing the Lower Level

(L-L). The peak for the lowest ploidy level (i.e. the peak on the left) was placed on lower channel 100 by varying the gain value. U-L (Upper Level) was filtered out unwanted signals from the higher channels. No signals above this threshold were measured. Where as with L-L (Lower Level) filtered out unwanted noise signals. No signals below this threshold were measured.

DNA ploidy analysis using external standard:-

The instrument was calibrated using nuclei isolated from a plant with known ploidy, e.g. 2n (the position of the RN_1 peak is recorded). All other samples were characterized by the relative position of their RN_1 peaks. Units were thus "C-values".

DNA ploidy analysis using internal standard:-

The nuclei of the standard with known ploidy and the nuclei of the sample were isolated, stained and analysed simultaneously. The DNA ploidy of the sample is then estimated using the ratio of RN_1 peak (units were "C-values")

3.9.3 Anatomical method:-

Estimation of ploidy level by stomatal area and stomatal density:

Chromosome counting in mitotic cells of root tips is an accurate procedure to determine the ploidy, but it is time consuming and requires much experience. Therefore, indirect methods for ploidy determination were found helpful. Ploidy determination using stomatal measurements, *viz.*, area and density of stomata used as criteria in the distinction between haploid and diploid/doubled haploid plants

The ploidy level of the plants regenerated from anther culture was estimated by stomatal guard cells measurements. The stomata were measured from imprint of the upper leaf surface, obtained by painting FEVICOL® (adhesive) on the leaf surface, allowing it to dry and peeling it off (Hamill *et al*., 1992). The imprint was laid on a clean microscope slide to allow the analysis of the guard cells. The stomata were measured at 40 x magnification under an Olympus microscope equipped with camera. In order to obtain mean stomatal area, twenty stomata per plant were measured. The measurements were recorded using µm unit. The stomata density average was calculated for each plant by counting stomata in 10 fields of view (124929 μm^2). The stomatal area and density were evaluated using the Image Pro express 6.0 software.

3.10 ACCLIMATIZATION

The rooted plantlets were removed from test tubes for hardening. Plantlets were washed thoroughly in distilled water to remove semi solid media. The plantlets were then subjected to a sequential treatment of 200 ppm Blitox solution (w/v) for 2 minutes, 200 ppm Bavistin solution (w/v) for 2 minutes and 2 - 3 washes with sterile water.

In first two treatments plants were kept into plastic cups containing different proportion of soil rite (Synthetic product of Karnataka Explosive Ltd., Bangalore as a substitute of soil) and soil. The two different potting mixtures soil alone, soil + soil rite (3:1) were sterilized at 1.0 kg/cm^2 for 60 minutes. Each cup was added with 5 ml solution of 200 ppm Ridomil (w/v), 5 ml single distilled water and spray of 100 ppm Carbendezim solution (w/v) on plant. These cups covered with transparent plastic bags and were kept in diffused light. Plastic bags were replaced with new one whenever moisture deposition was found inside the bags.

In the third treatment, plantlets were placed in nutrition water (White's medium (1963) - excluding vitamins) for 3 to 4 days under diffuse light condition, and then transferred for 10 to 15 days under controlled humidity (85 – 90%), temperature (25 ± 1 ºC) and light conditions (35 µmol m^{-2}s^{-1}, 16 h photoperiod) When the young plantlets formed new root system, they were transplanted into soil under 50 % shading then after under natural condition.

Observation on number of plantlets survived up to primary and secondary hardening was recorded.

3.11 OBSERVATIONS RECORDED DURING INVESTIGATION

(1) Percentage contamination (%):

Percentage contamination was recorded after one month of anthers inoculation as the number of bottles contaminated by microorganisms to the total number of bottles inoculated and expressed as percentage contamination.

(2) Days to callus initiation:

From the date of inoculation of anthers to days to initiation of callus was counted as days to callus initiation.

(3) Percentage callusing (%):

Cultures were scored for percentage of callus induction. The percentage callusing was calculated as the ratio of the number of anthers initiated callus to the total number of anthers inoculated and expressed as percentage callusing.

(3) Texture and colour of callus:

The morphological appearance of the callus was recorded by judging visually callus type (i.e. compact, friable or loose). Colour of callus was recorded at the end of callus induction (Y - Yellowish, White - W, Cream - Cr).

(4) Days to plantlet/shoot primordia initiation:

From the date of inoculation of callus to days to initiation of shoot primordia was counted as days to plantlets/shoot initiation.

(5) Per cent calli differentiation (%):

Per cent calli differentiation indicates the number of calli regenerated/differentiated compared to number of calli inoculated for regeneration.

(6) Per cent plantlet regeneration (%):

Per cent plantlet regeneration indicates the number of plantlets compared to number of calli inoculated for regeneration/shoot induction.

(7) Percentage green plantlets regeneration (%):

Percentage green plantlets regeneration indicates the number of green plantlets compared to number of calli inoculated for regeneration/shoot induction**.**

(8) Percentage albino plantlets regeneration (%):

Percentage albino plantlets regeneration indicates the number of albino plantlets compared to number of calli inoculated for regeneration/shoot induction**.**

(9) Anther culture capacity (%):

Anther culture capacity indicates the number of plantlets compared to number of anthers inoculated.

(10) Percentage rooting (%):

Percentage rooting calculated as the number of rooted plantlets compared to number of shoots inoculated for root induction**.**

(11) Percentage haploid plantlets (%):

Percentage haploid plantlets calculated as the number of haploid plantlets compared to total number of plantlets emerged/studied

3.12 STATISTICAL ANALYSIS

The experiments were laid in completely randomized design. There were experiments comprising different levels of more than one factor. The mean data for each treatment were subjected to statistical analysis as per CRD (factorial) suggested by Snedecor and Cochran (1963). The form of ANOVA for 1, 2 and 3 factor CRD analysis are given in Table 3.12.1, 3.12.2 and 3.12.3, respectively.

Table: 3.12.1 (A) Structure of ANOVA for individual equal CRD

Source of variation	d.f.	MS	Cal “F”
Treatments	(a – 1)	Ma	Ma / Me
Error	a (r-1)	Me	-

Table: 3.12.1 (B) Structure of ANOVA for individual unequal CRD

Source of variation	d.f.	MS	Cal “F”
Treatments	(a – 1)	Ma	Ma / Me
Error	(r - a)	Me	-

Table: 3.12.2 Structure of ANOVA for 2 factor CRD

Source of variation	d.f.	MS	Cal “F”
Factor A	(a – 1)	Ma	Ma / Me
Factor B	(b – 1)	Mb	Mb / Me
Ax B interaction	(a – 1) (b – 1)	Mab	Mab / Me
Error	ab (r-1)	Me	-

Factor A: Genotypes, Factor B: Treatments

Table: 3.12.3 Structure of ANOVA for 3 factor CRD

Source of variation	d.f.	MS	Cal “F”
Factor A	(a – 1)	Ma	Ma / Me
Factor B	(b – 1)	Mb	Mb / Me
Factor C	(c – 1)	Mc	Mc / Me
Ax B interaction	(a – 1) (b – 1)	Mab	Mab / Me
A x C interaction	(a – 1) (c – 1)	Mac	Mac / Me
B x C interaction	(b – 1) (c – 1)	Mbc	Mbc / Me

A x B x C interaction	(a – 1) (b – 1) (c – 1)	Mabc	Mabc / Me
Error	abc (r-1)	Me	-

Factor A: Genotypes, Factor B: Basal media, Factor C: Treatments

Transformation of data was carried out whenever needed with appropriate transformation scale.

IV. RESULTS AND DISCUSSION

In the present investigation, attempts were made to study the response of cold pre-treatment, effect of different basal media in combination with phytohormones and carbon source on *in vitro* callus induction, organogenesis, genotypic response for callus induction as well as for plantlet regeneration, *in vitro* root induction, acclimatization and production of haploid plantlets using anthers of two parents and its F_1 (hybrid). Various experiments were planned to study all these aspects and results of each of these aspects are presented and discussed under the following heads.

4.1 Appropriate stage (cytological and morphological) of anther for *in vitro* studies

4.2 Surface sterilization

4.3 Cold pre-treatment

4.4 Media response for *in vitro* callusing

4.5 Optimization of cultural environment

4.6 *In vitro* rooting

4.7 Cytological and anatomical study of anther derived plantlets

4.8 Acclimatization

4.9 Morphological differences between anther culture derived haploid and diploid/doubled haploid plants

4.1 APPROPRIATE STAGE (CYTOLOGICAL AND MORPHOLOGICAL) OF ANTHER FOR *IN VITRO* STUDIES

As per the literature, main culm and primary panicles have been found appropriate showing maximum frequency of uninucleate stage of pollen, which is most suitable stage of microsporogenesis giving highest *in vitro* response for production of haploids. Accordingly, main culm/primary types of panicles were selected for the present study. As rice is a basipetal in flower opening nature, panicles were selected when the distance between the base of the flag leaf and the auricle of the penultimate leaf was 5-9 cm (Plate-I, B). All florets of selected panicle were examined for anther stage. The middle part of the collected panicles had high proportion of anthers with microspores of uninucleate stage (Plate-I, C). Additionally, when blooming reached up to

1/2 - 1/3 of spikelets length contained a high proportion of microspores of uninucleate stage and found as a morphological indicator for selection of the panicle for anther culture studies.

These results are in conformity with Tsay *et al*., 1988; Chen and Chen, 1979; Oono, 1975; Chaleff and stolarz, 1981; Chung and Sohn, 1995 and Mercy and Zapata, 1986.

Reiffers and Freire (1990) also established a relationship between the microspore stage and the panicle morphology. At the late uninucleate stage (when the distance between the base of the flag leaf and the auricle of the last leaf was 3-6 cm) anthers were used for culture.

Dhaliwal *et al*. (1997) also reported the comparative study of microspore stage and panicle morphology. Which was same as found in this study.

Mercy and Zapata (1986) reported pollen development stage and its relation to auricle distance in two rice varieties. In Taipei-309, panicles with
7.0 cm auricle distances were as efficient as those with 8.5 cm distance in Kulu.

4.2 SURFACE STERILIZATION OF PANICLES AND SPIKELETS:

Proper surface sterilization of the spikelets before inoculation of anthers is essential to avoid the external contaminants, and establish contamination free cultures.

Analysis of variance for effect of genotypes (G) and surface sterilization treatments (S) on per cent contamination (Table 4.2.1) revealed significant differences for surface sterilization treatments indicating the real differences in the effect of different surface sterilization treatments. However, non-significant genotypic effect indicated equal response of genotypes to different treatments. Significant genotypes x surface sterilization treatment interaction effects further indicated genotype specific surface sterilization treatment.

Table: 4.2.1 Analysis of variance for effect of genotypes (G) and surface sterilization treatments (S) on per cent contamination

Source	d.f.	Mean squares
Genotypes (G)	1	0.382
S.S. Treatments (S)	2	3347.29*
G x S	2	318.68*
Error	30	20.49

*Significant at 5% level of significance.

Per cent contamination ranged from 0.00 to 50.00 per cent (Table 4.2.2). Among the various treatments, S_3 reduced the contamination effectively

(2.50 %) as compared to other treatments.

Table: 4.2.2 Effect of genotypes (G) and surface sterilization treatments (S) on per cent contamination [a]

Treatments / Genotypes	S_1	S_2	S_3	Mean
IR-58025 (B-line)	34.98 (33.33)	23.88 (16.66)	11.89 (5.0)	23.58 (18.33)
GR-7	44.98 (50.00)	23.88 (16.66)	1.28 (0.00)	23.38 (22.22)
Mean	39.98 (41.66)	23.88 (16.66)	6.58 (2.50)	
Range			0.00 to 50.00	
S.Em. (Genotype)			1.06	
C.D. at 0.05 (Genotype)			NS	
S.Em. (Treatment)			1.30	
C.D. at 0.05 (Treatment)			3.77	
S.Em. (Interaction)			1.84	
C.D. at 0.05 (Interaction)			5.33	
C.V. %			19.28	

a – Data transformed through Arc sin transformation.
( ) – Values in parenthesis are original mean values.

Among the six genotype x surface sterilization treatment combinations, treatment S_3 (70 % Ethyl alcohol for 10 seconds to panicles + 0.1% $HgCl_2$ for

5 minutes to spikelets) found superior (2.50 %) among all the treatments across the genotypes, followed by S_2 (70 % Ethyl alcohol for 10 seconds to panicles + 0.1% $HgCl_2$ for 3 minutes to spikelets) and S_1 (0.1% $HgCl_2$ for 3 minutes to spikelets). Both the genotypes were at par

for per cent contamination across the treatments. In general, surface sterilization treatment S_3 using 70 % Ethyl alcohol for 10 seconds to panicles + 0.1% $HgCl_2$ for 5 minutes to spikelets was found most effective for both the genotypes. The results of the present study are in agreement with the results of Narasimman *et al*. (1994).

Krishna Raj and Shree Rangasamy (1993) also reported surface sterilization of panicles with 70 % ethanol for 30 seconds followed by 0.1 % $HgCl_2$ for 10 minutes. Balachandran *et al*. (1990) and Bishnoi *et al*. (2000) also used 70 % ethanol followed by 0.1 % $HgCl_2$ as a surface sterilization agent.

In contrast to this Reiffers and Freire (1990) surface sterilized panicles in a 10 % sodium hypochlorite (NaOCl) solution for 15 min. and rinsed three times in sterile distilled water.

4.3 COLD PRE-TREATMENT

(a) Per cent callusing:

Pre-treatment of panicles before inoculation of anthers is essential to alter the normal gametophytic pathway of pollen to sporophytic pathway, for induction of callus (Sunderland *et al*., 1984).

Analysis of variance for effect of genotypes (G) and cold pre-treatments (C) on per cent callusing revealed non-significant differences for cold pre-treatment indicating that the effect of various treatments for efficient callus induction was at par (Table 4.3.1). Genotypes and genotypes x cold pre-treatment interaction effects were also non-significant. This also

indicated that there was no genotype specific effect of different cold pre-treatments.

Table: 4.3.1 Analysis of variance for effect of genotypes (G) and cold pre-treatments (C) on per cent callusing

Source	d.f.	Mean squares
Genotypes (G)	1	1.820 NS
Cold pre-treatments (C)	2	2.161 NS
G x C	2	0.114 NS
Error	30	0.774

Non-Significant at 5% level of significance

Per cent callusing ranged from 0.00 to 3.33 per cent (Table 4.3.2). Among three cold pre-treatments studied, C_2 (12 Days cold pre-treatment to panicles at 7 ± 1 ºC) exhibited better callusing (2.49 %) across the genotypes. Among genotypes, studied IR-58025 showed better response to all three cold pretreatments under study. Present study suggested that 12 days cold pretreatment to panicles at 7 ± 1 ºC found most effective for callusing.

Table: 4.3.2 Effect of genotypes (G) and cold pre-treatments (C) on per cent callusing [a]

Treatments / Genotypes	C_1 4 Days	C_2 12 Days	C_3 22 Days	Mean
IR-58025	0.561 (0.83)	1.573 (3.33)	0.899 (1.66)	1.011 (1.94)
GR-7	0.224 (0.00)	0.898 (1.66)	0.561 (0.83)	0.561 (0.83)
Mean	0.393 (0.41)	1.236 (2.49)	0.729 (1.24)	
Range			0.00 to 3.33	
S.Em. (Genotype)			0.207	
C.D. at 0.05 (Genotype)			NS	
S.Em. (Treatment)			0.254	
C.D. at 0.05 (Treatment)			NS	
S.Em. (Interaction)			0.359	
C.D. at 0.05 (Interaction)			NS	
C.V. %			111.93	

a – Data transformed through square root transformation.
( ) – Values in parenthesis are original mean values.

Chen *et al*. (1982) also reported intermediate durations (8 to 15 days) of cold pre-treatment better than shorter ones (2 to 4 days). However, prolonged cold pre-treatment (beyond 15 days) appeared affecting callus differentiation. The results of the present study are in conformity with the findings of Chen *et al.* (1982).

The increase in efficiency due to cold shock treatment might be due to the synchronization of the pollen grains, retardation of senescence of somatic tissues, amino acids levels, induction of the pollen mother cell to produce two identical nuclei instead of one vegetative and one generative nucleus. (Zapata *et al*., 1982)

Cold pretreatment reported to have the advantage of reducing the percentage of abnormal microspores (Qu and Chen, 1983).

Fang and Liang (1985) have observed that cold pretreatment delayed the degeneration of tapetum and the middle layer of anther wall and stimulate the development of epidermis and endothecium. They also suggested that the cold pretreatment provided enough time for anther wall tissue to supply microspore with substances needed for androgenesis.

Xie *et al*. (1997) reported that a least requirement of 12 days for cold pretreatment of panicles at 6 ± 1 °C for inducing androgenesis and microspore responded well to 17-27 days of pretreatment at 6 ± 1 °C. The cold pretreatment time in their study was mostly longer than in

previous reports (Chen, 1986; Jia *et al.,* 1987; Cho and Zapata, 1988; Matsusima *et al.,* 1988)

(b) Days to callus initiation:

The mean values for days to callus initiation using single callus induction media (N_6 + 2.0 mg l^{-1} 2,4-D + 6 % Sucrose) with different genotypes are presented in Table 4.3.3.

Table: 4.3.3 Effect of genotypes (G) and cold pre-treatments (C) on days to callus initiation [a]

Cold pre-treatment	Genotype		Mean
	IR-58025	GR-7	
C_1 (4 days at 7 ±1 ºC)	60.00	NCI	60.00
C_2 (12 days at 7 ± 1 ºC)	50.75	56.50	53.62
C_3 (22 days at 7 ± 1 ºC)	59.00	65.00	62.00
Mean	56.58	60.75	

[a] Values in the table are original mean values
NCI- No Callus Induction

Days to callus initiation ranged from 50.75 to 65.0 days for six combinations studied. Among the various cold pre-treatment studied, C_2 exhibited minimum days (53.62) to callus initiation whereas, cold pre-treatment C_3 revealed maximum days (62.0) to callus initiation.

Both genotypes *viz*., IR-58025 and GR-7 exhibited early induction of callus in C_2 treated explants.

Mercy and Zapata (1987) also reported that callus induction was started 40 days after inoculation/plating of anthers.

This also suggested the suitability of intermediate duration of cold pretreatment for early callusing. Very short or very long exposure to cold pretreatment induced late callusing.

4.4 MEDIA RESPONSE FOR *IN VITRO* CALLUSING:

Production of doubled haploid plants in rice anther culture usually takes three developmental processes, *viz.*, dedifferentiation of microspore (callus induction), differentiation of callus (plant regeneration), and maturation of plantlets. Nutritional and hormonal requirement for all these three steps are different (Chu *et al*., 1998; Chu and Zhang, 1988).

(a) *Per cent callusing*

In vitro response of anthers of different genotypes, basal media, auxin and sucrose levels to callusing is presented in Table 4.4.1 to 4.4.2

The present investigation was initiated with an objective to study effect of various basal media *viz.,* N_6 and MS-1, auxin (2, 4-D) levels and sucrose levels in all possible combinations for various aspects of callusing using anthers of rice cv. IR-58025 and GR-7.

Analysis of variance revealed significant differences among the treatments (Auxin: Sucrose combinations) and basal media for percentage callusing, indicating real differences between treatments as well as between basal media. However, differences were non-significant for genotypes, genotypes x basal media, genotypes x treatments, basal media x treatments and genotypes x basal media x treatments which indicated consistence performance of genotypes and also of treatments across the genotypes and basal media for callusing. (Table 4.4.1)

Narasimman *et al*. (1994) also reported significant effect of treatments and media for callus induction as well as plantlet regeneration.

Table: 4.4.1 Analysis of variance for effect of genotype (G), different basal media (M), Treatments (T) and their interaction *viz.*, G x M, G x T, M x T and G x M x T on per cent callusing

Source	d.f.	Per cent callusing
		Mean Square
Genotype (G)	1	0.671
Basal Media (M)	1	1.863*
Treatments (T)	5	2.490*
G x M	1	0.298
G x T	5	0.179
M x T	5	0.388
G x M x T	5	0.075
Error	120	

* Significant at 5 % level of significance

Table: 4.4.2 Effect of genotype (G), different basal media (M), Treatments (T) and their interaction *viz.*, G x M, G x T, M x T and G x M x T on per cent callusing

(a) Effect of genotype (G) [a]:

Genotypes	IR-58025	GR-7
Mean	1.00 (0.90)	0.86 (0.48)
	S.Em. – 5.665	C.D. at 0.05 - NS

[a] Data transformed through square root transformation
( ) – Values in parenthesis are original mean values.

(b) Effect of basal media (M) [a]:

Basal media	N_6	MS-1
Mean	1.04 (1.04)	0.82 (0.34)
	S.Em. – 5.665	C.D. at 0.05 – 0.157

[a] Data transformed through square root transformation
( ) – Values in parenthesis are original mean values.

(c) Effect of growth regulator (2,4-D) in combination with carbon source
(sucrose) treatments (T)[a]

Treatment	T_1	T_2	T_3	T_4	T_5	T_6
Mean	0.70 (0.0)	0.70 (0.0)	0.91 (0.62)	0.70 (0.0)	1.04 (1.04)	1.52 (2.49)
	S.Em. – 9.812		C.D. at 0.05 – 0.271		C.V. % - 51.43	

[a] Data transformed through square root transformation
( ) – Values in parenthesis are original mean values

(d) G x T interaction effect[a]:

Treatments	Genotype	
	IR-58025	GR-7
T_1	0.70 (0.00)	0.70 (0.00)
T_2	0.70 (0.00)	0.70 (0.00)
T_3	0.98 (0.83)	0.84 (0.41)
T_4	0.70 (0.00)	0.70 (0.00)
T_5	1.25 (1.66)	0.84 (0.41)
T_6	1.66 (2.91)	1.39 (2.08)
	S.Em. – 0.138	C.D. at 0.05 - NS

[a] Data transformed through square root transformation
( ) – Values in parenthesis are original mean values.

(e) G x M interaction effect [a]:

Basal Media	Genotype	
	IR-58025	GR-7
N_6	1.16 (1.38)	0.93 (0.69)
MS-1	0.84 (0.41)	0.79 (0.27)
	S.Em. – 8.011	C.D. at 0.05 – NS

[a] Data transformed through square root transformation
( ) – Values in parenthesis are original mean values.

(f) M x T interaction effect[a]:

Basal media Treatments	N_6	MS-1

T_1	0.70 (0.00)	0.70 (0.00)
T_2	0.70 (0.00)	0.70 (0.00)
T_3	1.11 (1.24)	0.70 (0.00)
T_4	0.70 (0.00)	0.70 (0.00)
T_5	1.25 (1.66)	0.84 (0.41)
T_6	1.79 (3.33)	1.25 (2.08)
	S.Em. – 0.138	C.D. at 0.05 – NS

[a] Data transformed through square root transformation
( ) – Values in parenthesis are original mean values.

(g) G x M x T interaction effect[a]:

Treatments \ Basal Media	Genotype			
	IR-58025		GR-7	
	N_6	MS-1	N_6	MS-1
T_1 (1.0 mg l^{-1} 2,4-D + 3 % Sucrose)	0.70 (0.00)	0.70 (0.00)	0.70 (0.00)	0.70 (0.00)
T_2 (1.5 mg l^{-1} 2,4-D + 3 % Sucrose)	0.70 (0.00)	0.70 (0.00)	0.70 (0.00)	0.70 (0.00)
T_3 (2.0 mg l^{-1} 2,4-D + 3 % Sucrose)	1.25 (1.66)	0.70 (0.00)	0.98 (0.83)	0.70 (0.00)
T_4 (1.0 mg l^{-1} 2,4-D + 6 % Sucrose)	0.70 (0.00)	0.70 (0.00)	0.70 (0.00)	0.70 (0.00)
T_5 (1.5 mg l^{-1} 2,4-D + 6 % Sucrose)	1.52 (2.50)	0.98 (0.83)	0.98 (0.83)	0.70 (0.00)
T_6 (2.0 mg l^{-1} 2,4-D + 6 % Sucrose)	2.07 (4.16)	1.25 (1.66)	1.52 (2.50)	1.25 (1.66)
	S.Em. – 0.196		C.D. at 0.05 – NS	

[a] Data transformed through square root transformation
( ) – Values in parenthesis are original mean values.

The mean values for percentage callusing ranged from 0.0 to 4.16 % (Table 4.3.2(g)). Per cent callusing was at par for both the genotypes (Table 4.4.2 (a)). Between the two basal media, *viz.*, N_6 and MS-1, N_6 showed significantly higher per cent callusing. These results are in agreement with Chu (1982); Lenka and reddy (1994) who also reported that N_6 media was better than other media for callus induction. Narasimman *et al*. (1994) also reported superiority of N_6 media for higher callusing.

Among the treatments, T_6 (2.0 mg l^{-1} 2,4-D + 6 % Sucrose + 0.9 % Agar) showed significantly higher callusing (2.49 %). Higher callusing

with increase in auxin (2,4-D) concentration along with higher sucrose in the present study was also observed earlier by Balachandran *et al*. (1990) who reported more callusing at moderate levels of 2,4-D with higher concentration of Sucrose.

In contrast to higher sucrose concentration, Sun and Zhao (1992) reported maltose (5 %) increased callus induction by up to 10 fold.

In general, the N_6 medium gave better results for callus induction than MS-1. The better callusing in N_6 medium might be due to presence of ammonium sulphate, which was absent in MS-1 medium, lower concentration of vitamins in N_6 medium as compared to MS-1, as well as different sources of nitrogen (Halperin and Wetherel, 1965), concentration of NH^{4+} ions and the ratio between NH^{4+} and NO^{3-} ions (Chu *et al*., 1975).

The superiority of N_6 medium as compared to MS, Blayed's, SK-8 and R_2 was also reported by Chu, 1982; Lenka and Reddy, 1994.

Raina and Zapata (1997) reported that the use of N_6 medium with 31-34 mM KNO_3 and 2.0-2.5 mM of $(NH_4)_2$ SO_4 in the callus induction medium, appeared to be suitable for realizing higher frequency of microspore derived green plants in the cv. 'IR-43'.

In contrast to this, N_6 medium also had difficulty to apply for universal genotype, especially for indica type (Chen, 1986). In the evaluation of basal medium for rice anther culture, the N_6-Y1 basic media developed at Yeongnam Crop Experiment Station of Rural Development Administration in Korea gave better results in culturability than those of MS, Miller and N_6. The total nitrogen contents was composed of 28 mM KNO_3, 1.75 mM $(NH_4)_2$ SO_4 and

1.75 mM L-Glutamine instead of 28 mM KNO_3 and 3.5 mM $(NH_4)_2$ SO_4 of N_6 (Chung and Sohn, 1986; Chung, 1988).

Narrasimman *et al*. (1994) also reported that N_6 media was better for callus induction than MS for the cv. IR-50, ARC 6650(indica), Norin 8 (japonica), Norin 8/IR 50 (japonica/indica) and IR 50/ ARC 6650 (indica/indica).

Faruque *et al*. (1998) reported effect of different basal media response to different genotypes, genotype BA (local salt tolerant land race) responded best in N_6 medium, BR-7 (high yielding variety) in R_2 and IR-43 (well known indica variety) in He-2 medium. These results suggested role of genotype as well as basal medium.

Chen *et al*. (1982) reported that the medium containing inorganic salts of N_6 composition gave better results in callus induction than that of MS inorganic salts. About 40 % of cultured, Tainung 67 anthers produced callus from N_6 medium compared with 20 % from MS medium.

(b) *Days to callus initiation*

Table: 4.4.3 Effect of genotype (G), different basal media (M), Treatments (T) on days to callus initiation

Treatments \ Basal Media	Genotype				Mean
	IR-58025		GR-7		
	N_6	MS-1	N_6	MS-1	
T_1 (1.0 mg l^{-1} 2,4-D + 3 % Sucrose)	NCI	NCI	NCI	NCI	NCI
T_2 (1.5 mg l^{-1} 2,4-D + 3 % Sucrose)	NCI	NCI	NCI	NCI	NCI
T_3 (2.0 mg l^{-1} 2,4-D + 3 % Sucrose)	70.0	NCI	75.0	NCI	72.5
T_4 (1.0 mg l^{-1} 2,4-D + 6 % Sucrose)	NCI	NCI	NCI	NCI	NCI
T_5 (1.5 mg l^{-1} 2,4-D + 6 % Sucrose)	64.0	70.0	65.0	NCI	66.33
T_6 (2.0 mg l^{-1} 2,4-D + 6 % Sucrose)	59.8	66.5	64.0	67.5	64.45

Mean	64.6	68.25	68.0	67.5	67.08
Mean (Media)	66.30 (N_6)		67.87 (MS-1)		
Mean (Genotype)	66.42		67.75		

NCI- No Callus Induction

The mean values for days to callus initiation on different callus induction basal media fortified with three levels of 2,4-D and two levels of sucrose using two genotypes are presented in Table 4.4.3. Days to callus initiation ranged from 59.8 to 75.0 days. Among the genotypes studied, IR-58025 exhibited 66.42 days as compared to GR-7 (67.75) for callus initiation. Between the two media studied, medium N_6 exhibited minimum number of days (66.30) for callus initiation as compared to MS-1. Among the six growth regulator and sucrose combination treatments, T_6 (2.0 mg l^{-1} 2,4-D + 6 % Sucrose + 0.9 % Agar) exhibited early induction of callus (64.45) as compared to other growth regulator and sucrose combination treatments tried.

Balachandran *et al*. (1990) also reported that plated anthers started showing callus induction after 40 days of incubation in the dark.

Zhang and Qifeng (1993) who also reported calli emerged after 4-5 weeks in culture in the two varieties *viz.,* Lunhui 422 and Jinzao 5 & their F_1, F_2.

In contrast to this Rout and Sarma (1991) who reported callus induction was distinctly early in different combinations of potato-2 medium compared to the corresponding N_6 media. Potato-2 medium supplemented with NAA gave earliest callus formation.

(c) *Callus colour and texture:*

Calli obtained on N_6 and MS-1 medium supplemented with different levels of 2,4-D and sucrose combinations exhibited variation in

color of callus *viz.,* cream, yellowish (Table 4.4.4; Plate III, A-C) and texture (friable, loose and compact) of callus irrespective of the genotypes. However, yellowish with compact texture calli were observed in the treatment T_6 (2.0 mg l^{-1} 2,4-D + 6 % Sucrose + 0.9 % Agar). This exhibited role of media for imparting colour and texture.

Table: 4.4.4 Morphogenic effect of various concentration of auxin (2,4-D) and Sucrose added in N_6 and MS-1 medium on anthers of rice cv. IR-58025, GR-7

Treatment	Concentration		N_6		MS-1	
	2,4-D (mg l^{-1})	Sucrose (%)	Callus colour	Texture of callus	Callus colour	Texture of callus
T_1	1.0	3	W	L	W	L
T_2	1.5	3	W	L	W	F
T_3	2.0	3	Cr	F	Cr	F
T_4	1.0	6	W	F	Cr	F
T_5	1.5	6	Cr	F	Cr	F
T_6	2.0	6	Y	C	Y	C

W - White; Cr - Cream; Y - Yellowish
L - Loose; F - Friable; C – Compact

Marrasi *et al.* (1993) also reported two types of callus emerged from anthers cultured on N_6 + 2.0 mg l^{-1} NAA + 0.5 mg l^{-1} Kn + 5 % Sucrose. The first type was compact, uniform in structure, yellow and not embryogenic. The second type was firm, nodular, white and embryogenic. These embryogenic and non-embryogenic calli from anther cultures of rice was similar in structure and colour to those obtained from other graminaceous species (Vasil and Vasil, 1981; Vasil, 1982; Bovo and Mroginski, 1989).

Based on the results of various treatment combinations for morphogenetic response a superior basal media (N_6) in combination

with

2,4-D (2.0 mg l^{-1}) and Sucrose (6 %) was identified for further work on both the genotypes *viz.,* IR-58025 and GR-7.

In vitro response of the two genotypes to per cent callusing and days to callus initiation is presented in Table 4.4.5 and 4.4.5.1. The media used in this study was N_6 + 2.0 mg l^{-1} 2,4-D + 6 % Sucrose + 0.9 % Agar (callus induction media) so that media effect was eliminated.

Analysis of variance for effect of genotype on per cent callusing and days to callus initiation revealed significant differences among the genotypes for percentage callus induction as well as for days to callus initiation (Table 4.4.5) indicating real effect of genotype on these two traits. Here, high CV observed for per cent callusing suggesting the existence of high variation between the genotypes for callus induction response.

Table: 4.4.5 Analysis of variance for effect of genotype on per cent callusing and days to callus initiation

Source	Per cent Callusing [a]		Days to callus initiation	
	d.f.	MS	d.f.	MS
Genotype (G)	1	11.86 *	1	190.07*
Error	118	1.66	48	25.54
Total	119		49	

Table: 4.4.5.1 Effect of genotypes on per cent callusing and days to callus initiation.

a = Data transformed through square root transformation.

Genotype	Per cent Callusing [a]	Days to callus initiation
IR-58025	1.567 (4.5)	58.19
GR-7	0.938 (2.0)	62.21
S.Em. (Genotype)	0.166	1.041
C.D. at 5 % (Genotype)	0.466	2.962
C.V. %	102.90	8.46

( ) – Values in parenthesis are original mean values.

The mean values for per cent callusing and days to callus initiation with different genotypes (Table 4.4.5.1) showed early (58.19 days) and better callusing (4.5 %) with IR-58025 exhibiting the genotypic effect on callusing.

A total of 2400 anthers were plated on the medium N_6 containing 2.0

mg l^{-1} 2,4-D + 6 % Sucrose, but only 4.5 % and 2.0 % callusing was obtained from the cv. IR-58025 and GR-7 respectively.

These results are in agreement with Balachandran *et al.* (1990) who also reported poor (5.8 %) callus induction with F_1 (IR-58025 A x IR BB7), anthers subjected to 2.0 mg l^{-1} 2,4-D and 6 % Sucrose.

After 8-10 days of culture, anthers became swollen and changed their colour from yellowish to brownish (Plate-I, D-E). Some small globular structures gradually developed from one end, from both the ends or along the anthers. In the following 6 weeks, the continued growth of such structures led to the formation of asynchronous calli (Plate-II, A-D).

Genotype is an important factor for anther culture success in rice (Abe and Futsuhara, 1986; Miah *et al*., 1985; Mikami and Kinoshita, 1988). Many japonica cultivars showed a high capacity for callus production and plant regeneration when exposed to low concentrations of growth regulators. However, indica varieties have displayed a low capacity for both responses, even with high hormone concentrations. This variation might by endogenous factors (Sundaru *et al*., 1983).

(d) ***Calli differentiation/Regeneration:***

The capacity of rice pollen callus to differentiate into plants is largely dependent upon the quality of the callus before its transfer to the regeneration medium. Pollen callus of rice loses its morphogenetic potential very rapidly during culture; an early transfer to the plant regeneration medium has been suggested (Oono, 1975; Chen, C.C., 1977). Wang *et al*. (1974) established the most suitable time for callus transfer to be approximately 10-15 days after its emergence. However, early transfer (before 15 days) also results in death of the callus (Wang *et al*., 1974). Another advantage of early transfer is that the chances for change in ploidy level during callus proliferation (Chen, C.C. and Chen, C.M., 1979) could be minimized.

A total of 132 (IR-58025: 96 and GR-7: 36) anther callus pieces were sub cultured over different basal media *viz.*, MS (1962) and MS-2 fortified with 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn with 3 % Sucrose. All calli transferred for regeneration were healthy and friable or compactly growing, with a pale yellowish white appearance. But only 39.58 to 55.55 % differentiated into plantlets. Whereas, some calli turned brown in the early stages and on passage of time it dried and turned black.

The mean values for calli differentiation as affected by genotypes and basal media are presented in Table 4.4.6.

Table: 4.4.6 Effect of various basal media and genotypes on calli differentiation.

Basal media* / Genotypes	Calli differentiation (%)		Mean
	MS (1962)	MS-2 *	

IR-58025	39.58	41.66	40.62
GR-7	55.55	44.44	49.99
Mean	47.56	43.05	

*See Table 3.1 (M & M) for detail composition of basal media
Both the media were supplemented with 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar.

Among the genotypes studied, GR-7 showed highest average calli differentiation (49.99 %) across basal media used suggesting genotypic effect on calli differentiation. Between the two basal media studied, MS (1962) found superior (47.56 %) to MS-2 (43.03) for calli differentiation.

In general, media MS-2 was found superior for IR-58025 (B-line) whereas MS (1962) was superior for GR-7 (Fig. 1). This indicated genotype specific effect of basal media treatments.

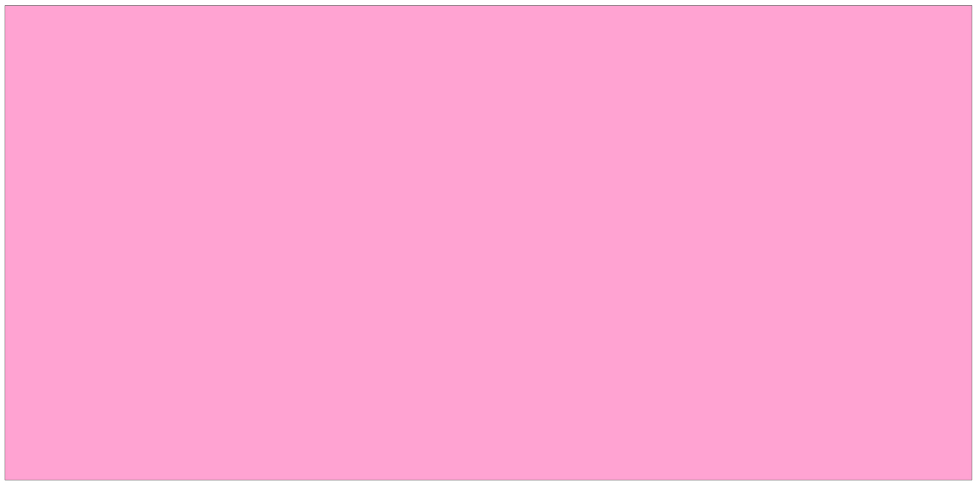


Fig. 1: Influence of genotypes (G) and basal media (M) on calli differentiation ability in rice

In IR-58025, 40.62 % of the calli exhibited differentiation and maximum

5 plantlets could be regenerated per regenerating callus (Plate-III, A) in

the media sequence of N_6 + 2.0 mg l^{-1} 2,4-D + 6 % Sucrose + 0.9 % Agar (Callus Induction) and MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar(Regeneration). In GR-7 calli differentiation of 49.99 % was attained and maximum 3 plantlets could be regenerated per regenerating callus, in the media sequence of N_6 + 2.0 mg l^{-1} 2,4-D + 6 % Sucrose + 0.9 % Agar (Callus Induction) and MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar (Regeneration).

The combination of NAA, BA and Kn that used in these studies had been previously used by Balachnadran *et al*. (1990) in rice anther culture using three varieties, IR-54, IR-72 (indica), Taipei 309 (japonica) and three indica/indica hybrids *viz.,* Ptb 21/ Leaung 152, Banglei/Eswarakora, Banglei/IET 7923-1 and one heterotic hybrid, IR-58025 A/ IR BB7.

The calli differentiation percentage in the present study is in agreement with Dhaliwal *et al.* (1997) who reported 27.3 to 70.1 % calli differentiation in indica/basmati crosses. These results are also in agreement with Wang *et al*. (1974) who reported 18.2 to 42.5 % calli regeneration.

Here, Shoot regeneration pattern was same as that has been mostly observed on the differentiation medium i.e. green spots first appeared on the callus surface (Plate-II, E) and shoots subsequently formed in these spots (Plate-II, F and G) and rhizogenesis occurred only in the cv. GR-7 (Plate-II, H).

Moreover, electron microscopic studies by Sun Ching-san *et al*. (1982) showed that, when the callus was transferred from the callus inducing medium to the differentiation medium, the numbers of mitochondria, plastid and ribosome increased in the epidermal meristematic cells, from which the shoot primordium may be differentiated. Meanwhile, the storage materials, such as starch grains in the plastids and lipid bodies in the cytoplasm, decreased rapidly or disappeared completely. The same events could be observed in the internal meristematic cells. This indicated that the callus transferred on the differentiation medium had passed a period of active metabolism and growth acceleration. The observations on histogenesis and organogenesis showed that the buds generally formed prior to the root formation, and the callus with only roots rarely differentiated further. The shoot primordium usually formed from the meristematic cells in the neighbourhood of the tracheary elements deep inside the callus, i.e. shoot primordia are exogenous while roots are endogenous in origin (Chang *et al*., 1978, Cheng, C. C., 1978)

(e) *Per cent green and albino plantlet regeneration:*

In vitro response of calli to per cent green and albino plantlet regeneration when subjected to two basal media *viz*. MS (Murashige and Skoog, 1962) and MS-2 fortified with 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 3 % Sucrose is presented in Table 4.4.7

The present investigation was initiated with an objective to study effect of two basal media incorporated with auxin (NAA), cytokinin (BA & Kn) and carbon source (Sucrose) for various aspects of regeneration of plantlets using calli of rice cv. IR-58025 and GR-7. Since MS media

fortified with phytohormone *viz.*, 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn with 3 % sucrose responded better (41.31 %) for induction of green shoots as compared to MS-2 media (35.41 %) fortified with 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn in combination with 3 % sucrose for both the genotypes.

Looking at the overall response, per cent green plantlet regeneration were 40.62 and 36.10 % for the cv. IR-58025 and GR-7, respectively indicating genotypic effect on the expression of this trait. Overall, average per cent green plantlet regeneration was of 38.36 % compared with lower albino plantlets (14.40 %) among all genotypes and media studied. (Table 4.4.7)

In general, basal media, MS (1962) was found superior for both the genotypes for percentage of green plantlet regeneration. These results are in agreement with the results of Dhaliwal *et al.* (1997) who reported 25.0 to 50.0 % green plantlet regeneration in indica/basmati crosses.

(f) *Days to shoot primordia/green and albino plantlet initiation*

The effect of different regeneration media fortified with growth regulators and Sucrose (in combination) on days to shoot primordia initiation using different genotypes is presented in Table 4.4.7. Days to shoot primordia initiation ranged from 17.50 to 29.33. Between the two media studied, MS basal medium exhibited early induction of green shoots (25.04 days) as compared to MS-2 basal medium (27.32). Whereas, among the genotypes studied, GR-7 exhibited early induction of albino shoots (19.25) as compared to IR-58025 (21.50).

Looking at the overall response, average days to green shoot primordia/green plantlet initiation were 23.86 and 28.49 for the cv. IR-58025 and GR-7 respectively, indicating genotypic effect for this trait. An overall, average days to green shoot primordia/green plantlet initiation was of 26.18 days as compared to albino plantlets initiation (20.37 days) among all genotypes and media studied. (Table 4.4.7)

Days to plantlets regeneration between 2-4 weeks has also reported by Lee *et al*. (2004) in japonica variety *viz*., Mankeumbyeo and a recalcitrant indica variety Ranta Emas.

Zhang and Qifeng (1993) also reported plantlet regeneration between 2-3 weeks in culture in the two varieties *viz.,* Lunhui 422 (P_1), Jinzao 5 (P_2) and their F_1, F_2, BC_1 (F_1/Lunhui 422) and BC_2 (F_1/Jinzao 5). It was observed that Lunhui 422 gave early and better plantlet regeneration as compared to others in the media MS (1962) supplemented with 2.0 mg l^{-1} Kn, 1.0 mg l^{-1} IAA and 3 % Sucrose indicated genotypic effect for this trait.

(g) *IN VITRO* ROOTING

The shoots obtained from callus on various basal media *viz.,* MS and MS-2 fortified with growth regulators and sucrose (1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar) were subjected to three root induction media consisting of half strength of MS (1962) basal media, Nitsch & Nitsch (1969) and White's (1963) media for induction of rooting. The excised shoots of different length obtained from previous experiment formed single root after 3-4 days on media and an attempt was made to find out best basal medium for rooting of *in vitro* regenerated shoots. Percentage rooting was recorded after 2 weeks (Table 4.4.8). Effect of different basal media on rooting is presented in Table 4.4.8.

Maximum percentage rooting (100.0 %) was observed on medium P_3 (White's + 2.0 % sucrose + 0.9 % Agar) irrespective of the genotypes followed by P_1 (½ MS + 0.9 % Agar) and P_2 (N & N + 1.0 % sucrose + 0.9 % Agar). Average per cent rooting for shoot induction media *viz.*, MS & MS-2 using cv. IR-58025 and GR-7 was found 83.33 %. These indicated absence of genotypic and shoot induction media effect on per cent rooting.

In this study, rooting media were devoid of any growth regulators suggesting that growth regulators are not essential for *in vitro* root induction on anther derived plantlets in rice. In general, it appeared that lower levels of salts (White's) in combination with sucrose induced efficient rooting.

(Plate-III, E).

These results are in agreement with Gun *et al*. (1978) who reported higher percentage of rooting in white's medium with 2 % sucrose.

These results are not in confirmation with the results of Balachandran

et al. (1990) who reported highest rooting in half strength MS media.

In contrast to this, Raina and Zapata (1997) used ¼ strength MS medium for rooting purpose.

Rohilla *et al*. (1997) reported one-half strength MS medium with 0.25

mg l^{-1} NAA and 2.5 mg l^{-1} MET for rooting purpose.

In contrast to the concentration of sucrose used in this study, Marrasi

et al. (1993) reported that basal medium MS with 8 % sucrose and no hormones promoted roots formation in rice. These results were in agreement with Maretzki and Hiraki (1980) in sugarcane, and Bovo and Mroginski (1986, 1989) in *Paspalum (Grameneae)*, where higher levels of sucrose promoted rooting.

(h) CYTOLOGICAL CONFIRMATION OF PLOIDY LEVEL:

Effect of genotypes and basal media (Shoot induction) on haploid plantlet regeneration was studied in the cv. IR- 58025 and GR-7.

In vitro raised plantlets from two basal media *viz*., MS and MS-2 fortified with 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 3 % Sucrose was studied for per cent haploid plantlets is presented in Table 4.4.9.

The present investigation was initiated with an objective to study effect of two basal media incorporated with auxin (NAA), cytokinin (BA & Kn) and carbon source (Sucrose) for regeneration of haploid plantlets using anthers of rice cv. IR-58025 and GR-7.

Chromosome counts performed from actively growing root tips by conventional cytological method (i.e. Acetocarmine squash preparation) (Plate-IV, C-D). Among the plantlets raised on media MS (1962), 20.71 % plantlets were found as haploids whereas it was only 6.66 % of the plantlets raised on media MS-2.

In general, medium MS (1962) was found superior for both the genotypes for percentage of haploid plantlet regeneration (20.71 %).

Largely, chromosome counts from root tips of plantlets regenerated in the two genotypes indicated 13.68 % haploid plantlets.

These results are not in agreement with the results of Narasimman and Sree Rangasamy (1993), who reported very high frequency (57.7 to 72.9 %) of haploid plants in the three indica-japonica interracial hybrids *viz.,* IR-50/Norin 8, ARC 6650/Norin 8 and CO 37/ Norin 6 and two japonica/indica hybrids *viz.,* Norin 8/IR 50 and Zuhio/ CO 37.

Regenerated haploid plantlets are indicator of their origin from haploid microspores. Frequency of diploid/doubled haploid plants was relatively higher (86.30 %) in both the cultivars, might be due to spontaneous diploidization or plantlets regenerated from somatic tissues. (Reiffers and Freire, 1990)

High percentage of diploid/doubled haploid plants regenerated from the pollen calli in rice anther culture is in fact advantageous. It resulted in spontaneous production of homozygous plants in a short period avoiding doubling chromosomes using colchicine.

Diploid plants were also regenerated from callus tissue obtained from haploid plants, owing to endomitosis or endoreduplication of callus cells (Chin *et al*., 1978; Chu, 1982).

Rout and Sarma (1991) also reported higher frequency of spontaneous double haploids (60.7 %). The frequencies of other chromosomal variants obtained were haploids (35.7%), polyploids (1.7 %) and aneuploids (1.7 %) in the interspecific hybrid, *Oryza sativa* L. x *Oyrza rufipogon* Griff.

(i) ACCLIMATIZATION

In vitro culture conditions affect anatomy and physiology of plantlet, which will be different from that of field grown one. Acclimatization is essential for *in vitro* raised plantlets before its establishment under field conditions. In the present study, plantlets of two rice cultivars *viz*., IR-58025 and GR-7 obtained after *in vitro* rooting were subjected to two potting mixtures, employing the methodology described earlier.

During the present study, it was observed that plantlets obtained after rooting when assigned to two potting mixtures *viz.,* soil alone (T_1) and soil: soil rite (3:1) (T_2) could survive for 25 days and very small number of plantlets could be taken for secondary hardening in green house. The values for per cent survival of the plantlets are presented in Table 4.4.10 to 4.4.11

Per cent survival of the anther culture raised plantlets ranged from 0.0 to 20.0 % up to secondary hardening. Among the potting mixtures studied, maximum survival of plantlets were seen with T_2 (3 soil: 1 soil rite) potting mixture (18.33 %). The average per cent survival was found to be quite superior in the cv. IR-58025 as compared to GR-7 among potting mixtures. The average per cent survival among the genotypes was of 13.57 %. This indicated that the fibrous root system of young rice plantlets developed in a synthetic medium could not be able to absorb their nutrients easily from the soil. These results are in agreement with Krishna Raj and Shree Rangasamy (1993) who reported poor survival of plantlets in sterile clay.

In contrast to this, Reiffers and Freire (1990) reported a mixture of soil and vermiculite (2:1) for plantlet hardening.

4.5 OPTIMIZATION OF CULTURAL ENVIRONMENT

The study covering various aspects of cultural environment was undertaken as a follow up of the preceding experiments in order to identify specific media sequence, media effect and genotype specific media sequence to obtain highest number of anther derived haploid plants efficiently.

CALLUS INDUCTION:

(a) Per cent callusing:

In vitro response of anthers to callusing using N_6 medium with various combinations of 2, 4-D/NAA, Kn and Sucrose using three rice cultivars,

IR-58025 (B-line) (P_1) and GR-7 (P_2), their single cross hybrid (F_1, IR-58025 A x GR-7 (GRH-1)) is presented in Table 4.5.1 to 4.5.2.

Table: 4.5.1 Analysis of variance for effect of genotypes (G) and callus induction media (CI) on per cent callusing

Source	d.f.	Mean Square
Genotype (G)	2	9.816 *
Callus induction media (CI)	14	6.080 *
G x CI	28	2.265 *
Error	360	0.775

* Significant at 5% level of significance

Analysis of variance revealed significant differences among the Genotypes, Callus induction media and Genotypes x Callus induction media interaction for percentage callusing, indicating real differences between genotypes, callus induction media and their interaction effects for per cent callusing. Genotype accounted for the greater variation in callus induction, although callus induction medium and callus induction medium x genotype interaction effects were significant. These results

revealed that abilities of callus induction in rice anther culture are strongly influenced by genotype and the composition of culture medium.

After 6-8 days of culture, anthers became swollen and changed their colour from yellowish to brownish (Plate-I, D-E). Some small globular structures gradually developed from one end, from both the ends or along the anthers. In the following five weeks, the continued growth of such structures led to the formation of asynchronous calli (Plate-II, A-D).

Wide range for percentage callusing (0.0 to 9.77 %) was observed. (Table 4.5.2) Among the different callus induction media, medium CI_{11}, CI_{10} (9.33, 6.22 % respectively) and CI_{7} (4.0 %) exhibited significantly higher percentage of callusing. whereas CI_{1}, CI_{2} and CI_{14} exhibited least percentage of callusing and were at par with each other in the cv. IR-58025. Medium CI_{13}, CI_{12} (9.77, 6.66 % respectively) and CI_{11} (4.0 %) exhibited significantly higher percentage of callusing. whereas CI_{4} and CI_{15} (1.33 %), exhibited least percentage of callusing and were at par with each other in the cv. GRH-1(Hybrid F_1). Medium CI_{9}, CI_{7} (4.44, 2.22 % respectively) exhibited significantly higher percentage of callusing. whereas, other treatments exhibited least percentage of callusing and were at par in the cv. GR-7. (Table: 4.5.2, 4.5.3). In these studies, usually one callus was noticed in a callusing anther. Since the size of anther is only 2-3 mm, multiple calluses, if present could not be distinguished.

Here, medium dependent response of pollen grains for androgenesis showed higher coefficient of variance (CV %). Under

similar conditions, different genotypes differ in their capacity to produce callus and express organogenic potential, in the same manner that anthers of the same genotype differ in their reaction to varying culture conditions. (Zapata *et al*., 1982)

Per cent callusing ranged from 0.44 to 9.33, 0.0 to 9.77 and 0.0 to 4.44 % for the genotype IR-58025, Hybrid(F_1) and GR-7 respectively.

Media CI_{11}, CI_7 and CI_9 were superior for callus induction across the genotypes.

Callus induction was found to be superior in the media CI_{11} in the cv. IR-58025, media CI_9 for the cv. GR-7. Where as, media CI_{13} was superior for their hybrid (F_1).

In the present study, higher callus induction was obtained from the media containing higher sucrose concentration (7.0 %).

In general, media CI_{11} exhibited significantly higher percentage of callusing followed by CI_7, CI_9, CI_{10}, CI_{12} and CI_{13} and were at par among all genotypes.

Per cent callusing was significantly increased in the F_1 (IR-58025 A x

GR-7) as compared to its parents.

Higher level of 2,4-D (3.0 mg l^{-1}) along with 0.5 mg l^{-1} Kn and 7 % Sucrose recorded highest callusing frequency (Table 4.8.2). However, the rate of increase was genotype dependent. The results revealed that the addition of growth regulators at the time of culture increased the percentage of calli as compared to control CI_1 (i.e. without growth regulators) indicating role of growth regulators for callus induction.

These results are in agreement with the earlier results reported by Narasimman *et al*. (1994) who reported more callusing at moderate levels of 2,4-D and lower levels of Kn.

Dhaliwal *et al*. (1997) reported 1.9 to 10.7 % callus induction in indica/basmati crosses using N_6 medium fortified with 2,4-D (2.5 mg l^{-1}) + Kinetin (0.5 mg l^{-1}) + 5 % Sucrose.

Dhaliwal *et al*. (1997) also reported highest callus induction in the media N_6 with 2.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 3 % maltose in the cross

Pusa Basmati 1/PAU 1198.

In contrary, Chen *et al.* (1982) reported the medium composed of N_6 inorganic salts, MS organic substances and supplemented with 4.0 mg l^{-1} NAA and 2.0 mg l^{-1} Kinetin with 6 % sucrose considered the best for callus induction in rice anther culture.

Chu *et al*. (1976) found that basal medium devoid of hormones produced plants from pollen grains of rice through somatic embryogenesis. However, inclusion of growth regulators in this study was necessary to obtain a high percentage response.

Krishna Raj and Sree Rangasamy (1993) also reported addition of 2,4-D and kinetin to the medium increased callus formation and proliferation, whereas when 2,4-D or Kn was added alone there was a comparatively lower frequency of callus formation.

Narasimman and Rangasamy (1993) reported significant genotypic differences for callus induction and regeneration among the different interracial hybrids of indica-japonica races.

Balachnadran *et al*. (1990) reported that the frequency of callus induction was rather low with the two indica varieties IR 54 and IR 72, whereas the response was as high as 20.5 % with the japonica variety, Taipei-309. The anther response of the three indica/indica hybrids was however, slightly better than the varieties and that of heterotic hybrid, IR-58025 A/IR BB7 was marginally higher.

Bishnoi *et al*. (2000) reported anther culture frequencies obtained in RZM medium was several fold higher than their earlier results with the similar indica x Basmati rice hybrids and comparable to those reported for responsive japonica varieties and hybrids (Chen *et al.,* 1991). Callus induction frequencies of 36-75 % and 16-49 % obtained for F_2 plants and F_1 hybrids, respectively in RZM medium.

Nguyen and Zapata (1993) reported significant genotype and medium interaction in 12 Vietnamese indica rice genotypes (including F_1, promising lines and varieties). All genotypes responded with callus induction being genotype and medium dependent.

In contrast to only one carbon source, Kavi-Kishore and Reddy (1986) have also reported the combination of 2 % sucrose and 3 % sorbitol to influence callus growth and plantlet regeneration.

The comparative picture of callus induction of all the 3 genotypes and all the 15 media based on an analysis of all three genotypes over all media treatments (Duncan’s New Multiple Range Test at 5 % level of significance) is presented in Table 4.5.3

Table: 4.5.3 Effect of genotype and callus induction media on per cent callusing of three indica rice genotypes (Duncan's New Multiple Range Test based on an analysis of all three genotypes over all media treatments at 5 % level of significance cv. IR-58025, Hybrid (F_1- GRH-1), GR-7 and average for all three genotypes)*.

Callus induction media	Treatment Mean			
	Genotype			
	IR-58025	Hybrid (F_1-GRH-1)	GR-7	Average
CI_1	0.422 f	0.223 e	0.223 c	0.289 e
CI_2	0.621 f	0.819 cde	0.621 bc	0.687 de
CI_3	0.819 ef	1.018 cde	0.621 bc	0.820 cd
CI_4	0.819 ef	0.712 de	0.621 bc	0.718 de
CI_5	0.911 def	1.018 cde	0.621 bc	0.850 cd
CI_6	1.614 bcde	0.981 cde	1.018 bc	1.205 bcd
CI_7	1.905 abc	1.706 bcd	1.217 ab	1.609 ab
CI_8	1.614 bcde	1.217 cd	1.018 bc	1.284 abc
CI_9	1.813 bcd	1.507 bcd	1.996 a	1.773 ab
CI_{10}	2.321 ab	1.217 cd	0.621 bc	1.387 ab
CI_{11}	2.855 a	1.798 bc	0.911 bc	1.855 a
CI_{12}	1.217 cdef	2.519 ab	0.621 bc	1.453 ab
CI_{13}	1.217 cdef	3.086 a	0.911 bc	1.739 ab
CI_{14}	0.621 f	1.110 cde	0.819 bc	0.850 cd
CI_{15}	0.911 def	0.712 de	0.819 bc	0.815 cd

* Data transformed through square root transformation
Note: Treatment means with the letter/letters in common are not significant by DNMRT at 5 % level of significance.

Here, dramatic differences were observed between genotypes and callus induction media.

(b) Days to callus initiation:

Table: 4.5.4 Analysis of variance for effect of callus induction media (CI_1-CI_{15}) on days to callus initiation for anthers of rice cv. IR-58025, GR-7 and their hybrid (F_1)

Source of variation	Genotypes					
	IR-58025		Hybrid (F_1)		GR-7	
	d.f.	MS	d.f.	MS	d.f.	MS
Callus induction media (CI)	14	329.84 *	13	348.88 *	13	203.69 *
Error	59	4.165	56	17.49	31	8.606
Total	73		69		44	

* Significant at 5 % level of significance

Analysis of variance revealed significant differences among the callus induction media for days to callus initiation indicating real differences between callus induction media for days to callus initiation.

The mean values for days to callus initiation on different callus induction media with different genotypes is presented in Table 4.5.5. Days to callus initiation ranged from 40.80 to 74.0 days. Among the various media studied, media CI_{13} (N_6 + 3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 7 % sucrose + 0.9 % agar) recorded minimum number of days (43.63) for callus initiation whereas, media CI_2 exhibited maximum number of days (71.66) for callus initiation for all the three genotypes studied. These results indicating higher concentration of auxin (2,4-D) and cytokinin (Kn) along with higher sucrose concentration gave early callus induction.

The average number of days from inoculation to callus formation was 56.23 days, with least for the cv. GRH-1 (Hybrid F_1) (53.74 days)

and the most for the cv. GR-7 (60.61 days). However, the time required for callus induction seemed to be genotype dependent. Days to callus initiation between 4-5 weeks and after 40 days also reported by Bishnoi *et al*. (2000) and Balachandran *et al*. (1990) respectively.

Faruque *et al*. (1998) reported callus emergence from anthers 4-8 weeks after culture initiation.

Jones *et al*. (1997) reported callus induction 4-5 weeks after inoculation and calli produced during this period had the greatest probability of yielding green plants.

Guiderdoni *et al.* (1992) reported microspore derived calli asynchronously emerged from inoculated anthers between the 4th and 8th weeks of incubation.

Bishnoi *et al*. (2000) reported that callus emerged asynchronously after 3-4 weeks in RZM medium and after 4-6 weeks in N_6M and Heh_5M media in the four indica cultivars *viz.*, Gobind, HKR 120, CH2 (DD), HKR86-3 and 2 Basmati rice (Basmati-370, Tarori Basmati) and their heterotic F_1 hybrids/F_2 plants. The F_2 plants were the earliest to respond when compared to the F_1 hybrids and parental rice cultivars.

(c) Callus colour and texture

Different combinations of phytohormones induced friable, loose and compact callus with cream and yellowish colour almost in all cases

in all the three genotypes as shown in Table 4.5.6. The low level of 2,4-D and Kn may have prompted to produce white/cream, loose/friable callus. Where as higher level of 2,4-D and Kn may have prompted to produce cream/yellowish, friable/compact callus. Whereas, inclusion of NAA in the media yielded yellowish coloured callus with compact texture.

In the present study, yellow coloured with compact texture calli showed higher calli differentiation.

Chen *et al*. (1982) reported callus induced from 2,4-D containing medium showed dark yellow colour, looser texture with dry weight content of 9-12 %. In contrary, callus induced from NAA and Kinetin containing medium showed lighter yellow colour, compact granule structure with dry weight content of 18-23 %.

Narciso and Hattori (1996) studied callus morphology of five indica

(IR-54, Re-2, Toboshi, 2757, 2764), two japonicas (Nipponbare, Tsutsu) & two javanica (Lemonte, Rinatte). Between these IR-54 and Toboshi were showed two callus masses: one was yellow, compact and smooth surfaced the other yellowish with hairy protuberance and green spots. Japonica and Javanica had almost similar morphology. Calli of both subspecies were yellowish, compact, and smooth surfaced.

PLANTLET REGENERATION:

Tsay *et al*. (1982) reported close correlation between the age and differentiation ability of callus. Fifteen to twenty day old callus produced the highest number of green plants. Where as, lower differentiation ability and the occurrence of more albino plants were associated with older callus. Plants differentiated from young callus were mostly haploid. The frequency of polyploidy, predominantly diploid, plants increased markedly when the age of callus cultured was increased.

(a) *Per cent calli differentiation:*

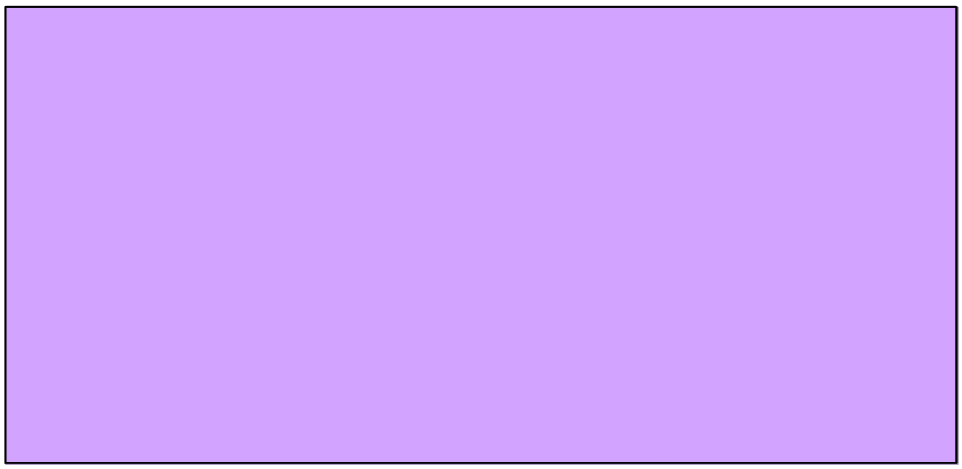


Fig. 2: Effect of callus age on differentiation

The effect of callus age on calli differentiation is presented in Fig. 2. It was found that calli emerged between 50-60 days (i.e. 60- 80 days old calli) showed better calli differentiation. The calli differentiation was 35.41, 27.08, and 25.0 per cent respectively for cv. IR-58025, Hybrid (F_1) and GR-7. Thereafter, the calli differentiation property decreased gradually. Such a decline, which has already been reported in japonica rice anther culture (Chen *et al*., 1984; Chung, 1987; Zhang, 1989), could be due to a depletion of the available nutrients in the un-renewed culture medium or/and due to release of toxic compounds from the senescent anther walls. However, these results showed that it was still

profitable to transfer late emerging calli to regeneration medium. Guiderdoni *et al.* (1992) also reported the effect of anther age on plantlet regeneration.

(i) Effect of genotypes and callus induction media on per cent callusing and calli differentiation

The statistical analysis for plantlet regeneration results was difficult to perform due to smaller number of callus induction from the media CI_1 to CI_{15} (due to relatively poor anther response).

The calli obtained from different callus induction media were sequentially subcultured over different regeneration media (R_1 - R_8) depending upon the availability of the calli mass to find out the effect of genotypes and callus induction media on per cent callusing and calli differentiation. The study showed wide differences among the genotypes and callus induction media on per cent callusing and calli differentiation

In the cultivar IR-58025, media N_6 + CI_{11} and CI_{10} exhibited higher percentage of callusing (9.33 %, 6.22%), where as, calli differentiation was found lower from these media (12.5, 25.0 %) when calli were transferred to different regeneration media (MS + R_1 to R_8). The highest calli differentiation (100 %) in IR-58025 attained when calli induced from N_6 + CI_{13} media and regenerated on different regeneration media (MS + R_1 to R_8). However, percentage of callusing in this medium was 2.22 % (Fig. 3)

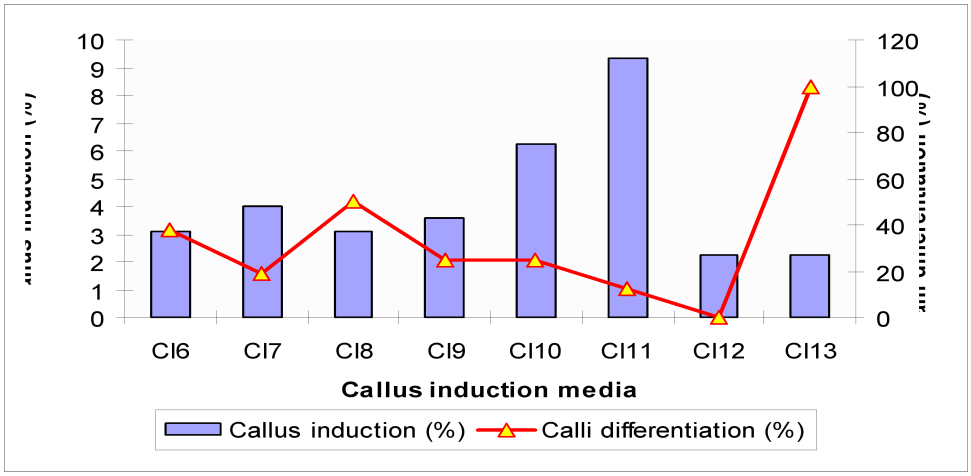


Fig. 3 Graph showing effect of callus induction media on callus induction and calli differentiation in the rice cv. IR-58025 (B-line)

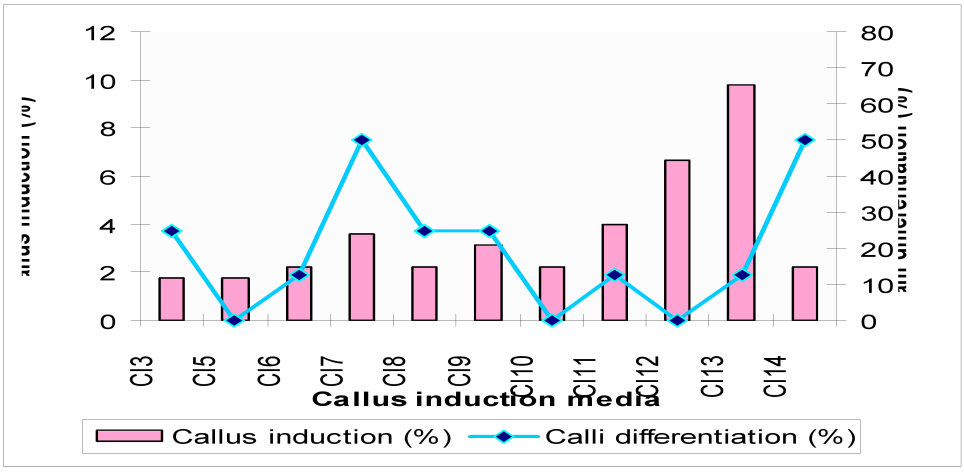


Fig. 4 Graph showing effect of callus induction media on callus induction and calli differentiation in the rice cv. IR-58025 A x GR-7 (F_1)

In cv. GRH-1 (Hybrid-F_1), medium N_6 + CI_{13} and CI_{12} showed higher callus induction (9.77, 6.66 %), where as, calli differentiation was found lower from these media (12.5, 0.0 %) when calli were transferred to different regeneration media (MS + R_1 to R_8). The best differentiation response (50 %) was attained when N_6 + CI_7 or N_6 + CI_{14} media was used as callus inducing medium and calli induced from these media were regenerated on different regeneration media (MS + R_1 to R_8). However, percentage of callusing in this medium was 3.55 and 2.22 % respectively. (Fig. 4)

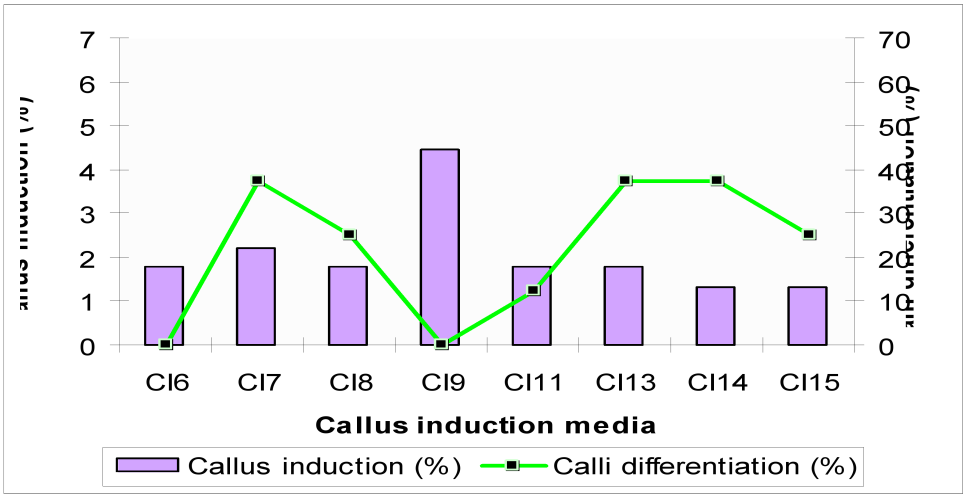


Fig. 5 Graph showing effect of callus induction media on callus induction and calli differentiation in the rice cv. GR-7.

In the genotype GR-7, media N_6 + CI_9, CI_7 exhibited higher percentage of callusing (4.44 %, 2.22%) , whereas, calli differentiation was found lowest from CI_9 media (0.0 %) when calli were subcultured to different regeneration media (MS + R_1 to R_8). Highest calli differentiation (37.5 %) was attained when calli induced from N_6 + CI_7 or CI_{13} or CI_{14} media were regenerated on different regeneration media (MS + R_1 to R_8) in this genotype. However, percentage of callusing in this medium was 2.22, 1.77 and 1.33 % respectively. (Fig. 5)

The results of the present study exhibited the extent of variation in callus induction and subsequent calli differentiation, which might be a consequence of medium x genotype interaction. This is an agreement with the results of Raina and Zapata (1997).

In general, dramatic differences were observed among genotypes as well as callus induction media for per cent callusing and calli differentiation (Fig. 6).

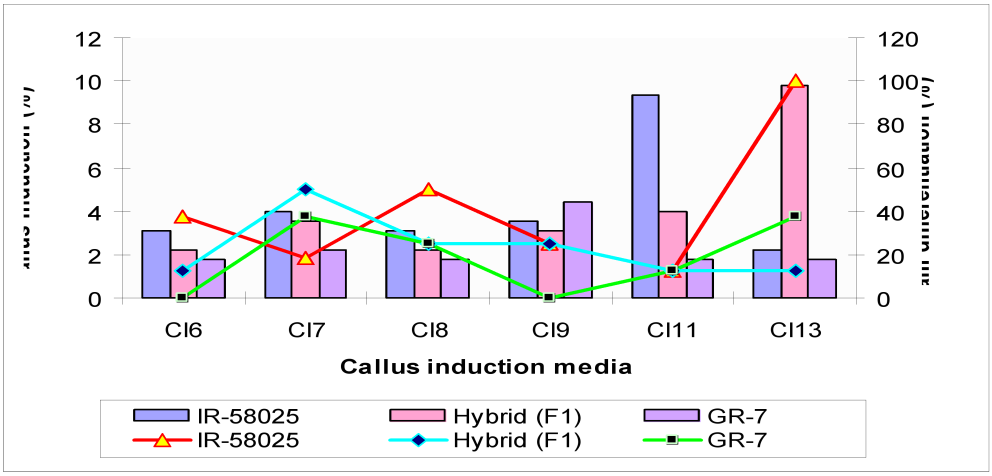


Fig. 6 Graph showing cumulative effect of callus induction media and genotypes on callus induction and calli differentiation (Bar represents per cent callus induction and line represents per cent calli differentiation)

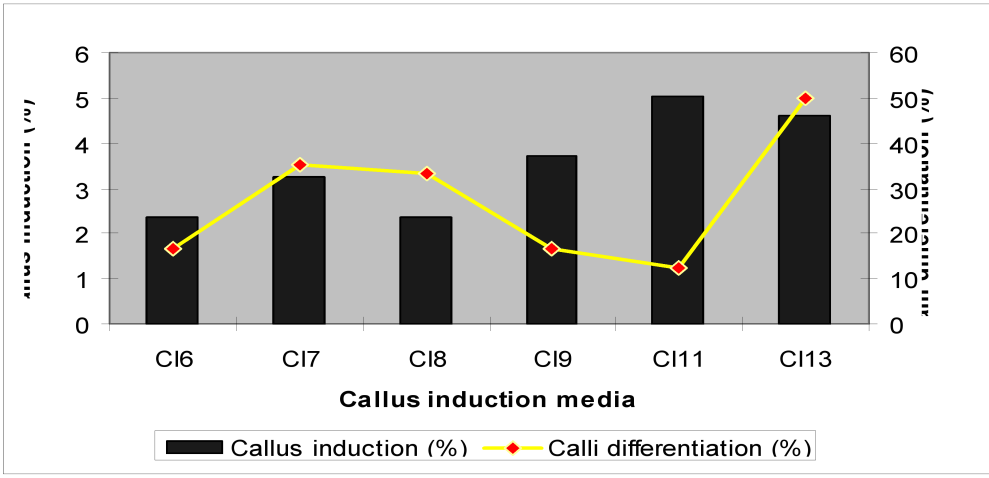


Fig. 7 Graph showing average effect of callus induction media on callus induction and calli differentiation among all genotypes

By and large, N_6 + CI_{13} medium (callus induction) was found to be superior over other combinations for calli differentiation/plantlet regeneration, when calli were transferred to different regeneration media (Fig. 7). However, media CI_7 and CI_8 were found superior for calli differentiation/plantlet regeneration, when calli transferred to different regeneration media (R_1 to R_8).

The response of all the three cultivars was quite low from sequences N_6 + CI_{11} media and were differentiated on different regeneration media (MS basal + R_1 to R_8 treatments) (IR- 58025:12.5%; GR-7: 12.5%) and N_6 + CI_6, CI_{11} and CI_{13} media and were differentiated on different regeneration media (MS basal+ R_1 to R_8 treatments) (GRH-1:12.5%).

In IR-58025 calli induced on N_6 + CI_{12} when transferred to different regeneration medium (MS basal+ R_1 to R_8 treatments), failed to produce any shoot buds, whereas in GRH-1 calli induced on N_6+CI_5, CI_{10} and CI_{12} also failed to regenerate on different regeneration media (MS basal + R_1 to R_8 treatments). In, GR-7 calli induced on N_6 + CI_6, CI_9 when transferred to different regeneration medium (MS basal + R_1 to R_8 treatments), also failed to produce any shoot buds.

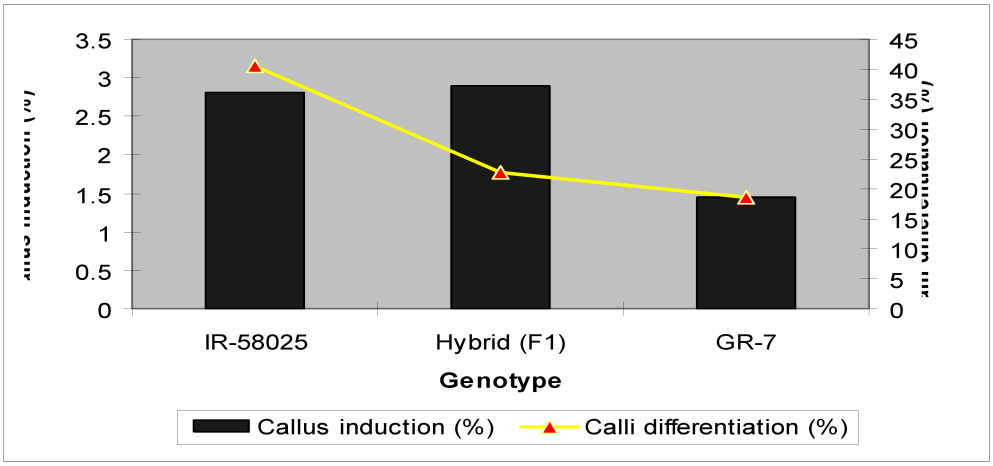


Fig 8 Graph showing average effect of genotype on callus induction and calli differentiation among all callus induction media

Looking to the overall response of all the calli inducing treatments, the average per cent callusing was 2.81, 2.90 and 1.44 % for the cv. IR-58025, Hybrid (F_1) and GR-7 respectively (Fig. 8). Overall response of all the calli inducing media treatments showed that average

per cent differentiation was 40.62, 22.91 and 18.75 per cent for the cv. IR-58025, Hybrid (F_1) and GR-7 respectively, invariable of the callus induction media (Fig. 8). The result thus showed the variability in genotypic influence on different calli induction and calli differentiation media which is in agreement with the study conducted by Wang *et al*. (1974).

Results further revealed that higher frequency of callus induction and subsequent green plantlet regeneration could be achieved by proper coordination of growth regulators in the callus induction medium. On an overall basis it appeared that higher level of 2,4-D (2.0-3.0 mg l^{-1}) with lower level of Kn (0.5-1.0 mg l^{-1}) along with higher sucrose concentration (5-7 %) exhibited higher callus induction as well as calli differentiation among all the genotypes. However, inclusion of NAA into the callus induction medium was found beneficial for calli differentiation in the cv. GR-7 and IR-58025 A x GR-7 (Hybrid).

These results are not in confirmation with the results reported by Goldsworthy and Mina (1991) who reported that as a synthetic plant growth regulator possessing strong auxin activity, 2,4-D was beneficial for callus induction in plant tissue culture, but it usually depressed the differentiation of calli.

Reddy *et al*. (1985) reported that NAA was better to induce callus, but 2,4-D was better for plantlet regeneration of Indica cultivars. However, high amounts of either NAA or 2,4-D enhanced callus formation but reduced plant regeneration.

Rout and Sarma (1991) also reported beneficial effect of NAA for callus induction as well as plantlet regeneration. Draz *et al*. (1991) reported the effect of callus induction media on plantlet regeneration.

Liang (1978) first realized the importance of combined effects of growth regulators in the callus induction and subsequent plantlet regeneration.

Reiffers and Freire (1990) also reported that crosses with low callus induction frequencies often show high regenerability frequencies. Indeed, for the cross Catetao precoce x Metica 1, the lowest callus induction frequency (0.2 %) and the highest plant regeneration frequency (144.4 %) observed. It thus seems essential to select those genotypes that are best able to regenerate plants rather than those which produce a high number of calli.

Faruque *et al*. (1998) reported that all F_1 hybrids with japonica cv. T-309 as one of the parents showed significantly increase in callus induction with respect to the indica parents but green plant regeneration did not necessarily increase.

Niizeki and Oono (1970) reported 2,4-D has inhibitory effects on callus differentiation and could be substituted for by NAA.

Some of the rice anther culture studies (Chen, 1986; Chung, 1987;

Hu and Huang, 1987; Raina, 1989) have indicated that a combination of auxins, 2,4-D and NAA with kinetin is better of overall green plant regeneration efficiency than a medium with 2,4-D or NAA alone or in combination with cytokinin (Kn or Zeatin).

Rout and Sarma (1991) reported a combination of this two auxin showing synergistic effects in callus induction, growth of callus and subsequent differentiation of green plants. Evidently, the combinations of both auxins played a critical role in determining the regeneration potential of the calli by balancing the callus growth. They also reported, the calli formed in the presence of NAA were the earliest to develop green points and differentiation of calli induced in the presence of 2,4-D was delayed. The calli induced in the presence of either 2,4-D or NAA showed low regenerability in contrast to the calli obtained in the presence of both auxin.

Huang *et al*. (1986) also reported that callus formed in the presence of 2,4-D was shown to be less capable of plant regeneration than callus formed on the medium with NAA.

Shahnewaz and Bari (2004) also reported that 2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 2.5 mg l^{-1} NAA + 4 % sucrose was suitable for inducing high frequency of callus induction and high green plant regeneration for the

cv. BRRI Dhan-29.

The results showed that the composition of media used for callus induction may not necessarily be favourable for calli differentiation/plant regeneration as the nutritional requirements of the two phases of development is always different. Furthermore, this differential requirement was found to be genotype specific. Transfer of calli from callus induction medium to plantlet regeneration medium involved either a change of only the growth regulators or a change of both, growth regulators as well as the basal medium.

In IR-58025, GR-7 and their hybrid (GRH-1–F_1), variations in plantlet differentiation ability were mainly due to callus induction media as well as regeneration media and had affected it most. Cultivars differed not only in their response to nutrient culture with different growth regulators media combination but also in their interactions with the media at different stages of growth.

It seemed obvious that the process of de-differentiation involves activation and/or suppression of several metabolic pathways and hence many genetic factors. In addition to the chemical composition of the culture medium and its ionic balance, the state of cell membrane permeability which are known to undergo changes through different stages of growth, would be a crucial factor affecting regenerability. (Khanna and Raina, 1998)

However, the medium constituents such as nitrate and ammonium ions are reported to affect uptake of growth regulators by affecting cell membrane permeability (Trewawas *et al*., 1983; Koetje *et al*., 1989). Thus, an *in vitro* response is the result of a complex interaction involving the physiological state of the explant, the genotype and the external environment.

The results also indicated that while the complexities of genetic control of *in vitro* response and regenerability limit the possibilities of developing high responding genotypes through breeding, manipulations of the constituents of the callusing as well as regeneration medium could enhance regenerability of a genotype of interest.

(ii) Effect of genotypes and shoot induction media on calli differentiation and green plantlet regeneration

In IR-58025, per cent calli differentiation ranging from 8.33 to 41.66 % and per cent green plantlet regeneration ranging from 16.66 to 58.33 % obtained in different treatments (R_1- R_8) are presented in Fig. 9. The Cultivar IR-58025 showed the highest calli differentiation (41.66 %) when calli regenerated on MS + R_3 and R_8 regeneration media. Maximum green plantlet regeneration (58.33 %) was also attained from the same media i.e. MS + R_3 and R_8.

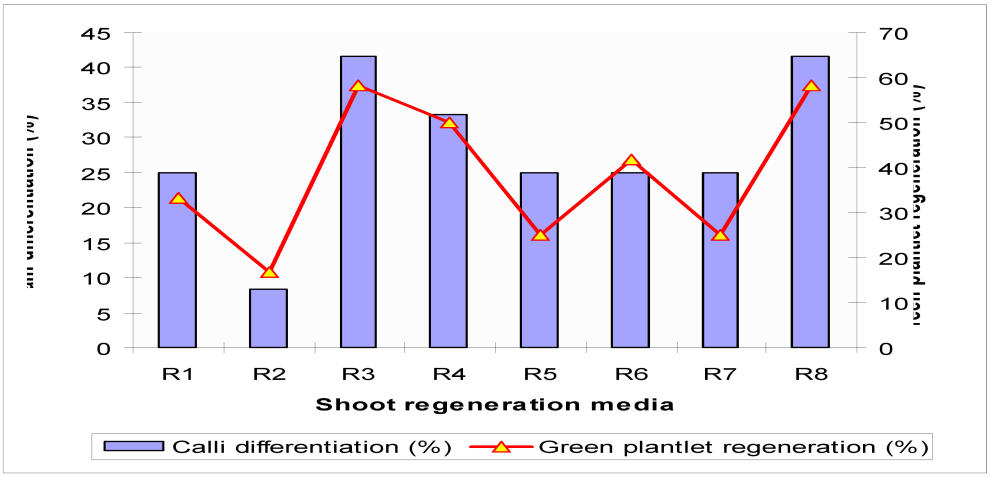


Fig. 9 Graph showing effect of shoot regeneration media on calli differentiation and green plantlet regeneration in the rice cv. IR-58025 (B-line)

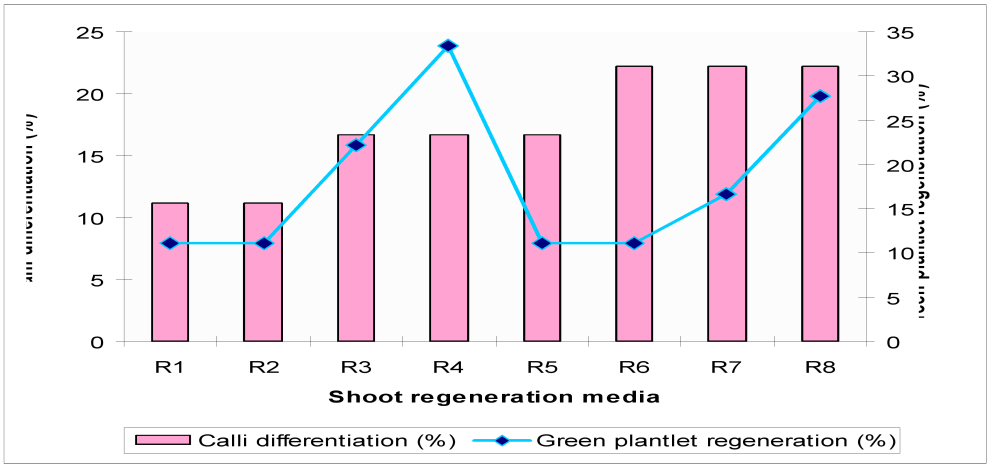


Fig. 10 Graph showing effect of shoot regeneration media on calli differentiation and green plantlet regeneration in the rice cv. IR-58025 A x GR-7 (F_1).

In hybrid, per cent calli differentiation ranging from 11.11 to 22.22 % and per cent green plantlet regeneration ranging from 11.11 to 33.33 % obtained across the treatments (R_1- R_8) are presented in Fig. 10. In hybrid (F_1), highest calli differentiation obtained in media MS + R_6, R_7 and R_8 (22.22 %) was resulted in to the lower green plantlet regeneration response (11.11, 16.66 and 27.77 % respectively). Conversely, lower calli differentiation response of 16.66 % obtained in MS + R_4 media, regenerated the highest (33.33 %) green plantlets.

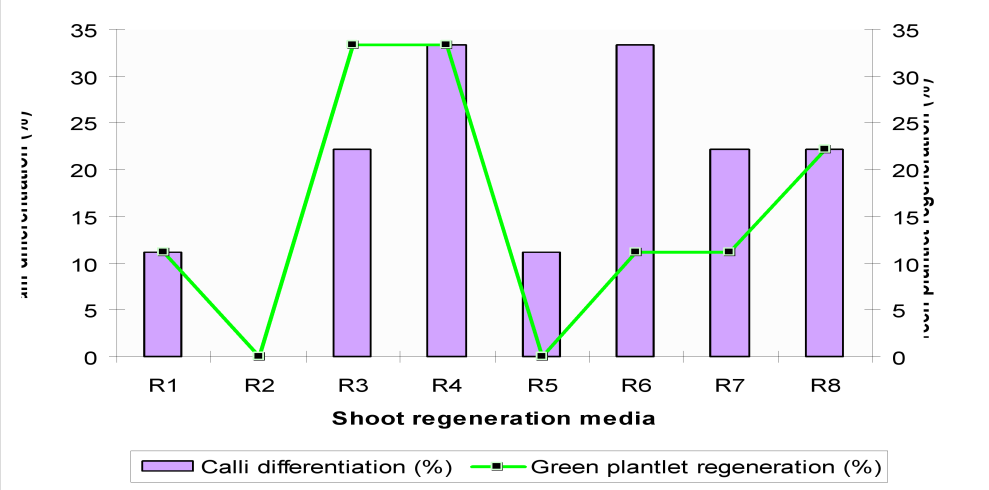


Fig. 11 Graph showing effect of shoot regeneration media on calli differentiation and green plantlet regeneration in the rice cv. GR-7

In GR-7, per cent calli differentiation ranging from 0.0 to 33.33 % and per cent green plantlet regeneration ranging from 0.0 to 33.33 % obtained across the treatments (R_1- R_8) are presented in Fig. 11. The media MS + R_4 and R_6 exhibited highest calli differentiation (33.33 %) was resulted into the lower green plantlet regeneration response in the media R_6 (11.11 %). Conversely, lower calli differentiation response of

22.22 % obtained in MS + R_3 media was exhibited the highest green plantlet regeneration (33.33 %).

These results revealed the extent of variation in calli differentiation and subsequent green plantlet regeneration might be because of medium x genotype interaction.

Raina and Zapata (1997) also reported variation in anther response and subsequent green plant regeneration, as a consequence of medium x genotype interaction in the cv. IR-9884 x Moroberekan using the media M-I, M-II and M-III. They also observed that larger calli especially when they were the loose and fast proliferating type, appeared to be less regenerable.

In general, dramatic differences were observed among genotypes and shoot regeneration media for per cent calli differentiation and green plantlet regeneration. (Fig.12)

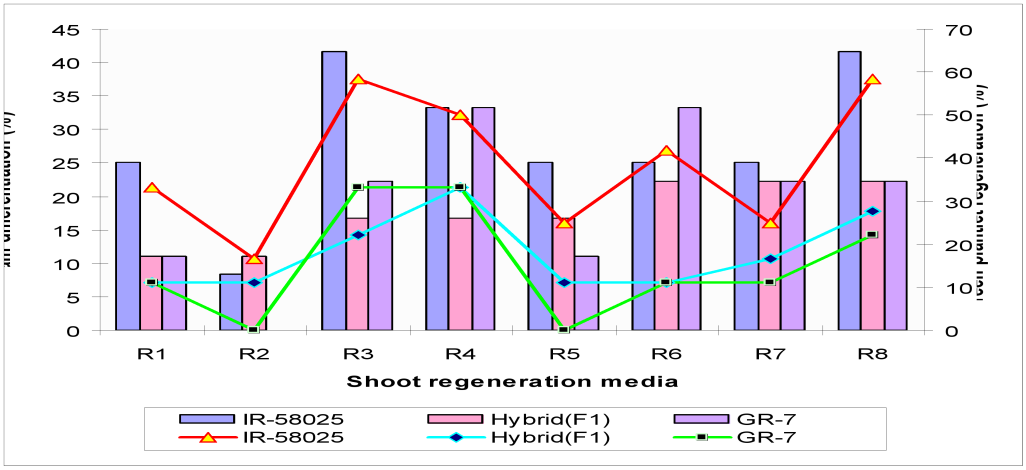


Fig. 12 Graph showing cumulative effect of genotypes and shoot regeneration media on calli differentiation and green plantlet regeneration (Bar represents per cent calli differentiation and line represents per cent green plantlet regeneration)

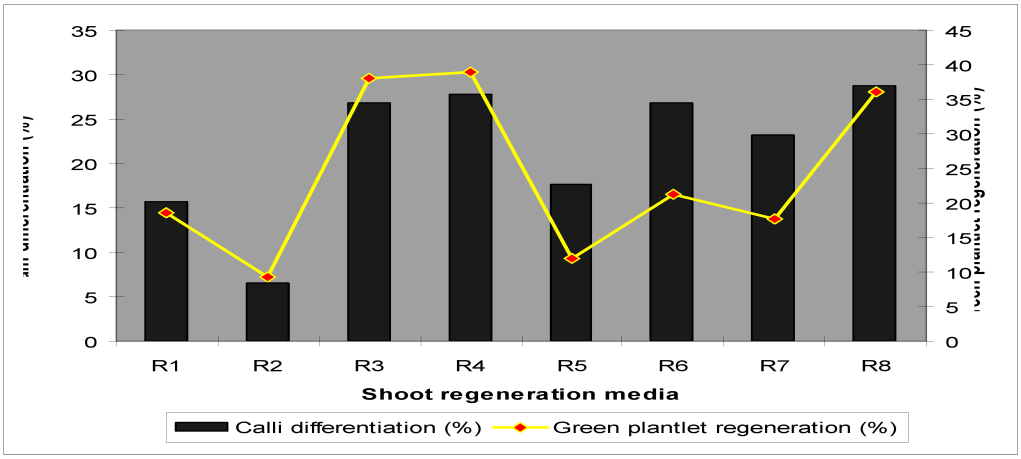


Fig. 13 Graph showing average effect of shoot regeneration media on calli differentiation and green plantlet regeneration among all genotypes

Largely, MS + R_4 medium was found to be superior over the other treatment combinations for green plantlet regeneration followed by MS + R_3 and R_8 among all the genotypes studied. (Fig.13)

The differentiation response of all the three cultivars was quite low on MS + R_2 regeneration media (IR- 58025: 8.33%; Hybrid (F_1): 11.11 % and GR-7: 0.0 %) whereas, the green plantlet regeneration response of all three cultivars was also quite low on the same regeneration media (IR- 58025: 16.66 %; Hybrid (F_1): 11.11 % and GR-7: 0.0 %).

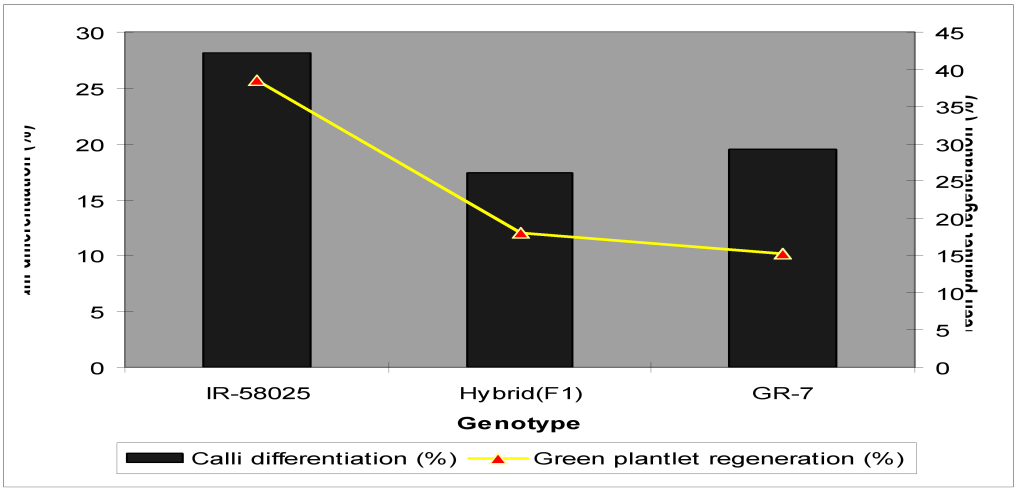


Fig. 14 Graph showing average effect of genotypes on calli differentiation and green plantlet regeneration among all shoot regeneration media

Overall response of all the differentiation treatments showed that the average per cent differentiation was 28.12, 17.35 and 19.44 per cent for the cv. IR-58025, Hybrid (F_1) and GR-7 respectively, invariable of the regeneration media. Per cent green plantlet regeneration was 38.53, 18.05 and 15.27 % for the cv. IR-58025, Hybrid (F_1) and GR-7 respectively. (Fig. 14) This indicated the genotypic effect on calli differentiation and shoot regeneration. (Fig.14). Overall response of the cv. IR-58025 was found quite satisfactorily as compared to cv. GR-7 and their hybrid. Overall, average per cent green plantlet regeneration among all genotypes and treatments was of 26.61 %. (Table 4.5.7)

These results are in agreement with Wang *et al.* (1974) who reported 13.2 to 64.7 per cent green plantlet regeneration. Several workers (Raina, 1997) have also reported genotypic differences in green plant regeneration from anther culture of rice and other cereals.

Results further revealed that higher frequency of green plantlet regeneration could be achieved by proper coordination of growth regulators in the regeneration medium.

On an overall basis it seems that lower level of NAA (0.5 mg l^{-1}) with moderate level of BA (1.0-2.0 mg l^{-1}) along with higher level of Kn (2.0 mg l^{-1}) with lower sucrose concentration (3.0 %) exhibited higher calli differentiation/green plantlet regeneration among all genotypes studied.

Zhang (1982) reported the different effect of 2,4-D and NAA on pollen callus growth may vary with the varieties used.

Strong influence of genotype on callus induction and plant regeneration have been reported by Ding *et al*. (1983), Miah (1983), Abe and Futsuhara (1986), Chen (1986), and Manimekalai and Sree Rangasamy (1987).

Faruque *et al*. (1998) reported callus induction, regeneration of the callus and specifically green plant regeneration significantly increased in F_1 cross of Taipei-309 with BR-7 and BA

Nguyen and Zapata (1993) reported M6 medium (Murashige and skoog) supplemented with 1.0 mg l^{-1} NAA and 8.0 mg l^{-1} Kn produced the most green plants.

Bishnoi *et al*. (2000) reported MSR-1 (MS+ BA + Kn+ NAA) and MSR-2 (MS + Kn+ NAA) media supported shoot regeneration from microspore derived calli, the frequencies of green plant regeneration were higher for MSR-1 medium. A combination of BA and Kinetin at the same concentration (1.0 mg l^{-1}) with NAA (0.5 mg l^{-1}) was effective for green plant regeneration.

Jones *et al*. (1997) reported that response to callus induction and green plant regeneration depended on the genetic origin of the crosses, with japonica/*Oryza glaberrima* hybrids responded best followed by pure japonica varieties and intra japonica hybrids, japonica/indica hybrids, and pure indica and intra indica hybrids.

(b) Days to shoot primordia initiation/plantlet regeneration:

Table: 4.5.8 Analysis of variance for effect of shoot/plantlet regeneration media treatments (R_1-R_8) on days to green plantlet regeneration of rice cv. IR-58025, GR-7 and their hybrid (F_1)

Source of variation	Genotypes

	IR-58025		IR-58025 A x GR-7 (F_1 : GRH-1)		GR-7	
	d.f.	MS	d.f.	MS	d.f.	MS
Shoot/plantlet regeneration media (R)	7	76.520*	7	38.908*	5	33.466*
Error	13	1.551	11	1.833	4	0.291
Total	20		18		9	

Analysis of variance revealed significant differences among the shoot/plantlet regeneration media treatments for days to green plantlet regeneration indicating real treatment differences between shoot/plantlet regeneration media for days to shoot primordia/plantlet initiation.

The mean values for days to green plantlet/shoot primordia initiation on different regeneration media (MS + growth regulators and Sucrose combination) with different genotypes are presented in Table 4.5.7 and 4.5.9

Days to green plantlet/shoot primordia initiation ranged from 15.0 to 34.0 days in parents (P_1 and P_2) & hybrid GRH-1 (Table 4.5.7). Among the various media studied, media R_8 recorded minimum number of days for green plantlet/shoot primordia initiation whereas, media R_1 exhibited maximum number of days (32.50) for green plantlet/shoot primordia initiation irrespective of genotypes indicating effect of media on the expression of this trait.

Among the three genotypes, studied Hybrid (F_1) exhibited early green shoot primordia initiation (22.39 days) across the different regeneration media. Where as, among the eight media studied, media

R_8 exhibited early induction of albino shoots (17.0) as compared to media R_5 (20.0). (Table 4.5.7)

Table: 4.5.9 Effect of shoot/plantlet regeneration media treatments (R_1-R_8) on days to green plantlet regeneration of rice cv. IR-58025, GR-7 and their hybrid (F_1)

Shoot/plantlet regeneration media (R)	Genotypes			Mean
	IR-58025	(Hybrid- F_1)	GR-7	
R_1	33.00	30.50	34.00	32.50
R_2	29.00	26.50	-	27.75
R_3	26.50	23.00	27.50	25.66
R_4	24.66	21.00	24.33	23.33
R_5	22.50	22.00	-	22.25
R_6	21.00	20.00	22.00	21.00
R_7	21.00	18.66	21.00	20.22
R_8	16.00	17.50	20.00	17.83
Mean	24.20	22.39	24.80	23.81
S.Em. (Treatment)	0.833	0.895	0.458	
C.D. at 5 % (Treatment)	2.581	2.787	1.801	
C.V. %	5.169	6.110	2.204	

(-) represents No Response Obtained

Looking at the overall response of all media treatments, mean days to green plantlet regeneration was 24.20, 22.39 and 24.80 for the cv. IR-58025, Hybrid (F_1) and GR-7 respectively. Overall, mean value for green plantlet regeneration among all genotypes and treatments was 23.81 days. Where as, days to albino plantlet regeneration was 18.0, 20.0 and 17.0 for the cv. IR-58025, Hybrid (F_1) and GR-7 respectively (Table 4.5.7). Overall, mean

value for albino plantlet regeneration among all genotypes and treatments was 18.33 days.

Days to plantlet regeneration ranged between 2-4 weeks, as reported early by Zhang and Qifeng (1993); Yan *et al*. (1996) and Lee *et al*. (2004)

The results of the present study revealed that regeneration media

R_1 (MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9 % agar) induced late regeneration whereas R_8 (MS + 1.0 mg l^{-1} NAA +

2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9% agar) induced early regeneration for all three genotypes under study.

(iii) Effect of genotypes and shoot induction media on calli differentiation and albino plantlet regeneration

In IR-58025, per cent calli differentiation ranging from 8.33 to 41.66 % and per cent albino plantlet ranging from 0.0 to 16.66 % obtained across the treatments (R_1-R_8) are presented in Fig. 15. The cultivar IR-58025 showed the highest calli differentiation (41.66 %) when calli regenerated on MS + R_3 and R_8 regeneration media resulted into lower albino plantlet regeneration (8.33 %) in the media MS+ R_3. Conversely, lowest calli differentiation response of 8.33 % obtained in MS + R_2 was followed by lowest (0.0 %) albino plantlet regeneration response, whereas, albino plantlet regeneration was found higher from the media R_3 and R_8 (8.33, 16.66 %). Highest albino plantlet regeneration attained from the media R_8

(16.66 %). Media MS + R_1, R_2 and R_6 were found superior for reduction in albino shoots in this cultivar.

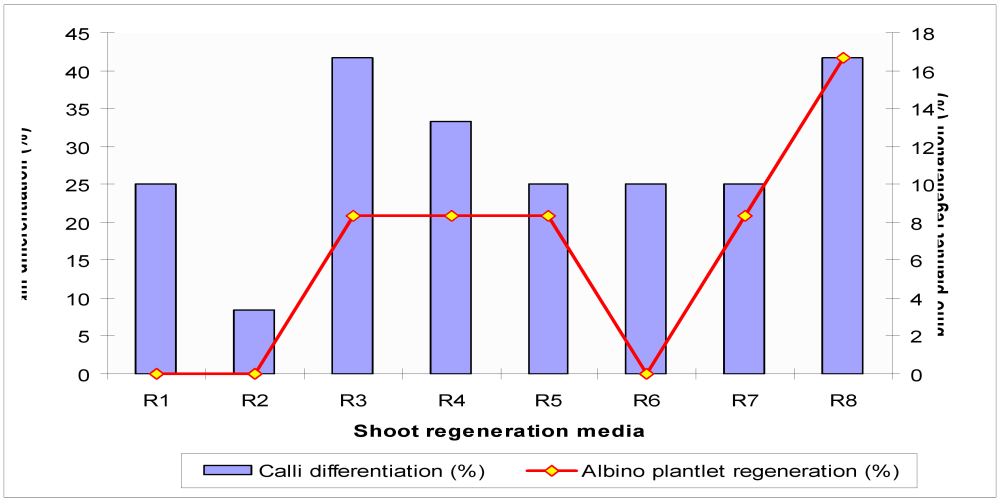


Fig. 15 Graph showing effect of shoot regeneration media on calli differentiation and albino plantlet regeneration in the rice cv. IR-58025 (B-line)

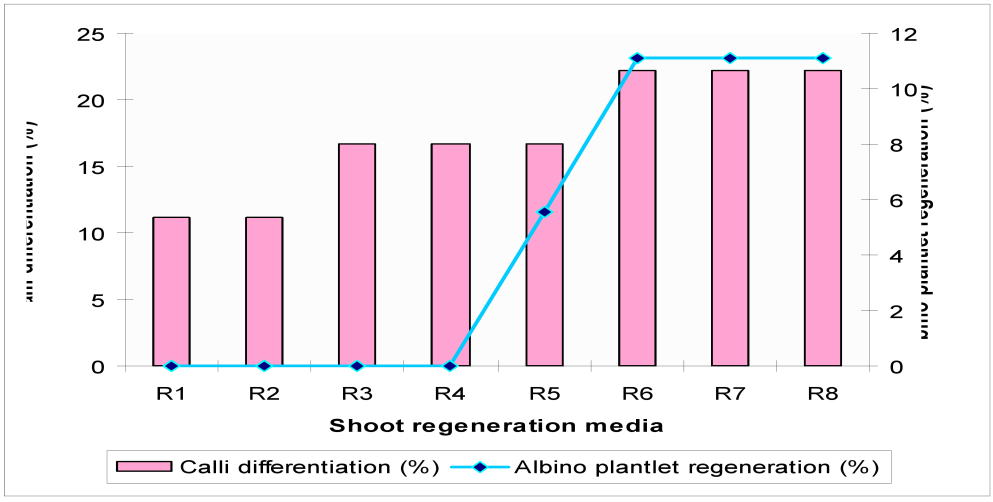


Fig. 16 Graph showing effect of shoot regeneration media on calli differentiation and albino plantlet regeneration in the rice cv.
IR-58025 A x GR-7(F_1)

In hybrid (F_1), per cent calli differentiation ranging from 11.11 to 22.22 % and per cent albino plantlet ranging from 0.0 to 11.11 % obtained across the treatments (R_1-R_8) are presented in Fig. 16. The media MS + R_6, R_7 and R_8 exhibited highest calli differentiation (22.22 %), whereas, albino plantlet regeneration was found highest from all

these media (11.11 %). Conversely, lowest calli differentiation response of 11.11 % obtained in MS + R_1 and R_2 were followed by lowest (0.0 %) albino plantlet regeneration response. Media MS + R_3 and R_4 were also found superior for reduction in albino shoots development in this genotype.

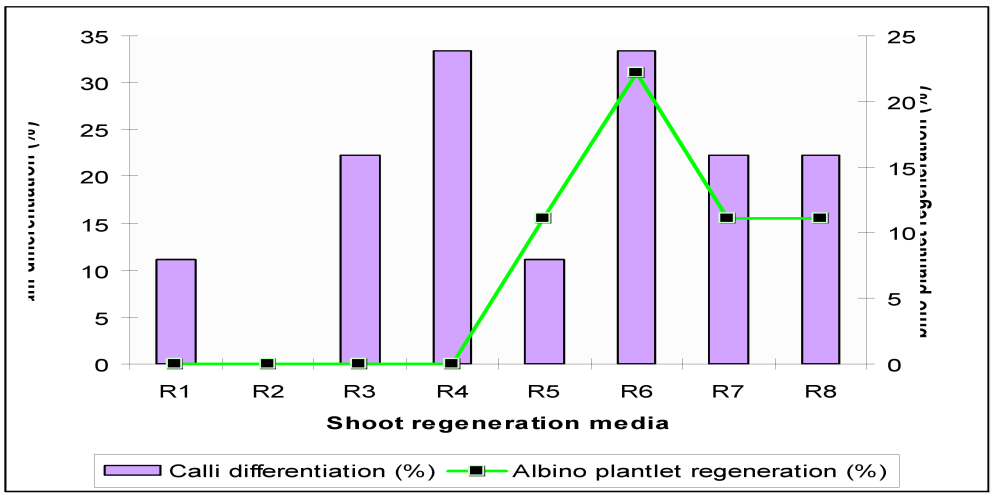


Fig. 17 Graph showing effect of shoot regeneration media on calli differentiation and albino plantlet regeneration in the rice cv. GR-7

In the cv. GR-7, per cent calli differentiation ranging from 0.0 to 33.33 % and per cent albino plantlet ranging from 0.0 to 22.22 % obtained across the treatments (R_1-R_8) are presented in Fig. 17. In GR-7, highest calli differentiation obtained in media MS + R_4 and R_6 (33.33 %) was followed by the lowest albino plantlet regeneration (0.00 %) in the media MS+ R_4. whereas, albino plantlet regeneration was highest from the media R_6 (22.22%).

Media MS + R_1, R_2, R_3 and R_4 were found superior for reduction of albino shoots development.

In general, dramatic differences were observed among genotypes and shoot regeneration media for per cent calli differentiation and albino plantlet regeneration. (Fig.18)

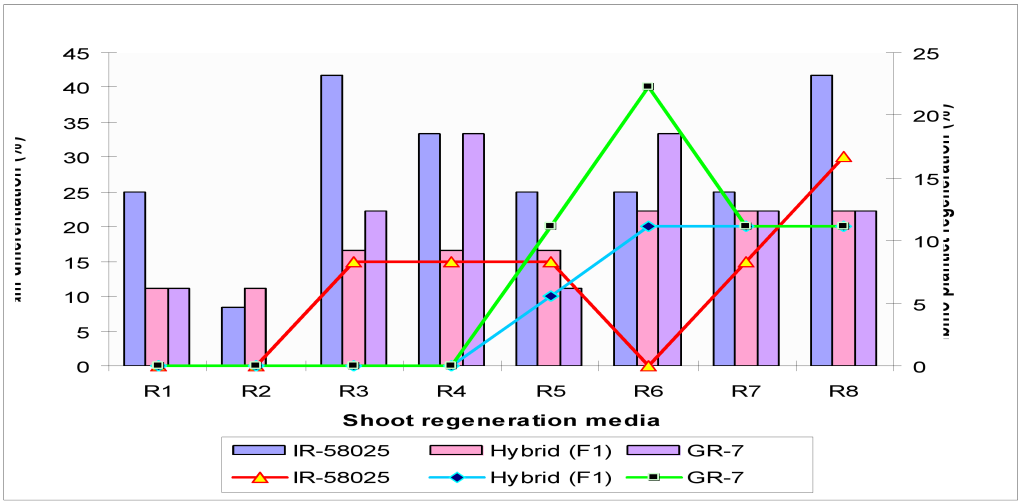


Fig. 18 Graph showing cumulative effect of genotypes and shoot regeneration media on calli differentiation and albino plantlet regeneration (Bar represents per cent calli differentiation and line represents per cent albino plantlet regeneration)

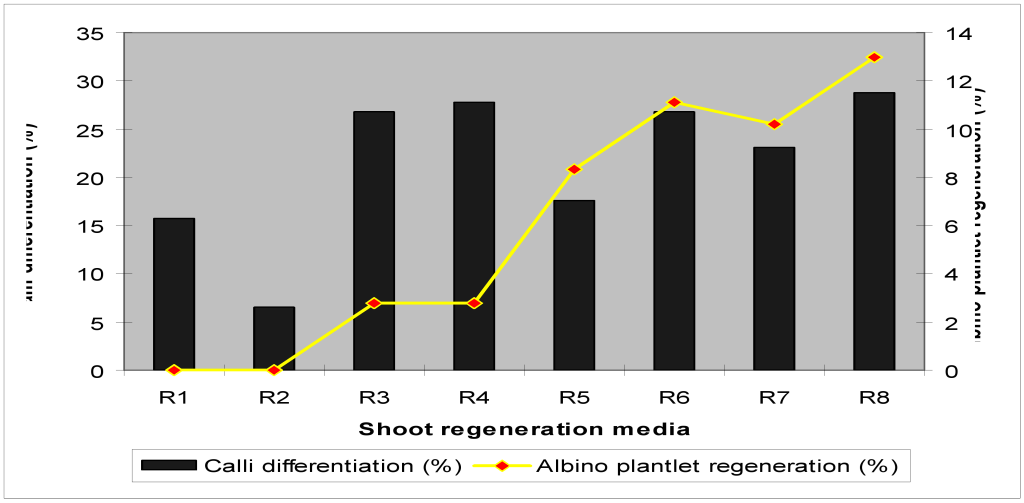


Fig. 19 Graph showing average effect of shoot regeneration media on calli differentiation and albino plantlet regeneration among all genotypes

By and large, MS + R_1 (MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % sucrose + 0.9% agar) and R_2 (MS + 0.5 mg l^{-1} NAA + 1.0

mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % sucrose + 0.9% agar) medium were found to be superior over the other media combinations for reduction in albino plantlet regeneration. (Fig. 19) Regeneration media, R_1 and R_2 did not regenerate albino shoots/plantlets in all the cultivars. Results thus revealed that lower frequency of albino plantlet regeneration could be achieved by proper combination of growth regulators (i.e. type & combination) in the regeneration medium.

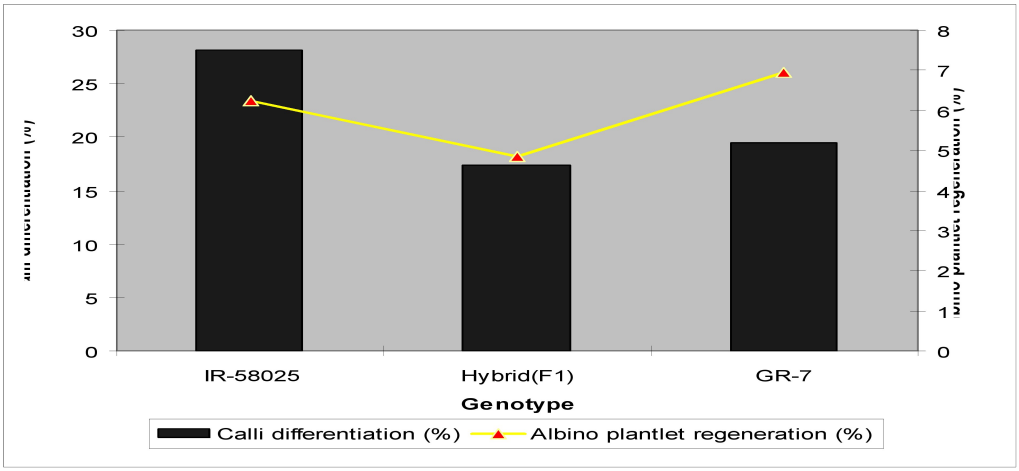


Fig. 20 Graph showing average effect of genotypes on calli differentiation and albino plantlet regeneration among all shoot regeneration media.

Looking to the overall response of all the treatments, the average per cent albino plantlet regeneration was 6.24, 4.86 and 6.94 % for the cv.

IR-58025, Hybrid (F_1) and GR-7 respectively.(Fig. 20) These results indicated the influence of genotypes in albino plant regeneration. Overall, average albino plantlet among all the genotypes and treatments was 6.01 %. (Table 4.5.7) These results are in contrast with Wang *et al*. (1978), where they reported 35.3 to 100 % albino plantlet regeneration in rice.

On an overall basis it seems that lower level of NAA (0.5 mg l^{-1}) with moderate level of BA (1.0 mg l^{-1}) and Kn (1.0-2.0 mg l^{-1}) along with lower sucrose concentration (3.0 %) exhibited lowest albino plantlet regeneration among all genotypes studied.

In contrast to these, Guiderdoni *et al*. (1992) reported the mean frequency of albino recovery in IR 64/Azucena, IR 64/IRAT216 and Kele/IRAT216 represented respectively, 53, 63, and 68 % of the regenerating calli.

Tsay *et al*. (1981) also reported a contrasting 100 % frequency of albino pollen plant in anther culture of japonica x indica materials.

In general, *in vitro* androgenesis is under genetic control and high responding cultivars may carry more of the genes responsible for this process. Therefore, one way to improve the response in anther culture is by genetic manipulation of the genotype of the donor plants (Foroughi-Wehr

et al., 1982). Jacobsen and Sopory (1978) reported high responding genotypes of potato obtained from crosses of low responding genotypes.

4.6 *IN VITRO* ROOTING*:*

On the basis of preliminary experiments for *in vitro* induction of rooting, the shoots obtained from callus on basal media *viz*. MS fortified with different growth regulators and sucrose combinations (R_1 to R_8) were subjected to root induction media (White's + 2 % Sucrose) for induction of rooting. The excised shoots of different length obtained from previous experiment formed single root after 3-4 days on root induction media and was found as best medium for rooting of *in vitro* regenerated shoots. Percentage rooting was recorded after 2 weeks are presented in Table 4.6.1.

Results showed that shoots of all three genotypes (i.e. parents and hybrid) induced from callus, when subjected to root induction media induced roots with higher percentage rooting. Average per cent rooting for the cv. IR-58025, GR-7 and Hybrid (F1) was 83.92, 100 and 95.83 % respectively. However, maximum percentage rooting (100.0 %) was

observed when shoots produced from on several shoot induction medium were subjected for rooting irrespective of the genotypes. Shoots of IR-58025 produced on shoot induction media R_3 and R_8 formed moderate percentage rooting (85.71 %). However, Hybrid (F_1) shoots raised on shoot induction media R_7 formed moderate percentage rooting (66.66 %). Overall, mean per cent rooting was 97.41 % in the genotypes and treatments studied.

These results are in contrast with the results of Balachnadran and Sarma (1990), who reported higher rooting in half strength MS medium.

4.7 CYTOLOGICAL AND ANATOMICAL STUDY OF ANTHER DERIVED PLANTLETS

The ploidy of the cells in the anther callus usually is unstable. That is one of the main reasons that non-haploid plants are produce from the anther culture. The cause presumed to be endomitosis of callus cells. High percentage of diploid/double haploid plants regenerated from the pollen calli in rice anther culture is in fact advantageous. It resulted in spontaneous production of homozygous plants in a short period. (Loo and Xu, 1986)

In cereal crops, chromosome instability has been reported in regenerants obtained from somatic tissue (Bayliss, 1973; Fedak *et al.,* 1987 a, b) or anther cultures (de Buyser *et al.,* 1985; Charmet *et al.,* 1986 and Metz *et al.,* 1988).

In vitro response of anther culture to per cent haploid plantlet regeneration among genotypes and shoot induction media is presented in Table 4.7.1

The present investigation was initiated with an objective to study effect of genotype and plantlet regeneration media (R_1 to R_8) to regenerate haploid plantlets using anthers of rice cv. IR-58025, GR-7 and their hybrid (F_1). The average per cent haploid plants produced using different regeneration media were 29.06, 22.21 and 30.20 % for the genotype IR-58025, GR-7 and their hybrid (F_1) respectively. In general, media R_1 appeared superior for all the three genotypes for percentage of haploid plantlet regeneration (72.20 %).

A total of 37 green regenerants derived from 96 calli pieces obtained from anther culture of the cv. IR 58025 were transferred from tube to soil, of these, 9 and 23 plants were observed to be haploid and diploid/doubled haploids, respectively. Whereas, in GR-7 a total of 11 green regenerants derived from 72 calli pieces were transferred from tube to soil, of these, 2 and 8 plants were observed to be haploid and diploid/doubled haploids, respectively.

A total of 26 green regenerants derived from 144 calli pieces obtained from anther culture of the heterotic hybrid (IR 58025A x GR-7) were transferred from tube to soil, of these, 6 and 16 plants were observed to be haploid and diploid/doubled haploids, respectively.

Largely, among the anther culture regenerants, the frequency of haploids ranged from 22.21 to 30.20 % and diploid/doubled haploids ranged from 69.78 to 77.76 %. The average percentage of haploids and diploid/doubled haploids obtained were 27.14 and 72.86 % respectively,

in the different genotypes studied. (Table 4.7.1)

These results are in agreement with McComb, J. A. (1978); Huang *et al*. (1978) and (Chen *et al*., 1983; Mercy and Zapata, 1986; Rout and Sarma, 1990), reported frequency of haploid plantlet in the range of 30-35 %.

In contrast to this, haploids ranged from 57.7 to 72.9 per cent and doubled haploid androgenic lines ranged from 27.1 to 42.3 per cent in anther culture of different indica-japonica hybrids. (Narasimman and Rangasamy, 1993)

Tsay *et al*. (1982) reported more haploid and polyploidy than naturally occurred double haploid plants from anther callus of japonica x indica (F_1) rices. On the contrary, anther callus from japonica rice varieties tended to produce more naturally double haploid plants.

The average values of spontaneous chromosome doubling frequencies reported by different author vary 59 % (Woo *et al.,* 1978), 48 % (Guiderdoni *et al.,* 1986), 69 % (Pulver, 1986) and 41.7 % (Demarly, 1983).

Guiderdoni *et al.* (1992) reported the frequency of spontaneous double haploid averaged 46 % and was similar to values obtained in anther culture of tropical japonicas (Reiffers and Freire, 1990; Guiderdoni *et al.,* 1991).

Tsay *et al.* (1982) contrastingly claimed that japonica x indica hybrids tend to produce more haploid plants than japonica crosses.

Rapid analysis of ploidy levels of plants:

In order to have a more practical method of analyzing the ploidy levels, two methods, *viz.,* (1) DNA ploidy analysis and (2) Stomatal measurements were studied.

(1) DNA ploidy analysis:

Attempts were made to check the *DNA ploidy* level of the anther culture derived plantlets of parents and their hybrid (F_1) using ploidy analyzer.

The initial run of the sample using the mother plant (2n = 2x = 24) for calibration showing single peak at channel 400 (Fig. 21) assured the constant diploid ploidy level of the mother plant under study. The same standard diploid mother plant was used for the ploidy

determination of the *in vitro* derived plantlets for haploid checking. Ploidy comparison of these R_1 plants showed two peaks wherever, the testing samples were of haploids (Fig. 22). Haploids appeared at channel number 200 representing the reduction of ploidy level in comparison to the mother plant, which appeared at channel number 400 representing the diploid ploidy level. Those samples, which were diploids /doubled haploids, appeared along with the standard mother plant samples at channel number 400 (Fig. 23). Small deviations in peak position could be due to instrument instability as well as due to variation in sample preparation.

Cauchy (1997) confirmed ploidy status through flow cytometric analysis by using microspore derived calli of the rice cv. Miara and Ariete.

Hedtrich and Hunter (1996) also reported ploidy determination by flow cytometric analysis in chicory leaves (*Cichorium intybus* L.) and observed most of the plants had polyploidised spontaneously *in vitro* leading to 1n + 2n, 2n, 2n + 4n and other mixoploid plants.

Gilissen *et al*. (1994) found diploids, tetraploids and mixoploid plants in tobacco, using flow cytometric analysis. However, when root tips were analysed by conventional cytology, only diploids and tetraploids identified.

Mix *et al*. (1978) reported endoreduplication phenomenon from microspore culture in *Hordeum vulgare* L.

Dolezel *et al.* (1997) also reported use of flow cytometry for rapid ploidy determination in *Musa* species.

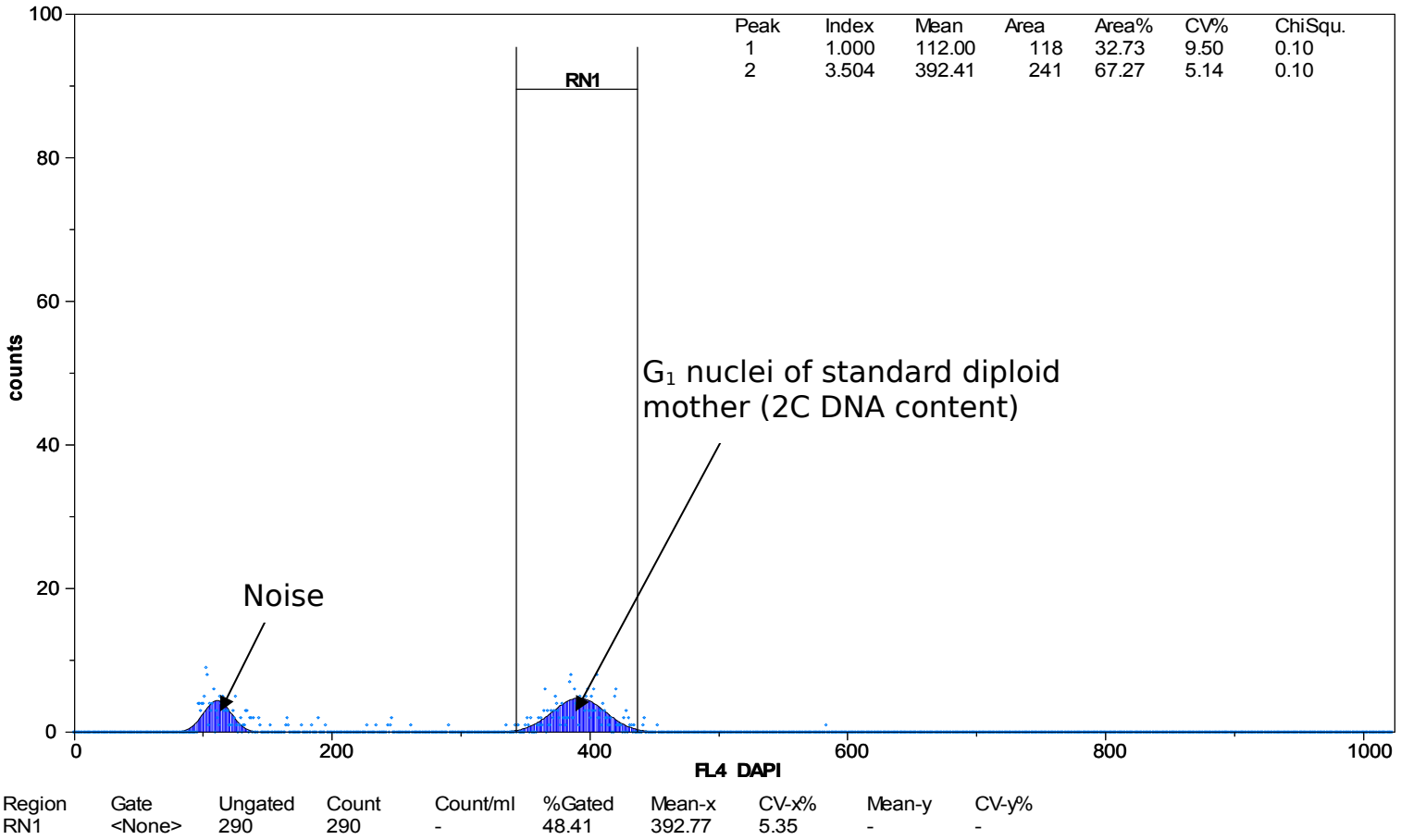


Fig. 21 Histogram of calibration peaks using diploid mother plant as standard showing single peak at channel

400 in ploidy

analyzer

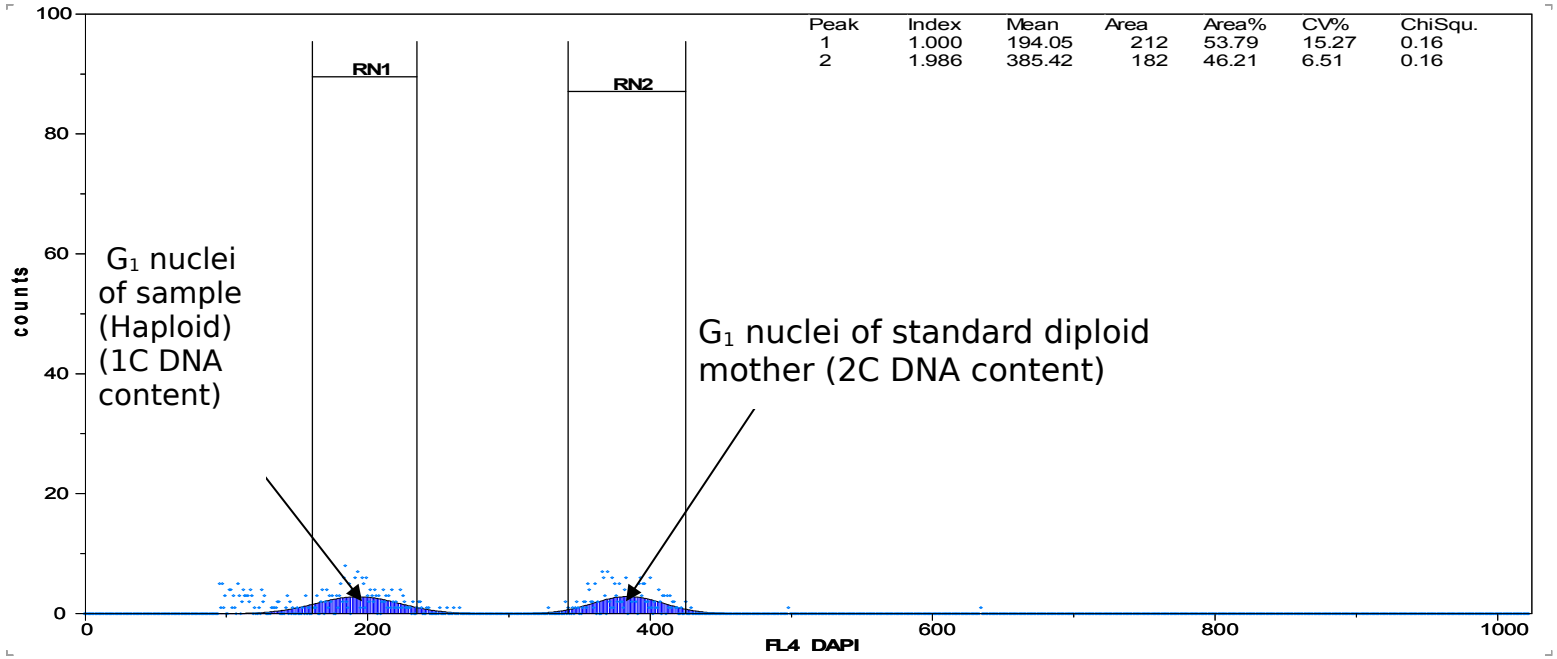


Fig. 22: Histogram showing two peaks confirming haploid nature of the tested sample in comparison to diploid standard in ploidy analyzer

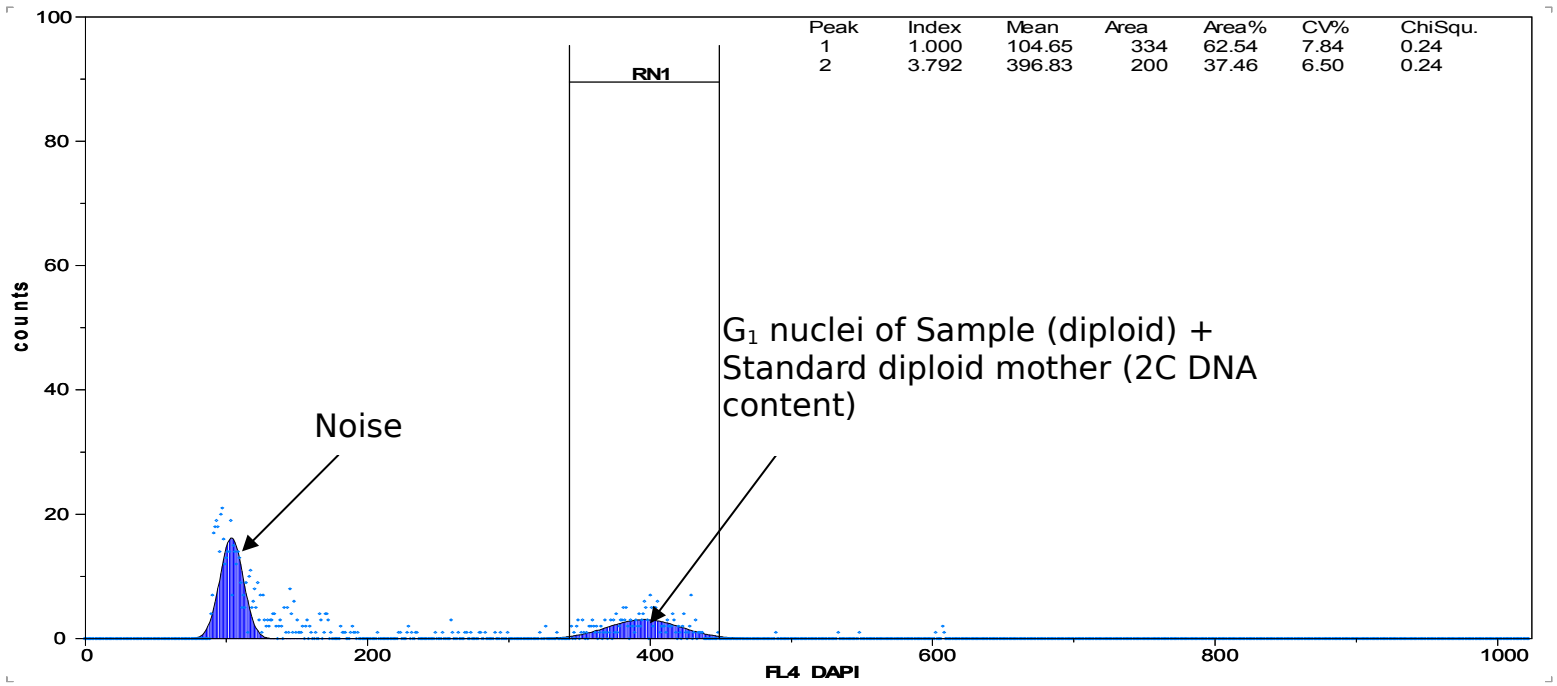


Fig. 23: Histogram showing single peak at channel 400 confirming the same ploidy level of the tested sample and the standard diploid mother in ploidy analyzer

(2) *Ploidy determination using stomatal measurements:*

The stomatal density is a useful tool for rapid pre-screening of anther derived regenerants (Silva *et al*., 2000). Stomatal density is not as influenced as the stomatal area by external factors such as temperature and water content of the plant tissue and the counting method does not demand sophisticated equipment and skills. The suitability of ploidy determination using stomatal measurements, *viz*. area and density of stomata (Plate-IV, E-F), was evaluated.

Haploid plants presented the lowest stomatal area (188.56 μm^2) while diploid showed the highest one (390.02 μm^2). The stomatal density for haploid plants was 40.90 and diploid showed 30.09 stomata per field of view (124929 μm^2) at 40 x. (Table: 4.9, p. 157)

Silva *et al.* (2000) determined ploidy level using stomatal measurements *viz.,* area and density of stomata, evaluated by associating these traits with chromosome counts. Significant differences among levels of ploidy found for both the area and density. Diploid plants represented the lowest stomatal area (1269) while tetraploidy showed the highest one (1916), mixoploidy presented intermediate values (1441). The stomata density was 8.0, 6.6 and 4.7 for diploids, mixoploids and tetraploid respectively in *Cattleya intermedia* (2n= 40).

(i) Effect of genotypes and callus induction-shoot regeneration media sequence on frequency of haploid plantlet (green and albino)

The Effect of genotypes and callus induction-shoot regeneration media sequence on regeneration of haploid plantlet (green and albino) per regenerating callus in rice cv. IR-58025, GR-7 and their Hybrid (F_1) are presented in Fig. 24, 25 and 26.

In IR-58025, green plantlet regeneration of 58.33 % was obtained on R_3 and R_8 media and maximum three plantlets regenerated per regenerating callus, in the media sequences of CI_{13} (callus induction medium) and R_3 (regeneration medium) or CI_{10}, CI_{13} (callus induction medium) and R_8 (regeneration medium) or CI_7 (callus induction medium) and R_4 (regeneration medium). But all the plantlets were found to be diploid/doubled haploid. Whereas, haploid plantlets were obtained from the best media sequences of CI_{13} (callus induction medium) and R_1 (regeneration medium) or CI_7, CI_{10} and R_3, CI_{13} and R_4, CI_{10} and R_5, CI_{13} and R_6, CI_{11} and R_7 & CI_{11} and R_8. (Fig. 24),
In IR-58025, maximum of one albino plantlet regenerated per regenerating callus, in the media sequence of CI_9, CI_{13} (callus induction medium) and R_8 (regeneration medium).

In GR-7, 33.33 % green plantlet regeneration was attained on R_3 and R_4 media and maximum two plantlets regenerated per regenerating callus, in the media sequences of CI_7 (callus induction medium) and R_3

(regeneration medium) but all plantlets were found to be diploid or doubled haploid. Haploid plantlets were obtained from the best media sequences of CI_{13} (callus induction medium) and R_1 (regeneration medium) & CI_8 and R_4 (Fig. 25). Maximum two albino plantlets regenerated per regenerating callus, in the media sequence of CI_{11} (Callus Induction) and R_6 (Regeneration) in the cv. GR-7. (Fig. 25)

Fig. 24: Effect of callus induction (CI) - shoot regeneration (R) media sequence on regeneration of green haploid plantlet per regenerating callus in rice anther culture cv. IR-58025 (B-line)

	CI_6	**CI_7**	**CI_8**	**CI_9**	**CI_{10}**	**CI_{11}**	**CI_{12}**	**CI_{13}**
R_1	●	O	● (-)	O	O	O	O	● *
R_2	O	O	O	● *	O	O	O	O
R_3	● (-)	●	O	O	●	O	O	● # ●
R_4	O	● #	O	O	● (-)	●	O	●
R_5	● *	O	●	O	●	O	O	O

R_6	O	● *	● *	O	O	O	O	●
R_7	O	O	●	O	O	●	O	● *
R_8	O	O	O	●	● #	●	O	● # ●

● Represents single green plantlet/callus; Cell with red coloured background represents green haploid plantlet and with clear background were diploid/doubled haploid;

represents three plantlets/shoots per callus, (-) represents not studied,

● Represents albino plantlet

O Represents no response/no plantlet regeneration

* Represents two plantlets/shoots per callus

Fig. 25: Effect of callus induction (CI)-shoot regeneration (R) media sequence on regeneration of green haploid plantlet per regenerating callus in rice anther culture cv. GR-7

	CI_6	CI_7	CI_8	CI_9	CI_{11}	CI_{13}	CI_{14}	CI_{15}
R_1	O	O	O	O	O	●	O	O
R_2	O	O	O	O	O	O	O	O
R_3	O	● *	O	O	O	O	● (-)	O
R_4	O	O	●	O	O	●	O	●
R_5	O	O	●	O	O	O	O	O
R_6	O	●	O	O	●	O	O	●
R_7	O	●	O	O	O	O	●	O
R_8	O	O	O	O	O	●	● (-)	O

Fig. 26: Effect of callus induction (CI)-shoot regeneration media (R) sequence on regeneration of green haploid plantlet per regenerating callus in rice anther culture cv. IR-58025 A x GR-7 (Hybrid-F_1)

	CI_3	CI_5	CI_6	CI_7	CI_8	CI_9	CI_{10}	CI_{11}	CI_{12}	CI_{13}	CI_{14}
R_1	O	O	O	O	O	O	O	O	O	●	●
R_2	O	O	O	●	O	O	O	O	O	●	O
R_3	O	O	O	● *	O	●	O	O	O	● (-)	O
R_4	O	O	O	● (-)	O	●	O	O	O	● #	O
R_5	●	O	●	●	O	O	O	O	O	O	O
R_6	O	O	O	● ●	●	O	O	O	O	O	●
R_7	O	O	O	● (-)	●	O	O	●	O	O	●
R_8	●	O	O	● #	O	O	O	● *	O	O	●

In Hybrid (F_1), green plantlet regeneration of 33.33 % was attained on R_4 media and maximum three plantlets regenerated per regenerating callus in the media sequences of CI_{13} (callus induction medium) and R_4 (regeneration medium) & CI_7 (callus induction medium) and R_8 (regeneration medium) and the regenerated plantlets were found to be diploid/doubled haploid. Haploid plantlets were obtained from the best media sequences of CI_{13} (callus induction medium) and R_1 (regeneration medium) or CI_7 and R_3, CI_9 and R_4, CI_6 and R_5, CI_{11} and R_7 (Fig. 26). In hybrid, maximum of two albino plantlets regenerated per regenerating callus, in the media sequence of CI_{14} (callus induction medium) and R_6 (regeneration medium). (Fig. 26) Regeneration media

(R_1 and R_2) did not produce any albino shoots/plantlets in all the three cultivars.

Results of the present study further revealed calli obtained on the callus inducing media containing higher concentration of sucrose when transferred to regeneration media produced higher numbers of albino plantlets. This observation is in agreement with the results of Shahnewaz and Bari (2004) who reported that highest concentration of sucrose (6 % and above) in the callus induction medium not only resulted in an increase in the percentage of callus formation but also promoted the regeneration of albino plants.

This observation is also in agreement with Chen (1978) who reported that the rates of callus formation and subsequent organogenesis increased with the sucrose concentration used (3-9 %), but the calli initiated on the medium with 9 % sucrose regenerated more albino plantlets. Thus, the after effect of sucrose considered for selecting the suitable concentration for callus initiation.

On an overall basis, the best callus induction – shoot regeneration media sequence was CI_{13}- (N_6 + 3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 7 % Sucrose + 0.9 % Agar) as callus induction media followed by R_1- (MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA +1.0 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar) as shoot regeneration for regeneration of higher number of green haploid plantlet.

Overall, differences were observed between genotypes and callus induction-shoot regeneration media sequences for regeneration of haploid plantlets.

In general, in IR-58025 among the 43 plants, regenerated 37 were green plantlets and six were of albino plantlets. Among the green plantlets, 29.06 % were found to be haploids by chromosome analysis from root tips as well as through ploidy analyzer, were obtained from 96 anther derived calli pieces of rice with 28.12 %, 44.79 %, 38.54 % and 2.38 % of calli differentiation, plantlet regeneration, green plantlet regeneration and anther culture capacity, respectively. whereas, In GR-7, total 16 plants, including 11 green and 5 albino plants, among which 22.21 % were found to be haploids by chromosome analysis from root tips, were obtained from 72 anther derived calli pieces of rice with 19.44 %, 22.22 %, 15.27 % and 0.88 % of calli differentiation, plantlet regeneration, green plantlet regeneration and anther culture capacity, respectively.

In Hybrid (F_1) total 33 plants, including 26 green and 7 albino plants, among which 30.20 % were found to be haploids by chromosome analysis from root tips as well as through ploidy analyzer. Plants obtained from 144 anther derived calli pieces of rice with 17.36 %, 22.91 %, 18.05 % and 1.33 % of calli differentiation, plantlet regeneration, green plantlet regeneration and anther culture capacity, respectively.

Considering the ratios of green plantlet regeneration versus plantlet regeneration, which includes both green and albino plantlets, the following inferred. This ratio was 0.86, 0.78 and 0.68 for the cv. IR-58025 (B-line), IR-58025 A x GR-7 and GR-7 respectively. The green plant regeneration ratio for the hybrid was increased compared to GR-7.

Hou *et al.* (1994) have also reported intermediate as well as values closer to low response genotype in barley anther culture of F_1 hybrids of highly and poorly responsive genotypes.

Per cent green plantlet regeneration relative to the calli differentiation was higher for the cv. IR-58025 (B-line) and its hybrid (F_1). This high percentage was due to the ability of many calli to give origin to multiple shoots ranging from 1-3 green plantlets.

These results are in agreement with Khanna and Raina (1998) who reported multiple green shoot induction in indica rice. Draz *et al*. (1991) also reported the effect of callus induction media on plantlet regeneration.

These results are also in agreement with the results of Zapata *et al*. (1982) who reported higher percentage of green plantlet regeneration than calli differentiation for Taipei 309 and Giza 170. These high percentage results were due to the ability of many calluses to give origin to multiple shoots ranging from 2-15 green plants. On an overall basis, number of green plantlets and regeneration of haploid plantlets was varying in all the three genotypes with respect to all media sequences studied.

The results further revealed that when calli were initiated between

40-55 days after anthers inoculation on different callus induction media and were transferred after 10-20 days of its emergence (i.e. 50-75 days after inoculation) to different regeneration media produced higher number of haploid plantlets, showing early callus induction as well as

early callus transfer to regeneration media beneficial to produce haploid plants.

Chen and Chen (1979) also reported advantage of early transfer for haploid plantlet regeneration and change in ploidy level during callus proliferation could be minimized with early transfer.

Therefore, it is concluded that response for green plantlet regeneration was remarkably higher when calli were sub cultured/transferred on to MS medium supplemented with 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar. Whereas, media MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar was found superior for percentage of haploid plantlet regeneration. The best media sequence was N_6 + 3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 7 % Sucrose + 0.9 % Agar as callus induction followed by transfer on MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar as shoot regeneration medium for production of maximum number of green haploid plantlets among all genotype.

4.8 ACCLIMATIZATION

As could be seen, lower survival rate in preliminary hardening experiments using different potting mixtures, the liquid nutrient as reported by Raina and Zapata (1997) was evaluated to check whether it would result in enhanced survival rate.

In the present study, plantlets of three cultivars *viz.*, IR-58025, GR-7 and their hybrid (F_1) obtained through anther culture with well

developed root system were transferred to pots of 250 ml containing three potting mixtures. The values for per cent survival of the plantlets are presented in Table 4.8.1 Per cent survival of the anther derived plantlets ranged from 36.36 % to 45.94 % up to secondary hardening (up to 25 days). Among the different potting mixtures studied, maximum survival (70.83 %) of plantlets were seen with T_3 (liquid nutrient + soil: soil rite (3:1)) potting mixture followed by T_2 (3 soil: 1 soil rite) whereas, least survival of plantlets were observed when

T_1 (Soil alone) was used as a potting mixture (14.76 %).

The per cent survival was found superior in IR-58025 as compared to GR-7 and their hybrid (F_1) among three potting mixtures. The average per cent survival in three genotypes studied was 40.25 %.

Lower survival rate in soil, indicated that the fibrous root system of young rice plantlets developed in a synthetic medium could not be able to absorb their nutrients easily from the soil. This problem of plant establishment in soil necessitated to formulate an intermediary liquid system, so that the delicate roots could easily absorb their nutrients from liquid medium. Liquid medium/hydroponic medium was formulated by considering the basic concept of nutrients requirement present in White's medium (Yoshida *et al*., 1986). The plantlets regenerated were transferred to liquid medium (contained macro and micro salts of white's media with iron source from MS media and lower pH- 5.5) for their better establishment. Within two weeks, regenerated plantlets produced elaborated roots in liquid medium (Plate-III, G). After proper

hardening, when plantlets developed new root system, they were transferred from liquid medium to soil: soil rite (3:1) and finally into soil. (Plate-V, A)

Superiority of liquid nutrient (Plate III, G) for the increased survival of anther derived plantlets observed in the present study is in confirmation with the results reported earlier by Raina and Zapata (1997) in rice. A total of 31 anther derived plantlets were transferred to green house conditions (daylight, 25-34 °C), 70 – 80 % relative humidity). However, the success of transplantation of regenerated green plants to soil conditions during late February to end of March was remarkably higher. (Plate- V)

Table: 4.8.1 Effect of genotypes and different potting mixtures (T) on per cent survival of anther culture raised plantlets in rice
cv. IR-58025(B-line), GR-7 and their Hybrid (F_1)

Genotypes / Potting mixtures	IR-58025		GR-7		Hybrid (F_1)		Mean
	Green plantlets transferred #	Green plantlets survived %	Green plantlets transferred #	Green plantlets survived %	Green plantlets transferred #	Green plantlets survived %	
T_1 (Soil alone)	10	3 (30.00)	3	0 (0.00)	7	1(4.28)	14.76
T_2 Soil : Soil rite (3:1)	15	5 (33.33)	4	1 (25.00)	11	4 (36.36)	31.56
T_3 Liquid nutrient + soil: soil rite (3:1)	12	9 (75.00)	4	3 (75.00)	8	5 (62.50)	70.83
Total/Mean	37	17 (46.11)	11	4 (33.33)	26	10 (37.71)	40.25

( ) Values in the parenthesis shows percentage of plantlets survived up to secondary hardening

Table: 4.8.2 Per cent survival of the anther derived green plantlets of rice cv. IR-58025, GR-7 and their Hybrid (F_1)

Genotype	Green plantlets obtained #	Plantlets survived up to primary hardening #	Plantlets survived up to secondary hardening #
IR-58025	37	33 (89.18)	17(45.94)
GR-7	11	10 (90.90)	4 (36.36)
Hybrid(F_1)	26	22 (84.61)	10 (38.46)
Total	74	31 (88.23)	31 (40.25)

( ) Values in the parenthesis shows percentage of plantlets survived

4.9 Morphological differences between anther cultures derived haploid and diploid/doubled haploid plants:

The haploid and diploid plants obtained through anther culture in the present investigation were further studied for differences in morphological traits. (The details is given in table 4.9)

The anther derived haploid plants showed morphological differences in their plant height, flag leaves, panicle and spikelets as compared to anther derived diploid/doubled haploid plants. The morphology of the haploid was not vigorous as compared to dihaploids. The height of the haploid was 43 cm whereas it was 95 cm in the diploid. Flag leaf, panicle and spikelets characters in haploid also showed considerable reduction in size *viz.,* flag leaf (area, length, width), panicle length and spikelets (length, width) (Table: 4.9) (Plate – IV, V).

Table: 4.9 Morphological differences between anther culture derived haploid and diploid/doubled haploid plants

Character	**Haploid**	**Diploid**
Plant height	43 cm	95 cm
Flag leaf		
Area	3.90 mm^2	17.76 mm^2
Length	12.08 cm	38.40 cm
Max. Width	0.6 cm	1.3 cm
Panicle length	28 cm	55 cm
Spikelets		
Avg. Length	7.58 mm	9.23 mm
Avg. Width	2.07 mm	2.36 mm
Stomatal Area*	188.56 μm^2	390.02 μm^2
Stomatal Density [a]	40.90	30.09
Chromosome number	12	24

* Mean stomatal area obtained from 20 stomata per plant were measured

The measurements were recorded using µm unit.

[a] The stomata density average was calculated by counting stomata in 10 fields of view (124929 μm^2 at 40x view)

Morphological differences between haploid and diploid/doubled haploid plants produced through anther culture were also reported by Nakamura *et al.* (1994) in rice.

Raina and Zapata (1997) also reported haploids have reduced plant height, stunted growth, narrower leaves, short cigar shaped panicles and complete pollen sterility as compared to diploid/doubled haploid plants.

V. SUMMARY AND CONCLUSION

The investigation embodied in the present thesis was aimed at: (i) Standardization of surface sterilization technique (ii) To study the effect of cold pre-treatment on callus induction (iii) To study the response of basal media for *in vitro* callus induction (iv) Optimization of cultural environment (v) *In vitro* rooting (vi) *In vitro* haploid plantlets regeneration (vii) Acclimatization by using two parental lines of indica rice *viz*., IR- 58025 (B-line), GR-7 and their heterotic hybrid i.e. IR-58025 A x GR-7 (F_1) at Plant Tissue Culture Laboratory, Department of Agricultural Biotechnology, Anand Agricultural University, Anand during 2005-2008.

The characters studied were percentage contamination, days to callus initiation, percentage callusing, colour and texture of callus, per cent calli differentiation, days to shoot primordia/plantlet initiation, percentage green plantlet regeneration, percentage albino plantlet regeneration, percentage rooting, percentage haploid plantlets regeneration, and percentage survival of plantlets.

Anthers contain healthy microspores from mid uninucleate to early binucleate stages were used for different experiments of the present study. N_6 medium (1975), MS medium (1962) and its modifications, Nitsch and Nitsch medium (1969), White's basal medium (1963) were used as basal media for different studies.

Various phytohormones (2,4-D, NAA, BA and Kn) and carbon source (Sucrose) were supplemented to different basal media based on objective of

APPROPRIATE STAGE (CYTOLOGICAL AND MORPHOLOGICAL) OF ANTHER FOR *IN VITRO* STUDIES

High proportions of microspores at uninucleate stage were observed when the distance between the base of the flag leaf and the auricle of the penultimate leaf was 5-9 cm and anther development/blooming reached up to 1/2 - 1/3 of spikelets length.

SURFACE STERILIZATION

Analysis of variance revealed significant differences for the surface sterilization treatments. Genotype x surface sterilization treatment interactions was also significant. Where as, genotype effect was non-significant. Among the various treatments, S_3 (70 % Ethyl alcohol for 10 seconds to panicle + 0.1 % $HgCl_2$ for 5 minutes to spikelets) significantly reduced (2.50 %) the contamination.

COLD PRE-TREATMENT

Analysis of variance revealed non-significant differences for cold pre-treatments indicating the effect of various treatments for efficient callus induction was at par.

Genotypes and genotypes x cold pre-treatment interaction effects were also non-significant. This indicated that there was no genotype specific effect of different cold pre-treatments. The per cent callusing for all the genotypes studied, indicating the superiority of C_2 (12 Days cool pre-treatment to panicles at 7 ± 1 ºC) among all cold pre-treatments tried. Among the genotypes studied, cold pre-treatment C_2 exhibited early induction of callus (53.62 days) as compared to other cold pre-treatment tried.

MEDIA RESPONSE FOR *IN VITRO* CALLUSING, PLANTLET REGENERATION, *IN VITRO* ROOTING AND *IN VITRO* HAPLOID PLANTLET REGENRATION

(i) Callus induction:

Even though responses to callusing were attempted using two basal medium *viz*., N_6 and MS-1, preliminary studies demonstrated that the medium N_6 gave significantly higher performance for callus induction than basal medium MS-1.

Analysis of variance revealed significant differences among the treatments (Auxin: Sucrose combinations) and basal media for percentage callusing, indicating real differences between treatments as well as between basal media. However, differences were non-significant for genotypes, genotypes x basal media, genotypes x treatments, basal media x treatments and genotypes x basal media x treatments which indicated consisted performance of genotypes and also of treatments across the genotypes and basal media for callusing. Nitrogen sources in inorganic form supported the callus induction, Inorganic nitrogen such as ammonium sulphate, potassium nitrate and concentration of NH^{4+} ions and the ratio between NH^{4+} and NO^{3-} ions supported the callus induction and its growth.

It appeared that moderate levels of 2, 4-D (2.0 mg l^{-1}) with higher concentration of sucrose (6 %) induced more callusing.

Between the two media studied, medium N_6 exhibited minimum number of days (66.30) as compared to MS-1 for callus initiation.

Among the genotypes, IR-58025 exhibited early callus initiation (66.42) as compared to GR-7.

Calli obtained on N_6 and MS-1 medium supplemented with different levels of 2,4-D and Sucrose combinations yielded variation in color of callus *viz.,* cream, yellowish with friable, loose and compact texture callus irrespective of the genotypes.

Based on further studies, analysis of variance revealed significant differences among the genotypes for percentage callus induction as well as for days to callus initiation indicating real effect of genotype on these two traits.

Among the genotypes studied, IR-58025 showed early (58.19 days) and better callusing (4.5 %) response as compared to GR-7.

(ii) Calli differentiation:

Even though responses to plantlet regeneration were attempted using two basal medium *viz*., MS and MS-2, MS basal medium was found to be superior (47.56 %) over MS-2 for calli differentiation when medium fortified with 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 3 % Sucrose + 0.9% Agar.

Among the genotypes studied, GR-7 showed highest average calli differentiation (49.99 %) irrespective of the basal media used suggesting presence of genotypic effect.

(iii) Plantlet regeneration:

MS media fortified with phytohormone *viz*., 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn in combination with 3 % sucrose responded better (41.31%) for induction of green shoots as compared to MS-2 media (35.41 %) fortified with 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn in combination with 3 % sucrose for both the genotypes.

Per cent green plantlet regeneration were 40.62 and 36.10 % for the

cv. IR-58025 and GR-7 respectively, indicating genotypic effect

Between the two media studied, MS basal medium with growth regulator and sucrose combination exhibited early induction of green shoots

(25.04 days) as compared to MS-2 basal medium with growth regulator and sucrose combination tried.

(iv) *In vitro* rooting:

Attempts to bring about induction of root were successful. Even though responses to rooting attempted using three basal medium *viz*., ½ MS,

Nitsch & Nitsch and White's, studies revealed that the medium white's gave better results for *in vitro* root induction than MS and Nitsch & Nitsch.

Maximum percentage rooting (100.0 %) was observed on medium White's + 2.0 % Sucrose + 0.9 % Agar on both the genotypes.

(v) *In vitro* haploid plantlet regeneration:

Medium MS was found to be superior (21.42, 20.0 %) for haploid plantlet regeneration for the genotype IR-58025 and GR-7 respectively. when supplemented with 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 3 % Sucrose.

Chromosome counts from root tips of plantlets regenerated in the two genotypes indicated that 13.68 % were haploid

(vi) Acclimatization:

Among the potting mixtures studied, maximum survival of plantlets were seen with T_2 (3 soil: 1 soil rite) potting mixture (18.33 %). The average per cent survival found to be quite superior in the cv. IR-58025 as compared to GR-7 among potting mixtures. The average per cent survival among genotypes was of 13.27 %.

OPTIMIZATION OF CULTURAL ENVIRONMENT

The growth hormones are the essential factors for the growth of the plant cells as they trigger metabolic pathways through their effects on nucleic acid metabolism.

(i) Callus induction:

Analysis of variance revealed significant differences among the Genotypes, Callus induction media and Genotypes x Callus induction media interaction for percentage callusing, indicating real differences between genotypes, callus induction media and their interaction effects for per cent callusing. Genotype accounted for the greater variation in callus induction, although callus induction medium and callus induction medium x genotype interaction effects were significant. These results revealed that abilities of callus induction in rice anther culture strongly influenced by genotype and the composition of culture medium.

Effect of auxin: cytokinin (2,4-D/NAA, Kn) and carbon source (Sucrose) at different concentration showed that the higher concentration of 2,4-D (3.0 mg l^{-1}) with lower concentration of Kn (0.5 mg l^{-1}) combined with higher sucrose concentration (7.0 %) was a major determinant of

response, for callus induction. However, the rate of increase was genotype dependent.

To confirm whether the growth hormone activity is the cause/consequence of the callus induction process, control (CI_1) was performed.

The responses for days to callus initiation to various combinations of

2,4-D, NAA, Kn and sucrose revealed that the early callus induction was attained in the medium(N_6) containing 3.0 mg l^{-1} 2,4-D in combination with

1.0 mg l^{-1} Kinetin and 7.0 % sucrose.

At their optimal concentrations auxin (2,4-D) and cytokinin (Kinetin) with sucrose together supported higher callus induction than auxin (2,4-D) and sucrose combination.

Analysis of variance revealed significant differences among the callus induction media treatments for days to callus initiation indicating real differences between callus induction media for days to callus initiation.

The results indicating higher concentration (3.0 mg l^{-1}) of auxin (2,4-D) and cytokinin (Kn) (1.0 mg l^{-1}) along with higher sucrose concentration (7 %) gives early callus induction.

Callus was friable/compact and creamish/yellowish in colour irrespective of the different genotypes studied. However, inclusion of NAA in the media yielded yellowish coloured callus with compact texture. Yellow coloured with compact texture calli showed higher calli differentiation.

(ii) Calli differentiation:

The effect of callus age on calli differentiation observed for the two parents and their hybrid, calli emerged between 50-60 days (i.e. 60- 80 days old calli) showed better calli differentiation and thereafter decreased gradually.

Calli differentiation was varying with respect to genotypes and callus induction-regeneration media combination. These results exhibited the extent of variation in callus induction and subsequent calli differentiation might be because of medium x genotype interaction.

Higher level of 2,4-D (3.0 mg l^{-1}) with lower level of Kn (1.0 mg l^{-1}) along with higher sucrose concentration (7 %) exhibited higher callus induction as well as calli differentiation among all genotypes. However, inclusion of NAA in to the callus induction medium appeared to be suitable for realizing higher per cent of calli differentiation in the cv. GR-7 and IR-58025 A x GR-7 (Hybrid).

(iii) Plantlet regeneration (Green and Albino):

Per cent green plantlet regeneration was varying with respect to genotypes and regeneration media combination. These results revealed the extent of variation in calli differentiation and subsequent green plantlet regeneration might be as a consequence of medium x genotype interaction.

The average per cent calli differentiation was 28.12, 17.35 and 19.44 % for the cv. IR-58025, Hybrid (F_1) and GR-7 respectively and the average

per cent green plantlet regeneration was 38.53, 18.05 and 15.27 % for the

cv. IR-58025, Hybrid (F_1) and GR-7 respectively, indicating genotypic effect for these traits

Among the media, response for green plantlet regeneration was remarkably higher when calli were sub cultured/transferred on to MS medium supplemented with 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3% Sucrose + 0.9 % Agar.

Among the media, response for albino plantlet regeneration was remarkably lower when calli were sub cultured/transferred on to MS medium supplemented with 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar and 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar.

Analysis of variance revealed significant differences among the shoot/plantlet regeneration media treatments for days to green plantlet regeneration.

Among the media studied, MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar exhibited early induction of green shoots (17.83 days) as compared to other growth regulator and sucrose combination treatments tried.

(iv) *In vitro* rooting:

Medium White's (1963) with low level of sucrose (2 %) induced efficient rooting.

(v) Cytological and anatomical study of anther derived plantlets:

Cytological study:

Chromosome analysis from root tips as well as through ploidy analyzer revealed that per cent haploid plantlet regeneration was varying with respect to genotypes and regeneration media combination.

MS media fortified with phytohormones *viz*., (NAA, BA/Kn) in combination with sucrose responded better (29.06 %, 22.21 % and 30.20 %) for the genotype IR-58025, GR-7 and their hybrid (F_1) respectively for haploid plantlet regeneration.

Among the eight media studied, MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar was found superior for percentage of haploid plantlet regeneration (72.20 %) as compared to other growth regulator and sucrose combination treatments tried.

The average percentage of haploids and diploid/doubled haploids was 27.14 % and 72.86 % respectively, over the different genotypes.

Among the various callus induction – shoot regeneration media sequences studied, N_6 + 3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 7 % Sucrose +

0.9 % Agar as callus induction medium followed by transfer on MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA +1.0 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar as shoot regeneration medium was found better for production of higher number of green haploid plantlets among all genotype.

Anatomical study:

Haploid plants presented the lowest stomatal area (188.56 μm^2) while diploid showed the highest one (390.02 μm^2). The stomatal density for haploid plants was 40.90 and diploid showed 30.09 stomata per field of view (124929 μm^2) at 40 x.

(vi) Acclimatization:

In vitro grown rooted plantlets transferred to small plastic container filled with different potting mixture (i.e. soil alone, soil: soil rite - 3: 1 and liquid nutrient + soil: soil rite (3:1)).

Per cent survival of the anther culture raised plantlets ranged from 36.36% to 45.94 % up to secondary hardening. Among the different potting mixtures studied, maximum survival of plantlets was seen with liquid nutrient followed by soil: soil rite (3:1) potting mixture. The average per cent survival among three genotypes was of 40.25 %.

The success of transplantation of regenerated green plants to soil conditions during late February to end of March was remarkably higher.

(vii) Morphological differences between anther cultures derived haploid and diploid/doubled haploid plants:

The anther derived haploid plants showed morphological differences in their plant height, flag leaves, panicle and spikelets as compared to anther derived diploid/doubled haploid plants. The morphology of the haploid was not vigorous in comparison to diploid.

Based on these results following conclusions can be drawn:

1. High proportions of microspores at uninucleate stage were observed when the distance between the base of the flag leaf and the auricle of the penultimate leaf was 5-9 cm and anther development/blooming reached up to 1/2 - 1/3 of spikelets length.
2. Cold pretreatment of 7 ± 1 °C for 12 days was found superior for callusing.

3. 70 % Ethyl alcohol for 10 seconds to panicle + 0.1 % $HgCl_2$ for 5 minutes to spikelets was found effective in reducing percentage contamination in three genotypes viz. IR- 58025 (B-line), GR-7 and their hybrid IR- 58025 A x GR-7.
4. N_6 basal medium (1975) was found superior for *in vitro* callus induction of all the three genotypes.
5. MS basal medium (1962) was found superior for green haploid plantlet regeneration of all the three genotypes.
6. Among the various auxin (2,4-D/NAA) : Cytokinin (Kn) and carbon source (Sucrose) combinations, higher concentration of 2,4-D

   (3.0 mg l^{-1}) with lower concentration of Kn (0.5 mg l^{-1}) combined with higher sucrose concentration (7.0 %) induced maximum callusing. However, the rate of increase was genotype dependent.
7. The plant regenerating capabilities of the calli increased slightly between 50-60 days of incubation (i.e. 60-80 days old calli) of the anthers.
8. Among the various auxin (2,4-D/NAA) : Cytokinin (Kn) and carbon source (Sucrose) combinations, higher concentration of 2,4-D

   (3.0 mg l^{-1}) with lower concentration of Kn (1.0 mg l^{-1}) combined with higher sucrose concentration (7.0 %) showed maximum calli differentiation. However, the rate of increase was genotype dependent.

9. Among the eight media, response for green plantlet regeneration was remarkably higher when calli were sub cultured/transferred on to MS medium supplemented with 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar. However, genotypic differences were found in green plantlet regeneration.
10. Among the eight media studied, MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar was found superior for percentage of haploid plantlet regeneration.
11. Among the various callus induction – shoot regeneration media sequences studied, N_6 + 3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 7 % Sucrose + 0.9 % Agar as callus induction medium followed by transfer on MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA +1.0 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar as shoot regeneration medium was found better for production of maximum number of green haploid plantlets among all genotype.
12. Higher levels of auxin, 2,4-D (3.0 mg l^{-1}) along with cytokinin (Kn) and sucrose induced better callusing in all the media combinations studied.
13. Significant differences among the genotype for various characters studied *in vitro* revealed the role of the genotype in development of rapid reproducible regeneration system.
14. Genotypic differences for various traits under study revealed the genetic control of these traits for its expression.
15. Among the different rooting media, White’s + 2.0 % Sucrose + 0.9 % Agar induced efficient rooting.

16. Acclimatization of anther culture raised plantlets showed the superiority of liquid nutrient followed by soil: soil rite (3:1) as a potting mixture. However, the success of transplantation of regenerated green plants to soil conditions during late February to end of March was remarkably higher.
17. The anther derived haploid plants showed morphological differences in their plant height, panicles, flag leaves, spikelets, stomata area and stomatal density as compared to anther derived diploid/doubled haploid plants.
18. *In vitro* androgenesis is under genetic control and high responding cultivars may carry more of the genes responsible for this process. Therefore, one way to improve the response in anther culture is by genetic manipulation of the genotype of the donor plants.
19. Conclusion suggest that in the development of anther culture as a breeding technique, emphasis should be on selecting responsive genotypes rather than on attempting to define culture media that enhance the response. Once this approach adopted, germplasm collections screened for both responsiveness of anthers to culture and frequency of regeneration of green plants, and those capabilities incorporated into cultivated varieties through hybridization.
20. It should be pointed out that the above conclusions may not be applied to all rice cultivars as genotypic differences in hormone requirement have been reported (Liang, 1978; Cornejo-Martin and Primo-Millo, 1981).

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* Original not seen

Table: 4.4.7 Effect of genotype and basal media on per cent plantlet (green and albino) regeneration and days to shoot primordia or plantlet initiation (green and albino) [a]

Media*	Days to shoot primordia/plantlet initiation [a]						Per cent plantlet regeneration					
	Genotype						Genotype					
	IR-58025		GR-7		Mean		IR-58025		GR-7		Mean	
	Green	Albino	Green	Albino	Green	Albino	Green	Albino	Green	Albino	Green	Albino
MS(1962)	22.42	20.50	27.66	21.00	25.04	20.75	43.75	8.33	38.88	22.22	41.31	15.27
MS-2	25.31	22.50	29.33	17.50	27.32	20.0	37.50	10.41	33.33	16.66	35.41	13.53
Mean (Media)	23.86	21.50	28.49	19.25	26.18	20.37	40.62	9.37	36.10	19.44	38.36	14.40
Mean (Genotype)	22.68		23.87		23.27		24.99		27.77		26.38	

[a] Values in the table are original mean values

* Both media were supplemented with 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 3 % Sucrose + 0.9 % Agar

Table: 4.4.8 Effect of genotype, shoot induction basal media and rooting media treatments on percentage rooting [a]

Treatment	Genotype												Mean
	IR-58025						GR-7						
	MS			MS-2			MS			MS-2			
	Shoots inoculated #	Shoots rooted #	Rooting %	Shoots inoculated #	Shoots rooted #	Rooting %	Shoots inoculated #	Shoots rooted #	Rooting %	Shoots inoculated #	Shoots rooted #	Rooting %	
P_1	6	5	83.33	6	5	83.33	2	2	100.0	2	2	100.0	91.66
P_2	6	4	66.66	6	4	66.66	2	1	50.0	2	1	50.0	58.33
P_3	6	6	100.0	6	6	100.0	2	2	100.0	2	2	100.0	100.0
Mean			83.33			83.33			83.33			83.33	83.33

[a] Values in the table are original mean value

Table: 4.4.9 Effects of genotypes and basal media (Shoot induction) on haploid plantlet regeneration in rice anther culture cv. IR- 58025 and GR-7

Shoot induction Media	Plantlets Studied #		Total Plantlets Obtained #		Haploid %			Diploid/Doubled haploid %		
	IR-58025	GR-7	IR-58025	GR-7	IR-58025	GR-7	Mean	IR-58025	GR-7	Mean
MS (1962)	14	5	21	7	21.42(3)	20.00(1)	20.71	78.57(11)	80.00(4)	79.28
MS-2	15	4	18	6	13.33(2)	0.00(0)	6.66	86.66(13)	100.00(4)	93.33
Total	29	9	39	13	5	1		24	8	
Mean					17.37	10.0	13.68	82.61	90.0	86.30

( ) Values in the parenthesis are the number of plantlets

Table: 4.4.10 Effect of genotype and different potting mixtures (T) on per cent survival of anther culture raised plantlets in rice cv.IR-58025(B-line) and GR-7

Genotypes / Potting mixtures	IR-58025		GR-7		Mean
	Green plantlets transferred #	Green plantlets survived #	Green plantlets transferred #	Green plantlets survived #	
T_1 (Soil alone)	17	3 (17.64)	5	0 (0.00)	8.82
T_2 (Soil : Soil rite (3:1))	20	4 (20.00)	6	1 (16.66)	18.33
Total/Mean	37	7 (18.82)	11	1 (8.33)	13.57

( ) Values in the parenthesis shows percentage of plantlets survived up to secondary hardening.

Table: 4.4.11 Per cent survival of the anther derived green plantlets of rice cv. IR-58025 (B-line) and GR-7

Genotype	Green plantlets obtained #	Plantlets survived up to primary hardening #	Plantlets survived up to secondary hardening #
IR-58025	39	27 (72.97)	7 (18.82)
GR-7	13	9 (81.81)	1 (8.33)
	52	36 (77.39)	8 (13.57)

( ) Values in the parenthesis shows percentage of plantlets survived.

Table: 4.5.2 Effect of genotype and callus induction media on per cent callusing from anthers of rice cv. IR-58025, GR-7 and their hybrid (F_1 - GRH 1) [a]

Media code	Callus induction media	Genotype			Mean
		IR-58025	Hybrid (F_1)	GR-7	
		Mean	Mean	Mean	
CI_1	(0.0 mg l^{-1} 2,4-D + 0.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 6 % Sucrose)	0.42 (0.44)	0.22 (0.00)	0.22	0.28
CI_2		0.62 (0.88)	0.82 (1.33)	(0.00)	(0.14)
CI_3	(1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 % Sucrose)	0.82 (1.33)	1.01 (1.77)	0.62 (0.88)	0.68
CI_4		0.82 (1.33)	0.71 (1.33)	0.62 (0.88)	(1.03)
CI_5	(1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 % Sucrose)	0.91 (1.77)	1.01 (1.77)	0.62 (0.88)	0.82
CI_6		1.61 (3.11)	0.98 (2.22)	0.62 (0.88)	(1.32)
CI_7	(1.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 % Sucrose)	1.90 (4.00)	1.70 (3.55)	1.01 (1.77)	0.71
CI_8		1.61 (3.11)	1.21 (2.22)	1.21 (2.22)	(1.18)
CI_9	(1.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 % Sucrose)	1.81 (3.55)	1.50 (3.11)	1.01 (1.77)	0.85
CI_{10}		2.32 (6.22)	1.21 (2.22)	1.99 (4.44)	(1.47)
CI_{11}	(2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 % Sucrose)	2.85 (9.33)	1.79 (4.00)	0.62 (0.88)	1.20
CI_{12}		1.21 (2.22)	2.52 (6.66)	0.91 (1.77)	(2.36)
CI_{13}	(2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 % Sucrose)	1.21 (2.22)	3.08 (9.77)	0.62 (0.88)	1.60
CI_{14}		0.62 (0.88)	1.11 (2.22)	0.91 (1.77)	(3.25)
CI_{15}	(2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 % Sucrose)	0.91 (1.77)	0.71 (1.33)	0.82 (1.33)	1.28
				0.82 (1.33)	(2.36)
	(2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 % Sucrose)				1.77
					(3.70)
	(3.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 % Sucrose)				1.38
					(3.10)
	(3.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 % Sucrose)				1.85
					(5.03)
	(3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 % Sucrose)				1.45
					(3.25)
	(3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 % Sucrose)				1.73
					(4.58)

	(2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 1.5 mg l^{-1} NAA + 6 % Sucrose) (2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 2.5 mg l^{-1} NAA + 6 % Sucrose)				0.85 (1.47) 0.81 (1.47)
Mean		**1.313(2.81)**	**1.311(2.90)**	**0.844(1.44)**	**1.15 (2.38)**
Range		0.42 - 2.85 (0.44 - 9.33)	0.22 - 3.08 (0.0 - 9.77)	0.22 - 1.99 (0.0 - 4.44)	
S.Em. (Genotype)		0.076			
C.D. at 0.05 (Genotype)		0.210			
S.Em. (Callus induction media)		0.169			
C.D. at 0.05 (Callus induction media)		0.470			
S.Em. (Interaction)		0.294			
C.D. at 0.05 (Interaction)		0.814			
C.V. (%)		76.19			

Data transformed through square root transformation, [a] Values in the parenthesis represents original mean values

Table: 4.5.5 Effect of callus induction media treatment (CI_1-CI_{15}) on days to callus initiation for anthers of rice cv. IR-58025, GR-7 and their Hybrid (F_1)

Media code	Callus induction media	Genotype			Mean
		IR-58025	Hybrid (F_1)	GR-7	
		Mean	Mean	Mean	
CI_1	(0.0 mg l^{-1} 2,4-D + 0.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 6 % Sucrose)	74.00	-	-	74.00
CI_2	(1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 % Sucrose)	73.00	69.00	73.00	71.66
CI_3	(1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 % Sucrose)	71.33	68.25	72.50	70.69
CI_4	(1.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 % Sucrose)	58.00	66.00	71.00	65.00
CI_5	(1.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 % Sucrose)	56.33	58.50	68.50	61.11
CI_6	(2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 % Sucrose)	53.85	55.33	66.25	58.47
CI_7	(2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 % Sucrose)	52.12	54.57	64.40	57.03
CI_8	(2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 % Sucrose)	51.14	54.00	63.50	56.21
CI_9	(2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 % Sucrose)	47.25	52.83	60.75	53.61
CI_{10}	(3.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 % Sucrose)	46.12	46.40	55.00	49.17
CI_{11}	(3.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 % Sucrose)	45.37	45.85	52.00	47.74
CI_{12}	(3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 % Sucrose)	41.80	44.11	50.00	45.30
CI_{13}	(3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 % Sucrose)	40.80	42.11	48.00	43.63
CI_{14}	(2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 1.5 mg l^{-1} NAA + 6 % Sucrose)	56.00	50.50	54.33	53.61
CI_{15}	(2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 2.5 mg l^{-1} NAA + 6 % Sucrose)	48.00	45.00	49.33	47.44

Mean	54.34	53.74	60.61	56.23
Range	40.80-74.00	42.11-69.00	48.00-73.00	
S.Em. (Treatment)	1.109	2.088	1.780	
C.D. at 0.05 (Treatment)	3.139	5.918	5.137	
C.V. (%)	4.02	8.12	4.85	

(-) represents No Response Obtained

Table: 4.5.6 Effect of different callus induction media on callus morphology in rice cv. IR-58025, GR-7 and their Hybrid (F_1)

Media code	Callus induction media	Genotype					
		IR-58025		Hybrid (F_1)		GR-7	
		Callus color	Callus texture	Callus color	Callus texture	Callus color	Callus texture

CI_1	(0.0 mg l^{-1} 2,4-D + 0.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 6 %	W	C	-	-	-	-
CI_2	Sucrose)	W	L	W	F	W	F
CI_3	(1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 %	W	F	W	L	W	F
CI_4	Sucrose)	W	F	W	F	W	F
CI_5	(1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 %	Cr	F	Cr	F	Cr	F
CI_6	Sucrose)	Y	C	Y	C	Y	C
CI_7	(1.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 %	Y	C	Y	C	Y	F
CI_8	Sucrose)	Y	C	Y	F	Y	C
CI_9	(1.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 %	Y	F	Y	F	Cr	F
CI_{10}	Sucrose)	Cr	C	Cr	C	Y	C
CI_{11}	(2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 %	Cr	F	Y	F	Y	C
CI_{12}	Sucrose)	Y	C	Y	C	Y	C
CI_{13}	(2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 %	Y	C	Y	C	Y	F
CI_{14}	Sucrose)	Y	C	Y	C	Y	C
CI_{15}	(2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 %	Y	C	Y	C	Y	C
	Sucrose)						
	(2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 %						
	Sucrose)						
	(3.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 %						
	Sucrose)						
	(3.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 %						
	Sucrose)						
	(3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 5 %						
	Sucrose)						
	(3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kn + 0.0 mg l^{-1} NAA + 7 %						
	Sucrose)						
	(2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 1.5 mg l^{-1} NAA + 6 %						
	Sucrose)						
	(2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn + 2.5 mg l^{-1} NAA + 6 %						
	Sucrose)						

W - White; Cr - Cream; Y - Yellowish
L - Loose; F - Friable; C - Compact

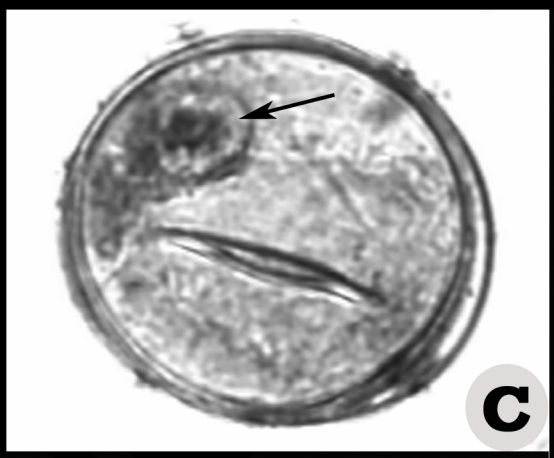
Table: 4.6.1 Effect of genotype and shoot induction media treatments on percentage rooting

Treatment	Genotype									Mean
	IR-58025			GR-7			Hybrid (F_1 GRH-1)			
	Shoots inoculated #	Shoots rooted #	Rooting %	Shoots inoculated #	Shoots rooted #	Rooting %	Shoots inoculated #	Shoots rooted #	Rooting %	
R_1	4	4	100.0	1	1	100.0	2	2	100.0	100
R_2	2	2	100.0	0	0	0.00	2	2	100.0	100
R_3	7	6	85.71	3	3	100.0	4	4	100.0	95.23
R_4	6	6	100.0	3	3	100.0	6	6	100.0	100
R_5	3	3	100.0	0	0	0.00	2	2	100.0	100
R_6	5	5	100.0	1	1	100.0	2	2	100.0	100
R_7	3	3	100.0	1	1	100.0	3	2	66.66	88.88
R_8	7	6	85.71	2	2	100.0	5	5	100.0	95.23
Mean			83.92			100.0			95.83	97.41

Values in the table are original values, Rooting media: White’s basal media + 2% Sucrose + 0.9 % Agar







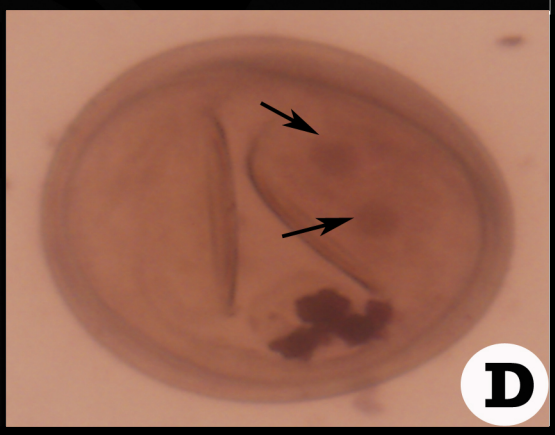






PLATE - I

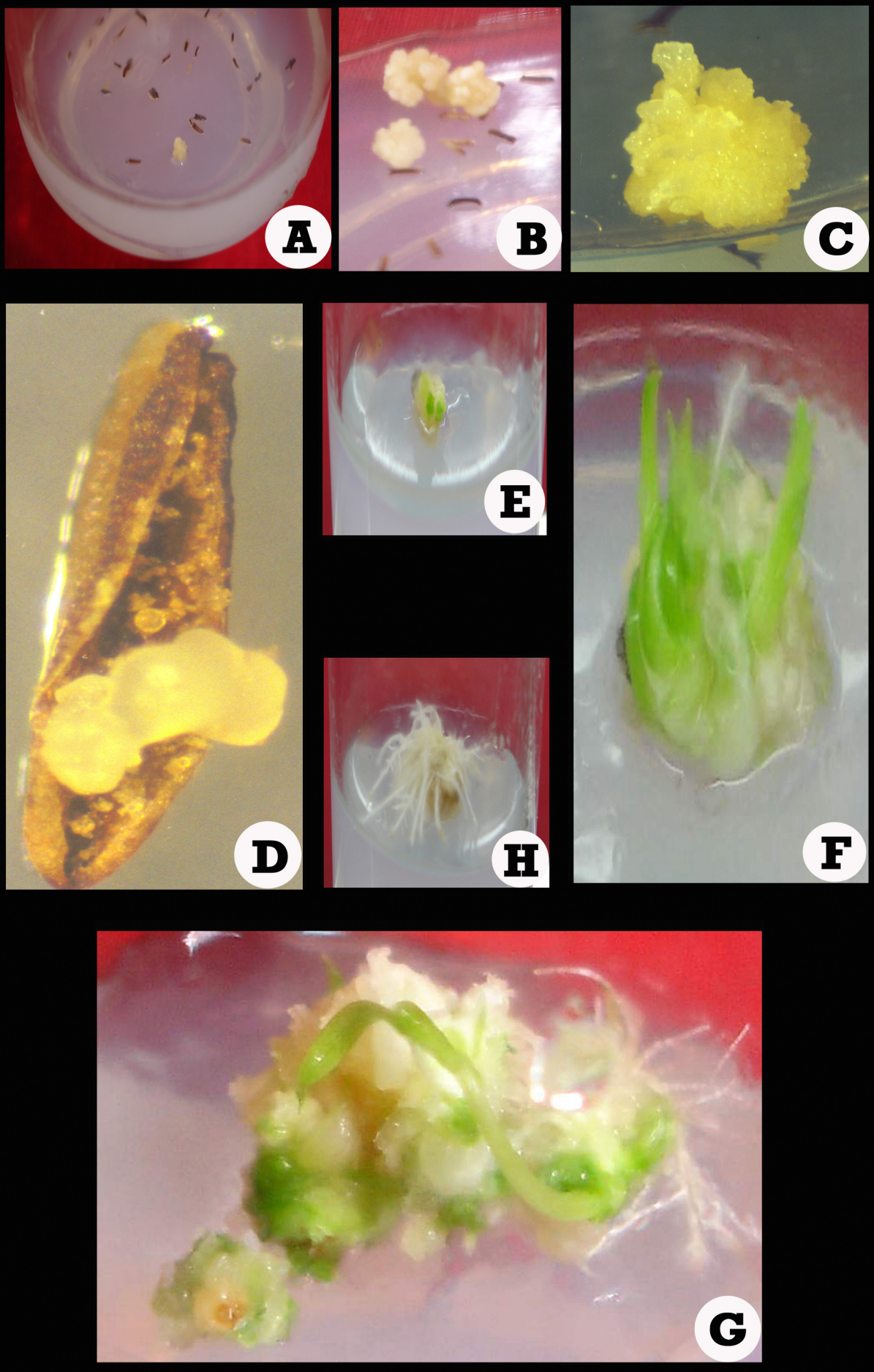
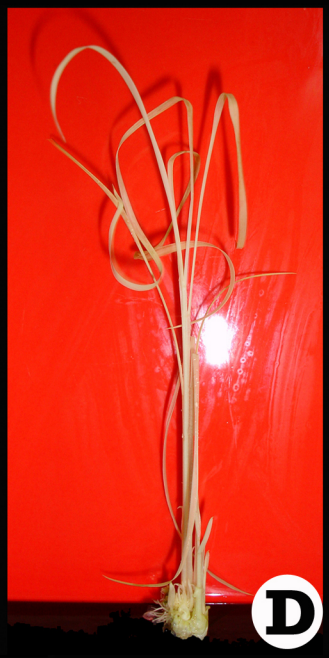


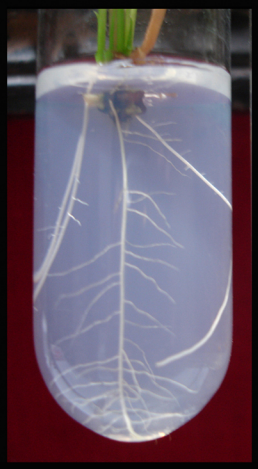
PLATE - II













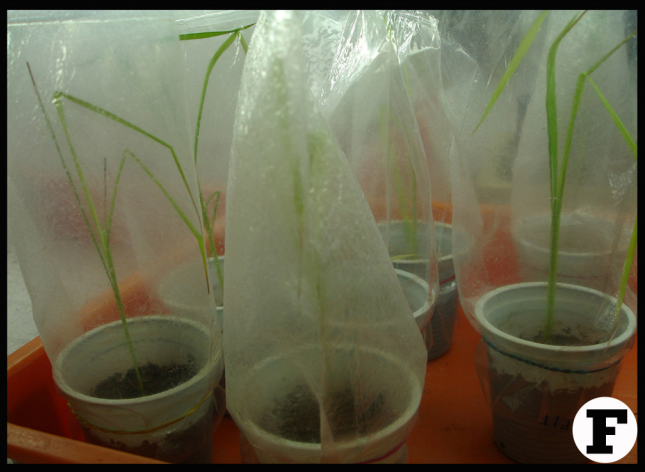




PLATE - III





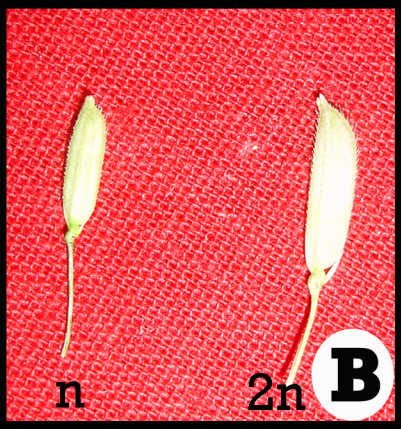


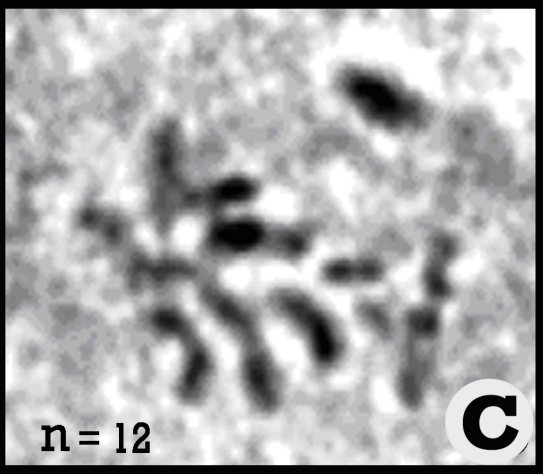


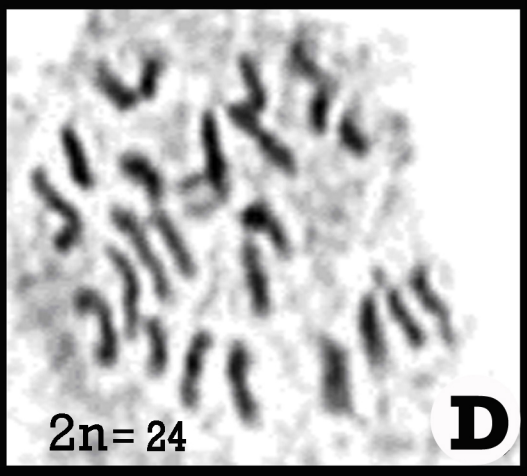
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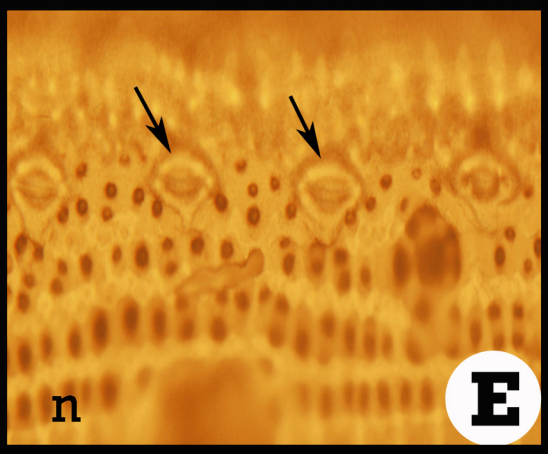
PLATE - V











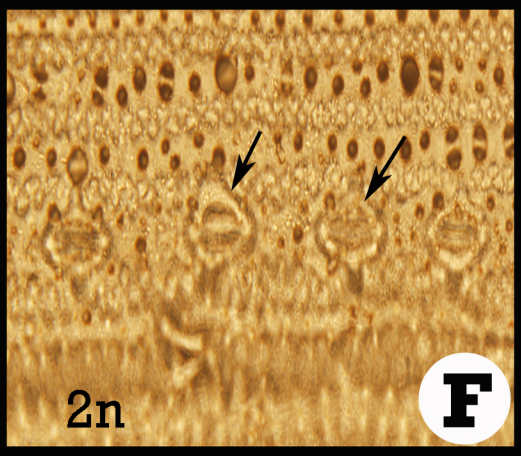


PLATE - IV