ANDROGENESIS IN RICE (*Oryza sativa* L.) BREEDING

By

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THESIS

submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

Faculty of Agriculture
Kerala Agricultural University, Thrissur

2004

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DECLARATION

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

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CERTIFICATE

Certified that the thesis entitled **"Androgenesis in rice (*Oryza sativa* L.) breeding"** is a record of research work done independently by Sri. V.T. Chandrahasan under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to him.

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ACKNOWLEDGEMENT

I wish to express my deep sense of gratitude and indebtedness to Dr.Dijee Bastian, Assistant Professor, Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara and Chairperson of my Advisory Committee for her meticulous guidance, concrete suggestions and constant encouragement throughout this investigation and preparation of thesis.

I express my heartfelt gratitude to Dr.Achamma Oommen, Professor and Head, Department of Plant Breeding and Genetics and member of my Advisory Committee for her kind concern and valuable help. My heartfelt thanks are expressed to Dr.K.Pushkaran, former member of my advisory committee for his help and suggestions.

I am grateful to the valuable advices received from Dr.V.V.Radhakrishan, Associate Professor, Department of Plant Breeding and Genetics and member of my Advisory Committee, during the course of my study.

I am extremely thankful to Dr.K.Aravindakshan, Associate Professor, Department of Pomology and Floriculture and member of my Advisory Committee for his help during various stages of the study. I express my sincere thanks to Dr.P.K. Rajeevan, Associate Professor and Head, Department of Pomology and Floriculture for providing lab facilities

I am thankful to the teaching and non-teaching staff of Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara for all the help rendered during the period of my research work. Research Associates and Lab Assistants, Tissue Culture Unit, Department of Pomology and Floriculture, Vellanikkara are gratefully acknowledged for all the help rendered by them during the course of my research.

A note of thanks to Dr.S.Lakshmi Narayanan, Research Associate, NBPGR, Dr.S.Manonmani, Paddy Breeding Station, TNAU and Dr. Sukumar, CPMB, TNAU for their help and valuable suggestions.

I shall always remain indebted to all my friends for their timely help and encouragement. I will always treasure their rich friendship.

Words can not express my gratitude to my parents, sister and brother without whose sacrifice the study would have never been possible.

The award of KAU research fellowship is deeply acknowledged.

V.T.Chandrahasan

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

A_1	-	Plants directly regenerated from callus
A_2	-	Self pollinated progeny of A_1
BAP	-	6- Benzylaminopurine
CH	-	Caseine Hydrolysate
CRD	-	Completely Randomized Design
CW	-	Coconut Water
df	-	degrees of freedom
2, 4- D	-	2, 4- Dichlorophenoxyacetic acid
DH	-	Doubled Haploid
edf	-	error degrees of freedom
FCRD	-	Factorial Completely Randomised Design
IAA	-	Indoleacetic acid
IBA	-	Indole-3- Butric acid
Kn	-	Kinetin
NAA	-	Napthaleneacetic acid
pDNA	-	plasdid DNA

INTRODUCTION

1. INTRODUCTION

Rice is the principal food of nearly half of mankind. Rice is grown in our country in an area of 43 million hectares with a production of 77.7 million tonnes (Anon, 2003). It is estimated that the rice demand in 2010 will be 100 million tonnes and in 2025, the demand will be 140 million tonnes (Mishra, 2004). In Kerala, rice occupies an area of 3.9 lakh hectares with an annual production of 7.7 lakh tonnes (FIB, 2002). In the era of ever increasing population and shrinking resources, including arable land and irrigation water, increasing the food grain production is highly challenging task to agricultural scientists. Any research programme carried out to increase the production and productivity of rice will be of great value in the service of mankind and nation.

Better management alone cannot lead to better yields from inferior varieties beyond a certain limit. The limit of yield is set by the genetic make up of the variety (Singh, 1983). Thus continuous favourable changes in the genotype of crop varieties are a must for increasing yields from crop plants. Conventional breeding methods are widely used for crop improvement. But in certain situations, these methods have to be supplemented with innovative techniques either to increase their efficiency or to be able to achieve the objective, which is not possible through conventional methods (Singh, 1998)

Totipotency an important quality of plant cells is not exploited in conventional breeding methods. The techniques of cell, tissue and organ culture have made available a new range of hitherto unavailable materials for genetic manipulation. The formation of a sporophyte directly from the gametophyte has often been described as one of the most significant developments in plant biology, in the last three decades. At, the haploid level, the normal gametophytic development of both the microspore and megaspore of rice can be diverted to a sporophytic pathway through *in vitro* techniques. The culture of anthers to induce sporophytic development of microspores has become more important than the

culture of ovaries or ovules because the latter generally exhibited too a low response for application to rice breeding.

Anther culture is used to produce haploids by inducing the development of a single haploid pollen grain into plantlet. Anther culture in rice (Niizeki and Oono, 1968) bears enormous promise in developing varieties within a short period of time as evidenced in China, where hundreds of varieties derived through anther culture are under large scale cultivation. Anther culture is preferred over conventional breeding methods because of its increased speed and selection efficiency and involvement of a unique form of gametic selection (Kasperbauer *et al.*, 1980). It lowers cost (Sanint *et al.*, 1996), reduces space and labour requirements in experimental fields (Zheng *et al.*, 1984) widens gene pools due to induction of heritable variation (Raina, 1989), enables early expression of recessive genes and reduces time required for variety development. In most plants, *in vitro* androgenesis has been achieved by a judicious adjustment of microspore stage and culture medium.

Application of anther culture technique to highly heterotic hybrids helps in exploiting the advantage of superior gametic genotypes. It is possible to obtain homozygotes with fixed heterosis provided that partial to complete dominance predominates (Sprague and Eberhart, 1977). Anther culture of potential hybrids makes it possible to develop superior gametic genotypes that possess the vigour and fitness of the hybrid (Bong and Swaminathan, 1995)

However, inspite of all these merits, anther culture could not be exploited to its full potential owing to some handicaps such as strong genotype dependence, low culturability especially in indica rices, high percentage of albino plants and abundance of haploids in the primary regenerants. There is also concern regarding the gametic spectrum representation by the anther culture derived doubled haploids.

The present investigation was carried out with the following objectives

i) To produce a hybrid of IR 36 and PTB 45 and culturing the F_1 anther to produce doubled haploids.

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

iii) To study the influence of carbon source viz., sucrose and maltose on callus induction.

iv) To study the effect of embryogenic and non-embryogenic calli on plant regeneration

2. REVIEW OF LITERATURE

Rice is the most important food crop of Kerala. It occupies an area of 3.9 lakh hectares with annual production of 7.7 lakh tonnes (FIB, 2002), which is sufficient to meet only 30 per cent of the requirement. Since the prospect of bringing more area under rice is very remote, increase in production should primarily come through enhanced productivity. The cultivation of rice is extremely sensitive to different biotic and abiotic stress factors especially salinity, drought and submergence (Grover, 1999). These situations necessitate continuous flow of high yielding varieties possessing biotic and abiotic stress resistance.

Hybridization followed by anther culture is a very useful method of crop improvement, in which breeding cycles can be reduced considerably. The present study entitled "Androgenesis in rice (*Oryza sativa* L.) breeding" was carried out with a view to produce a hybrid and fixing of hybrid performance in doubled haploid lines using anther culture techniques. The available literature on hybridization and anther culture in rice are reviewed here.

2.1 HYBRIDIZATION

Hybridization is the most potent technique for breaking yield barriers and evolving varieties having built in high yield potential. When two genotypically different plants are crossed, the genes from both parents are brought together in the F_1. The importance in selecting genetically different parents either to exploit heterosis or to get desirable recombinants has been stressed upon by many workers (Joshi and Dhavan, 1966; Anand and Murthy, 1968). Cultivars chosen for crossing in this study IR 36 and PTB 45 are genetically dissimilar with respect to many characters such as yield, duration, amylose content, BPH resistance (Vanaja, 1998).

Sprague and Eberhart (1977) suggested the possibility of obtaining homozygotes with fixed heterosis provided that partial to complete dominance predominates. Chen and Li (1978) were the first to use anther culture to fix heterosis of F_1 hybrids, some have been claimed to be more ideal than the hybrid itself.

Bong and Swaminathan (1995) opined that it is possible to exploit hybrid vigour in rice through the development of doubled haploid lines from anther culture of potential hybrids.Study conducted by them revealed that the performance of some doubled haploid lines are almost comparable to that of the hybrids for the characters days to 50 per cent flowering, plant height, number of panicles per plant, 1000-grain weight and harvest index. The variance component due to additive effect for these characters was highly significant. As compared to the better parents, yield improvement of promising doubled haploid lines was encouraging with a yield advantage up to 20 per cent. This improvement was brought about through the combination of desirable traits in doubled haploid lines.

Heterosis refers to the superiority of F_1 hybrid over both its parents in yield and other yield component traits. Heterosis over better parent was significant in IR 36 x PTB 45 with respect to various characters such as grain yield / plant (43.27), 1000 grain weight (24.0) (Biju, 2001). Based on estimation of standard heterosis, he concluded that hybrid IR36x PTB 45 can be exploited as commercial rice hybrid.

The combining ability analysis gives useful information regarding selection of parents in terms of performance of their hybrids (Dhillon, 1975).The concept of combining ability helps to determine the nature of gene action involved in the expression of qualitative traits of economic importance. For PTB 45, Vanaja (1998) recorded desirable significant GCA effects in many characters such as number of days to 50 per cent flowering, plant height, and yield. She also

recorded desirable significant GCA effects for IR 36 in characters such as plant height, L/B ratio of grain, yield.

2.2 ANTHER CULTURE

2.2.1 Use of Anther Culture in Crop Improvement

Anther culture is used to produce haploids by inducing the development of a single haploid pollen grain into plantlet. In haploids, induced mutations are readily detected and doubled haploids provide immediate homozygosity and the recovery of alleles easily detected. Haploid plants can be used to detect the extend of genic interaction, linkage, estimation of gene number affecting quantitative traits and location of quantitative trait loci (Dunwell, 1985). Hence,the production of haploids and doubled haploids in large number is of greater value to plant breeders.

Plant breeders have worked intensively to obtain haploids (sporophytes of higher plants with gametophytic chromosome constitution) either *in vivo* or *in vitro*. The *in vivo* methods like distant hybridization, irradiated pollen, *etc* are employed to induce haploid production. However, *in vivo* methods are time consuming and have haploids with irregular and low frequency (Razdan, 1993).

The success achieved using *in vitro* methods has been spectacular and there are reports on haploids being induced by anther culture (or) pollen culture from about 250 species and hybrids. Anther culture in rice (Niizeki and Oono, 1968) bears enormous promise in developing varieties within a short period of time as evidenced in China and Korea where more than hundred of varieties derived through anther culture are under large scale cultivation.

Use of anther culture for generating large populations of doubled haploids from F_1 hybrids appear attractive because of problems hindering the conventional

breeding (i.e. slow fixation of the lines, frequent partial sterility of the progenies and low recovery of useful recombinants) (Guiderdoni *et al.*, 1992). Sanint *et al.* (1993) studied to compare anther culture costs with the cost of pedigree breeding. They concluded that under certain assumptions made in that study anther culture was a cost effective way of producing new varieties.

Narasimman and Rangaswamy (1993b), based on their experimental results suggested that the sterility barriers for realizing genetic recombinants and fixation of fertile homozygous lines in indica-japonica hybridization programme could be overcome through F_1 anther culture technique.

Doubled haploids have long been recognized as a valuable tool in plant breeding since it not only offers the quickest method of advancing heterozygous breeding lines to homozygosity, but also increases the selection efficiency over conventional procedures due to better discrimination between genotypes within any one generation (Chen *et al.*, 2001). Doubled haploids provide a unique system to attempt the fixing of hybrid performance in homozygous lines and avoid the step of hybrid seed production (Maluszynski *et al.*, 2001).

2.2.2 The Evolution of Anther Culture Breeding of Rice

Tulecke (1953) was the foremost scientist who observed that mature pollen grains of a gymnosperm *Ginkgo biloba* could be cultured to proliferate and form haploid callus. In line with this, Guha and Maheshwari (1964) were the first to report on the direct development of embryos from microspores of *Datura innoxia*. Bourgin and Nitch (1967) obtained successful haploid plants of *Nicotiana tabacum*.

The first successful report on the induction of haploid plantlets in rice was by Niizeki and Oono (1968). Other successful reports include those of Guha *et al.* (1970), Iyer and Raina (1972), Chen *et al.* (1985), Maheswaran (1985), Mercy

and Zapata (1986), Manimekalai and Rangaswamy (1988), Jayanthi (1989), Krishnaraj (1989), Raina (1989), Gu *et al.* (1992), Hanttue *et al.* (1995) and Maiti and Mandal (1998).

New varieties of tobacco and rice were developed using anther culture technique in 1974. China is the first country in which the rice bred through anther culture has been widely used in commercial cultivation. Many protocols of rice breeding by anther culture have been generated and anther culture is now widely included in conventional breeding (Zhenhua, 1992).

2.2.3 Factors Affecting Androgenesis

Although anther culture has been successfully used to hasten the breeding programmes in several crop species, including rice, there still remain some handicaps to realize its full potential. Unlike the highly responsive model systems, most of the indica rice cultivars respond rather poorly in anther cultures. Both the callus induction and plantlet regeneration steps require different cultural conditions and are affected by the genetic make-up of the plants. The literatures pertaining to the factors affecting androgenesis in rice, particularly indica rice, are reviewed under the following heads.

2.2.3.1 Genotypes

Callus induction and regeneration ability of the pollen vary greatly with the genotypes of the anther-donor plants. Miah *et al.* (1985) reported that anther culture response varied from 41 per cent for a japonica cultivar to 0 per cent for an indica cultivar. In general, indica cultivars of rice exhibit poorer androgenic response than Japonica cultivars (Hu, 1985; Raina, 1997).

Nine japonica x indica F_1 hybrids of rice involving six indica and three japonica tropical varieties were anther cultured. The frequency of callusing

anthers averaged 18.71 per cent. The microspore derived calli produced green plants with a mean frequency of 8.7 per cent (Guiderdoni *et al.*, 1992).

Among the indica cultivars a considerable variation of pollen callusing and plant regeneration has been observed. Guha and Mukherjee (1973) reported that only 5 out of the 18 indica cultivars showed pollen callusing and in only 4 cases did the calli differentiate into plants. The purple pigmented, coarse-grained indica cv crossa performed significantly better (40%) than the fine grained, aromatic cv Basmati – 370 (<10%) (Raina, 1989).

Lentini *et al.* (1995) reported that only one out of 35 indica cultivars exhibited pollen callusing on N_6 medium. Several researchers like Zapata (1985), Mikami and Kinoshitra (1988), Boyadzhiev and Kong (1989), Quimio and Zapata (1990), and Bhojwani and Razdan (1996) have observed genotypic differences for culturability in rice.

The time required for callus induction also genotype dependant. Reddy *et al.* (1985) studied anther culture response of eight indica cultivars of rice. They found that in Small Fruits and Tetep, callus appeared within three weeks, whereas in Megna and Radhunipagal callus appeared only after five weeks.

F_1 hybrids performed better than their inbred parents in rice anther culture (Manimekalai and Rangaswamy, 1987). Chu *et al.* (2003) reported that F_1 generation showed lower anther culture response (3.1%) than F_2 (15.9%) generation.

Genotypic variation to the extent of green plant or albino plant regeneration was also reported in rice *in vitro* haploid production (Datta *et al.*, 1990).

Bullock and Baenziger (1982) compared the anther callus production ability of wheat F_1's and their reciprocal crosses. No differences were noted between

reciprocal crosses. The results indicate that the transfer of *in vitro* androgenic ability to F_1 hybrids is not dependant upon the maternal cytoplasm source. Studies on the genetics of anther culturability revealed that this trait had independent inheritance (Davoyan, 1987) and had dominant gene action (Chen and Chen, 1993). Narasimman and Rangaswamy (1993a) also reported that this trait was heritable.

2.2.3.2 Developmental Stage of Explant

In rice, anthers inoculated at mid to late uninucleate pollen stage have been found to be the most suitable for culture. Oono (1975), Chen and Lin (1976), Chen (1977), Cornejo-Martin and Primo–Millo (1981) conducted detailed studies on the effect of developmental stage of pollen grains for anther culture response. They concluded that anthers at the tetrad stage do not respond at all, and early uninucleate pollen may respond poorly. Mid-to-late uninucleate pollen responds the best. Anther response falls sharply after the first pollen mitosis.

Chaleff and Stolarz (1981) found that panicles harvested prior to emergence from the flag leaf sheath when the base of the flag leaf was 3 cm to 5 cm above the base of the next lower leaf, corresponds to late uninucleate stage of pollen in the anthers. Zapata *et al.*(1983) reported that a distance of 6 cm to 10 cm between the auricle of the flag leaf and the subtending leaf was having anthers at mid-to-late uninucleate stage suitable for culturing.

Reiffers and Freire (1990) found that a distance of 4 to 8 cm between the base of the flag leaf and the auricle of the penultimate leaf have anthers at late uninucleate stage. Shahjahan *et al.* (1992) reported that yellowish green colour of spikelets corresponds to mid uninucleate stage of the pollen where maximum response was observed.

According to Chung (1992), best cultures were obtained with anthers that have attained 1/2 to 1/3 of the spikelet length. 10 cm to 12 cm distance between auricle of flag leaf and next leaf was found as a good morphological marker to select mid-to-late uninucleate stage of anthers for culture (Mandal and Bandyopadhyay, 1996).

2.2.3.3 Temperature Pre Treatment

In most of the published works on androgensis in rice, panicles were given a cold pre-treatment but the temperature and duration varied. Gupta and Borthakur (1987) had selected pre-treatment at 10°C for 11 days for anther culture of indica cultivar Khonovullo.

Matsushima *et al.* (1988) had reported that a pre-treatment at 10°C for 10 to 18 days was necessary to induce sporophytic divisions in microspores of the japonica cv Nipponbare. Pande (1997) observed that cold treatment was essential for androgensis in anther cultures of the indica cv IR 43, and 10^0C for 10 days was most suitable.

Ogawa *et al.* (1995) observed that 25 days of pre-treatment at 10°C was optimum for indica cv IR 24. Although the frequency of anthers showing pollen callusing after cold-treatment for 25 days was fairly high, most of the plants regenerated from the calli formed such a long cold pre-treatment were albinos.

Many researchers opined that pre treatment at 10^0C for 10 to 12 days was most suitable (Hue and Chae, 1987; Manimekalai and Rangaswamy, 1987; Moon *et al.*, 1989). Reddy *et al.* (1985) reported that a brief (10 min) exposure to high temperature (35^0C) before cold-treatment was better for pollen callusing but it adversely affected the green plant production.

2.2.3.4 Culture Media

Chu *et al.* (1975) demonstrated that the level of ammoniacal nitrogen in culture medium is critical for androgensis in rice. On that basis he developed the N_6 medium, which is most widely used for rice anther culture. Yin *et al.* (1976) noticed more callus induction frequency on N_6 medium than MS or Miller's. Boyadzhiev and Kong (1989) reported that N_6 was better for obtaining callus induction. Lenka and Reddy (1994) demonstrated that N_6 medium induced 46 per cent callus in rice anther culture.

Narayanan (1997) studied the anther culture response of indica japonica crosses and reported that N_6 medium supplemented with 2,4-D and kinetin proved to be better for enhanced callus induction. Mandal *et al.* (2000) obtained *in vitro* anther culture response in indica rice variety PTB 28 using N_6 medium. Several researchers like Mandal and Gupta (1996), Maiti and Mandal (1998), Balachandran *et al.* (1999)), Manonmani (1999), and Lee *et al.* (2003) have used N_6 medium for callus induction and MS medium for obtaining regeneration.

Reddy *et al.* (1985) studied the callusing efficiency of He_2 and N_6 medium on eight indica cultivars and found He_2 medium to be better than N_6 medium. He_2 medium is derived from N_6 medium by reducing NH_4^+ to half strength and $MgSO_4$ to $1/50^{th}$ level and doubling the concentration of KH_2PO_4 (Huang *et al*, 1981).

Ogawa *et al.* (1995) studied the effect of nitrogen source on androgenesis of indica cultivar IR 24, using R-2 medium as the control. R-2 has 40 *mM* KNO_3 and 2.5 *mM* $(NH_4)_2SO_4$. When 20 *mM* KNO_3 was combined with a small amount of $(NH_4)_2SO_4$, glutamine or alanine all treatments induced pollen callusing but alanine was the best supplement. It not only induced high frequency androgenesis but also showed maximum regeneration of green plants.

A anther culture medium, consisting of a higher KNO_3 content (31*mM*) and CH (500 mg/l) but without ammonium salts, was tested in comparison with a medium consisting of the widely used N_6 medium nitrogen background, using four indica x indica F_1 hybrids as test materials. Green plant regeneration frequency was at least three-fold higher in the microspore-calli derived from the former medium than in those derived from the modified N_6 medium (Raina and Zapata, 1997).

Raina and Zapata (1997) carried out another study using indica x japonica and indica x javanica F_1 hybrids. The results indicated that a medium with higher (3.5*mM*) ammonium sulphate may induce a higher frequency of anthers with microspore-calli but not necessarily lead to a larger number of green plants regenerating calli.

Another study carried out by Raina and Zapata (1997) using indica cv IR43 as the test material revealed that use of a lower level (1.75*mM*) of $(NH_4)_2SO_4$ in addition to KNO_3 (31*mM*) to be better than CH (500 mg/l) for anther response as well as green plant regenerability of the derived microspore calli.

Tang *et al.* (1998) found that NK medium containing suitable concentrations auxin and cytokinin may be successfully applied for anther culture of selected wild *Oryza* species. NK medium (Chen *et al.*, 1982b) contains N_6 inorganic salts, MS organic salts, CH (300 mg/l), activated charcoal (0.5%).

Many reports point out that media with a relatively high content of inorganic salts are more suitable for the differentiation of callus. Water stress induced by mannitol incorporation in the medium (Jain *et al.*, 1996; Lai and Liu, 1998) and partial tissue desiccation (Tsukahara and Hirosawa, 1992; Rance *et al.*, 1994; Zhao *et al.*, 1997) enhanced regeneration in indica rice calli.

Rice haploids generated through tissue culture are usually produced by a two-step procedure involving callus induction and then plantlet regeneration. One-step procedure was worked out where plants could be regenerated in the same plates without transferring to a separate medium. Two media namely P2 (modified B5) and M10 (modified potato) which contained NAA was compared to B5 medium containing 2, 4-D. Both P2 and M10 produced significantly higher number of direct plantlets without transferring calli to differentiation medium (Karim and Zapata, 1990).

Use of modified MS medium containing reduced nitrogen (glutamine and CH) and 10 per cent (w/v) ficoll might favour microspore embyogenesis and green plant regeneration in rice (Datta *et al.*, 1990; Priyamvada, 1992). Zhuo *et al.* (1995) reported that they got indirect and direct plant regeneration when they used M8 medium.

2.2.3.4.1 Carbon Source

Various concentrations of sugar ranging from 1.5 to 12 per cent have been tested. The literature available on sucrose concentration has been reviewed in table 1.

Maltose has been shown to be a superior source of carbohydrate than sucrose for androgensis in several species, including cereals (Finnie *et al.*, 1989; Last and Brettel, 1990; Pande and Bhojwani, 1999).

In cultures, sucrose rapidly breaks down to glucose and fructose, so than after three weeks of barely anther culture medium did not contain any sucrose. On the other hand, the hydrolysis of maltose during the same period was below the detectable level (Last and Brettel, 1990).

Table 1.Reviews on effect of sucrose concentration in rice anther culture

Carbon source	Anther response	Reference
Sucrose 3 and 6 per cent	Differentiation frequency of green plantlets higher at 6 per cent than at 3 per cent	Clapham (1973) Chen *et al.* (1974)
Sucrose 3, 6 and 9 per cent	Sucrose 6 and 9 per cent enhanced both callus formation and organogenesis but plantlets differentiated from callus with media containing 9 per cent sucrose were albinos.	Chen (1978)
Sucrose 3 and 6 percent	6 per cent sucrose increased the differentiation frequency of green plantlets from 18.8 to 45.5 per cent over 3 per cent.	Wakasa (1982)
Sucrose 5 and 8 per cent	Low concentration (5%) favoured androgenic initiation and callus induction, while a higher concentration (8%) inhibited callus formation.	Rangaswamy *et al.*,1982
Sucrose 3,5,7,9 and 11 per cent	Callus induction and plantlet yield was highest at 5%	Reddy *et al.*,1985
Sucrose 3 per cent + Sorbitol 2 per cent	Increased frequency of regeneration	Krishnaraj (1989)
Sucrose 3,6 and 9 per cent	At 6 per cent level callus induction and plant regeneration were high and the calli were mostly embryogenic	Narasimman (1989)
6 per cent sucrose	Regeneration frequency highest	Lenka and Reddy (1994)

Sun *et al.* (1993) reported that maltose promoted callus induction than sucrose, fructose, galactose, glucose, mannose, and serbose. Lentini *et al.* (1995) reported that on N_6 medium with 146 *mM* sucrose only one out of 23 indica cultivars exhibited pollen callusing and green plant production. Substitution of sucrose by equimolar amount of maltose enhanced pollen callusing from 6.3 per cent to 10.1 per cent and green plant regeneration from 0.6 per cent to 1 per cent.

Xie *et al.* (1995) found that no significant effect of maltose in place of sucrose on plantlet regeneration was seen in regeneration medium. Raina (1997) observed that a significant increase in anther culture efficiency and green plant formation in indica rice when sucrose was replaced by maltose.

2.2.3.4.2 Exogenous Hormones

2.2.3.4.2.1 For Callus Induction

A wide variety of growths stimulating hormones have been tried, singly and in numerous combinations. Chaleff and Stolarz (1981) reported that R3 medium containing NAA of 2 mg/l was most effective (33.6 %) in promoting callus formation in rice. The frequency of callus formation on R3 medium was not increased significantly by addition of 0.5 mg/l 2,4-D and was inhibited by IAA.

Reddy *et al.* (1985) found that NAA (10.5 μm) was more effective in rice anther callus induction than 2, 4-D (9.0μm). However, plantlet yield was high in a medium containing 2, 4-D. Both 2.4-D and NAA at high concentrations (22.5μm and 27.0 μm respectively) increased callus induction but plantlet yield was very poor.

Patel and Nerkar (1991) recorded 0.01 per cent callus induction when 2 mg/l 2, 4-D was used in the callus induction medium N_6. Rout and Sharma (1991)

reported that highest callus induction (27%) was in the N_6 media containing1 mg/l 2, 4-D, 2 mg/l NAA and 1 mg/l Kn.

Among the various combinations for callus induction and plantlet regeneration tried by Lenka and Reddy (1994), the combination 2 mg/l 2, 4-D and 0.5 mg/l Kn gave highest callus induction frequency and combination 1 mg/l IAA and 1 mg/l NAA gave highest regeneration frequency.

Mandal and Bandhyopadhyay (1996) reported that the induction of callus was best (35%) on callus induction medium supplemented with 2 mg/l 2, 4-D and good regeneration (60 %) in the medium containing 1.5 mg/l BAP, 1 mg/l Kn and 0.5 mg/l NAA.

Anther culture response of an intervarietal hybrid (Pankaj x FR-BA) rice was studied using modified N_6 medium supplemented with different concentrations (1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) of NAA and 2, 4-D along with 1.0 mg/l Kn to determine the optimum concentration of auxin for induction of callus. Callusing frequency was maximum in presence of 2, 4-D 2mg/l (Mandal and Gupta, 1996).

Narayanan (1997) conducted an experiment to study the effect of 2, 4-D and Kn combination on anther culture response of three different hybrids of rice. He concluded that 2mg/l 2, 4-D and 0.5 mg/l Kn was best invariably in all hybrids.

According to Raina and Zapata (1997), an increase in level of 2, 4-D and Kn from 0.5mg/l to 1.0 mg/l did not enhance the anther response or the regenerability of the derived calli, whether under a low or high ammonium background.

Maiti and Mandal (1998) studied the anther culture response of rice using N_6 medium supplemented with 0.05 mg/l Kn and three different concentrations of

2,4-D, viz 1mg/l, 2 mg/l and 3 mg/l. They found that 1 mg/l have better response (6.24%) than 2mg/l (5.44%) and 3 mg/l (5.24 %) in all rice varieties.

Tang *et al.* (1998) investigated the effects of four combinations of auxin and cytokinin on callus induction from anthers of wild rice species. They reported that preferred medium for *O.punctata* and *O.ridleyi* was medium containing 2 mg/l 2, 4-D +2 mg/l Kn. Callus induction percentage was 0.84 and 0.59 respectively.

2.2.3.4.2.2 For Regeneration

In rice, all pollen calli do not regenerate and shoot differentiation from 50 per cent of the calli is considered very good (Rangaswamy *et al.*, 1982). Maheswaran (1985) reported that better regeneration was observed in differentiation medium supplemented with 1.0 mg/l Kn and 1.0mg/l NAA.

The regeneration medium with IAA 1mg/l and BAP 1 mg/l had the highest range of green plant regeneration (60 to 80 %) (Narasimman, 1989). Meifang (1992) reported that regeneration media containing 1mg/l NAA, 2mg/l Kn, 0.5 mg/l IAA or 2 mg/l NAA, 4 mg/l Kn were equally effective.

Among the various growth regulators combinations for plantlet regeneration tried by Xie *et al.* (1995), the combination 0.5mg/l IAA and 2mg/l Kn were beneficial for green plant regeneration.

2.2.4 Callus Age and Quality

The callus that emerges in 50 days has good competence for green plantlet regeneration, and those emerging after 70 days are usually larger with poor regeneration capacity. Callus with a diameter 2 mm showed the highest level of green plantlets regeneration, but most of the regenerated plants were haploids.

Callus smaller as well as larger than 2 mm will either die or encounter difficulty in regeneration (Meifang, 1992).

The callus quality could be identified by observing callus morphology, callus that is milky-white, compact, moist, smooth and slow growing had excellent ability of plantlet regeneration. On the contrary, callus that was cream coloured or dark yellow; friable, dry and fast growing was poor in plantlet regeneration. (Meifang, 1992; Xie *et al.*, 1995)).

2.2.5 Albinism

The occurrence of large-scale proportion of albino plants in the pollen plant population is probably the most frustrating feature of androgensis in its application to rice breeding. The frequency of albinos may vary from 5 per cent to 100 per cent. Indica rice cultivars are more prone to this problem than japonica rice (Bhojwani *et al., 2003*).

Many factors have been found to affect the degree of albinism, such as the genotype and physiological state of the anther donor plants (Bullock and Baenziger, 1982), developmental stage of microspore (Chen and Lin, 1976), culture temperature (Huang, 1984), cold pre-treatment (Genovesi and Magill, 1979) and sucrose concentration in combination with growth hormones (Clapham, 1973).

Reddy *et al.* (1985) observed that high sucrose containing media promoted the regeneration of albino plantlets. Eleven per cent sucrose was deleterious for callus induction and no green plants could be regenerated. Chen (1978) and Mercy and Zapata (1986) reported that high sucrose concentration (9 to 12 %) increased the frequency of albinos.

The anthers pre-treated at 12°C for more than 20 days increases the frequency of albinos (Chung, 1992). Kawata *et al.* (1995) opined that culture period is responsible for pDNA deletions. The plants regenerated from one-month-old pollen calli did not show pDNA deletion but those from one-year-old callus showed substantial pDNA deletion. Calli from anthers cultured at the early uninucleate stage of pollen produce only green plants, and frequency of albinism increased with advancing stage of pollen (Pande, 1997).

2.2.6 Identification of Ploidy Level

Nishi and Mitsuoka (1969) observed considerable differences in the phenotypes among the variated ploidy plants. Haploids were lowest in height and had slender leaves and stems and awnless ears. Shoots of triploid were taller than any others and stems were robust. Tetraploids were similar to diploid, in general, with exceptions of lower pollen fertility and of longer awn. Pentaploids had morphologically peculiar roots. Tiller numbers in haploid plants were about three times as many as compared with other ploidy plants.

Kamo and Griesboch (1993) studied ploidy changes in "Mitchell" petunia using three methods: chromosome counts of root tip cells, number of chloroplast per guard cell pair and microflurometry. They found that the majority (80%) of haploid plants propagated through tissue culture were chimeric rather than purely diploid or haploid.

Nakamura *et al.* (1994) attempted to establish a method of determining ploidy level in rice based on morphological traits, panicle length, plant height and glume length. Differences in glume length enabled the classification of regenerated plants into three distinct classes. Cytological analyses were correlated with three ploidy level haploids (short glumes), diploids (medium glumes) and triploids (long glumes). The two other traits did not give a clear classification. Similar results were obtained with regenerated plants derived from gamma-

irradiated callus. In contrast to panicle length and plant height, the distinction of glume length was not affected by irradiation. Thus glume length can be considered a stable index of ploidy level in rice.

2.2.7 Frequency of Spontaneous Diploids

Zapata *et al.* (1991) reported that among 57 plantlets obtained through microspore culture, 41 were homozygous diploids and the rest haploids. Guiderdoni *et al.* (1992) observed that the overall frequency of spontaneous doubling averaged 46 per cent in anther culture derived plants. Zhu *et al.* (1993a) reported that among 654 lines produced by anther culture, the homozygous diploids were 88.6 per cent.

In rice and barley the direct production of doubled haploids by androgenesis has been improved to the extent that colchicine treatment of the remaining haploids can be omitted (Wehr and Wenzel, 1993).

Zhenhua (1992) found that in addition to haploids and diploids, there were polyploids and a few microploids and aneuploids among anther derived plants of rice.

Many factors such as developmental stage of anthers inoculated, nature of cold pre-treatment, components of medium, genotype of donor plants, callus age and plant growth regulators affect the ploidy level of plants derived from anther culture (Chung, 1992).

The natural doubling could have occurred during different stages, including callus induction, callus redifferentiation and embryoid formation. It could also occur in tillers, branches of panicles or even individual spikelets. Hence, it is often observed that haploid and diploid cells co-exist in the same plant (Zhenhua, 1992).

2.2.7 Diploidization of Haploid Plants

The usual methods of induced chromosome doubling involve leaf sheath injection of colchicine, soaking roots or whole plants in a colchicine solution, culturing plantlets in colchicine containing medium (Chen *et al.*, 1994).

Chung (1992) reported that induced chromosome doubling was most effectively achieved by submerging the base of a haploid seedling in 0.1 per cent to 0.2 per cent colchicine solution for 12 to 24 hours.

Takashima *et al.* (1995) have found that high frequency chromosome doubling of anther-derived haploid tobacco plants can be accomplished by immersing anthers into a colchicine solution just before culturing them *in vitro*. Optimum diploidization (66.7%) was achieved by immersing the anthers in 0.4% colchicine for eight hours.

Zamani *et al.* (2000) observed that regenerants obtained from the colchicine supplemented media produced significantly higher percentages of fertile plants in all the three varieties of wheat. However, the level of fertility was significantly different among the fertile plants obtained.

Under field conditions seed set could be found in more than 50 per cent of haploid barely plants without colchicines treatment. The high occurrence of haploid plants setting seed implies the production of unreduced gametes on the haploid cell level (Wehr and Wenzel, 1993).

2.2.9 Rooting and Hardening of Green Plantlets

Hazarika (1983) reported that rooting and root quality of regenerated rice plantlets was enhanced by lower macro nutrient salt concentration media. Most pollen plants regenerated in the test tube have very weak adaptability to the

natural environment. Direct transplanting into soil results into their death. Therefore, the plants should be prepared to enable their adaptation to the *in vivo* climate (Chung, 1992). Zhenhua (1992) reported that survival rate can be improved by maintaining the high relative humidity following transplanting of plantlets.

2.2.10 Variability of Pollen Derived Plants

Chen *et al.* (1982a) reported that the recombination frequencies estimated from anther-derived plants were remarkably similar to those from F_2 progeny.

According to Meifang (1992), it was impossible to compare among A_1 plant populations because regeneration occurred asynchronously and plants were not grown in a normal season.

Wadhwari (1993) demonstrated that 90 per cent pollen derived plants showed homozygosity in subsequent generation.

Chung (1992) reported that most doubled haploid lines derived from anther culture of a specific variety appear the same phenotypically as the donor variety, whereas 6 to 8 per cent of the A_2 lines appear to have a different phenotypic pattern from the donor variety.

Zhenhua (1992) suggested that the main reasons for the segregation among A_2 lines may be somoclonal variations occurring in gene and chromosome structure, plants derived from somatic tissues instead of microspore, out cross taking place as a result of stigma extension or male sterility.

Zhu *et al.* (1993a) studied the progenies of doubled haploid plants of indica rice hybrids, produced by anther culture. They observed that characters with in one line were relatively uniform and showed stable heritability. Among different

lines, broad genetic recombination occurred. Hence, they suggested that use of anther culture technique to improve cultivars was an effective breeding path.

2.2.11 Achievements in Anther Culture

Selection from doubled haploids is said to be six times more efficient than the normal diploids. Shen *et al.* (1983) reported that 150 plants derived from F_1 anther culture to be equivalent to 4000 to 5000 F_2 plants. Zhu *et al.* (1990) successfully bred the variety Gan Zhao Xion through anther culture of hybrid Shan You 2. It showed early maturity, high yielding ability and cold tolerance at the seedling stage.

The Chinese indica rice line long Hua 83-079 was bred by pollen culture. It has strong lodging resistant straw and is resistant to blast (*Magnaporthe grisea*) (E, 1993). Kunihiro *et al.* (1993) developed a new rice variety 'Aya' by anther culture breeding. Derived from cross Eikci 842771 X Kitake, Aya carries the dull (du) gene which lowers the amylose content of endosperm starch and thus confers good eating quality.

Zhu *et al.* (1993b) created a good quality WCR (Wide Compatible Restorer) based HR 1004 through anther culture of the F_1 hybrid. Courtois (1993) conducted detailed study on comparison of single seed descent and anther culture derived lines of rice. He concluded that doubled haploid and single seed descent are equally effective techniques in rice to obtain high performance lines from single crosses.

Salinity tolerance with higher grain yield was obtained in the anther-derived line IR 51500-AC 11-1(Alejar *et al.*, 1995). Another variety Gan Zhao Xion 31(Ding *et al.*, 1995) has high yield potential and resistance to diseases and pests. This variety out yielded the control by 12.5 per cent.

Sixty-one anther derived doubled haploids derived from three different hybrids were evaluated. It was recorded that there were DH lines showing a short duration of growth and shorter plant height than the parents and hybrids. The upper limit of the range of DH lines for panicle number/plant, grain weight, proportion of high-density grain and harvest index more or less reached the heterotic level of the hybrids and often exceeded the value of the better parents (Bong and Swaminathan, 1995).

Selection efficiency of anther culture breeding compared with conventional breeding showed that 16.74 per cent of new line from anther culture recorded 1.49 times more yield than conventional breeding and the frequency of improved varieties obtained from anther culture was 0.93 per cent i.e. 3.32 times more than that from conventional breeding (Songs *et al.*, 1999).

Two-anther culture derived doubled haploid lines (DH7 and DH21) with cold tolerance at reproductive phase were evaluated along with improved cold tolerant checks in Meghalaya, India. The average unmilled rice yield of DH7 was significantly higher than that of the cold tolerant controls. DH7 and DH21 matured 11 and 21 days earlier than the improved cold tolerant checks (Pattanayak *et al.*, 2000).

Pattanayak and Gupta (2000) evaluated three anther culture derived doubled haploid lines of rice under dry seeded upland conditions in Meghalaya, India. They reported that all DH lines exhibited significantly higher yields per plant than improved and local controls. Yang *et al.* (2001) demonstrated that blast resistance genes could be transferred from wild rice species into cultivated varieties through crossing and anther culture. Tien and Long (2003) have developed good quality aromatic rice varieties by anther culture breeding.

3. MATERIALS AND METHODS

3.1 MATERIALS

Rice varieties (*Oryza sativa* L.) IR 36 and PTB 45 obtained from the Regional Agricultural Research Station, Pattambi were used in this study. The details of parents are furnished in Table 2.

3.2 METHODS

The experimental methods consists of following three parts

(i) Pot culture

(ii) Anther culture

(iii) Statistical analysis

3.2.1 Pot Culture

3.2.1.1 *Details of the Experiment*

The experiment was conducted during Kharif and Rabi, 2003 at College of Horticulture, Vellanikkara.

The ovule parent (IR36) and pollen parent (PTB45) were sown in pots in a staggered manner so as to get synchrony in flowering for effecting crossing. Twenty five days old seedlings were transplanted at the rate of two hills per pot. Hybridisation was carried out by hand emasculation and artificial pollination by the wet cloth method (Chaisang *et al.*, 1967). The F_1 plants derived from above cross were grown along with parents in pots with a regular supply of water and fertilizers under natural light conditions in glasshouse.

Table 2. Details of parents

Character	IR36	PTB45
Origin	IRRI	Kerala (India)
Parentage	IR1561-228/IR24-4 *Oryza nivara*/CR94-13	Selection from Triveni
Duration	115 days	100-105 days
Bran colour	White	Red
Grain type	Long slender	Medium bold
Yield	5.0t/ha	5.5t/ha
Amylose content (%)	23.3	25.8
Alkali grading value	2.0	2.5
Salient features	Multiple tolerant variety	Suited for kuttanad and kole land. Resistant to BPH.

3.2.1.2. *Observations Recorded*

The following metric characters were studied in each parent and hybrid.

(i) Days to flowering

The number of days taken from sowing to ear emergence in plants was recorded.

(ii) Plant height

In main culm, the height was measured from the soil surface to the tip of the primary panicle in centimetres at the time of maturity.

(iii) Panicles per plant

Number of ear bearing tillers (panicles) in each plant was counted at the time of harvest and recorded.

(iv) Panicle length

In the primary panicle the length was measured from the neck of the panicle to the tip of the panicle and expressed in centimetres.

(v) Grains per panicle

The number of well-filled grains in the primary panicle was counted and recorded.

(vi) Grain weight

A random sample of 100 well-filled grains from the produce of a plant was weighed in grams and recorded.

(vii) Pollen fertility

At the time of flowering ten spikelets from the early emerging panicles were collected during the early morning hours and fixed in 70 per cent alcohol. Anthers from the selected spikelets were separated, crushed and stained using one per cent potassium iodide solution and observed under compound microscope. The number of fully stained and unstained pollen grains was counted separately at ten different fields and the total in each category was worked out. The pollen fertility was calculated by the formula suggested by Chaudhary *et al.*, (1981).

$$\text{Pollen fertility} = \frac{\text{No. of fully stained pollen grains}}{\text{Total No. of pollen grains}} \times 100$$

The fertility status was classified according to the standards presented by IRRI (Govindaraj and Virmani, 1988) as follows.

Pollen fertility per cent	Fertility status
0 to 0.99	sterile
1 to 29.99	partial sterile
30 to 59.99	partial fertile
60 to 100	fertile

(viii) Spikelet sterility

The total number of well-filled and ill-filled spikelets in the primary panicle in each plant was counted separately. Spikelet sterility was calculated using the following formula and expressed in percentage

$$\text{Spikelet sterility} = \frac{\text{Number of ill-filled spikelets}}{\text{Total number of well-filled and ill-filled spikelets}} \times 100$$

(ix) Grain yield

The weight of fully ripened grains from a single plant was recorded in grams at 14 per cent moisture level.

3.2.2 Anther Culture

Anther culture experiment was conducted at the Tissue Culture Unit, Department of Pomology and Floriculture, College of Horticulture, Vellanikkara.

3.2.2.1 Details of the Experiment

3.2.2.1.1 Selection of Explant

Anthers at intermediary stage between mid uninucleate and late uninucleate were collected from F_1 plants when the distance between auricle of the flag leaf and next leaf was 6 cm to 10 cm as suggested by Reiffers and Freire (1990) and Mandal and Bandhyopadhyay (1996).

3.2.2.1.2 Cold Treatment of Anthers

The anthers collected at the appropriate stage were given cold pre-treatment at 10^0C for 10 to 12 days.

3.2.2.1.3 Media

The nutrient media used for the study was N_6 medium (Chu *et al.*, 1975) for callus induction and MS medium (Murashig and Skoog, 1962) for regeneration of anther. The composition of the media is given in Table 3.

3.2.2.1.3.1 Preparation of Stocks for Basal Media

The stock solutions for the media were prepared in double distilled water and kept in sterile glass stoppered bottles and stored in a refrigerator at 5^0C.The stock formulations and the quantity of stocks used per litre of the respective media are given in Table 4 and Table 5.

3.2.2.1.3.2 Preparation of Stocks for Growth Regulators

Auxins viz., 2,4-D, IAA and IBA were dissolved in few drops of ethanol, slightly heated and gradually diluted to 100 ml with double distilled water.

Table 3.Composition of tissue culture media

Constituents	MS (mg/l)	N_6 (mg/l)
Major inorganic nutrients		
NH_4NO_3	1650.0	463.0
$(NH_4)_2 SO_4$	-	2830.0
KNO_3	1900.0	185.0
$MgSO_4.7H_2O$	370.0	400.0
KH_2PO_4	170.0	166.0
$CaCl_2.2H_2O$	440.0	
Trace elements		
$MnSO_4.4H_2O$	22.3	4.4
$ZnSO_4.7H_2O$	8.6	1.5
H_3BO_3	6.2	1.6
KI	0.83	0.8
$Na_2MoO4.2H_2O$	0.25	-
$CuSO_4.5H_2O$	0.025	-
$CoCl_2.6H_2O$	0.025	-
Iron source		
Na_2EDTA	37.25	37.25
$FeSO_4.7H_2O$	27.85	27.85
Organic supplements		
Myo-inositol	100.0	-
Thiamine HCl	0.1	1.0
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Glycine	2.0	2.0
Carbon source		
Sucrose	30 000	60 000
pH	5.8	5.8

Table 4. Preparation of stock solutions for MS medium

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

*Iron stock preparation

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

Table 5.Preparation of stock solutions for N_6 medium

Sl.no	Ingredients	Quantity (mg)	Volume of stock solution preparation (ml)	Volume of stock solution per litre of medium(ml)
1.	**Macro elements**			
	$(NH_4)_2 SO_4$	4630		
	$MgSO_4.7H_2O$	1850		
	KNO_3	2830	500	50
	KH_2PO^4	4000		
	$Cacl_2.2H_2O$	1660		
2.	**Micro elements**			
	$MnSO_4.4H_2O$	880		
	$ZnSO_4.7H_2O$	300	500	2.5
	H_3BO_3	320		
3.	KI	160	200	2.5
4.	**Iron stock***			
	Na_2 EDTA	3725	250	2.5
	$FeSO4.7H_2O$	2785		
5.	**Organic supplements**			
	Thiamine HCl	100		
	Nicotinic acid	50	250	2.5
	Pyridoxine HCl	50		
	Glycine	200	100	1

*Stock solution for Fe-EDTA complex was prepared as for MS medium.

Cytokinins viz., Kinetin and BAP were dissolved in a few drops of 0.5N NaOH, slightly heated and gradually diluted to 100 ml with double distilled water.

3.2.2.1.3.3 Preparation of Media

For preparing one litre of solid medium, the required quantity of stock solutions and high purity sucrose or maltose (Analar grade) added as solid, were dissolved in double distilled water and the volume was made up to 500 ml. Agar (0.8 per cent) was added to 500 ml distilled water and then heated in a water both until the agar dissolved completely. The nutrient solution and the dissolved agar were mixed and the volume was made up to one litre with double distilled water. The pH was adjusted to the requirement with 0.1N NaOH or 0.1 N HCl. The medium was distributed in 10 ml to 15 ml quantities into 150 mm X 125 mm test tubes.

3.2.2.1.3.4 Media Sterilization

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

3.2.2.1.4 Culturing of Anther

3.2.2.1.4.1 Disinfestation of Explants

The cold treated spikelets were washed in tap water and transferred to laminar airflow cabinet. They were surface sterilized using 70 per cent ethanol for 2 to 3 minutes followed by 0.1 per cent mercuric chloride solution for 8 to 10 minutes. The spikelets were washed 2 to 3 times with sterile double distilled water.

3.2.2.1.4.2 Inoculation of Anthers

Anthers were carefully inoculated into the callus induction media with a density of 10 to 12 anthers per tube. Spikelets were cut near their basal end with sterile scissors. Each spikelet was picked up using forceps and their cut ends tapped onto the rim of the test tube. This vibrated the spikelets and anthers fell onto the surface of the medium. The culture was incubated in the dark at 25 ± 2^{0}C.

3.2.2.1.4.3 Callus Induction Medium

Callus induction response was studied in the *in vitro* cultured hybrid by the following experiments.

(i) Effect of 6 per cent sucrose and different combinations of growth regulators 2, 4-D and Kinetin on callus induction

1. N_6 + 2, 4 - D 2mg/l + Kn 0.5mg/l (T_1)
2. N_6 + 2, 4 - D 2mg/l + Kn1mg/l (T_2)
3. N_6 + 2, 4 - D 3mg/l + Kn 0.5mg/l (T_3)
4. N_6 + 2, 4 - D 3mg/l + Kn 1 mg/l (T_4)

(ii) Effect of 6 per cent sucrose and different combinations of growth regulators IAA and Kinetin on callus induction

1. N_6 + IAA 1mg/l + Kn 0.5mg/l (T_5)
2. N_6 + IAA 1mg/l + Kn1mg/l (T_6)
3. N_6 + IAA 2mg/l + Kn 0.5mg/l (T_7)
4. N_6 + IAA 2mg/l + Kn 1 mg/l (T_8)

(iii) Effect of 6 per cent sucrose and different combinations of IBA and kinetin on callus induction

1. N_6 + IBA 1mg/l + Kn 0.5mg/l (T_9)
2. N_6 + IBA 1mg/l + Kn1mg/l (T_{10})

3. N_6 + IBA 2mg/l + Kn 0.5mg/l (T_{11})
4. N_6 + IBA 2mg/l + Kn 1 mg/l (T_{12})

(iv) Effect of 6 per cent maltose and different combinations of growth regulators 2,4-D and Kinetin on callus induction

1. N_6 + 2, 4 - D 2mg/l + Kn 0.5mg/l (T_{13})
2. N_6 + 2, 4 - D 2mg/l + Kn1 mg/l (T_{14})
3. N_6 + 2, 4 - D 3mg/l + Kn 0.5mg/l (T_{15})
4. N_6 + 2, 4 - D 3mg/l + Kn 1 mg/l (T_{16})

v) Effect of 6 per cent maltose and different combinations of growth regulators IAA and Kinetin on callus induction

1. N_6 + IAA 1mg/l + Kn 0.5mg/l (T_{17})
2. N_6 + IAA 1mg/l + Kn1 mg/l (T_{18})
3. N_6 + IAA 2mg/l + Kn 0.5mg/l (T_{19})
4. N_6 + IAA 2mg/l + Kn 1 mg/l (T_{20})

vi) Effect of 6 per cent maltose and different combinations of IBA and kinetin on callus induction

1. N_6 + IBA 1mg/l + Kn 0.5mg/l (T_{21})
2. N_6 + IBA 1mg/l + Kn1 mg/l (T_{22})
3. N_6 + IBA 2mg/l + Kn 0.5mg/l (T_{23})
4. N_6 + IBA 2mg/l + Kn 1 mg/l (T_{24})

3.2.2.1.4.4 Callus Induction and Maintenance

Anthers were inoculated at the rate of 10 to12 anthers per tube in each treatment with two replications (20 tubes per replication). Cultures were examined for response after four weeks and every weeks thereafter upto 70 days. Number of calli produced was noted and the percentage of callus induction was calculated.

Embryogenic calli (milky-white, compact, moist, smooth and slow growing) and non-embryogenic calli (cream colour, friable, dry and fast growing) (Nabors *et al.*, 1983) were assorted from the calli mass before subculture. Percentage of embryogenic and non-enbyogenic callus was calculated.

Approximately two weeks old calli (both embyogenic and non embyogenic) were subcultured one time in N_6 medium and sub cultured calli were transferred to regeneration medium.

3.2.2.1.4.5 Plant Regeneration

The callus tissues were transferred to regeneration medium when calli attained diameter of 2 mm size (approximately 50 mg weight). Two regeneration media were used in this study.

1. MS + NAA 1mg/l + Kn 2mg/l + IAA 0.5mg/l + CW (5%) (T_{25})
2. MS + NAA 2mg/l + Kn 4mg/l + BAP 0.5mg/l + CW (5%) (T_{26})

The cultures were kept under continuous light (3000 lux intensity) at 25±2^{0}C and the percentage of plant regeneration was worked out.

3.2.2.1.5 Plant Transplanting

(i) First, the test tubes with green plantlets taller than 5 cm was exposed to sunlight in mist chamber.
(ii) After 3 days the tubes were opened and distilled water was added (up to 0.5 cm to 1.0 cm above the medium surface)
(iii) Chromosome numbers in the shoot tip cells of regenerated plants were examined by Feulgen's staining method after fixation with 3:1 aceto alcohol.
(iv) About three days later, plantlets were removed from tubes, medium adhering to roots were dislodged carefully with a needle.

(v) Base of the haploid seedlings were submerged in 0.1 to 0.2-per cent colchicine solution for 12 hours.

(vi) Then the plantlets were transplanted to plastic cups containing sterilized clay and kept in mist chamber for three weeks by which time, the seedlings had established well with fresh roots and leaves.

(vii) High relative humidity was maintained immediately following transplanting of plantlets.

(viii) The plants were then transferred to pot and grown to maturity.

3.2.2.1.6 Plant Management and Seed Harvest

Conventional crop management was practiced. They were provided with regular supply of water and fertilizers. The seeds were collected from both spontaneous doubled haploid plants and artificial doubled haploid plants (A_1 generation).

3.2.2.2 Observations Recorded

(i) Percentage of callus induction

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

(ii) Percentage of embryogenic callus

$$\text{Embrogenic calli percentage} = \frac{\text{Number of embryogenic calli produced}}{\text{Total number of calli produced}} \times 100$$

(iii) Percentage of non-embryogenic callus

$$\text{Non-embrogenic calli percentage} = \frac{\text{Number of non-embryogenic calli produced}}{\text{Total number of calli produced}} \times 100$$

(iv) Percentage of plant regeneration

$$\text{Percentage of plant regeneration} = \frac{\text{Number of plants produced (Green + Albino)}}{\text{Total number of calli plated}} \times 100$$

(v) Percentage of albinism

$$\text{Percentage of albinism} = \frac{\text{Number of albino plants produced}}{\text{Total number of plants produced}} \times 100$$

(vi) Percentage of green plantlets

$$\text{Percentage of green plants} = \frac{\text{Number of green plants produced}}{\text{Total number of plants produced}} \times 100$$

(vii) Percentage of panicle producing plants

$$\text{Percentage of panicle producing plants} = \frac{\text{Panicle producing plants}}{\text{Total plants produced}} \times 100$$

(viii) Seed setting percentage of doubled haploid plants

The total number of well-filled and ill-filled spikelets in the primary panicle in each plant was counted separately. Seed setting percentage was calculated using the following formula and expressed in percentage

$$\text{Seed setting percentage} = \frac{\text{Number of seeds per panicle}}{\text{Total number of spikelets}} X100$$

(ix) Grains per panicle

The number of well-filled grains in the primary panicle was counted and recorded.

3.2.3 Statistical Analysis

(i) The experimental results relating to the biometric observation of parents and hybrids and effect of different treatments on anther callus induction were statistically analysed in Completely Randomised Design

Analysis of variance for CRD when t treatments

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

S.Ed = $\sqrt{}$ 2EMS/r

CD at 5% = SEd x't' value at edf at 5%

(ii) The interaction effect of two growth regulators viz 2, 4-D and kinetin on anther callus induction was studied by subjecting the data into 2^2 Factorial Completely Randomised Design.

Analysis of variance for FCRD when t treatments.

Source	df	SS	MSS	F value
Treatment	t-1			
G1	1			
G2	1			
G1XG2	1			
Error	n-t			
Total	n-1			

CD for G1 means = $\sqrt{}$ 2EMS /r x G2 X't' value at edf

CD for G2 means = $\sqrt{}$ 2EMS /r x G1 X't' value at edf

CD for interaction effects = $\sqrt{}$ 2EMS /r X't' value at edf

(iii) The effect of carbon source was studied using paired 't 'test

$$Sd = \sqrt{\frac{\Sigma d^2 - (\Sigma d)^2 / n}{n-1}}$$

$$t = \frac{d}{Sd/\sqrt{n}} \sim t_{n-1}\, df$$

RESULTS

4. RESULTS

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

4.1 EVALUATION OF PARENTS AND HYBRID

The experimental results relating to the biometric observation of parents and hybrid were statistically analysed in completely randomized design. The analysis of variance revealed the presence of significant differences among parents and hybrid (Table 6). The mean value of parents and hybrid are given in Table 7.

The parents IR 36 (86.0) and PTB 45 (80.4) differed significantly for the character days to flowering. The hybrid recorded 84.6 days, which was on par with the female parent.

Hybrid was found to be taller (102.8 cm) than parents. Plant height of IR 36 was 95.5 cm while PTB 45 was 92.3 cm. There were significant differences among the parents and hybrid for plant height.

Hybrid (9.2) recorded significantly higher value for panicles per plant than PTB 45(6.6) while the parents and hybrid differed significantly for panicle length. The panicle length of IR 36, PTB 45 and hybrid was 20.3 cm, 22.5 cm and 27.3 cm respectively.

The grains per panicle ranged from 95.2 (IR 36) to 110.6 (hybrid). Hybrid recorded significantly higher value than both the parents. Maximum grain weight was recorded by hybrid (2.82g) and minimum by IR 36 (2.27g). There were significant differences among the parents and hybrid for grain weight.

Table 6. ANOVA for evaluation of parents and hybrids

Source	df	Mean square								
		Days to flowering	Plant height (cm)	Panicles per plant	Panicle length (cm)	Grains per panicle	Grain weight (g)	Pollen fertility (%)	Spikelet sterility (%)	Grain yield/ plant(g)
Treatment	2	42.467**	144.56**	8.867**	15.145**	296.867**	0.398**	0.429	0.979	65.598**
Error	12	1.367	0.859	0.767	0.314	17.933	0.005	0.241	1.413	0.140
Total	14									

** Significant at 1 per cent level
* Significant at 5 per cent level

Table 7. Mean performance of parents and hybrid

	Days to flowering	Plant height (cm)	Panicles per plant	Panicle length (cm)	Grains per panicle	Grain weight (g)	Pollen fertility (%)	Spikelet sterility (%)	Grain yield/ plant(g)
IR.36	86.0 a	95.5 b	8.4 a	20.3 c	95.2 c	2.27 c	96.7 a	14.2 a	5.69 c
PTB.45	80.4 b	92.3 c	6.6 b	22.5 b	102.4 b	2.64 b	96.0 a	14.7 a	8.31 b
IR.36 x PTB.45	84.6 a	102.8 a	9.2 a	23.7 a	110.6a	2.82 a	96.4 a	13.8 a	12.85 a
CD (5%)	1.607	1.277	1.207	0.772	5.835	0.097	0.677	1.638	0.516

a,b,c: means in a column followed by different letter are significantly different.

Plate 1. Rice variety - IR 36

Plate 2. Rice variety - PTB 45

Plate 3. Rice hybrid - IR 36 X PTB 45

Hybrid recorded significantly higher yield (12.85g) than both the parents. Between the parents higher grain yield was recorded by PTB 45 (8.31g) than IR 36 (5.69g)

4.2 ANTHER CULTURE

4.2.1 Callus Induction Response

The experimental results relating to the effect of growth regulators viz, 2, 4-D, Kn, IAA, IBA and carbon source viz sucrose, maltose on callus induction of anthers were statistically analysed in completely randomized design. The results of the effect of different treatments are given in Table 8.

The values of callus induction percentage ranged from 0 to 4.18. Maximum callus induction percentage was recorded by T_{13} (4.18%) followed by T_{14} (3.32%), T_{16} (2.59%). Among the 24 treatments callus induction was observed in 11 treatments.

4.2.1.1 Effect of Carbon Source

In order to study the influence of carbon source, sucrose was compared with maltose. Effect of carbon source on callus induction in the presence of 2, 4-D/Kn and IAA/Kn were separately analysed using paired't' test.

4.2.1.1.1 2, 4-D/Kn combination

Results indicated that when compared with sucrose, maltose enhanced callus induction frequency in the presence of growth regulators 2, 4-D and Kn (Table 9). Calculated't 'value was 3.86 while table't' value at 5 per cent was 3.182.

Table 8. Differences in response of rice anther to different treatments

Treatment No.	No.of anthers inoculated	No.of calli produced	Callus induction percentage	Significance test.
1	427	2	0.47	ef
2	426	0	0.00	f
3	420	4	0.95	e
4	421	1	0.24	f
5	447	0	0	f
6	431	2	0.46	ef
7	426	1	0.24	f
8	453	0	0	f
9	449	0	0	f
10	456	0	0	f
11	464	0	0	f
12	466	0	0	f
13	431	18	4.18	a
14	452	15	3.32	b
15	445	9	2.02	d
16	424	11	2.59	c
17	436	0	0	f
18	452	1	0.22	f
19	436	2	0.44	f
20	421	0	0	f
21	426	0	0	f
22	439	0	0	f
23	459	0	0	f
24	422	0	0	f

CD (5%) value: 0.5

Table 9. Effect of Carbon source on frequency of Callus induction in 2,4-D/Kn combination

Growth regulators combination	Sucrose			Maltose			d
	No.of anthers inoculated	No.of calli induced	Callus induction percentage	No.of anthers inoculated	No.of calli induced	Callus induction percentage	
2,4-D 2 mg/l + Kn 0.5 mg/l	427	2	0.47	431	18	4.18	-3.71
2,4-D 2 mg/l + Kn 1 mg/l	426	0	0.00	452	15	3.32	-3.32
2,4-D 3 mg/l + Kn 0.5 mg/l	420	4	0.95	445	9	2.02	-1.07
2,4-D 3 mg/l + Kn 1 mg/l	421	1	0.24	424	11	2.59	-2.35

Table 10. Effect of carbon source on frequency of callus induction in IAA/Kn combination

Growth regulators combination	Sucrose			Maltose			d
	No.of anthers inoculated	No.of calli induced	Callus induction percentage	No.of anthers inoculated	No.of calli induced	Callus induction percentage	
IAA 1mg/l +Kn 0.5 mg/l	447	0	0	436	0	0	0
IAA 1mg/l +Kn 1 mg/l	431	2	0.46	452	1	0.22	0.24
IAA 2 mg/l +Kn 0.5 mg/l	426	1	0.24	436	2	0.44	-0.2
IAA 2 mg/l +Kn 1 mg/l	453	0	0	421	0	0	0

4.2.1.1.2 IAA/Kn combination

The differences in the callus induction response when sucrose was replaced by maltose in the presence of IAA / Kn has been shown in Table 10. Analysis of paired't 'test revealed non-significant difference between the carbon souce viz., sucrose and maltose. Calculated't 'value was 0.1. Table't 'value at 5 per cent at 3 df was 3.182.

4.2.1.2 Effect of Growth Regulator Combination

The results obtained from various growth regulators combination are summarized in Table 11 and Table 12. Callus induction frequency was high in 2, 4-D/Kn combination followed by IAA/Kn combination irrespective of carbon source. There was no response in IBA/Kn combination.

4.2.1.3 Main and Interaction effects of 2, 4-D and Kn.

The main and interaction effects of 2, 4-D and Kn in two different carbon sources were separately analyzed using 2^2 factorial completely randomized design. Analysis of variance has been shown in Table 13.

4.2.1.3.1 Sucrose

The F-test indicated that the main effects of 2, 4-D and kinetin were significant. But the interaction was non-significant indicating that the data did not reveal definite evidence of differential response of 2,4-D to the different level of kinetin.

Least significant difference test was used to compare the observations and results are presented in Table 14. Between two levels of 2, 4-D, 3mg/l (0.6 %)

Table 11. Effect of growth regulators on frequency of callus induction (Carbon source: Sucrose).

Growth regulator combination	No. of anthers inoculated	No. of calli produced	Callus induction percentage
2,4-D and Kn	1694	7	0.41
IAA and Kn	1757	3	0.17
IBA and Kn	1835	0	0

Table 12. Effect of growth regulators on frequency of callus induction (Carbon source:Maltose).

Growth regulator combination	No. of anthers inoculated	No. of calli produced	Callus induction percentage
2,4-D and Kn	1752	53	3.01
IAA and Kn	1745	3	0.17
I BA and Kn	1746	0	0.00

Table 13. ANOVA for main and interaction effects of 2, 4-D and Kn

Source	df	Mean square	
		Sucrose	Maltose
2,4-D	1	0.259*	4.185**
Kn	1	0.696**	0.04
Interaction	1	0.029	1.03
Error	4	0.029	0.179

** Significant at 1 per cent level
* Significant at 5 per cent level

was superior to 2 mg/l (0.24%). Between two levels of kinetin 0.5 mg/l (0.71%) was superior to 1 mg/l (0.12%).

4.2.1.3.2 Maltose

The F test indicated that the main effect for 2, 4-D is significant. The results are summarized and presented in Table 15. The critical difference is given for marginal difference corresponding to the main effect of 2, 4-D which have proved significant. The concentration of 2, 4-D at 2 mg/l (3.75%) was superior to 3 mg/l (2.31%).

4.2.2 Embryogenic Vs Non-Embryogenic Calli.

Among the 11 response giving treatments, six treatments combinations produced both embryogenic and non-embryogenic calli. Number of embryogenic calli and non-embryogenic calli produced in each treatment are presented in Table 16.

In the total 66 calli obtained through anther culture, 57 were embryogenic calli and the rest non-embryogenic calli. The percentage of embryogenic calli (86.4 %) was higher than non-embryogenic calli (13.6 %).

4.2.3. Plant Regeneration Studies.

The various results obtained from regeneration experiments are presented in Table 17. In total 66 calli plated, 19 regenerated in T_{25}. Regeneration efficiency was 28.79 per cent. Among 66 calli plated in T_{26}, 16 regenerated. Regeneration frequency was 24.24 per cent.

Among 35 plants regenerated, 30 plants were green plants rest albinos. The frequency of green plant and albinos were 85.71 per cent and 14.29 per cent

Table 14. Main and interaction effects of 2,4-D and Kn in callus induction when sucrose was carbon source

	2,4-D 2mg/l (D_1)	2,4-D 3mg/l (D_2)	Mean
Kn 0.5 mg/l (K_1)	0.47	0.95	0.71
Kn 1 mg/l (K_2)	0	0.24	0.12
Mean	0.24	0.6	0.42

CD for 2,4-D means = 0.33
CD for Kn means = 0.33

Table 15. Main and interaction effects of 2,4-D and Kn in callus induction when maltose was carbon source

	2,4-D 2mg/l (D_1)	2,4-D 3mg/l (D_2)	Mean
Kn 0.5 mg/l (K_1)	4.18	2.02	3.1
Kn 1 mg/l (K_2)	3.32	2.59	3.0
Mean	3.75	2.31	3.03

CD for 2, 4-D means = 0.83

Table 16. Embryogenic calli vs non-embryogenic calli

Treatment	No.of calli produced	No. of embryogenic calli	No.of non-embryogenic calli
T_1	2	1	1
T_3	4	3	1
T_4	1	1	0
T_6	2	2	0
T_7	1	0	1
T_{13}	18	16	2
T_{14}	15	13	2
T_{15}	9	8	1
T_{16}	11	11	0
T_{18}	1	1	0
T_{19}	2	1	1
Total	66	57 (86.4)	9 (13.6)

Note: Figures in parenthesis are in percentage

Table 17. Plant regeneration studies

Treatment	Type of calli	No.of Calli plated	No. of regenerated plants	No. of green plants	No. of albinos
T 25	Embryogenic	57	17 (29.82)	15 (88.24)	2 (11.76)
	Non-embryogenic	9	2 (22.22)	1(50)	1(50)
	Sub total	66	19 (28.79)	16 (84.21)	3(15.79)
T26	Embryogenic	57	14 (24.56)	14 (100)	0(0)
	Non-embryogenic	9	2 (22.22)	0 (0)	2 (100)
	Sub total	66	16 (24.24)	14 (87.5)	2(12.5)
	Total	132	35 (26.52)	30 (85.71)	5 (14.29)

Note: Figures in parenthesis are in percentage.

Plate 4. Calli induced from anthers

Plate 5. Embryogenic and non-embryogenic calli

respectively. The ratio of green plants to albinos in T_{25} and T_{26} were 16:3 and 14:2 respectively.

4.2.4. Ploidy Level of Green Plants.

Ploidy level of each green plant was examined using Feulgen's staining method. Details of ploidy level of plants obtained from each treatment are presented in Table 18. Among 30 green plantlets obtained, 21 were homozygous diploids and rest haploids. Frequency of spontaneous doubling was 70 per cent

4.2.5 Doubled Haploid Lines

Anther culture derived plants (A_1) were observed to identify panicle producing plants, grains per panicle and seed setting percentage. Totally 24 lines survived after hardening of which 13 plants were derived from T_{25} and 11 from T_{26}.

Each plant was considered as a single line and number has been given based on the treatment and the test tube number on which it regenerated. Seed setting percentage and grains per panicle of all the lines are presented in Table 19.

Table.18. Ploidy level of Anther culture derived plants.

Treatments	No.of green plants obtained	Ploidy level	
		Haploid	Diploid
T 25	16	4 (25)	12 (75)
T 26	14	5 (35.7)	9 (64.3)
Total	30	9 (30)	21 (70)

Note: Figures in parenthesis are in percentage.

Table19. Studies on doubled haploid lines

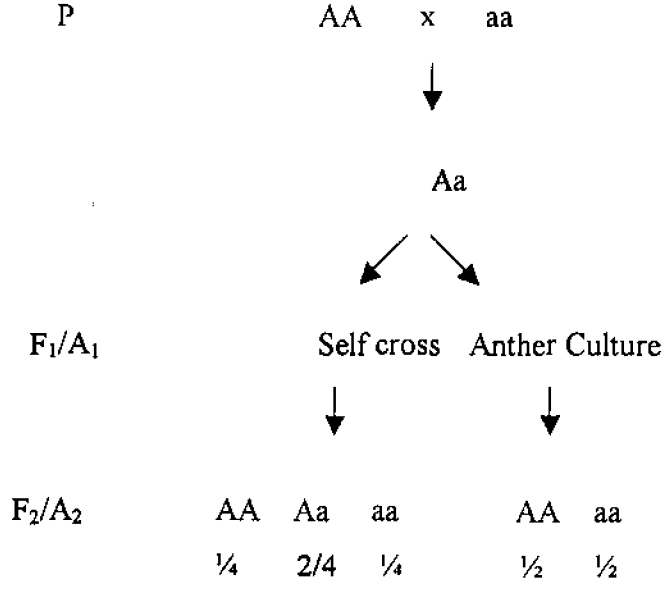
D.H line No.	Grains per panicle	Seed setting percentage	D.H.Line No.	Grains per panicle	Seed setting percentage	D.H.line No.	Grains per panicle	Seed setting percentage
T 25 -2	95	90.48	T25-22	103	91.15	T 26 –11	88	89.79
T 25 –3	98	93.33	T25-24	88	89.79	T 26 –12	87	90.63
T 25 –6	95	91.35	T25-25	94	90.38	T 26 –13	96	94.12
T 25 –9	102	95.33	T 25–32	97	94.17	T 26-24	102	95.33
T 25 –11	90	94.12	T 25–46	95	88.79	T 26-25	96	92.31
T 25 –12	97	90.65	T 26- 2	99	95.28	T 26-37	99	90.83
T 25 –13	89	93.68	T 26- 6	108	94.73	T 26-46	94	92.57
T 25 –19	106	91.38	T 26- 9	94	2.16	T 26-49	103	94.49

DISCUSSION

5. DISCUSSION

Varietal breeding programme in the context of agricultural production is an important factor of technological progress. Providing a better variety than the existing one has always been the aim of plant breeders. The production of new cultivars by haploid breeding cycle, raises the efficiency of selection, and saves space and labour in the experimental field. Great achievements have been made in crop improvement through anther culture in the last 20 years. However, as a breeding method, anther culture is only at its early stage of development.

Gene segregation patterns are different between the usual sexual generation and the anther-derived generation.



If n is the number of loci segregating, the probabilities of fixation of all desirable alleles are $(1/4)^n$ and $(1/2)^n$ for traditional and doubled haploid breeding, respectively. This means that the frequency of fixation in an A_1-derived doubled haploid population is the square root of the probability in an F_2 population, i.e, the

selection efficiency in anther-derived population increases significantly, and especially when n is large.

Thus, it has great potential for increasing yield, and will play an important role in elevating traditional agriculture to high technology agriculture. In this context, the goal of present investigation was to develop a new cultivar from crosses of diverge origin using anther culture technique. The results obtained from various experiments conducted in this study are discussed below.

5.1 EVALUATION OF PARENTS AND HYBRIDS

The basic idea of hybridization is to combine favourable genes present in different parents into a single genotype. The hybrid obtained in the present investigation was evaluated along with parents based on mean performance for agronomic traits. The hybrid recorded significantly different values for plant height, panicle length, grains per panicle, grain weight and grain yield/plant, which is consistent with earlier reports (Vanaja, 1998; Biju, 2001).

5.2 ANTHER CULTURE

The anthers showing mid-uninucleate to late-uninucleate stage of development alone were used for culture. In rice, microspore in the uninucleate stage has been reported as giving the best callus induction response (Wang *et al.*, 1974; Chen, 1977; Chaleff and Stolarz, 1981). The higher response at the uninucleate stage may be because, at that stage, the microspores were still undifferentiated enough to be easily diverted to sporophytic development (Maheshwari *et al.*, 1982) and, as development proceeded, becomes more committed to entering the gametophytic pathway. On the

other hand, at the tetrad stage they may not yet be ready for a totipotent development and hence no androgenic response was obtained.

Pretreatment at 7^0C for 10-12 days was given in this study since its necessity was stressed by several workers (Datta *et al.*, 1990; Ogawa *et al.*, 1992; Raina and Irfan, 1998). The most likely explanation for cold treatment is that the degeneration of the tapetum was preferentially accelerated at low temperatures severing the connection between the microspores and tapetum and the normal developmental sequence was prevented (Sunderland and Xu, 1982).

5.2.1 Callus Induction Response

The degree of callus induction response varies in relation to different media composition. Several publications have confirmed that high induction frequencies obtained for indica cultivars are largly due to the media composition (Torrizo and Zapata, 1986; Rout and Sharma, 1987; Karim, 1987; Zapata *et al.*, 1986; Shahjahan *et al.*, 1985; Karim *et al.*, 1985; Bishnoi *et al.*, 2000; Yang *et al.*, 2001). N_6 medium (Chu *et al.*, 1975) was chosen for the present study as its superiority has been reported earlier (Chen *et al.*, 1986; Raina, 1989; Lynch *et al.*, 1991).

The callus induction frequencies were variable and dependant upon the carbon source and growth regulators combination. The response in the present study varies from 0 per cent to 4.18 per cent. The low level of response may also be due to genotype (Guha and Mukherjee, 1973; Miah *et al.*, 1985; Lentini *et al.*, 1995) and physiology of the donor plant (Raina, 1997; Sun, 1999).

5.2.1.1 Effect of Carbon Source

There was an increased rate of callus induction when sucrose was replaced by maltose in the presence of growth regulator combination 2,4-D and Kn (Fig 1) which is consistent with that of other previous investigations(Finnie *et al.*, 1989; Last and Brettel,1990; Pande and Bhojwani, 1999; Raina,1997; Lentini *et al.*, 1995).

The beneficial effect of maltose was connected with either its ability to stabilize the initial culture medium osmolatity (Kuhlmann and Foroughi- wehr, 1989) or with a slow rate of maltose degradation to glucose (Finnie *et al.*, 1989; Last and Brettel, 1990). As compared to maltose, sucrose seems to break down much more rapidly, especially in autoclaved media, its degradation products being glucose and fructose.

The effect of maltose could not be realized in the medium supplemnted with IAA and Kn (Fig 1). This may be due to low level of response (0% to 0.46%) showed by these treatments.

5.2.1.2 Effect of Growth Regulator Combination

Presence of appropriate concentrations of growth regulators in the medium played a critical role in callus formation from cultured anthers was found by Chu (1982). Concentration of 2, 4-D 2mg/l and Kn 0.5 mg/l was most effective in promoting callus formation. Manonmani and Khan (2004) observed this same result when they tried with 12 treatments involving four levels of 2,4-D and three levels of kinetin. 2, 4-D in the callus induction medium was most suitable for anther culture of rice was confirmed by Liang (1981). Maiti and Mandal (1998) reported that while comparing callus induction percentage between 2, 4-D treatments, 1 mg/l found to have better response (6.42%) than 2 mg/l (5.44%). Contrary to this Chaleff and

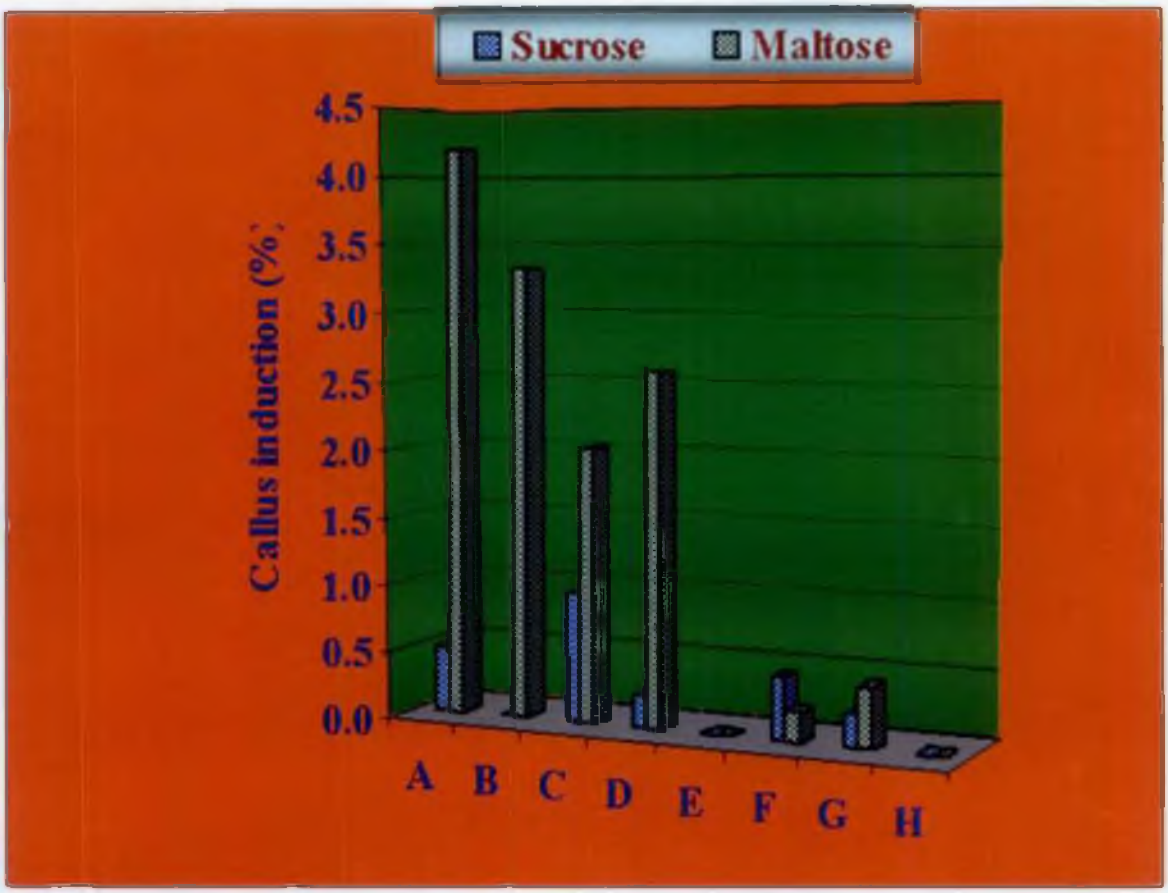


Fig 1. Effect of carbon source on callus induction

A - 2,4-D 2 mg/l + Kn 0.5 mg/l
B - 2,4-D 2 mg/l + Kn 1 mg/l
C - 2,4-D 3 mg/l + Kn 0.5 mg/l
D - 2,4-D 3 mg/l + Kn 1 mg/l
E - IAA 1 mg/l + Kn 0.5 mg/l
F - IAA 1 mg/l + Kn 1 mg/l
G - IAA 2 mg/l + Kn 0.5 mg/l
H - IAA 2 mg/l + Kn 1mg/l

Stolarz (1981) reported that there was no significant difference in callus formation on addition of 0.5 mg/l 2, 4-D to the basal R3 medium while addition of IAA inhibited callus induction. Desamero *et al.* (1998) found that N_6 basal medium supplemented with 2, 4-D 2mg/l was best for anther callus induction. According to Maiti and Mandal (1998) there is interaction between 2, 4-D and genotype. Hence, optimum level of 2, 4-D may vary with the genotypes.

5.2.2 Embryogenic Vs Non-embryogenic Calli

Among the 66 calli obtained in this study, 57 were embryogenic calli and the rest non-embryogenic. Growth regulator combination is said to influence the type of calli produced. Effect of 2, 4-D/Kn and IAA/Kn on callus quality is presented in Table 20.

2, 4-D/Kn combinations produced more number of embryogenic calli (88.3%) than IAA/Kn combination (66.7%). Lin *et al.* (1984) and Reynolds (1986) reported that in rice anther culture, higher concentration of 2,4-D often lead to embryogenic callus while weaker auxins (NAA, IAA) promote non-embryogenic callus which is confirmed by the present study.

5.2.3 Plant Regeneration Studies

In the present study the regeneration frequency was 26.52 per cent. Results of several studies have indicated that the frequency of plant regeneration from calli is highly variable: 8.9 per cent (Pulver, 1986), 153.4 per cent (Zapata *et al.*, 1982), 15 per cent (Mercy and Zapata, 1987) or 2.1 per cent (Guiderdoni *et al.*, 1986).

Table 20. Effect of growth regulator combination on callus quality

Growth regulator combination	Total no. of calli produced	Embryogenic calli produced	Non-embryogencic calli produced
2,4-D/Kn	60	53(88.3)	7(11.7)
IAA/Kn	6	4(66.7)	2(33.3)

Note: Figures in parenthesis are in percentage

Table 21. Effect of callus quality on plant regeneration

Type of calli plated	No. of calli plated	No. of plants regenerated	No.of green plants	No. of albino plants
Embryogenic	114	31(27.19)	29 (94.12)	2(5.88)
Non-embryogenic	18	4(22.22)	1(25)	3(75)

Note: Figures in parenthesis are in percentage

Regeneration frequency of embryogenic and non-embryogenic calli was 27.19 per cent and 22.22 per cent respectively (Table 21). The green plantlet regeneration ability seems to be affected by the type of calli plated (Fig 2). Embryogenic calli tend to produce more number of green plants (94.12%) than the non-embryogenic calli (25%). Zhu *et al.* (1991) and Xie *et al.* (1995) also observed that the green plantlet regeneration ability was affected by the type of calli plated.

5.2.4 Ploidy Level of Anther Derived Plants

The frequency spontaneous doubling in the present study was 70 per cent. The average value of spontaneous chromosome doubling frequencies reported by different authors vary: 59 per cent (Woo *et al.*, 1978), 48 per cent (Guiderdoni *et al.*, 1986), 69 per cent (Pulver, 1986), 41.1 per cent (Demarly, 1983).The spontaneous chromosome doubling of the androgenic plants, frequent in rice might be due to endomitosis or cellular fusion (Niizeki and Oono,1970).

5.2.5 Doubled Haploid Lines

Owing to different microspore origin and somoclonal variations during in vitro culture, variations in morphogenic and physiologic traits are common in A_1 generation. This is at the basis of anther culture breeding.

It is impossible to compare among the A_1 plant populations because of asynchronous regeneration and plants were not grown under normal cultural practices. So all seeds were collected plant by plant to evaluate in the next generation.

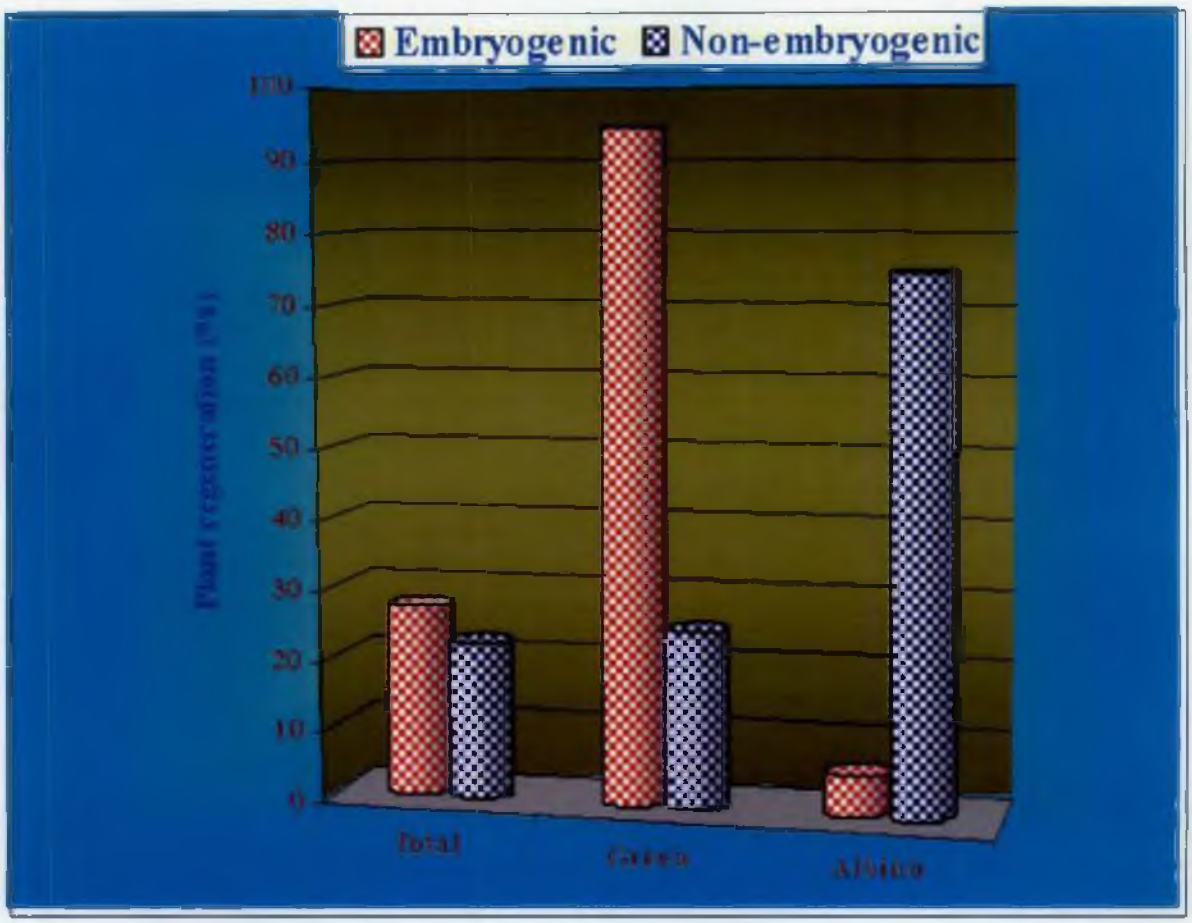


Fig 2. Influence of callus quality on plant renegeration

5.2.6 Future Line of Work

The immediate future objective of the present investigation is to evaluate the stability of anther derived lines in A_2 generation.

Screening can be done for biotic and abiotic stress resistance in subsequent generation.

We can try to raise the anther culturability, i.e callus formation and green plantlet regeneration frequencies, so that a great number of green plantlets can be obtained.

SUMMARY

6. SUMMARY

The present study entitled "Androgenesis in rice (*Oryza sativa* L.) breeding" was undertaken at the Department of Plant Breeding and Genetics, College of Horticulture, Kerala Agricultural University, Vellanikkara to produce a hybrid between IR36 and PTB 45 and culturing the anthers of the F_1 to produce doubled haploids.

The experimental materials consisted of two rice varieties IR36 (ovule parent) and PTB 45 (pollen parent). The anthers from the hybrid of IR 36 x PTB 45 were used for *in vitro* culture. N_6 and MS medium were used for callus induction and regeneration respectively. Callus induction response was studied using different carbon sources (sucrose and maltose) and different growth regulators combination (2, 4-D, IAA, IBA and Kn). The salient results of the study are summarized and presented in this chapter.

The analysis of variance revealed the presence of significant differences among the parents and hybrid. The hybrid recorded significantly different values for plant height, panicle length, grains per panicle, grain weight and grain yield/plant.

Among the 24 treatments used for anther culture, callus induction was observed in 11 treatments. Callus induction percentage ranged from 0 to 4.18. Maximum callus induction percentage (4.18%) was recorded in N_6 medium supplemented with maltose 60 mg/l, 2, 4-D 2 mg/l and Kn 0.5 mg/l followed by N_6 medium supplemented with maltose 60 mg/l, 2, 4-D 2 mg/l and Kn 1 mg/l (3.32%).

Effect of carbon source viz., sucrose and maltose on callus induction in the presence growth regulators combination 2, 4-D/Kn and IAA/Kn were analysed using

paired 't' test. Results indicated that when compared with sucrose, maltose enhance callus induction frequency in the presence of growth regulators combination 2, 4-D/Kn.

Different plant growth regulators viz., 2, 4-D, IAA, IBA and Kn were tested in various concentrations and combinations. Callus induction frequency was high in 2, 4-D/Kn combination followed by IAA/Kn combination irrespective of carbon source. IBA/Kn combination was totally non-responsive.

The main and interaction effects of 2, 4-D and Kn in medium with two different carbon sources were analysed using 2^2 factorial completely randomized design.

Main effects of 2, 4-D and Kn were significant, but interaction was non-significant when sucrose was used as carbon source. Among the two levels of 2, 4-D, 3 mg/l (0.6%) was superior to 2 mg/l (0.24%) and among the two levels of Kn 0.5 mg/l (0.71%) was superior to 1 mg/l (0.12%).

Main effects of 2, 4-D only significantly differed when maltose was used as carbon source. Main effects of 2, 4-D indicated that there was significant reduction in callus induction response when level of 2, 4-D increased from 2mg/l (3.75%) to 3 mg/l (2.31%).

Sixty six calli were obtained through anther culture of which 57 were embryogenic calli and the rest non-embryogenic calli. The percentage of embryogenic calli (86.4%) was higher than non-embryogenic calli (13.6%). 2, 4-D/Kn combination produced more number of embryogenic calli (88.3%) than IAA/Kn combination (66.7%).

The calli were plated for regeneration in two medium (MS + NAA 1mg/l + Kn 2mg/l + IAA 0.5mg/l + CW (5%) (T_{25}) and MS + NAA 2mg/l + Kn 4mg/l + BAP 0.5mg/l + CW (5%) (T_{26})). 19 plants regenerated in T_{25} (28.79%) and 16 plants in T_{26} (24.24%).

Thirty green plants and 5 albinos were obtained from regeneration medium (T_{25} and T_{26}). The frequency of green plant and albinos were 85.71 per cent and 14.29 per cent respectively. The ratio of green plants to albinos in T_{25} was 16:3 and 14:2 in T_{26}. Regeneration frequencies of embryogenic and non-embryogenic calli were 27.19 per cent and 22.22 per cent respectively.

Among 30 green plantlets obtained, 21 were homozygous diploids and the rest haploids. Frequency of spontaneous doubling was 70 per cent. 24 plants survived after hardening. These anther culture derived plants were observed for grains per panicle and seed setting percentage. The values of grains per panicle and seed setting percentage ranged from 87 to 106 and 89.79 to 95.33 respectively.

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* Originals not seen

APPENDIX

APPENDIX

Composition of various media for rice anther culture (mg/l)

Composition	Miller	R2	He2	P2	B5	M8	M10
KNO_3	1000	253	3181.5	1250	2500	2830	3000
NH_4NO_3	1000	400					
$(NH_4)_2SO_4$			231.0	67	134	231	150
$NaH_2PO_4.H_2O$					150		160
$NH_4H_2PO_4$							
$Ca(NO_3)_2.4H_2O$	347						
KH_2PO_4	300	170	800			641	
KCl	65						
$CaCl_2.2H_2O$		440	166.0	75	150	166	160
$MgSO_4.7H_2O$	35	370	3.5	125	250	370	260
Na_2-EDTA	37.3	37.2	223.5	37.3	37.3	74.5	44.7
$FeSO_4.7H_2O$	27.8	27.8	167.1	27.8	27.3	55.7	33.4
$MnSO_4.4H_2O$	4.4	16.8	4.4	2.5	10.0	11.2	10.0
$ZnSO_4.7H_2O$	1.5	6.62	1.5	0.5	2.0	4.3	2.5
H_3BO_3	1.6	6.2	1.6	1.0	3.0	6.2	3.0
KI	0.8	0.83	0.8	0.25	0.75	0.8	0.80
$CuSO_4.5H_2O$		0.02			0.025	0.025	0.030
$NaMoO_4.2H_2O$		0.25		0.025	0.025	0.25	0.030
$CoCl_2.6H_2O$		0.02			0.025	0.025	0.030
Inositol		100		50	160.0		160
Glycine	2	1		1.0			
B_1	0.1		0.5	5.0	10.0	5.0	10.0
B_6	0.1		0.5	1.5	0.5	2.5	1.0
Nicotinic acid	05		2.5	0.5	0.5	3.0	1.0
Yeast extract			1000.0			10.0	
Casein			250.0				
Hydrolysate							
Glutamine				0.15			0.2
L-cysteine							0.5
Potato extract				100000			
DL-Alanine						10.0	

ANDROGENESIS IN RICE (*Oryza sativa* L.) BREEDING

By

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ABSTRACT OF THE THESIS

submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

Faculty of Agriculture
Kerala Agricultural University, Thrissur

2004

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ABSTRACT

An investigation was carried out in rice, at the College of Horticulture, Vellanikkara to produce doubled haploid lines through anther culture technique. The study included production of a hybrid between IR 36 (ovule parent) and PTB 45 (pollen parent), evaluation of parents and hybrid, culturing the anthers of F_1 to produce doubled haploids and analyse the effect of growth regulators (2,4-D, Kn, IAA and IBA) and carbon source (sucrose and maltose) on callus induction.

There was significant difference among the parents and hybrid. The hybrid recorded significantly different values for plant height, panicle length, grains / panicle, grain weight and grain yield / plant.

Among the 24 treatments used for anther culture, callus induction was observed in 11 treatments. Callus induction percentage ranged from 0 to 4.18. Maximum callus induction percentage was recorded in N_6 medium supplemented with maltose 60 mg/l, 2, 4-D 2mg/l and Kn 0.5 mg/l (4.18%) followed by N_6 medium supplemented with maltose 60 mg/l, 2, 4-D 2 mg/l and Kn 1mg/l (3.32%).

A significant increase in anther culture efficiency was observed when sucrose was replaced by maltose in the presence of growth regulators 2,4-D/Kn but not in the presence of IAA/Kn. Callus induction frequency was high in 2,4-D/Kn combination followed by IAA/Kn combination irrespective of carbon source. IBA/Kn combination was totally non-responsive.

Main effects of 2, 4-D and Kn were significant, but there was no interaction between 2, 4-D and Kn when sucrose was used as carbon source. Among the two levels of 2, 4-D, 3 mg/l (0.6%) was superior to 2 mg/l (0.24%) and among the two levels of kinetin 0.5 mg/l (0.71%) was superior to 1 mg/l (0.12%).

Main effects of 2, 4-D only was significant when maltose was used as carbon source. Main effect of 2, 4-D indicated that there was significant reduction in callus induction response when level of 2, 4-D increased from 2mg/l (3.75%) to 3mg/l (2.31%).

Sixty six calli were obtained through anther culture of which 57 were embryogenic calli and the rest non-embryogenic calli. The percentage of embryogenic calli (86.45%) was higher than non-embryogenic calli (13.6%). 2, 4-D/Kn combination produced more number of embryogenic calli (88.3%) than IAA/Kn combination (66.7%).

Thirty green plants and five albinos were obtained from regeneration medium. The frequency of green plant and the albinos were 85.71 per cent and 14.29 per cent respectively. Regeneration frequency of embryogenic and non-embyogenic calli were 27.19 per cent and 22.22 per cent respectively. Among 30 green plants obtained , 21 were homozygous diploids and the rest haploids. Frequency of spontaneous doubling was 70 per cent. Of the 30 regenerated plants taken for hardening 24 survived and all were observed for grains per panicle and seed setting percentage. The values of grains per panicle and seed setting percentage ranged from 87 to 106 and 89.79 to 95.33 respectively.