DEVELOPMENT OF DOUBLED HAPLOIDS FOR IRON TOXICITY TOLERANCE IN RICE (*Oryza sativa* L.)

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(2016 - 11 - 015)

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

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DEPARTMENT OF PLANT BREEDING AND GENETICS
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KERALA, INDIA
2020

DECLARATION

I, Chakravarthi Marri (2016 -11- 015) hereby declare that this thesis entitled **"Development of doubled haploids for iron toxicity tolerance in rice (*Oryza sativa* L.)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Vellanikkara

Date: 21/01/2021

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(2016-11-015)

CERTIFICATE

Certified that this thesis entitled **"Development of doubled haploids for iron toxicity tolerance in rice (*Oryza sativa* L.)"** is a record of research work done independently by **Mr. Chakravarthi Marri (2016-11-015)** 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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We, the undersigned members of the advisory committee of **Mr. Chakravarthi Marri (2016-11-015)**, a candidate for the degree of **Master of Science in Agriculture** with major field in **Plant Breeding and Genetics**, agree that this thesis entitled **"Development of doubled haploids for iron toxicity tolerance in rice (*Oryza sativa* L.)"** may be submitted by **Mr. Chakravarthi Marri** (2016-11-015) in partial fulfillment of the requirement for the degree.

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Chakravarthi Marri

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS

%	per cent
µM	Micro molar
a.i.	active ingredient
ANOVA	Analysis of Variance
BAP	6 – Benzyl aminopurine
CEC	Cation exchange capacity
CH	Casein hydrolysate
cm	Centimetre
CRD	Completely Randomized Design
CW	Coconut Water
df	Degrees of freedom
2,4–D	2, 4–Dichlorophenoxy acetic acid
DH	Doubled haploid
DMSO	Dimethyl Sulphoxide
edf	error degree of freedom
FCRD	Factorial Completely Randomized Design
g	Gram
ha	Hectare
HCl	Hydrochloric acid
IAA	Indoleacetic acid
KAU	Kerala Agricultural University
Kg	Kilogram
Kn	Kinetin
L	Litre
LS	Linsmaier and Skoog
mg	Milligram
mm	Millimetre
mM	Milli molar
MS	Murashige and Skoog
MHa	Million Hectare

MT	Million Tonnes
NAA	Naphthalene acetic acid
NaOCl	Sodium hypochlorite
PAA	Phenyl acetic acid
POP	Package of Practice
ppm	Parts per million
PVP	Polyvinylpyrrolidone
QTL	Quantitative Trait Loci
RH	Relative Humidity
UN	United Nations

Introduction

1. INTRODUCTION

Rice is the staple food and important source of carbohydrates for more than half of the population in the World (Cassman, 1999; Khush, 2005; Sasaki, 2005). Along with wheat and maize, rice is one of the most important food crops grown in the world. During 2001-02 in India, it was cultivated in an area of 44.9 M Ha with a production of 93.34 MT and productivity of 2079 Kg/ha, while the yield and production in 2018-19 increased to 2659 Kg/ha and 116.42 MT respectively, the area under production of rice dwindled to 43.79 M Ha. (GOI, 2019). Even though there was an increase in productivity and production in rice during the period, the growth is very minimal compared to the rapid rise in population. According to the UN (United Nations) projections, the population of the world will reach 9.15 billion by the year 2050 (Alexandratos and Bruinsma, 2012). Hence, in order to keep up with the nutritional needs and food security of the population, the yields of the food crops have to be increased substantially than what it is now. To increase the food availability, the acreage, productivity and production has to increase. However, the production is not increasing at the pace required to cope up with the situation. In addition, the acreage of food crops is decreasing considerably because of rapid urbanisation that calls for conversion of agricultural lands to residential areas. Moreover, the crops grown are marred with many problems like biotic and abiotic stresses which are posing a threat to food security.

In the state of Kerala, which is situated in the south western part of India, rice is the staple food for its populace. It was grown in an area of 8.76 lakh Ha. during 1975-76. The area dwindled to 3.22 lakh Ha. during 2001-02 and decreased further considerably during 2018-19 (2.02 lakh Ha) with a production of 0.57 MT compared to 0.70 MT in 2001-02 which is 18 per cent decrease in production (GOK, 2019). Conversion of agricultural lands to non-agricultural lands due to rapid urbanisation, biotic stresses like pest and diseases are the reasons attributed to such a decrease. Added to this, because of high rainfall, acidic soils are a problem in Kerala resulting in aluminium toxicity and iron toxicity in low land paddy cultivation, thereby reducing the production and yield drastically. According to Becker and Asch (2005), iron toxicity reduces the yield by 15 to 30 per cent on an average, while in extreme cases it can lead to a yield loss up to 100 per cent. Even though the effects of iron toxicity can be reduced

by resorting to agronomic practices such as zinc and other micronutrients spray, balanced nutrition, lime application, digging of ditches around fields, proper irrigation followed by draining *etc*., developing varieties which have innate tolerance to iron toxicity is an appropriate solution to realise good yields in an economically feasible manner.

Through previous studies taken up in the Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara, the genotypes PTB-53 (Mangala Mahsuri), PTB-57 (Swetha) and Tulasi were identified as tolerant to iron toxicity and the genotypes Cul-8709 and Cul-90-03 were reported to be susceptible to iron toxicity (Joseph, 2015; Reddy, 2016). To develop varieties tolerant to iron toxicity or to introgress the character of iron toxicity tolerance in otherwise high yielding varieties, marker assistant selection (MAS) breeding approach can be used if the markers for the QTLs responsible for iron toxicity tolerance in rice are known. For achieving this, QTL mapping has to be done to identify the markers linked to iron toxicity tolerance. For QTL mapping, mapping populations like doubled haploid populations (DH populations) which are immortal fine mapping population are highly invaluable. Once QTL mapping is done and markers are identified, it can be used in various breeding programmes to develop iron toxicity tolerant rice varieties. Apart from it, if any of the doubled haploids obtained from the various gametic recombinations possess characteristics like iron toxicity tolerance and better yield compared to their parents, they can be forwarded to generate advanced breeding lines and develop new varieties rapidly. Unlike in conventional breeding methods, in which the degree of homozygosity increases at the rate of 50 per cent per breeding cycle and may take 8-10 years to develop nearly homozygous lines, through doubled haploidy, recombinant homozygous lines can be generated within one generation after hybridisation. Production of doubled haploids has the advantages of high selection efficiency as both the dominant and recessive genes express in homozygous conditions without any masking and consequent expression of masked characters in later generations (Devaux and Pickering, 2005). Since the DH plants are homozygous recombinants, will be stable in further generations (Datta, 2005).

In these circumstances, we have set out to utilise iron toxicity tolerant and susceptible varieties as found out in the previous experiments in our department, with the following objective:

- To develop doubled haploids in rice for iron toxicity tolerance

Review of Literature

2. REVIEW OF LITERATURE

Rice is the staple food of the people of Kerala which is located in the south west part of India. In Kerala, the area and production under rice is decreasing considerably because of many biotic and abiotic stresses. Out of the many abiotic stresses affecting the production of rice, Iron toxicity is one of the major stresses causing exorbitant loss of yields especially in parts of Kerala where the soils are more acidic. To deal with this situation, development of varieties tolerant to iron toxicity is an ideal option. To develop tolerant varieties within a short span of time, methods like marker assisted selection can be used which needs the genes to have been identified and characterised for which mapping populations are required. To develop such mapping populations which includes doubled haploids, the present study **"Development of doubled haploids for iron toxicity tolerance in rice (*Oryza sativa* L.)"** has been taken up which will be useful to do further breeding programmes on iron toxicity. Therefore, available literature on anther culture with an introduction to hybridisation and major effects of iron toxicity is presented here.

2.1 Hybridisation

Hybridisation is one of the best techniques to increase the innate yield potential of varieties by obtaining F_1s after combining two unrelated parents which brings together the genes from both the parents. Hybridisation can be used to exploit the heterosis of the resultant F_1s over their parents for many characters as well as to generate novel recombinants to increase the variation for different characters for further breeding programmes.

The term heterosis was first used by Shull in 1914. Heterosis may be defined as the superiority of the F_1s over both their parents in terms of yield or any other character. The concept of heterosis in rice started with Jones (1926) who has observed more number of culms and increased grain yield in some F_1s compared to its parents. Heterosis in rice can be exploited to increase the rice yield and yield attributes (Virmani *et al.,* 1982). Many Scientists have found heterosis for yield and yield characters in rice, some of which are listed in the table below:

Range of Heterosis for yield and yield attributes

Character	Relative heterosis	Heterobeltiosis	Reference
Days to flowering		-2.16 to 11.54	Tirkey *et al.* (2006)
	-11.50 to 10.24	-18.67 to 4.03	Palaniraja *et al.* (2010)
	-11.50 to -5.32	-15.61 to -11.43	Nayak *et al. (2012)*
	-10.43 to 12.35	-11.43 to 8.88	Latha *et al.* (2013)
Plant Height (cm)	-17.10 to 21.20	-31.80 to 11.80	Biswas and Julfiquar (2006)
	-27.25 to 16.37	-35.38 to 5.08	Palaniraja *et al.* (2010)
	-10.43 to 12.35	-11.43 to 8.88	Latha *et al.* (2013)
Panicles per plant	-22.80 to 62.60	-30 to 55.20	Biswas and Julfiquar (2006)
	9.68 to 69.57	30.77 to 69.57	Nayak *et al. (2012)*
Panicle length (cm)	-14.10 to 18.70	-25.00 to 10.00	Biswas and Julfiquar (2006)
		-25.38 to 27.81	Tirkey *et al*. (2006)
	-21.05 to 15.33	-29.66 to 9.05	Palaniraja *et al.* (2010)
	-2.05 to 18.81	-7.75 to 18.50	Latha *et al.* (2013)
Grains per panicle	-55.90 to 68.20	-60.20 to 62.40	Biswas and Julfiquar (2006)
	-11.66 to 8.16	-21.50 to 5.25	Palaniraja *et al.* (2010)
Test Weight(g)	-7.10 to 20.10	-20.80 to 9.10	Biswas and Julfiquar (2006)
		-27.57 to 29.12	Tirkey *et al.* (2006)
	-21.40 to 13.91	-27.45 to 4.01	Latha *et al.* (2013)
Spikelet fertility (%)	-50.20 to 273.70	-55.70 to 203.00	Biswas and Julfiquar (2006)
	-98.92 to -6.27	-98.99 to -10.04	Latha *et al.* (2013)
Grain yield per plant (g)		11.98 to 63.01	Satheesh kumar and Sarvanan (2011)
	51.11 to 89.25	44.42 to 87.24	Nayak *et al*. (2012)

Anther culturability is also a character governed by the genotype. Although the gene action of anther culturability is not deciphered properly, many scientists are of the opinion that anther culturability is governed by nuclear genome after conducting diallel analysis (Miah *et al.*1985; Quimio and Zapata, 1990). Many of the scientists reported that both additive effects and non-additive effects (mainly dominant effects) of genes govern both callus induction and plant regeneration (Miah *et al.*, 1985; Zhang and Qifeng, 1993; He *et al.*, 2006). Zhang and Qifeng (1993) and Yan *et al.* (1996) opined that the trait callus induction is governed by genes which have high additive effects with high narrow sense heritability, therefore this trait can be transferred easily from highly responsive cultivars to recalcitrant indica types by hybridisation and selection. But plant regeneration is governed by genes having low additive effects and low narrow

sense heritability. Some workers opine that the genotypes which show high callus induction ability also show high plant regeneration ability (Shahnewaz *et al.*, 2003; Javed *et al.*, 2007) while some scientists observed that the genotypes that show high callusing ability show low plant regeneration ability (He *et al.*, 1998; Talebi *et al.,* 2007). Guideroni *et al.* (1992) have opined that japonica varieties show more callus response than indica varieties. Even within indica varieties, the hybrids of indica types show more anther response than the anthers from the indica varieties alone. Therefore, a good approach to increase the anther culturability of indica rice is to do sexual hybridisation of indica varieties which may also be followed by marker assisted selection for QTLs responsible for anther response (Silva, 2010).

2.2. Iron toxicity

Iron toxicity is a widespread nutritional disorder in wetland rice in acid sulphate soils, clayey acid soils, peat soils, poorly drained sandy valley-bottom soils (Becker and Asch, 2005). It is mostly observed in soils with low CEC, moderate to high acidity, low to moderately high organic matter and active Fe (easily reducible Fe) (Sahrawat, 2005). Iron toxicity is one of the leading abiotic stresses responsible for reduction of yield in paddy crop in Kerala. More than 90 per cent of midland laterite soils of Kerala, mainly northern Kerala, which are the drainage basins of hills and hillock are affected by the accumulation of reduced iron and thereby iron toxicity can be seen in these places. Many leading varieties in Kerala are susceptible to iron toxicity making them unsuitable for cultivation in such conditions. Even though many agronomic practices are recommended to reduce the effects of iron toxicity, many workers are of the opinion that burdening low income farmers with these extra agronomic practices is not economically feasible as compared to the use of varieties tolerant to iron toxicity (Sikirou *et al*., 2015). Many scientists have screened for varieties which are resistant to iron toxicity and utilised them for further breeding programmes as well as development of QTL mapping populations to find the genes responsible for iron toxicity tolerance (Wu *et al*, 1997; Audebert and Sahrawat, 2000; Gridley *et al*., 2006; Onaga *et al*., 2013). DH populations are also used as QTL mapping populations to identify the genes responsible for iron toxicity tolerance in rice (Wu *et al.*, 1997).

2.3. Doubled haploids

Haploids are plants (sporophytes) that contain a gametic chromosome number (n). Haploids produced from diploid species (2n=2x), known as monoploids, contain only one set of chromosomes in the sporophytic phase (2n=x). They can originate spontaneously in nature or can be induced using various induction techniques like modified pollination methods (wide hybridisation, chromosome elimination, pollination with irradiated pollen, etc.) or by *in vitro* culture of immature gametophytes (Germana, 2011). Spontaneous development of haploid plants has been known since 1922, when Blakeslee first described this phenomenon in *Datura stramonium* (Blakeslee *et al*., 1922). This was subsequently followed by similar reports in tobacco (*Nicotiana tabacum* L.), wheat (*Triticum aestivum* L.) and several other species (Forster *et al*., 2007). However, spontaneous occurrence is a rare event and therefore of limited practical value. The potential of haploidy for use in plant breeding arose in 1964 with the achievement of haploid embryo formation from *in vitro* culture of *Datura* anthers (Guha and Maheshwari, 1964, 1966) which was followed by successful *in vitro* haploid production in tobacco (Nitsch and Nitsch, 1969). Many attempts have been made since then to produce haploids, resulting in published protocols for over 250 plant species belonging to almost all families of the plant kingdom (Maluszynski *et al*., 2003). Generally, haploids are smaller and exhibit a lower plant vigour compared to donor plants and are sterile due to the inability of their chromosomes to pair during meiosis. In order to propagate them through seed and to include them in breeding programs, their fertility has to be restored with spontaneous or induced chromosome doubling. The obtained DHs are homozygous at all loci and can represent a new variety (self-pollinated crops) or parental inbred line for the production of hybrid varieties (cross-pollinated crops). Doubled haploids have been used in plant breeding for several years, but their common use is limited to only selected species. This limitation is because of the lack of efficient haploid induction protocols in many species and the induction protocols vary among genotypes within the species.

Using DH technology, completely homozygous plants can be established in one generation thus saving several generations of selfing compared to conventional methods (Germana, 2011), by which also only partial homozygosity is obtained. Doubled haploids thus represent recombinant products of parental genomes in a completely homozygous state. They can be propagated as true breeding lines,

facilitating large-scale testing of agronomic performance over the years. Due to complete homozygosity, the efficiency of selection for both qualitative and quantitative characters is increased since recessive alleles are fixed in one generation and directly expressed. Additionally, doubled haploids can be used in a recurrent selection scheme in which superior doubled haploids of one cycle represent parents for hybridization for the next cycle. Several cycles of crossing, doubled haploid production and selection are performed and gradual improvement of lines is expected due to the alternation of recombination and selection (Griffings, 1975). Doubled haploids can be produced from haploids generated through either male gametes (anther or microspore culture) or female gametes (gynogenesis). But generally, gynogenesis is used only in species where a proper anther culture protocol is not available, as the efficiency of obtaining haploids through ovule is very low (Forster *et al.*, 2007)

2.4. Anther culture

Anther culture started with the works of Guha and Maheswari in *Datura* and that of Nakata and Tanaka in tobacco to develop haploids (Niizeki and Oono, 1968). Later Niizeki and Oono (1968), succeeded in developing haploids in rice through anther culture. They believed that anther culture will be useful in plant breeding as well as in studies on cell physiology, morphogenesis and genetics. According to Serrat *et al.* (2014) genetic recombination occurs during meiosis I which results in the formation of gametes which are genetically unique and they result in the formation of doubled haploids which are unique and stable.

2.4.1. Factors affecting anther culture response

2.4.1.1. Physiological status of Donor

The conditions in which donor plants are grown has a great influence on the anther culture response. According to Szarejko (2003), donor plants should be grown in optimal controlled conditions as the factors which impinge stresses upon the donor plants have a profound effect on the androgenic response of the donor plant. According to him, factors of growth like temperature, light intensity, photoperiod, nutrition, water relations and plant protection measures have an effect on the anther response. In his studies, the plants grown in fully or partially controlled growth conditions in growth houses have given more anther response than the anthers collected from the field grown plants. But according to Veeraraghavan (2007), donor plants

grown in fields are more responsive for anther culture than the green house grown plants. While studying the relation of culture temperature with growth conditions of donor plants in four varieties of wheat (*Triticum aestivum* L.), Ouyang *et al.* (1987) found that the optimum culture temperature of field grown plants is 2^0C more than that of the plants grown in green house conditions irrespective of the variety. They also observed that the development of anthers in green house grown plants is not as good as that of the plants grown in field conditions.

Kristiansen and Andersen (1993) studied the effect of donor plant growth temperature and photoperiod on the embryo formation in anther culture of *Capsicum annuum* L. They have grown the plants at minimum temperature regimes of 16 to 30^0C and photoperiods between 11 and 19 h. They have also studied the effect of donor plant age on anther culture by collecting the anthers from individual plants from five to nine weeks of age.

They obtained embryos from plants grown in all the temperature and photoperiod regimes but the anther culture response decreased significantly with increase in age of the donor plants. Zhang (1989) opined that rice plants grown above 35^0C during meiosis of anthers show more albinos but plants having more nitrogen content show more green plantlet regeneration during anther culture.

Zongxiu *et al.* (1993) have studied the effect of thermo-photo periods on the anther culture response of donor plants by growing them in 9 phytotrons with 3 thermoperiods of 29.7^0C , 25.7^0C , 23.5^0C x 3 photoperiods of 14.75 h, 14.00 h, 13.25 h and found that callus induction was highest when the thermo-photo period was 25.7^0C/14.00h and green plant regeneration frequency was more than 20 per cent when the thermo-photo period was 23.5^0C or 25.7^0C/13.25 h indicating that mid or low levels of thermo-photo periods are suitable for more callus induction as well as green plant regeneration.

Mayakaduwa and Silva (2018) have studied the effects of seasonal variations and nitrogen stresses on *in vitro* anther culture response in rice. When six *indica* rice varieties are grown in two seasons namely *yala* (2012 and 2014) and *maha* (2012/13, 2013/14), they found that anther culture response could be improved up to five folds (17.6 %) during *yala* compared to *maha* season in some varieties. They opined that this response may be due to the stress exerted by the harsh environment

during *yala* season (in terms of temperature, rainfall and sunshine hours) compared to the better environment of *maha* season. In the same study, they have also observed the effect of nitrogen starvation during plant growth as well as Ammoniacal nitrogen starvation in the media used. The anthers obtained from the plants grown in fields with half the recommended dose of nitrogen have given more anther culture response (10.38%) compared to the control (2.88%) when the excised anthers were grown in N_6 medium devoid of ammoniacal nitrogen, the response has increased from 3.10 % in control to 10.40 % in starved medium. When its concentration was reduced to half, the response was 5.44 %. This shows that inorganic nitrogen stress both in field as well as *in vitro* will enhance the anther culture response.

Many scientists have studied the effect of tiller position on the anther culture response in various crops. They found that anthers from primary tillers are more responsive than secondary tillers in callus induction as well as green plantlet regeneration (Jacquard *et al.*, 2006; El Goumi *et al.*, 2017)

2.4.1.2. Genotype

According to Gueye and Ndir (2010), anther culture response varies with the species, sub species and varieties. *Oryza glaberrima* varieties have more potential to callus and generate green plantlets than that of *Oryza sativa*. Among the *O. sativa* sp., japonica varieties were more responsive to callus induction than indica varieties. Guideroni *et al.* (1992) observed a general trend of anther culture response in japonica, indica varieties and their hybrids as follows: japonica/japonica > japonica > japonica/indica >indica/indica > indica. Talebi *et al.* (2007) found that callus induction is genotype specific among indica varieties.

Niroula and Bimb (2009) studied anther culture response of 6 varieties of which two varieties Chandranath-3 and Chomrong Local are *japonica* varieties and four varieties Khumal-4, Pravat, Bindeswori and Hardinath-1 are *indica* varieties. Among these, the 2 japonica varieties and Khumal-4 are temperate (hill rice) varieties and the remaining 3 indica varieties are tropical (terai rice) varieties. In their study, they found that all the three hill rice varieties have given good callus induction and only the varieties Chandranath-3 and Khumal-4 were able to regenerate into green plantlets. Thus, they observed that hill rice varieties are more responsive to anther culture than terai varieties. When four Indonesian local varieties were studied for their

anther culture response by Nurhasanah *et al.* (2015), they found that anther culture response varied across the varieties even when the media and other culture conditions were same. Among the cultivars Buyung, Geragai, Mayas Kuning and Serai Gunung, Serai Gunung has reported highest callus induction followed by Geragai, Buyung, Mayas Kuning. Among the cultivars, Geragai has highest anther culture efficiency followed by Serai Gunung, Buyung, Mayas Kuning in terms of the total number of plants regenerated from the given number of anthers.

2.4.1.3. Stage, size, position and time of collection of explant

The developmental stage of anther is crucial for callus induction and plant regeneration. Generally, the distance between auricles of flag leaf and the penultimate leaf is utilised as a morphological marker to correlate with the developmental stage of microspores. Many scientists consider microspore stage from mid uninucleate to early binucleate stage as ideal for anther culture response. As observing microspore stage of each and every pollen in anthers of the spikelets is difficult to, a correlation will be made by workers depending on the genotype between the flag leaf distance and the microspore nuclear mitotic stage. The stage of the microspores can be obtained by nuclear staining method as suggested by some scientists like Gupta and Borkathur (1987). As per the protocol suggested by them, the anthers will be fixed in 1:3 ratio solution of glacial acetic acid and absolute ethanol for 24 hours and then the anthers are stained with iron alum-haematoxylin. The nuclear stage of the microspore can be observed after staining with a microscope. The uninucleate stage of the microspore can be determined with the observation of a single large nucleus which will be stained darkly. The presence or absence of a vacuole in the uninucleate microspore corresponds to the early or late uninucleate stage respectively. In the early uninucleate stage because of the presence of a large vacuole, the nucleus will be pushed to the periphery of the microspore. As the microspore enters late uninucleate stage, the vacuole will diminish and the microspore will be occupied with a dense cytoplasm with a single nucleus. This nucleus will divide to form a binucleate nucleus (Mayakaduwa and Silva, 2017).

Several workers have used different lengths of the distance between the flag leaf and penultimate leaf in their studies as a marker to identify stage of the microspore. The distance between the auricles of flag leaf and penultimate leaf, stage of microspore,

position of anthers and the time of collection of the explant as followed by different workers are given in the table below.

Distance between auricles of flag leaf and penultimate leaf (cm)	Stage of microspore	Timing of panicle collection	Size/position of anthers	Reference
4-8 cm		8:00 and 9:30 am		Serrat *et al.* (2014)
	late uninucleate			He *et al.* (1998)
	Uninucleate			Andaya *et al.* (1998)
4-5 cm		9:00 and 10:00 am	middle spikelets	Bishnoi *et al.* (2000)
	mid and late-uni-nucletae stage			Chen *et al.* (2001)
4-5 cm	mid uni-nucleate to early bi-nucleate	8:30 and 9: 00 am		Chowdary and Mandal (2001)
	uninucleate	8 am and 9 am		Mandal and Gupta (1997)
	mid to late uni-nucleate stage			Otani *et al.* (2005)
	mid to late uni-nucleate stage			Lee *et al.* (2003)
	mid to late uni-nucleate stage			Lee and Lee (2002)
	mid to late uni-nucleate stage	7 am and 8 am	half to one third of spikelet length	Rout *et al.* (2016)
7-10 or 11-13 cm			basal spikelets	Afza *et al.* (2000)
5 -9 cm				Bagheri *et al.* (2009)
7-10 cm	mid to late uni-nucleate stage			Cha-um *et al.* (2009)
4-7 cm	uninucleate stage			Grewal *et al.* (2006)
4-8 cm	mid to late uni-nucleate stage	8:30 and 10:00 am	middle spikelets	Guzman and Arias (2000)
5-15 cm	mid to late uni-nucleate stage			He *et al.* (2006)

5-7 cm	uninucleate stage	9:00 and 10:00 am		Herath *et al.* (2009)
8-11 cm	late uninucleate	9:00 and 10:00 am		Mayakaduwa and Silva (2017)
3-6 cm	late uninucleate	8:00 and 10:00 am		Islam *et al.* (2004)
	mid to late uni-nucleate stage	7:00 and 8:00 am		Niroula and Bimb (2009)
4-8 cm	mid to late uni-nucleate stage	8:00 and 10:00 am		Lentini *et al.* (1995)
	Uninucleate stage			Nguyen *et al.* (2016)
14-16 cm	mid to late uni-nucleate stage	7:00 and 8:00 am		Naik *et al.* (2016)
6-12 cm			position is genotype dependent	Nurhasanah *et al.* (2015)

2.4.1.4. Pre-treatments

As per Shariatpanahi *et al*. (2006) stresses of various kinds like cold, heat, osmotic stress, sugar starvation, gamma irradiation, and chemical treatments before anther inoculation enhances callus induction and reduces callus formation from other somatic tissues like anther wall and tapetum. These pre-treatments make the pollen to enter into sporophytic pathway of development instead of continuing their gametophytic pathway. Generally cold pre-treatment is given to anthers in case of rice compared to any other treatment.

2.4.1.4.1. Cold Pre-treatment

According to Matsushima *et al.* (1988) cold pre-treatment induces and sustains the sporophytic mode of microspore formation instead of the next stage of gamete formation. According to Usenbekov *et al.* (2014), cold pre-treatment induces synchronisation of cell division and also maintains the viability of embryogenic microspores. The cold pre-treatments used by several workers are listed in the table below

Cold Pre- treatment temperature and duration	Reference
7◦C for 7 -12 days	Serrat *et al.* (2014)
10◦C for 10 days	He *et al.* (1998)
10±2◦C for 10 days	Bishnoi *et al.* (2000)
7◦C for 5 days	Chen *et al.* (2001)
10◦C for 4 days	Mandal and Gupta (1997)
8◦C for 8 days	Mandal *et al.* (2000)
8◦C for 7 days	Otani *et al.* (2005)
10◦C for 10 days	Lee *et al.* (2003)
10◦C for 10 days	Lee and Lee (2002)
4◦C, 8◦C, 10◦C, 12◦C for 2, 4, 6, 8, 10, 12 days and 10◦C for 2 and 8 days were found to be best	Rout *et al.* (2016)
8◦C for 8 days	Afza *et al.* (2000)
8±2◦C for 8-10 days	Mishra *et al.* (2015)
8◦C for 8 days	Bagheri *et al.* (2009)
8◦C for 7 to 10 days	Cha-um *et al.* (2009)
4◦C for 10 days	Grewal *et al.* (2006)
8◦C for 8 to 10 days	Guzman and Arias (2000)
7◦C and 9◦C for 7 days	He *et al.* (2006)
8◦C for 14 days	Herath *et al.* (2009)
10◦C for 7 to 10 days	Mayakaduwa and Silva (2017)
4-8◦C for 8 days	Islam *et al.* (2004)
10◦C for 4-8 days	Lapitan *et al.* (2009)
8±2◦C for 7 days	Niroula and Bimb (2009)
10±2◦C for 7 days	Lentini *et al.* (1995)
8◦C for 3 days	Usenbekov *et al.* (2014)
5◦C for 5-7 days	Nguyen *et al.* (2016)
12◦C for 5 days	Kaushal *et al.* (2014)
4, 8, 10, 12◦C for 2, 4, 6, 7, 8, 10, 12 days and 10◦C for 7-8days was best	Naik *et al.* (2016)

2.4.1.5. Surface sterilisation

The surface sterilisation treatments used by various workers is as follows:

Sterilisation	Reference
a.) 70 % Ethanol for 3 mins	Serrat et al (2014)
b.) 10% sodium hypochlorite + Tween 20 + HCl 35% (50 drops/L) + 1 min in 70 % Ethanol	
70 % Ethanol for 40 s + 0.1 % mercuric chloride for 8 mins	Bishnoi *et al.* (2000)
70% Ethanol for 20s + 0.5 % sodium hypochlorite for 10 mins	Chen *et al.* (2001)
0.1 % Mercuric chloride for 5 mins	Chowdary and Mandal (2001)
0.1 % Mercuric chloride for 7 mins	Mandal and Gupta (1997)
70 % Ethanol for 30 s	Otani *et al.* (2005)
70 % Ethanol for 30 s	Lee *et al.* (2003)
70 % Ethanol for 30 s	Lee and Lee (2002)
70 % Ethanol for 4 min + 4% commercial bleach for 2min	Rout *et al.* (2016)
70 % Ethanol for 20sec + 5.2 % NaOCl for 10 min	Afza *et al.* (2000)
4% NaOCl for 5 min	Mishra *et al.* (2015)
1% NaOCl for 20 mins	Bagheri *et al.* (2009)
10 % Chlorox (5.25 % NaOCl) for 20 mins followed by wash with 3% Chlorox for 20 mins	Cha-um *et al.* (2009)
70 % Ethanol for 2 min + 0.1 % mercuric chloride for 10-12 min	Grewal *et al.* (2006)
70 % Ethanol for 5 mins + 5.25 % NaOCl for 20 mins	Guzman and Arias (2000)
0.1% Mercuric chloride for 7 mins	He *et al.* (2006)
70 % Ethanol for 20 sec + 0.2 % mercuric chloride for 10 min	Islam *et al.* (2004)
5.25 % NaOCl for 20 mins	Lapitan *et al.* (2009)
70 % Ethanol for 1 min + 5.25 % NaOCl for 3 mins	Lentini *et al.* (1995)
70% Ethanol for 30s + 20 % NaOCl for 15 minutes	Nguyen *et al.* (2016)
70 % Ethanol for 4 minutes + 4% NaOCl for 2 minutes	Naik *et al.* (2016)
0.1% Mercuric chloride for 5 minutes	Kaushal *et al.* (2014)

2.4.1.6. Inoculation

Lentini *et al.* (1995) cut the base of the individual spikelets after holding their tips with the forceps and dusted the anthers on the side of the culture jar by holding the uncut ends of the spikelets with forceps and gently tapping the forceps on the sides of the jars to dust the anthers. Naik *et al.* (2016) have dusted approximately 40-50 anthers in a test tube.

2.4.1.7. Media

Among the factors influencing the anther culture response, culture media plays a major role. The type of media suitable for anther culture depends on the species, subspecies as well as cultivars within the sub species. The culture media supplies the microspores with macro and micro salts which along with the carbon source and growth hormones helps in the initiation of androgenesis. These nutrients not only provide nutrition to the microspores but also direct them towards embryonic development (Kaushal *et al.*, 2014). The different media used by several workers for callus induction and green plantlet regeneration are given in the tables below

Media for callus induction	Reference
N_6 + CH (1g/L) + l- Proline (250mg/L) + 2,4 - D (2mg/L) + Kn (1mg/L) + MES (500 mg/L) + 30 g/L sucrose + 3g/L Gelrite	Serrat *et al.* (2014)
SK3 media + 2,4-D (2mg/L) + NAA (2mg/L)	He *et al.* (1998)
N6+2,4-D (2mg/L) + 30g/L Sucrose + agar (0.8 %)	Andaya *et al.* (1998)
RZM + NAA (2mg/L) + Kn (2mg/L) + 2,4- D (0.5 mg/L) + AgNO3 (10mg/L) + 40g/L maltose	Bishnoi *et al.* (2000)
N6 + 2,4 - D (2mg/L) + 60 g/L sucrose + 1.8g/L Gelrite	Chen *et al.* (2001)
He2 + NAA (2mg/L) + Kn (0.5mg/L) + Sucrose (5%)	Mandal and Gupta (1997)
N6 + 2,4- D (1-4 mg/L)	Mandal *et al.* (2000)
N6 + 2,4-D (4mg/L) + sucrose (5% w/v) + gellan gum (0.25% w/v)	Otani *et al.* (2005)
N6 + NAA (2mg/L) + CH (1g/L) + gelrite (0.6%)	Lee *et al.* (2003)

N6 + 2,4-D (4mg/L) + sucrose (5% w/v) + gellan gum (0.3% w/v)	Yamagishi *et al.* (1998)
N6 + NAA (2mg/L) + CH (1g/L) + Proline (250mg/L) + gelrite (0.6%)	Lee and Lee (2002)
MS, N6, SK1 + BAP (0.25-1.0mg/L) + Kn (1mg/L) + 2,4-D (1.5-2.5mg/L) + maltose (30g/L)	Rout *et al.* (2016)
N6 + 2,4-D (2mg/L) + sucrose (6%) + agarose (0.5%)	Afza *et al.* (2000)
N6, MO19, SK1 + 2,4-D (2mg/L) + Kn (0.5mg/L) + myo-inositol (100mg/L) + agar (0.8%)	Mishra *et al.* (2015)
N6 + 2,4-D (2mg/L) + maltose (4%) or sucrose (4%) or maltose + sucrose (4%)	Bagheri *et al.* (2009)
N6 + Kn (0.5mg/L) + 2,4-D (1mg/L) + NAA (2.5mg/L) + maltose (3%) + phytagel (2.5%w/v)	Cha-um *et al.* (2009)
N6 + 2,4-D (2.5mg/L) + Kn (0.5mg/L) + cysteine (40mg/L) + agarose (0.4%)	Grewal *et al.* (2006)
N6 + 2,4-D (2mg/L) + ABA (2,5,10mg/L) + sucrose (6%)	Guzman and Arias (2000)
N6 + 2,4-D (2mg/L) + Kn (0.5mg/L) + sucrose (5%)	Herath *et al.* (2009)
N6 + 2,4-D (0.5mg/L) + NAA (2.5mg/L) + Kn (0.5mg/L) + maltose (6%) + agar (0.8%)	Mayakaduwa and Silva (2017)
N6 + 2,4-D (1mg/L) + NAA (2mg/L) + Kn (1mg/L)	Islam *et al.* (2004)
N6 + 2,4-D (2mg/L) + NAA (1mg/L) + Kn (1mg/L) + maltose (5%)	Lapitan *et al.* (2009)
N6 + myo-inositol (100mg/L) + 2,4-D (2.5mg/L) + Kn (0.5mg/L) + $AgNO_3$ (10mg/L) + maltose (5%) + agar (0.7%)	Niroula and Bimb (2009)
He2 + 2,4-D (2mg/L) + picloram (0.07mg/L) + Kn (0.5mg/L) + maltose (5%) + AgNO3 (10mg/L)	Lentini *et al.* (1995)
Liquid MS + PAA (10mg/L) + Ficoll 400 (12%) + maltose (9%)	Usenbekov *et al.* (2014)
N6 + 2,4- D (2mg/L) + BAP (0.5%) + maltose (3%)	Naik et al. (2016)

Media for Plant regeneration	**Reference**
N_6 + CH (1g/L) + L- Proline (250mg/L) + NAA (1mg/L) + Kn (2mg/L) + MES (500 mg/L) + 30 g/L sucrose + 3g/L Gelrite	Serrat *et al.* (2014)
MS + Kn(2mg/L) + IAA (1mg/L) + NAA (1mg/L)	He *et al.* (1998)

MS + NAA (2mg/L) + 30g/L Sucrose + agar (0.8%)	Andaya *et al.* (1998)
MS + Kn (1-2mg/L) + BA (1mg/L) + NAA (0.5mg/L) + agarose (1% w/v)	Bishnoi *et al.* (2000)
MS + BAP (2mg/L) + NAA (0.5mg/L) + myo-inositol (100mg/L) + CH (100 mg/L) + thiamine (200mg/L) + 30g/L sucrose + 9g/L agar	Chen *et al.* (2001)
MS + NAA (0.5mg/L) + Kn (2mg/L) + sucrose (3%)	Mandal and Gupta (1997)
MS + Kn (1-3 mg/L) + NAA (0.5mg/L)	Mandal *et al.* (2000)
LS + NAA (2mg/L) + Kn (1mg/L) + MES (1g/L) +CH (2g/L) + D-sorbitol (3% w/v) + gellan gum (0.32%)	Otani *et al.* (2005)
N6 + NAA (1mg/L) + Kn (2mg/L) + CH (1g/L) + gelrite (0.6%)	Lee *et al.* (2003)
N6 + NAA (1mg/L) + Kn (2mg/L) + CH (1g/L) + Proline (250mg/L) + gelrite (0.6%)	Lee and Lee (2002)
MS + BAP (1.5 and 0.5mg/L) + Kn (0.5 and 1.5) + NAA (0.5) + sucrose (3%) + agar-agar (0.8%) (shooting media) + MS + NAA(2mg/L) + Kn (0.5mg/L) + sucrose (5%) + agar-agar (0.8%) (rooting media)	Rout *et al.* (2016)
MS + BAP (1mg/L) + NAA (0.5mg/L) + sucrose (3%) + agarose (0.5%)	Afza et al. (2000)
MS + Kn (0.25mg/L) + BAP (0.75mg/L) + NAA (0.25mg/L) for shooting + MS + Kn (0.25mg/L) + NAA (1mg/L) for rooting	Mishra *et al.* (2015)
MS + NAA (1mg/L) + Kn (1mg/L) + maltose (4%) or sucrose (4%) or maltose + sucrose (4%)	Bagheri *et al.* (2009)
MS + sucrose (3%) + phytagel (2.5%w/v) for one week followed by MS + Kn (2mg/L) + NAA (1mg/L) + sucrose (3%) + maltose (3%) + putrescine (0.5mM) + Spermidine or Spermine (0.5mM)	Cha-um *et al.* (2009)
MS + BAP (4mg/L) + sucrose (3%) followed by MS + sucrose (3%) for rooting	
MS + NAA (0.5mg/L) + BAP (2mg/L) + agar (0.8%)	Grewal *et al.* (2006)
MS + BAP (2mg/L) + NAA (1mg/L) + Kn (2mg/L) + sucrose (3%) + agarose (0.45%)	Guzman and Arias (2000)
MS + Kn (2mg/L) + NAA (0.5mg/L) + sucrose (5%)	Herath *et al.* (2009)
MS + NAA (1mg/L) + Kn (0.5mg/L) + BAP (1mg/L) + sucrose (3%) +agar (0.8%)	Mayakaduwa and Silva (2017)
MS + NAA (0.5mg/L) + Kn (3mg/L) + sucrose (5%) + agar (0.8%)	Islam *et al.* (2004)

MS + NAA (0.5mg/L) + Kn (1mg/L) + BAP (0.5mg/L) + sucrose (3%)	Lapitan *et al.* (2009)
Half strength MS + NAA (1mg/L) + BAP (2mg/L) + Kn (0.5mg/L) + sucrose (2%) + 0.7% agar	Niroula and Bimb (2009)
MS + NAA (1mg/L) Kn (4mg/L) + sucrose (3%) + Phytagel (0.15%)	Lentini *et al.* (1995)
MS + NAA (0.5mg/L) + BAP (1mg/L) + proline (500mg/L)	Usenbekov *et al.* (2014)
MS + NAA (0.5mg/L) + Kn (0.5mg/L) + BAP (1.5mg/L) + sucrose (3%) + agar (0.8%) (shoot regeneration media)	Naik *et al.* (2016)
MS + NAA (2mg/L) + Kn (0.5%) + sucrose (5%) + agar (0.8%) (rooting medium)	

2.4.1.8. Growth hormones

Niroula and Bimb (2009) studied the effect of 2,4-D and NAA on anther culture response in six varieties which includes 2 *japonica* varieties and 4 *indica* varieties. They found that the media supplemented with 2,4-D has obtained more calli than the NAA supplied media but the plant regenerability is high in the calli obtained from the media supplemented with NAA compared to 2,4-D. Nguyen *et al.* (2016) have used only 2,4-D (1.5 mg/L) for callus induction and BAP (3 mg/L) and NAA (1 mg/L) for green plant regeneration media.

Naik *et al.* (2016) used various concentrations and combinations of growth hormones like BAP (0.1-0.5 mg/L), kinetin (0.1-0.5 mg/L), 2,4-D (1-2mg/L) and thidiazuron (0.5mg/L). They obtained the best callus induction response when 2,4-D (2mg/L) and BAP (0.5mg/L) were used in the media. When kinetin was used instead of BAP, the callus induction efficiency is reduced by one third. Also, addition of thidiazuron reduced callus induction.

Lentini *et al*. (1995) got high callus induction response when they used a combination of 2mg/L 2,4-D, 0.07mg/L Picloram and 0.5mg/L Kinetin. Islam *et al*. (2004) tried anther culture with various types of growth hormones and obtained 35.5% callus induction when they used N6 medium supplemented with 1mg/l of 2,4-D, 2mg/L of NAA and 1mg/l of Kinetin. This medium has also shown the highest green plant regeneration of 69.3 % when the calli obtained from it were transferred to MS medium supplemented with 0.5 mg/L NAA and 3 mg/L Kinetin out of which 56.14 % were

green whereas the callus induction media with 1 mg/L NAA and 1 mg/L Kinetin has directly produced 70 % green plants without being transferred to another media for regeneration.

When Usenbekov *et al.* (2014) used 0.5mg/L of NAA + 1mg/L of BAP and 500 mg/L of proline for regenerating calli, they obtained albinos. When the BAP level in the media increased to 2mg/L of BAP along with addition of casein hydrolysate and glutamine, the regeneration of green plants with proper roots and aerial parts increased.

Bishnoi *et al.* (2000) used NAA (2mg/L), Kinetin (2mg/L) and 2,4-D at 2mg/L for N6M and Heh5M media whereas only 0.5mg/L of 2,4-D for RZM media. They added Picloram at 0.7 to 1mg/L for different media. For callus regeneration, they used two types of media. MS media supplemented with Kn (1mg/L), BAP (1mg/L), NAA (0.5mg/L) and MS media supplemented with Kn (2mg/L) and NAA (0.5mg/L).

Otani *et al.* (2005) supplemented N_6 media with 2,4-D at 4mg/L and the calli induced were transferred onto LS media supplemented with NAA (2mg/L) and Kn (1mg/L). Lee *et al.* (2003) obtained better callus induction when N_6 media was supplemented with NAA (2mg/L) and higher plant regeneration in N_6 + NAA (1mg/L) + Kn (2mg/L).

Yamagishi *et al.* (1998) used N_6 supplemented with 4mg/L of 2,4-D for callus induction and for regeneration they have used LS media supplemented with NAA (2mg/L) and Kn (1mg/L). Afza *et al.* (2000) utilised N_6 media supplemented with 2,4-D (2mg/L) for callus induction and for plant regeneration, MS media supplemented with BAP (1mg/L) and NAA (0.5mg/L) was used. Cha-um *et al.* (2009) used N_6 media supplemented with 2,4-D (1mg/L), NAA (2.5mg/L) and Kn (0.5mg/L) for callus induction and MS media supplemented with Kn (2mg/L) and NAA (1mg/L) for plantlet regeneration.

2.4.1.9. Supplements and other additives

Many workers used many types of supplements and other additives to enhance the anther culture response. These supplements maybe organic or chemical additives. Generally, scientists use organic supplements like coconut water, yeast extract and chemicals like $AgNO_3$, sorbitol, mannitol, activated charcoal, proline, polyamines etc; to enhance the anther culture efficiency.

Roy and Mandal (2005) studied the effect of yeast extract and casein hydrolysate on anther culture response in rice. They supplemented N6 medium with yeast extract (YE) at 200, 400, 1000mg/L and casein hydrolysate (CH) at 50, 250 and 500mg/L and coconut water at 5,10,15%. The highest callus induction was obtained at 200mg/L of yeast extract. The callus induction was obtained at all concentrations of casein hydrolysate. When CH was used, the green plant regeneration varied from 30.77-100%. According to them, the beneficial effect of CH might be due to the influence of undefined nitrogenous compounds which favour embryogenic callus induction and the dose of CH varies according to the genotype. Different genotypes gave different response to coconut water at different concentrations. Among the varieties used, IR 72 showed highest callus induction. Green plantlet concentration was significantly enhanced in some varieties (66.66 %).

Silver nitrate ($AgNO_3$) is reported to have positive effect in induction of morphogenic callus in rice anther culture (Sarao and Gosal, 2018). According to Niroula and Bimb (2009) addition of $AgNO_3$ at the rate of 5-10 mg/L increases the callus induction as well as green plant regeneration considerably. This may be due to the ethylene synthesis inhibitory activity of $AgNO_3$. Lentini *et al.* (1995) observed that addition of 10mg/L of $AgNO_3$ doubles the callus induction and green plant regeneration of *indica* rice varieties. Kaushal *et al.* (2014) studied the effect of four types of media He2, SK1, B5 and N6 on anther culture response. All the four media except N6 media contains $AgNO_3$.They responded better to callus induction and green plant regeneration in the order of N6<SK1<B5<He2. This shows that among many other factors, presence of $AgNO_3$ in the media enhances the overall anther culture response. Callus browning was reduced, and subsequently the androgenic response was significantly enhanced, when PVP (2 mg/L), an anti-oxidant was added to culture media in anther culture of borage (*Borago officinalis* L.) (Abdollahi *et al*., 2017).

Kaushal *et al.* (2014) studied the effect of Mannitol treatment on the anther culture response by adding mannitol at the rate of 0 mg/L (control), 100 mg/L, 200mg/L and 300mg/L. They observed that 100mg/L of mannitol resulted in a callus induction and green plant regeneration of 18.60% and 16.33% respectively followed by control (17.96% and 14.93%), 200mg/L (13.87% and 11.34%) and 300mg/L (9.14% and 5.41%). They observed a significant genotype x mannitol interaction. Raina and

Irfan (1998) observed callus induction when 0.4M mannitol treatment was supplemented in the media.

Grewal *et al.* (2006) studied the effect of addition of cysteine on the androgenesis and plant regeneration in rice. When cysteine was applied at 40 mg/L, the callus induction and green plant regenerability increased considerably. Casein hydrolysate is thought to be a source of calcium, phosphate, several microelements, vitamins and amino acids. Instead of using individual amino acids which may have positive effects on callus induction, casein hydrolysate can be used for anther culture (Ageel and Elmeer, 2011). Nguyen *et al.* (2016) have used casein hydrolysate (0.3g/L) in callus induction as well green plant regeneration media.

Polyamines are a group of additives that can be used to improve gametophytic embryogenesis. Putrescine, spermidine, and spermine are the polyamines that are present in all living organisms and considered as key modulators of plant growth and development. They act like plant growth regulators (PGRs) and therefore can be involved in the embryogenesis process, through interaction with nitric oxide (Tiburcio and Alcazar, 2018). In addition to their hormonal role, they act as carbon and nitrogen reserves and therefore can control many critical developmental processes. Polyamines are ethylene inhibitors that can improve the efficiency of anther culture experiments by delaying the senescence of cultured anthers (Sarao and Gosal, 2018). Ahmadi *et al.* (2014) investigated the effects of different concentrations of putrescine like 2.26, 5.67, 11.34, 22.68, and 56.72 µM/L, for 12, 24, and 48 h on the microspore embryogenesis of *B. napus* and discovered that the application of 2.26 µM/L putrescine for 48 h increased microspore embryogenesis three-fold. In addition, a normal plantlet regeneration of 92% was achieved by the application of 5.67 µM/L putrescine for 48 h. Ahmadi *et al.* (2015) have found a positive effect of an application of 32.76 µM/L chitosan on callogenesis and shoot regeneration in the shed microspore culture (SMC) of tomato. The effects of different concentrations of proline (434.29, 868.58, 173,716, and 4342.91 µM/L) and chitosan (6.55, 13.10, 32.76, and 65.53 µM/L) were assessed on microspore embryogenesis efficiency of *B. napus* and the application of 868.58 µM/L of proline and 6.55 µM/L of chitosan led to significant increase in microspore embryogenesis (Ahmadi and Shariatpanahi, 2015). Application of calcium in culture medium can improve the efficiency of microspore embryogenesis (Ahmadi *et al*., 2018)

2.4.1.10. Carbon source

Many carbon sources were used by many scientists. Powell (1990) opined that carbon sources are important in production of embryos in anther culture because of their nutritional and osmotic effects. Naik *et al.* (2016) studied the effect of maltose, glucose, fructose and sucrose at 3% on callus induction. They got a callus induction of 29.35% with maltose followed by sucrose (19.61%), fructose (15.73%) and glucose (5.26%). Maltose has been considered to be a good source of carbon for anther culture in rice by Xie *et al.* (1995) because of its ability to keep a high proportion of microspores in swollen condition and thereby increasing its division rate. Last and Bretell (1990) are of the opinion that maltose is more beneficial than sucrose because of its ability to osmotically stabilise the culture medium rather than plasmolysing the microspores as done by sucrose. They proposed that the degradation of maltose into glucose is slow compared to sucrose and that the fructose obtained by degradation of sucrose inhibits androgenesis.

Lentini *et al.* (1995) observed that maltose along with silver nitrate increases callus induction from 6.3 to 20.6 % compared to sucrose in many highly recalcitrant genotypes. The induction increased 15 times when the concentration of maltose is increased from 29 to 351mM. They opined that glucose drastically reduces callus induction compared to mannitol. They have found that maltose not only increased the callus induction percent but also green plant regeneration as well as green plants /albinos ratio.

Bishnoi *et al.* (2000) when working with indica parents and indica x basmathi hybrids obtained 34 % and 78 % of callus induction respectively when they used RZ medium with maltose (4-5%) as source of carbon instead of sucrose. Nguyen *et al.* (2016) have used 60g/L of sucrose for callus induction media and 30g/L for green plantlet regeneration.

Roy and Mandal (2005) have studied the effect of different types of carbon sources namely sucrose, glucose, maltose, dextrose, galactose and cane sugar. They obtained a callus induction of 4.47% in Pusa basmati on N6 with 3% maltose. Callus induction was observed in all the varieties with all the carbon sources but the best callus induction was obtained when the carbon source was 3 and 6% of maltose. The varieties Karnal local 95 variety supplemented with 3% glucose and Taraori basmati

supplemented with 6% maltose during regeneration have majorly turned to be albinos while varieties Pusa basmati and Karnal local-95 supplemented with 6% maltose, recorded a green plantlet regeneration of 13.04% and 20 % respectively.

2.4.1.11 Culture conditions

The culture conditions play an important role in inducing calli from anthers. Culture conditions were altered with different lighting conditions, temperature etc., in the laboratories to enhance the callus induction. Serrat *et al.* (2014) inoculated the anthers in petri dishes and allowed them to incubate in the dark at 24^0C for 6 to 8 weeks until the first microcalli were obtained from the anthers. Nguyen *et al.* (2016) incubated anthers in the dark at 25^0C. After 25 days, they have transferred 2-3 mm calli to regeneration media and cultured them in 14h day with light intensity of 66 µE m^{-2} s^{-1} and 8 hrs dark at 28^0C for differentiation and regeneration. The regenerated plants were transferred to rooting medium supplemented with half strength N_6 medium supplemented with sucrose (20mg/L), colchicine (4mg/L), agar (7g/L) at pH 5.8. The plantlets with good root system were transferred to paddy field after acclimatization.

Kaushal *et al.* (2014) incubated the anthers for callus induction at 25 ± 1^0C and RH of 65 % in dark for callus induction, transferred the calli of at least 2-3mm to the MS regeneration medium. Naik *et al.* (2016) cultured the anthers at 25 ± 2^0C in dark and later the calli were transferred to culture conditions of 25 ± 2^0C under a light intensity of 45µmol m^{-2}/s^{-1}.from cool white fluorescent lights under 16 h photoperiod.

2.4.1.12 Chromosome doubling treatment

Usenbekov *et al.* (2014) used colchicine along with various hormones and cryoprotectants to double the chromosomes in haploids. They used 0.05% colchicine + 2 % DMSO + 10mg/L Gibberellin as first treatment and 0.25% colchicine + 2 % DMSO + 20 drops of Tween 80/L later. After these treatments, 20 out of 26 green plantlets survived which is nearly 77 percent. Chen *et al.* (2001) immersed the roots of haploid plants in 1.25mM colchicine or 25µM oryzalin for 12h at tillering stage for chromosome doubling.

2.4.1.13 Hardening and Field Transfer

After colchicine treatment, Usenbekov *et al.* (2014) transplanted the surviving plantlets into mild sunlight and sprayed a solution of Gibberellic acid (2mg/L)

+ Nicotinamide (3mg/L) + Kinetin (0.5 mg/L) for 5-6 days. Naik *et al.* (2016) removed the agar from green plantlets with well formed roots and kept them in normal tap water for 3-4 days at 27-29^{0}C along with RH of 50-70% for acclimatization. These plants were transferred to 12 inch pots containing field soil and kept in net house and necessary culture measures were taken for proper growth and development of the plants.

Materials and Methods

3. MATERIALS AND METHODS

The present investigation was conducted in Kerala Agricultural University during 2017-19. The details of the materials used and the methods employed in the present study are given below:

3.1 MATERIALS

Seeds of the rice varieties (*Oryza sativa* L.) Swetha (PTB-57), Mangala Mahsuri (PTB-53), Cul 8709, Cul-90-03 and Tulasi obtained from Department of Seed Science and Technology, College of Horticulture, Vellanikkara were used as parents in the hybridisation programme.

3.2 METHODS

The research programme consisted of three experiments namely

a.) Experiment I: Production of F_1s

b.) Experiment II: Raising of F_1s

c.) Experiment III: Production of doubled haploids

3.2.1 Experiment I: Production of F_1s

The seeds of parents obtained from Department of Seed Science and Technology, College of Horticulture, Vellanikkara were sown in pots in staggered manner to obtain synchronized flowering. Seedlings were transplanted into pots at the rate of two hills per pot with two plants per hill. The plants were grown as per the recommendation of package of practices of KAU (KAU, 2016). The following cross combinations were attempted in this study:

Parents		**Hybrids (F_1)**
Female	**Male**	
Swetha (P1)	Tulasi (P5)	H1
Mangala Mahsuri (P2)		H2
Cul-90-03 (P3)		H3
Cul 8709 (P4)		H4

3.2.1.1 Emasculation

Clipping method was followed. Emasculation of spikelets was done late in the afternoon after 3 pm from the panicles which have emerged partially from the leaf sheath. The leaf sheath from such panicles were detached so as to expose the basal spikelets and also for ease of emasculation. The basal spikelets whose anthers are less than half in size of the spikelets and the top spikelets which have already dehisced were removed from the panicle. Only those spikelets having anthers more than half in length of the spikelet were used for emasculation. In such spikelets, the top one third portion of the spikelet was cut off using scissors and the six anthers in each spikelet were removed carefully using forceps, without injuring the ovary. After emasculation, each panicle was bagged in butter paper cover and tagged.

3.2.1.2 Pollination

Pollination of the female parents with pollen from desired male parent was done immediately on the next day of emasculation. The panicles of the male parent which were ready to dehisce were selected and the pollen from such anthers were collected in a petri dish by gentle tapping. The collected pollen was then dusted on the receptive stigma using a thin painting brush. The pollinated panicles were then re-bagged with butter paper cover to avoid contamination with foreign pollen. The bags were removed after five days and seed setting was observed (Joseph, 2015).

3.2.2 Experiment II – Raising of F_1s

The F_1s were raised during 2018-19 in pots at the rate of two hills per pot with one plant per hill.

3.2.3 Experiment III – Production of Doubled haploids

This section consists of the preliminary studies done in anther culture for standardising the protocol and subsequent inoculation of anthers of the actual F_1s.This study was conducted from 2017 to 2019 at the Tissue culture Laboratory, Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara.

3.2.3.1 Selection of Explant

The panicles in booting stage when the distance between the auricle of the flag leaf and penultimate leaf is between 6 and 10 cm, was used for inoculation as

suggested by Reiffers and Freire (1990), Afza *et al.* (2000) and Chandrahasan (2004). The developmental stage of some of the anthers was determined by using the protocol suggested by Gupta and Borthakur (1987). In this method, the anthers from the top, middle and basal spikelets of the panicle were fixed in 1:3 glacial acetic acid and ethanol solution with 2 per cent $FeCl_3$ for 24 hours and later stained with 2 per cent acetocarmine solution and observed under microscope. Since all anthers cannot be subjected to cytological examination, anthers which were 1/3 to 1/2 of the length of the spikelet were used for anther inoculation (Lapitan *et al.,* 2009).

3.2.3.2 Collection of Explant

The panicles at booting stage collected on sunny days between 8.00 am and 9:00 a.m. (Mandal and Gupta, 1997) were cleaned with cotton soaked in 70 % ethanol, wrapped in moist muslin cloth and sealed in aluminium foil. The panicles were then packed in polythene bags for cold pre-treatment (Mayakaduwa and Silva, 2017).

3.2.3.3 Cold Pre-treatment of anthers

The packed panicles were subjected to cold pre-treatment. To identify the best cold pre-treatment duration for callus induction, callus induction was attempted at intervals of three days from the zeroth to fifteenth day as listed below:-

Treatment No	Cold pre-treatment days (10^{o}C)
C1	0 day
C2	3 days
C3	6 days
C4	9 days
C5	12 days
C6	15days

The basal medium used - N_6 + 2,4-D (2mg/L) + Kn (0.5mg/L) + Maltose (3%) + Agar (0.8%)

3.2.3.4 Media

In this study, N_6 (Chu *et al.*, 1975) and B_5 (Gamborg *et al*, 1968) media were used for callus induction studies. For plant regeneration studies, MS (Murashige and Skoog, 1962) medium was used. The composition of the media is given in the Table 1.

3.2.3.5 Preparation of media

The stock solutions for the media were prepared in doubled distilled water and stored in screw capped amber coloured glass bottles in a refrigerator. The stock formulations and quantity of stocks used for preparing media are given in the Tables 2, 3, 4.

3.2.3.6 Preparation of stock solutions of growth regulators

Auxins like 2,4-D, NAA were dissolved in few drops of ethanol, slightly heated and the volume made up to 100 ml by gradual dilution using doubled distilled water. Similarly, cytokinins like Kinetin and BAP were dissolved in a few drops of 0.5N NaOH, gently heated and the volume was made up to 100ml gradually using doubled distilled water. All growth regulator stock solutions were prepared as 25 mg/100 ml.

3.2.3.7 Preparation of media

For Preparation of one litre of media, the stock solutions were pipetted out into a vessel and sucrose or maltose (Analar grade), growth hormones, myo-inositol and other supplements as necessitated were added and the volume made up to 500ml. In another vessel, the required amount of Agar (tissue culture grade) at 0.8% (8g/L) or Gelrite gellan gum at 2.5g/L was dissolved in 500ml of double distilled water and heated with continuous stirring until the gelling agent dissolved. The nutrient solution and the gelling agent solution were mixed immediately and the volume made up to one litre. The pH of the solution was adjusted to 5.6 - 5.8 range using 0.1N NaOH or 0.1N HCl solution. The medium was dispensed into test tubes at the rate of 10 ml to 15 ml quantity in 15 x 125 mm test tubes and stoppered with non-adsorbent cotton.

3.2.3.8 Sterilisation of media

The test tubes containing the media were sterilised in an autoclave at 121^0C for 20 minutes at 1.01 Kg/cm^2 pressure. The media is then allowed to cool at room temperature and stored at cooler temperature in the tissue culture laboratory in slanting position for proper solidification.

3.2.3.9 Sterilisation of explant

An experiment has been conducted to study the best method for sterilising the explant. The following treatments have been applied:

Treatment No.	Treatment
S1	70 % Ethanol for 30s
S2	70 % Ethanol for 5 mins
S3	70 % Ethanol for 20s + 0.5% Sodium Hypochlorite for 10 mins
S4	70 % Ethanol for 2 to 3 mins + 0.1 % $HgCl_2$ for 8 to 10 mins
S5	0.1 % $HgCl_2$ for 5 mins
S6	5.25 % Sodium hypochlorite for 5 minutes
S7	5.25 % Sodium hypochlorite for 20 minutes

3.2.3.10 Inoculation of anthers

Spikelets were cut at their base with the help of sterile scissors and tapped onto the rim of the test tube with the cut open end pointing towards the media. Anthers were inoculated into the test tube at the rate of 10-12 anthers per test tube.

3.2.3.11 Preliminary studies on media

3.2.3.11.1 Effect of media and growth hormone on callus induction

A preliminary study was conducted to assess the effect of two different media *viz.,* N_6 and B_5 and different combinations of 2,4-D and Kinetin at different levels on callus induction. The combination of the media used for this study are as follows:

Treatment No.	Medium	Growth Hormones	
		2,4-D (mg/L)	Kn (mg/L)
D1	N_6	1	0.5
D2	N_6	2	0.5
D3	N_6	1	1.0
D4	N_6	2	1.0
D5	B_5	1	0.5
D6	B_5	2	0.5
D7	B_5	1	1.0
D8	B_5	2	1.0

Table 1. Composition of different media used in this study

Components	N6 (mg/L)	B5 (mg/L)	MS (mg/L)
Major inorganic nutrients			
NH_4NO_3	-	-	1650.0
$(NH_4)_2SO_4$	463.0	134.0	-
KNO_3	2830.0	2500.0	1900.0
KH_2PO_4	400.0	-	170.0
$NaH_2PO_4.H_2O$	-	150.0	-
$MgSO_4.7H_2O$	185.0	250.0	370.0
$CaCl_2.2H_2O$	166.0	150.0	440.0
Trace elements			
H_3BO_3	1.6	3.0	6.2
$MnSO_4.H_2O$	4.4	10.0	22.3
$ZnSO_4.7H_2O$	1.5	2.0	8.60
$Na_2MoO4.2H_2O$	-	0.25	0.25
KI	0.8	0.75	0.83
$CuSO_4.5H_2O$	-	0.025	0.025
$CoCl_2.6H_2O$	-	0.025	0.025
Iron source			
$FeSO_4.7H_2O$	27.85	27.8	27.8
Na_2EDTA	37.25	37.25	37.25
Organic supplements			
Thiamine-HCl	1.0	10.0	0.1
Nicotinic acid	0.5	1.0	0.5
Pyridoxine-HCl	0.5	1.0	0.5
Glycine	2.0	0	2.0
Inositol	-	100.0	100.0

Table 2. Preparation of Stock solutions for MS Medium

SI No.	Components	Quantity(mg)	Volume of Stock Solution prepared(ml)	Volume of stock solution taken per litre of medium(ml)
1	**Major inorganic nutrients (20 X)**			
	NH_4NO_3	16500		
	$(NH_4)_2SO_4$	-		
	KNO_3	19000		
	KH_2PO_4	1700	500	50
	$NaH_2PO_4.H_2O$	-		
	$MgSO_4.7H_2O$	3700		
	$CaCl_2.2H_2O$	4400		
2	**Micro elements**			
	H_3BO_3	620		
	$MnSO_4.4H_2O$	2230		
	$ZnSO_4.7H_2O$	860		
	$Na_2MoO4.2H_2O$	25	500	5
	$CuSO_4.5H_2O$	2.5		
	$CoCl_2.6H_2O$	2.5		
3	**Iron stock***			
	$FeSO_4.7H_2O$	2785	250	2.5
	Na_2EDTA	3725		
4	**KI**	166	200	1
5	**Organic supplements**			
	Thiamine-HCl	10		
	Nicotinic acid	50	250	2.5
	Pyridoxine-HCl	50		
	Glycine	200	100	1
	Inositol#			

*Iron stock solution

Myo-inositol (100 mg/L) was added fresh during media preparation.

To prepare the solution, 3.725 g of Na_2EDTA and 2.785 g of $FeSO_4.7H_2O$ were dissolved separately in 100ml of double distilled water. These two solutions are warmed up slightly. The hot solution of Na_2EDTA is added to the hot solution of $FeSO_4.7H_2O$ and the final volume of the combined solution is made up to 250 ml using double distilled water

Table 3. Preparation of stock solution for N_6 (Chu *et al.*, 1975) medium

SI No.	Components	Quantity(mg)	Volume of Stock Solution prepared(ml)	Volume of stock solution taken per litre of medium(ml)
1	**Major inorganic nutrients (20 X)**			
	NH_4NO_3	-		
	$(NH_4)_2SO_4$	4630		
	KNO_3	28300		
	KH_2PO_4	4000	500	50
	$NaH_2PO_4.H_2O$	-		
	$MgSO_4.7H_2O$	1850		
	$CaCl_2.2H_2O$	1660		
2	**Micro elements**			
	H_3BO_3	320		
	$MnSO_4.H_2O$	880	500	2.5
	$ZnSO_4.7H_2O$	300		
3	**Iron stock***			
	$FeSO_4.7H_2O$	2785	250	2.5
	Na_2EDTA	3725		
4	**KI**	160	200	1
5	**Organic supplements**			
	Thiamine-HCl	100		
	Nicotinic acid	50	250	2.5
	Pyridoxine-HCl	50		
	Glycine	200	100	1

*Iron stock solution is prepared as mentioned for MS stock solution

Table 4. Preparation of stock solution for B_5 (Gamborg *et al.*, 1968)

SI No.	Components	Quantity(mg)	Volume of Stock Solution prepared(ml)	Volume of stock solution taken per litre of medium(ml)
1	**Major inorganic nutrients (20 X)**			
	NH_4NO_3	-		
	$(NH_4)_2SO_4$	1340		
	KNO_3	25000		
	KH_2PO_4	-	500	50
	$NaH_2PO_4.H_2O$	1500		
	$MgSO_4.7H_2O$	2500		
	$CaCl_2.2H_2O$	1500		
2	**Micro elements**			
	H_3BO_3	300		
	$MnSO_4.4H_2O$	1000		
	$ZnSO_4.7H_2O$	200		
	$Na_2MoO4.2H_2O$	25	500	5
	$CuSO_4.5H_2O$	2.5		
	$CoCl_2.6H_2O$	2.5		
3	**Iron stock***			
	$FeSO_4.7H_2O$	2785	250	2.5
	Na_2EDTA	3725		
4	**KI**	150	200	1
5	**Organic supplements**			
	Thiamine-HCl	1000		
	Nicotinic acid	100	250	2.5
	Pyridoxine-HCl	100		
	Glycine	200	100	1
	Inositol#			

*Iron stock solution is prepared as mentioned for MS stock solution

Myo-inositol (100 mg/L) is added freshly during media preparation

3.2.3.11.2 Effects of carbon source on callus induction

In this study, the effects of carbon sources *viz.*, maltose and sucrose at different levels and that of growth hormones -2,4-D and Kn were observed. The treatment details are as follows:

Treatment No.	Carbon Source (CS)	Quantity of CS (g/L)	2,4-D(mg/L)	Kn(mg/L)
CS1	Maltose	30	1	0.5
CS2		30	2	0.5
CS3		30	1	1
CS4		30	2	1
CS5		40	1	0.5
CS6		40	2	0.5
CS7		40	1	1
CS8		40	2	1
CS9		50	1	0.5
CS10		50	2	0.5
CS11		50	1	1
CS12		50	2	1
CS13	Sucrose	30	1	0.5
CS14		30	2	0.5
CS15		30	1	1
CS16		30	2	1
CS17		40	1	0.5
CS18		40	2	0.5
CS19		40	1	1
CS20		40	2	1
CS21		50	1	0.5
CS22		50	2	0.5
CS23		50	1	1
CS24		50	2	1

3.2.3.11.3 Effect of growth hormones on callus induction

The effect of different combinations of four growth hormones *viz.,* 2,4-D, Kn, NAA, BAP on callus induction was assessed by preparing treatment combinations as follows

Treatment No.	Media	Growth Hormones			
		2,4-D (mg/L)	Kn (mg/L)	NAA (mg/L)	BAP (mg/L)
G1	N6	1	0.5	0	0
G2	N6	2	0.5	0	0
G3	N6	1	1	0	0
G4	N6	2	1	0	0
G5	N6	0	0.5	1	0
G6	N6	0	0.5	2	0
G7	N6	0	1	1	0
G8	N6	0	1	2	0
G9	N6	1	0	0	0.5
G10	N6	2	0	0	0.5
G11	N6	1	0	0	1
G12	N6	2	0	0	1
G13	N6	0	0	1	0.5
G14	N6	0	0	2	0.5
G15	N6	0	0	2	1
G16	N6	0	0	2	1

3.2.3.11.4 Effect of silver nitrate on callus induction

The effect of silver nitrate ($AgNO_3$) on callus induction was assessed using anthers. The basal medium used is N_6 + 2,4- D (2mg/L) + Kn (0.5mg/L) + Maltose (3%) and Agar (0.8%). The various levels of silver nitrate ($AgNO_3$) used are as follows

Treatment No.	$AgNO_3$ (0.1 N) in ml
A1	0.0
A2	0.1
A3	0.2
A4	0.3
A5	0.4
A6	0.5
A7	0.6
A8	0.7
A9	0.8
A10	0.9
A11	1.0

3.2.3.11.5. Effect of Casein hydrolysate on callus induction

The effect of casein hydrolysate (CH) on callus induction was assessed using the medium N_6 + 2,4D (2mg/L) + Kn (0.5mg/L) + Agar (0.8%) in the following concentrations: -

Treatment	CH (mg/L)	Maltose (%)
H1	0	3
H2	0	4
H3	0	5
H4	250	3
H5	250	4
H6	250	5
H7	500	3
H8	500	4
H9	500	5
H10	750	3
H11	750	4
H12	750	5
H13	1000	3
H14	1000	4
H15	1000	5

3.2.3.11.6. Effect of Proline on callus induction

The effect of Proline on callus induction was adjudged at various levels as follows

Treatment No.	Proline (mg/L)
P1	0
P2	250
P3	500
P4	750
P5	1000

The basal medium used - N_6 + 2,4-D (2mg/L) + Kn (0.5mg/L) + Maltose (3%) + Gelrite gellan gum (2.5g/L).

3.2.3.11.7. Effect of activated charcoal on callus induction

The effect of activated charcoal on callus induction has been studied using the anthers of Swetha variety and the basal media- N_6 + 2,4-D (2mg/L) + Kn (0.5mg/L) + Maltose (3%) + Agar (0.8%)

The treatments are as follows

Treatment	Concentration
AC1	0 mg/L of Activated charcoal (0%)
AC2	25 mg/L of Activated charcoal (0.0025%)
AC3	50 mg/L of Activated charcoal (0.005%)
AC4	75 mg/L of Activated charcoal (0.0075%)
AC5	100 mg/L of Activated charcoal (0.01%)

3.2.3.11.8. Effect of yeast extract on callus induction

The effect of yeast extract (YE) on callus induction has been studied using the anthers of Swetha variety and the basal media-N_6+2,4D (2mg/L) + Kn (0.5mg/L) + Maltose (3%) + Agar (0.8%). The treatments are as follows

Treatment No	Yeast Extract (YE) (mg/L)
Y1	0
Y2	250
Y3	500
Y4	750
Y5	1000

3.2.3.11.9. Inoculation of F_1 anthers

3.2.3.11.9.1. Effects of cold pre-treatment on callus induction in F_1s

The anthers were inoculated on a basal medium containing N_6+2,4-D (2mg/L) +Kn (0.5mg/L) + L-Proline (250mg/L) + CH (250mg/L) + 0.1N$AgNO_3$ (0.5ml) + Gelrite Gellan gum (2.5g/L) as per the treatments given below

Treatment No.	Hybrid	Cold Pre-treatment(10°C) days
T7	H1	0
T8	H1	3
T9	H1	6
T10	H1	9
T11	H1	12
T12	H1	15
T13	H3	0
T14	H3	3
T15	H3	6
T16	H3	9
T17	H3	12
T18	H3	15
T19	H4	0
T20	H4	3
T21	H4	6
T22	H4	9
T23	H4	12
T24	H4	15

3.2.3.11.9.2 Effect of maltose levels on callus induction

The panicles were subjected to a cold pre-treatment of 10ºC for 10 days and inoculated on a basal medium containing N_6+2,4-D (2mg/L) + Kn (0.5mg/L) + L-Proline (250mg/L) + CH (1g/L) + 0.1N$AgNO_3$ (0.5 ml) + Gelrite Gellan gum (2.5g/L). The panicles were subjected to different treatments of maltose as follows:

Treatment	Hybrid	Maltose
T1	H1	3%
T2	H1	4%
T3	H1	5%
T4	H2	3%
T5	H2	4%
T6	H2	5%

3.2.3.11.9.3 Effects of media and growth hormone on callus induction in F_1 hybrids

The anthers were inoculated on a basal medium containing Maltose (30g/L) + L-Proline (250mg/L) + CH (250mg/L) + 0.1N$AgNO_3$ (0.5ml) +Gelrite Gellan gum (2.5g/L) along with different treatment combinations as detailed below: -

Trt. No	Hybrid	Media	Growth hormones(mg/L)				Trt. No	Hybrid	Media	Growth hormones(mg/L)			
			2,4-D	Kn	NAA	BAP				2,4-D	Kn	NAA	BAP
T25	H1	N_6	2	0.5	0	0	T41	H4	N_6	2	0.5	0	0
T26			2	0	0	0.5	T42			2	0	0	0.5
T27			2	0.5	0.5	0	T43			2	0.5	0.5	0
T28			2	0.5	1	0	T44			2	0.5	1	0
T29		B_5	2	0.5	0	0	T45		B_5	2	0.5	0	0
T30			2	0	0	0.5	T46			2	0	0	0.5
T31			2	0.5	0.5	0	T47			2	0.5	0.5	0
T32			2	0.5	1	0	T48			2	0.5	1	0
T33	H3	N_6	2	0.5	0	0	T49	H2	N_6	2	0.5	0	0
T34			2	0	0	0.5	T50			2	0	0	0.5
T35			2	0.5	0.5	0	T51			2	0.5	0.5	0
T36			2	0.5	1	0	T52			2	0.5	1	0
T37		B_5	2	0.5	0	0	T53		B_5	2	0.5	0	0
T38			2	0	0	0.5	T54			2	0	0	0.5
T39			2	0.5	0.5	0	T55			2	0.5	0.5	0
T40			2	0.5	1	0	T56			2	0.5	1	0

Trt. No-treatment Number

3.2.3.12 Callus culture

The anthers inoculated in the test tubes were maintained in the dark at a temperature of 27 ± 2ºC. The observations on callus induction were after four weeks and every week thereafter until 90 days. Embryogenic calli (milky-white, compact, moist, smooth and slow growing) and non-embryogenic calli (cream colour, friable, dry and fast growing) were sorted before sub culture as followed in Chandrahasan (2004) and as suggested by Nabors *et al.* (1983). The percentage of embryogenic and non-embryogenic calli was calculated.

3.2.3.13 Plant regeneration

The calli were transferred to regeneration media when the calli attained 2mm diameter size. The calli were transferred to the following media:

Treatment	Media composition	Reference
R1	MS+NAA (1mg/L)+Kn(2mg/L)+IAA(0.5mg/L)+CW(5%)	Chandrahasan (2004)
R2	MS+NAA (0.25mg/L)+BAP(0.75mg/L)+Kn(0.25mg/L)	Mishra *et al.* (2015)

The cultures were kept in light at an intensity of 3000 Lux for 14 hrs at 25±2°C and the per cent of plant regeneration was calculated.

3.2.4 Observations recorded

The observations on yield and other biometric traits were recorded in five randomly selected plants as follows:

3.2.4.1. Days to flowering

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

3.2.4.2. Plant height (cm)

In main culm, the height was measured from the ground level to the tip of the flag leaf in centimetre at the time of maturity.

3.2.4.3. Panicles per plant

Number of ears bearing tillers (panicles) were counted at the time of harvest and recorded.

3.2.4.4. Panicle length (cm)

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

3.2.4.5. Grains per panicle

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

3.2.4.6. Spikelet fertility (%)

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

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

3.2.4.7. Test weight (g)

A random sample of 1000 well-filled grains of a plant were weighed and recorded

3.2.4.8. Grain yield per plant (g)

The weight of grains from individual plants were recorded and is expressed in grams.

3.2.4.9. Callus induction per cent

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

3.2.4.10. Embryogenic calli per cent

$$\text{Embryogenic calli per cent} = \frac{\text{No. of embryogenic calli produced}}{\text{Total No. of calli produced}} \times 100$$

3.2.4.12.Non-embryogenic calli per cent

$$\text{Non-Embryogenic calli per cent} = \frac{\text{No. of non-embryogenic calli produced}}{\text{Total No. of calli produced}} \times 100$$

3.2.4.13. Plant regeneration per cent

$$\text{Plant regeneration per cent} = \frac{\text{No. of plants produced (green + albino)}}{\text{Total No. of calli plated}} \times 100$$

3.2.4.13 Per cent albinism

$$\text{Per cent albinism} = \frac{\text{No. of albino plants produced}}{\text{Total No. of plants produced}} \times 100$$

3.2.4.14 Per cent green plantlets

$$\text{Per cent green plantlets} = \frac{\text{No. of green plants produced}}{\text{Total No. of plants produced}} \times 100$$

3.2.5 Statistical Analysis

The statistical analysis is carried out using IBM® SPSS® Statistics software version 22 and Microsoft excel. The ANOVA is carried out in Completely Randomised Design (CRD) for comparison of treatments and Factorial Completely Randomised Design (FCRD) is used in factorial experiments to know the significance of main effects and interaction effects. DMRT was used at 5 percent probability to compare the means of the treatments which is calculated by the software. The structure of the ANOVA followed is given below:

3.2.5.1. ANOVA for CRD

Source	df	SS	MSS	F value
Treatment	t-1	TrSS	TrMS=TrSS/(t-1)	TrMS/EMS
Error	n-t	ESS	EMS=ESS/(n-t)	
Total	n-1	Total SS		

Where r = number of replications

t= number of treatments

n = total number of observations

TrSS = Treatment sum of squares; TrMS= Treatment mean sum of squares

ESS = Error sum of squares; EMS = Error mean sum of squares

3.2.5.2. ANOVA for two factors CRD

Source	df	SS	MSS	F value
Factor A	a-1	Factor A SS	MSa	Msa/Mse
Factor B	b-1	Factor B SS	MSb	MSb/Mse
AxB interaction	(a-1) (b-1)	Interaction SS	MSaxb	MSaxb/Mse
Error	ab(r-1)	ESS	Mse	
Total	(abr-1)	Total SS		

Where r = number of replications; a = number of factor A; b = number of factor B

3.2.5.3. ANOVA for three factor CRD

Source	df	SS	MSS	F value
Factor A	a-1	Factor A SS	MSa	Msa/Mse
Factor B	b-1	Factor B SS	MSb	MSb/Mse
Factor C	c-1	Factor C SS	MSc	MSc/Mse
AxB	(a-1) (b-1)	Interaction AxB SS	MSaxb	MSaxb/Mse
AxC	(a-1) (c-1)	Interaction AxC SS	MSaxc	MSaxc/Mse
BxC	(b-1) (c-1)	Interaction BxC SS	MSbxc	MSbxc/MSe
AxBxC	(a-1) (b-1) (c-1)	Interaction AxBxC SS	Msaxbxc	MSaxbxc/Mse
Error	abc (r-1)	ESS	Mse	
Total	(abcr-1)	Total SS		

Where r = number of replications; a = number of factor A;

b = number of factor B; c = number of factor C

3.2.5.4. Pair wise comparison using DMRT

Duncan's multiple range test (DMRT) was used for experiments that require the evaluation of all possible pairs of treatment means, especially when the total number of treatments is large. Computation of numerical boundaries that allow for the classification of difference between any two treatments or means as significant or non-

significant is done. However, unlike the LSD test in which only a single value is required for any pair comparison at a prescribed level of significance, the DMRT requires computation of a series of values, each corresponding to a specific series, of pair comparisons. The following steps are followed for ranking the data (Gomez and Gomez, 1976).

Step 1: Rank all the treatment means in decreasing (or increasing) order. It is customa ry to rank the treatment means according to the order of preference.

Step 2: The standard error of error mean sum of squares (SEm) was calculated by using the formula

$$SEm = \sqrt{EMS/r}$$

where, 'EMS' is the error mean sum of squares and 'r' is the number of replications.

Step 3: Compute the (t-1) values of the shortest significant ranges as:

$$Rp = (r_p)\ (SEm)$$

For p = 2, 3⋯, t using significant studentized ranges (r_p) from statistical tables

Where, 't' is the total number of treatments, 'SEm' is the standard error of the mean difference computed in step 2, 'r' values are the tabular values of the significant ranges, and 'p' is the distance in rank between the pairs of treatment means to be compared (i.e., p = 2 for the two means with consecutive rankings and p = t for the highest and lowest means).

Step 4: Identify and group together all treatment means that do not differ significantly from each other.

Step 5: Use the alphabet notation according to the ranking to present the test results.

3.2.5.5 Estimation of Heterosis

The Magnitude of heterosis of the hybrids was estimated over the mid-parent and better parent (Nadarajan and Gunasekaran,2008)

Relative Heterosis (di)

The superiority of F_1 over the mid-parent was estimated as

$$di = \frac{(F\text{-}MP) \times 100}{MP}$$

Where F is mean value of hybrid

MP is mid-parent value

Heterobeltiosis (dii)

The superiority of F_1 over the better parent was estimated as

$$dii = \frac{(F-BP) \times 100}{BP}$$

Where F is mean value of hybrid

BP is mean value of better-parent

Test of significance

Significance of estimates of heterosis was tested at error degrees of freedom as suggested by Turner (1953)

$$\text{'t' value for relative heterosis} = \frac{(F-MP)}{\sqrt{(Me/r \times 3/2)}} \times 100$$

$$\text{'t' value for heterobeltiosis} = \frac{(F-BP)}{\sqrt{(Me/r \times 2)}} \times 100$$

Results

4. RESULTS

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

4.1 Evaluation of Parents and Hybrids

The biometrical observations recorded on the five parents and the four F_1s were statistically analysed. The results of ANOVA (Table 5) revealed significant differences for all the characters under study.

4.1.1. Evaluation of Parents and Hybrids

4.1.1.1. Days to Flowering

There was significant variation among the genotypes for days to flowering. The days to flowering varied from 76 days in P5 and H3 to 116.20 days in H2 (Table 6). The hybrid H3 exhibited a negative relative heterosis of -1.43 while all the other hybrids exhibited positive and significant relative heterosis ranging from 7.94 (H4) to 24.66 (H1). H3 recorded a negative heterobeltiosis of -2.81 while all the other hybrids recorded positive heterobeltiosis [7.73 (H4), 6.64 (H1), 1.66 (H2)] (Table 7).

4.1.1.2. Plant height (cm)

Plant height varied from 70.2 cm (P3) to 102.8cm (H1). Among the parents, P3 registered the least height of 70.20 cm and P1 recorded the highest value of 95.7cm. Among the hybrids, H4 recorded a height of 74.8cm while, H1 recorded a height of 102.8cm (Table 6). Significant positive relative heterosis and heterobeltiosis was recorded by hybrids H1 (17.15 and 7.42) and H3 (32.8 and 41.88), while the hybrid H4 has recorded significant negative relative heterosis as well as heterobeltiosis of -7.19 and -8.10 respectively. The hybrid H2 exhibited a non-significant positive relative heterosis of 1.09 while it recorded a negative heterobeltiosis of -6.68 which is significant (Table 7).

4.1.1.3 Panicles per plant

Significant variation was observed among the genotypes for panicles per plant (Table 6). The panicles per plant varied from 5.5 (P3) to 10.4 (P2) among the parents and it ranged from 9.4 (H4) to 13.2 (H1). All the hybrids recorded significant values for relative heterosis and heterobeltiosis for this character (Table 7). The relative

heterosis ranged from 45.10 (H2) to 73.00 (H1). The heterobeltiosis ranged from 13.90 (H2) to 62.70 (H4) (Table 7).

4.1.1.4 Panicle length (cm)

A significant variation was observed between the genotypes for panicle length. Among the genotypes, the panicle length ranged from 17.6 cm (H4) to 27cm (H1) with a range of 21.59 (P4) to 24.38 (P1) among parents and a range of 17.6cm (H4) to 27cm (H1) between hybrids. The hybrid H1 recorded a positive and significant relative heterosis as well as heterobeltiosis of 13.61 and 10.75 respectively. The hybrid H2 exhibited a non-significant relative heterosis as well as heterobeltiosis of 4.16 and 2.95 respectively, whereas the hybrid H3 recorded a non-significant negative relative heterosis as well as heterobeltiosis of -1.42 and -2.38 respectively. The hybrid H4 has shown a significant negative relative heterosis and heterobeltiosis of -21.31 and -23.97 respectively.

4.1.1.5 Grains per panicle

The grains per panicle varied from 127 (P3) to 246.4 (P1) and from 130.4 (H4) to 215 (H2) among the parents and hybrids respectively (Table 6). Except H2, all the hybrids recorded negative relative heterosis for this character. The relative heterosis varied from -14.58 (H4) to 8.72 (H2). All the hybrids registered negative heterobeltiosis, ranging from -25.57 (H4) to -2.41(H2) (Table 7).

4.1.1.6 Test weight (g)

The test weight varied from 18.3 (P5) to 25.2 (P4) which is also the range among all the genotypes. Among the hybrids, the test weight varied from 21.5 (H3) to 22.5 (H1). All the hybrids recorded a positive relative heterosis for this trait ranging from 3.23 (H4) to 13.75 (H2). In case of heterobeltiosis, the hybrid H4 recorded a negative heterosis of -11.11 and the remaining hybrids have shown a positive heterobeltiosis ranging from 0.94 (H3) to 9.69 (H2).

4.1.1.7 Spikelet fertility (%)

The spikelet fertility varied from 75.4 (H3) to 95.4 (P2). Among the parents, it varied from 77.3 (P4) to 95.4 (P2). Among the hybrids, it varied from 75.4 (H3) to 85.4 (H1). All the hybrids have shown a negative relative heterosis for this character which varied from -1.47 (H4) to -12.27 (H3). In case of heterobeltiosis, the heterosis varied from -6.19 (H4) to -12.27(H3) (Table 7).

4.1.1.8 Grain yield/ plant (g)

The grain yield per plant varied significantly among all the genotypes. The mean performance of the genotypes for this character varied from 11.47 (P3) to 35.6 (H2) (Table 6). Among the parents this value varied from 11.47 (P3) to 28.02 (P1). Among the hybrids, it varied from 16.66 (H4) to 35.6 (H2). All the genotypes recorded a positive heterosis for both relative heterosis as well as heterobeltiosis. The relative heterosis varied from 27.86 (H4) to 76.41 (H2) and in case of heterobeltiosis, the value ranged from 16.91 (H1) to 52.13 (H3) (Table 7).

4.2 Anther culture studies

4.2.1 Preliminary studies:

The results of various preliminary experiments done for callus induction are given below

4.2.1.1 Cold pre-treatment

The studies on cold pre-treatment of anthers at 10^{o}C for various days has shown a significant difference between days of treatment for callus induction (Table 8). The mean value of callus induction among the treatments varied from 0% (C2) to 7.5 % (C4) (Table 9). The treatments C3 (2.7 %) and C1 (1.1%) were on par with each other. The treatments C1 (1.1%), C2 (0%), C5 (0.5%) and C6 (0.6%) were on par with each other. The treatment C4 was the best among all the treatments with 7.5% callus induction followed by C3 which has recorded 2.7% of callus response. The treatment C2 has not given any callus induction response.

4.2.1.2 Explant sterilisation

The different explant sterilisation methods tried had shown a significant difference (Table 10). The treatment S6 (4.17%) registered the least contamination followed by S7 (5.83%) and the highest contamination was recorded by the treatment S1 at 19.17% (Table 11). The DMRT showed that the treatments S6 and S7 were on par with each other while the contamination in case of S6 was less than that of S7. The treatments S3 (11.67%), S4 (14.17%), S5 (13.33%) were on par with each other and also S4 and S5 were on par with each other along with S2 (17.50%). The treatments S1 (19.17%) and S2 (17.50%) were also on par with each other.

Table 5. ANOVA for yield and biometrical traits in rice

Source	df	Mean sum of squares							
		Days to Flowering	Plant height (cm)	Panicles per plant	Panicle length (cm)	Grains per panicle	Test weight (g)	Spikelet fertility (%)	Grain yield/ plant (g)
Treatment	8	1761.026**	657.24**	33.91**	32.351**	10257.73**	0.182**	239.044**	426.419**
Error	36	3.492	16.314	1.189	1.766	277.858	0.02	11.169	1.253
Total	44								

** Significant at 1% level

Table 6. Mean performance of genotypes for biometric characters

	Days to Flowering	Plant height (cm)	Panicles per plant	Panicle length (cm)	Grains per panicle	Test weight (g)	Spikelet fertility (%)	Grain yield/plant (g)
Parents								
Swetha(P1)	106.90	95.70	9.40	24.38	246.40	21.6	94.40	28.02
MM(P2)	114.30	94.27	10.40	23.70	220.30	19.6	95.40	27.91
Cul-90-03(P3)	78.20	70.20	5.50	22.73	127.00	21.3	86.40	11.47
Cul 8709(P4)	76.30	81.40	6.90	21.59	130.10	25.2	77.30	13.61
Tulasi(P5)	76.00	79.75	5.90	23.15	175.20	18.3	85.49	12.47
Hybrids								
Swetha/Tulasi (H1)	114.00	102.80	13.20	27.00	196.60	22.5	85.40	32.76
MM /Tulasi (H2)	116.20	88.02	11.80	24.40	215.00	21.6	81.80	35.60
Cul-90-03/Tulasi (H3)	76.00	99.60	9.60	22.60	145.00	21.5	75.40	18.94
Cul 8709/Tulasi (H4)	82.20	74.80	9.40	17.60	130.40	22.4	80.20	16.66
SEm (±)	2.67	1.71	0.39	0.40	6.81	0.03	1.08	1.32

Table 7. Estimation of heterosis for yield and yield components

Hybrid	Days to Flowering		Plant height (cm)		Panicles per plant		Panicle length (cm)		Grains per panicle		Test weight (g)		Spikelet fertility (%)		Grain yield/plant (g)	
	di	dii	di	dii	di	dii	di	dii	di	dii	di	dii	di	dii	di	dii
Swetha/Tulasi (H1)	24.66**	6.64**	17.15**	7.42**	73.00**	41.00**	13.61**	10.75**	-6.74	-20.21**	11.94**	2.27	-5.05**	-9.53**	61.89**	16.91**
MM /Tulasi (H2)	22.12**	1.66	1.09	-6.68*	45.10**	13.90*	4.16	2.95	8.72	-2.41	13.75**	9.69*	-9.56**	-14.25**	76.41**	27.55**
Cul-90-03/Tulasi (H3)	-1.43	-2.81	32.80**	41.88**	46.90**	36.20**	-1.42	-2.38	-4.04	-17.23**	8.86**	0.94	-12.27**	-12.73**	58.36**	52.13**
Cul 8709/Tulasi (H4)	7.94**	7.73**	-7.19*	-8.10*	67.70**	62.70**	-21.31**	-23.97**	-14.58 **	-25.57**	3.23	-11.11**	-1.47	-6.19**	27.86**	22.41**
SEm(±)	1.02	1.18	2.21	2.55	0.60	0.69	0.73	0.84	9.13	10.54	0.08	0.02	1.83	2.11	0.61	0.71

** Significant at 1 per cent level; * Significant at 5 per cent level di- Relative Heterosis: dii-Heterobeltiosis

4.2.1.3 Effect of media and growth hormone combination

The results of the study on different combinations of 2,4-D and Kn with the media N_6 and B_5 revealed significant differences between the different media, growth hormone combinations and their interaction on callus induction (Table 12). The treatment combination D2 had given the highest callus induction of 7.8%. (Table 13). The callus induction percentage varied for remaining treatments from 0.6 (D1) to 1.3% (D5). Significant interaction was observed between the media and growth hormone combination. The N_6 media had a mean performance of 2.3% while the B_5 recorded only 0.8% (Table 14). The growth hormone combination 2,4-D (1mg/L) + Kn (1mg/L) had a mean callus induction of 0.3% for both the media combined and the combination 2,4-D (2mg/L) + Kn (0.5 mg/L) had contributed to 4.5% of the mean callus induction for both the media combined which was the highest among the growth hormone combinations. N_6 medium recorded a high callus induction of 7.8% with the growth hormone combination 2,4-D (2 mg/L) + Kn (0.5mg/L). For B_5 media, the highest callus induction of 1.3% was obtained with the growth regulator combination of 2,4-D (1mg/L) + Kn (0.5mg/L)

4.2.1.4 Effect of different carbon sources and growth hormones on callus induction

The study on the effect of different combinations of carbon sources namely maltose and sucrose at 30,40,50 g/L combination along with 2,4-D (1mg/L and 2mg/L) and Kinetin (0.5mg/L and 1mg/L) revealed that there existed significant differences among the main effects of carbon source levels and growth hormones as well as their interaction (Table 15).The callus induction varied from 7.9% for the treatment CS2 to 0.0% in case of many treatments (Table 16).Between maltose and sucrose at various levels , the callus induction ranged from 2.15% for maltose at 30g/L to 0.16% when sucrose was used at 30g/L. The carbon source maltose gave highest callus induction of 2.15% at 30g/L whereas it was only 0.33% at 50g/L. Sucrose recorded a callus induction of 0.16% at 30g/L whereas it had a callus induction percent of 0.34% at both 40g/L and 0.33% at 50g/L (Table 18). 2,4-D (2mg/L) and Kn (0.5mg/L) was the best combination of growth hormones for callus induction with a mean

Table 8. ANOVA for effect of cold pre-treatment on callus induction

Source of Variation	df	SS	MSS	F -value
Treatment	05	121.92	24.38	27.09*
Error	12	10.80	0.9	
Total	17	132.72		

* - Significant at 5 per cent level

Table 9. Callus induction at different levels of cold pre-treatment

Treatment	Cold pre-treatment days	Total		
		AI	CI	Per cent CI
C1	**0 day**	181	2	1.1^{bc}
C2	**3 days**	179	0	0^{c}
C3	**6 days**	183	5	2.7^{b}
C4	**9 days**	186	14	7.5^{a}
C5	**12 days**	182	1	0.5^{c}
C6	**15 days**	181	1	0.6^{c}

AI: No of Anthers inoculated; CI: No of calli induced from the anthers; per cent CI: Callus induction percentage ((AI/CI) *100)

Treatments having mean of % callus induction containing at least one letter common among a, b, c are not significantly different using DMRT method.

Table 10. ANOVA for sterilisation treatments for anther culture in rice

Source of Variation	df	SS	MSS	F -value
Treatment	6	561.31	93.55	13.09**
Error	14	100.00	7.14	
Total	20	661.31		

** Significant at 1 per cent level

Table 11. Effect of sterilisation treatments on anther culture

Treatment No.	Treatment	Test tubes inoculated	Contaminated	Per cent contamination
S1	**70 % Ethanol for 30s**	120	23	19.17^{d}
S2	**70 % Ethanol for 5 mins**	120	21	17.50^{cd}
S3	**70 % Ethanol for 20s+0.5% Sodium hypochlorite for 10 mins**	120	14	11.67^{b}
S4	**70 % Ethanol for 2 to 3 mins + 0.1 % $HgCl_2$ for 8 to 10 mins**	120	17	14.17^{bc}
S5	**0.1 % HgCl2 for 5 mins**	120	16	13.33^{bc}
S6	**5.25 % Sodium hypochlorite for 5 minutes**	120	5	4.17^{a}
S7	**5.25 % Sodium hypochlorite for 20 minutes**	120	7	5.83^{a}

Treatments having % of contamination containing at least one letter common among a. b. c, d are not significantly different.

Table 12. ANOVA for the effects of media and growth hormones on callus induction

Source of Variation	df	SS	MSS	F-value
Treatment	**7**	143.299	20.471	14.5**
Media	**1**	13.575	13.575	9.615*
2,4-D+Kn	**3**	74.275	24.758	17.536*
Media*2,4-D+Kn	**3**	55.449	18.483	13.091*
Error	**16**	22.589	1.412	
Total	**23**	165.888		

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

Table 13. Mean performance of different media and growth hormones on callus induction

Treatment No	Medium	Growth Hormones		Total			
		2,4-D	Kn	AI	CI	Per cent	CI
D1	**N_6**	**1**	**0.5**	155	1	0.6[b]	
D2	**N_6**	**2**	**0.5**	153	12	7.8[a]	
D3	**N_6**	**1**	**1**	151	0	0.0[b]	
D4	**N_6**	**2**	**1**	153	1	0.7[b]	
D5	**B_5**	**1**	**0.5**	154	2	1.3[b]	
D6	**B_5**	**2**	**0.5**	162	2	1.2[b]	
D7	**B_5**	**1**	**1**	157	1	0.6[b]	
D8	**B_5**	**2**	**1**	155	0	0.0[b]	

Table 14. Combined effect of media and growth hormones on callus induction

Growth Hormone combination	N_6	B_5	Mean
	Per cent CI	Per cent CI	
2,4-D (1mg/L) + Kn (0.5mg/L)	0.6	1.3	1.0
2,4-D (2mg/L) + Kn (0.5 mg/L)	7.8	1.2	4.5
2,4-D (1mg/L) + Kn (1mg/L)	0.0	0.6	0.3
2,4-D (2mg/L) + Kn (1 mg/L)	0.7	0.0	0.35
Mean	2.3	0.8	1.5

Table 15. ANOVA for the effects of carbon sources and growth hormones on callus induction

Source of Variation	df	SS	MSS	F -value
Carbon source (CS)	5	22.932	4.586	9.285**
2,4-D + Kn	3	26.036	8.679	17.569**
CS*2,4-D + Kn	15	82.431	5.495	11.125**
Error	24	11.855	0.494	
Total	47	143.253		

**Significant at 1 percent level

Table 16. Effect of different carbon sources with growth hormones on callus induction

Treatment No.	Carbon source (CS)	CS(g/L)	Growth Hormones		Mean		
			2,4-D(mg/L)	Kin (mg/L)	AI	CI	Per cent CI
CS1	**Maltose**	**30**	**1**	**0.5**	153	0	0.0[c]
CS2		**30**	**2**	**0.5**	151	12	7.9[a]
CS3		**30**	**1**	**1**	151	0	0.0 [c]
CS4		**30**	**2**	**1**	152	1	0.7 [c]
CS5		**40**	**1**	**0.5**	147	0	0.0 [c]
CS6		**40**	**2**	**0.5**	151	5	3.3[b]
CS7		**40**	**1**	**1**	151	0	0.0 [c]
CS8		**40**	**2**	**1**	153	1	0.7 [c]
CS9		**50**	**1**	**0.5**	154	1	0.6 [c]
CS10		**50**	**2**	**0.5**	156	0	0.0 [c]
CS11		**50**	**1**	**1**	151	0	0.0 [c]
CS12		**50**	**2**	**1**	150	1	0.7 [c]
CS13	**Sucrose**	**30**	**1**	**0.5**	152	0	0.0 [c]
CS14		**30**	**2**	**0.5**	151	0	0.0 [c]
CS15		**30**	**1**	**1**	154	1	0.6 [c]
CS16		**30**	**2**	**1**	150	0	0.0 [c]
CS17		**40**	**1**	**0.5**	152	1	0.7 [c]
CS18		**40**	**2**	**0.5**	154	0	0.0 [c]
CS19		**40**	**1**	**1**	146	1	0.7 [c]
CS20		**40**	**2**	**1**	151	0	0.0 [c]
CS21		**50**	**1**	**0.5**	150	0	0.0 [c]
CS22		**50**	**2**	**0.5**	148	1	0.7 [c]
CS23		**50**	**1**	**1**	151	1	0.7 [c]
CS24		**50**	**2**	**1**	156	0	0.0 [c]

AI: No of Anthers inoculated; CI: No of call induced from the anthers; per cent CI: Callus induction percentage ((AI/CI) *100)

The letters a, b, c in the last column indicate that the means with common letters are not significantly different by using DMRT method

Table 17. Effects of different carbon sources and growth hormone combinations on callus induction.

Growth Hormones (GH)		Maltose				Sucrose				GH mean	CS (3%) mean	CS (4%) mean	CS (5%) mean
2,4-D(mg/L)	Kn(mg/L)	3%	4%	5%	Mean	3%	4%	5%	Mean				
1	0.5	0.00	0.00	0.65	0.22	0.00	0.66	0.00	0.22	0.22	0.00	0.33	0.32
2	0.5	7.95	3.31	0.00	3.75	0.00	0.00	0.68	0.23	1.99	3.97	1.66	0.34
1	1	0.00	0.00	0.00	0.00	0.65	0.68	0.66	0.67	0.33	0.32	0.34	0.33
2	1	0.66	0.65	0.67	0.66	0.00	0.00	0.00	0.00	0.33	0.33	0.33	0.33
Mean		2.15	0.99	0.33	1.16	0.16	0.34	0.33	0.28	0.72	1.16	0.66	0.33

induction percentage of 1.99% whereas the least mean callus induction percentage was 0.22% when 2,4-D (1mg/L) and Kn (0.5mg/L) were used (Table 17). 2,4-D (2mg/L) and Kn (0.5mg/L) with maltose at 30mg/L and 40mg/L had given the best callus induction of 7.95% and 3.31% respectively while the least callus response of 0% was obtained in 13 out of 24 treatments.

4.2.1.5 Effects of different growth hormone combinations on callus induction

There was a significant difference between the different auxin levels and cytokinin levels and their interaction on callus induction (Table 18). Among the treatments, the highest callus induction was obtained for G2 (5.9%) followed by G6 (1.6%) (Table 19). Among the auxins, 2,4-D (1.05%) performed better than NAA (0.39%) in terms of mean callus induction (Table 20). Among the cytokinins, Kn at 0.5mg/L has given the highest mean performance of 2.00% callus induction whereas BAP at 1mg/L gave only 0.13%. There was a significant interaction between auxins and cytokinins. The combination 2,4-D (2mg/L) and Kn (0.5mg/L) had the highest callus induction (5.9%). 2,4-D yielded a response at both levels of Kn but NAA didn't yield any response when Kn is applied at 1mg/L. When BAP is used as cytokinin, 2,4-D recorded callus induction only when BAP is used at 0.5mg/L and all the other combinations of 2,4-D and BAP yielded nil response. When NAA is combined with BAP, all the combinations except NAA (2mg/L) +BAP (1mg/L) didn't record callus induction response.

4.1.2.6 Effect of Silver nitrate ($AgNO_3$) on callus induction

The ANOVA (Table 21) of the effects of various $AgNO_3$ treatments indicates that there was a significant difference among the treatments for callus induction. The treatments A6, A7, A10 had given the highest callus induction of 3.36% (A6, A7) and 3.38% (A10) which was on par with each other and the treatment A3 has given 0% callus induction (Table 22). The treatments A1, A2, A4, A5 and A10 were on par with each other ranging from 1.36% (A1) to 2.68% (A8).The treatments A1, A2, A4, A5, A9 and A11 were on par with each other ranging from A9 (0.66%) to A2 (2.05%). A1, A3, A9, A11 were on par with each other ranging from A3 (0.0%) to A11 (0.67%).

The average number of days for callus induction with silver nitrate treatment varied from 41 days (A6) to 75 days (A1). Among the best performing treatments in terms of callus induction, the minimum number of days for callus induction was taken by treatments A6 and A7 at 41 and 53 days respectively and A10 took 62 days for callus induction.

Table 18. ANOVA for the effect of different growth hormones on callus induction

Source of Variation	df	SS	MSS	F -value
Treatment	15	64.266	4.284	14.008*
Auxin	3	17.627	5.876	19.21**
Cytokinin	3	18.086	6.029	19.711**
Auxin x Cytokinin	9	28.554	3.173	10.373**
Error	16	4.894	0.306	
Total	31	69.106		

Table 19. Per cent callus induction for various growth hormone combinations

Treatment No	Media	Growth Hormones				Total		
		2,4-D	Kn	NAA	BAP	AI	CI	Per cent CI
G1	**N6**	**1**	**0.5**	**0**	**0**	185	1	0.5^{bc}
G2	**N6**	**2**	**0.5**	**0**	**0**	186	11	5.9^{a}
G3	**N6**	**1**	**1**	**0**	**0**	192	0	0.0^{c}
G4	**N6**	**2**	**1**	**0**	**0**	195	2	1.0^{bc}
G5	**N6**	**0**	**0.5**	**1**	**0**	189	0	0.0^{c}
G6	**N6**	**0**	**0.5**	**2**	**0**	193	3	1.6^{b}
G7	**N6**	**0**	**1**	**1**	**0**	188	0	0.0^{c}
G8	**N6**	**0**	**1**	**2**	**0**	190	0	0.0^{c}
G9	**N6**	**1**	**0**	**0**	**0.5**	185	0	0.0^{c}
G10	**N6**	**2**	**0**	**0**	**0.5**	193	2	1.0^{bc}
G11	**N6**	**1**	**0**	**0**	**1**	197	0	0.0^{c}
G12	**N6**	**2**	**0**	**0**	**1**	192	0	0.0^{c}
G13	**N6**	**0**	**0**	**1**	**0.5**	190	1	0.5^{bc}
G14	**N6**	**0**	**0**	**2**	**0.5**	197	1	0.5^{bc}
G15	**N6**	**0**	**0**	**1**	**1**	192	1	0.5^{bc}
G16	**N6**	**0**	**0**	**2**	**1**	191	0	0.0^{c}

Table 20. Combined effects of different growth hormones at various levels on callus induction

Cytokinins (mg/L)		Auxins (mg/L)						Cytokinin mean
		2,4-D (1)	2,4-D (2)	Mean	NAA (1)	NAA (2)	Mean	
	Kn (0.5)	0.5	5.9	3.2	0	1.6	0.8	2.00
	Kn (1)	0	1	0.5	0	0	0	0.25
	BAP (0.5)	0	1	0.5	0.5	0.5	0.5	0.50
	BAP (1)	0	0	0	0.5	0	0.25	0.13
	Mean	0.13	1.98	1.05	0.25	0.53	0.39	0.72

*Values in parentheses indicate respective growth hormone concentration in mg/L

Table 21. ANOVA of the effects of silver nitrate treatments on callus induction

Source of Variation	df	SS	MSS	F -value
Treatments	10	27.997	2.8	4.530*
Error	11	6.799	0.618	
Total	21	34.796		

* Significant at 5 percent level

Table 22. Mean performance of different silver nitrate concentrations on callus induction

Treatment No.	$AgNO_3$ (0.1 N) in ml	Total			DTI
		AI	CI	Per cent CI	
A1	**0.0**	147	2	1.36 ^{bcd}	75
A2	**0.1**	146	3	2.05 ^{abc}	51
A3	**0.2**	147	0	0.00 ^{d}	00
A4	**0.3**	151	3	1.99 ^{abc}	64
A5	**0.4**	154	3	1.95 ^{abc}	45
A6	**0.5**	149	5	3.36^{a}	41
A7	**0.6**	149	5	3.36 ^{a}	53
A8	**0.7**	149	4	2.68 ^{ab}	50
A9	**0.8**	151	1	0.66 ^{cd}	64
A10	**0.9**	148	5	3.38 ^{a}	62
A11	**1.0**	149	1	0.67 ^{cd}	60

DTI-Days to callus induction

Table 23. ANOVA for casein hydrolysate with maltose on callus induction

Source of Variation	df	SS	MSS	F -value
CH	4	2.531	0.633	1.699^{NS}
Maltose	2	8.75	4.375	11.749**
CH*Maltose	8	10.422	1.303	3.499*
Treatment	14	21.703	1.55	4.163**
Error	15	5.585	0.372	
Total	29	27.288		

**Significant at 1 per cent level; * Significant at 1 per cent level

Table 24. Average performance of different casein hydrolysate concentrations and maltose on callus induction

Treatment No.	CH (mg/L)	Maltose (%)	Total			DTI
			AI	CI	Per cent CI	
H1	**0**	**3**	182	3	1.6 [b]	84
H2	**0**	**4**	187	2	1.1 [bc]	87
H3	**0**	**5**	185	0	0.0 [c]	-
H4	**250**	**3**	186	6	3.2[a]	48
H5	**250**	**4**	190	0	0.0 [c]	-
H6	**250**	**5**	192	0	0.0 [c]	-
H7	**500**	**3**	186	1	0.5 [bc]	75
H8	**500**	**4**	190	2	1.1 [bc]	77
H9	**500**	**5**	186	1	0.5 [bc]	70
H10	**750**	**3**	186	2	1.1 [bc]	77
H11	**750**	**4**	192	0	0.0 [c]	-
H12	**750**	**5**	193	0	0.0 [c]	-
H13	**1000**	**3**	192	1	0.5 [bc]	80
H14	**1000**	**4**	186	1	0.5 [bc]	76
H15	**1000**	**5**	187	0	0.0 [c]	-

DTI-Days to callus induction

Table 25. Effects of casein hydrolysate and maltose on callus induction

CH (mg/L)	Maltose (g/L)			Mean
	30	40	50	
0	1.6	1.1	0.0	0.9
250	3.2	0.0	0.0	1.1
500	0.5	1.1	0.5	0.7
750	1.0	0.0	0.0	0.3
1000	0.5	0.5	0.0	0.3
Mean	1.4	0.5	0.1	0.7

Table 26. ANOVA for the effect of proline treatment on callus induction

Source of Variation	df	SS	MSS	F -value
Proline	4	68.689	17.172	3.413^{NS}
Error	10	50.316	5.032	
Total	14	119.005		

NS: Non-significant

Table 27. Mean performance of various levels of proline (mg/L) on callus induction

Treatment	Proline (mg/L)	Total		
		AI	CI	Per cent CI
P1	**0**	158	2	1.27^{b}
P2	**250**	156	9	5.77^{a}
P3	**500**	160	8	5.00^{ab}
P4	**750**	158	2	1.27^{b}
P5	**1000**	165	1	0.61^{b}

Table 28. ANOVA for different concentration of activated charcoal on callus induction

Source of Variation	df	SS	MSS	F -value
Treatments	4	1.116	0.279	0.75^{NS}
Error	10	3.719	0.372	
Total	14	4.834		

NS- Non Significant

Table 29. Mean performance of activated charcoal on callus induction

Treatment	Treatment	Total		
		AI	CI	Per cent CI
AC1	0 mg/L of Activated charcoal (0%)	180	1	0.56[a]
AC2	25 mg/L of Activated charcoal (0.025%)	180	1	0.56 [a]
AC3	50 mg/L of Activated charcoal (0.05%)	180	0	0 [a]
AC4	75 mg/L of Activated charcoal (0.075%)	180	0	0 [a]
AC5	100 mg/L of Activated charcoal (0.1%)	180	0	0 [a]

Table 30. ANOVA for the effect of yeast extract on callus induction

Source of Variation	df	SS	MSS	F -value
Treatments	4	3.431	0.858	1.462[NS]
Error	10	5.866	0.587	
Total	14	9.298		

NS : Non-significant at 5 per cent level

Table 31. Mean performance of yeast extract on callus induction

Treatment No	Yeast extract (YE) (mg/L)	Total		
		AI	CI	Per cent CI
Y1	0	149	1	0.67[a]
Y2	250	147	0	0.00 [a]
Y3	500	150	1	0.67 [a]
Y4	750	151	0	0.00 [a]
Y5	1000	154	2	1.30 [a]

4.1.2.7 Effect of casein hydrolysate (CH) in combination with Maltose on callus induction

The ANOVA for the effect of casein hydrolysate (CH) in combination with maltose showed that there was no significant difference in the main effects of CH on callus induction while there was significant difference in the effect of different levels of maltose and there was an interaction between the levels of CH and maltose on callus induction (Table 23).While the treatments H3, H5, H6, H11, H12 and H15 registered no callus induction , the treatment H4 registered the highest callus induction of 3.2% (Table 24). The treatments H1, H2, H7, H8, H9, H10, H13, H14 were on par with each other while all treatments except H1 and H4 were on par with each other. There was significant effect of interaction between the maltose and CH levels on callus induction. Maltose induced a mean callus induction of 0.1% at 50g/L and 1.4% at 30g/L. CH at 250mg/L recorded the highest mean callus induction of 1.1% while it was only 0.3% at both 750mg/L and 1000mg/L which was the lowest (Table 25). The minimum number of days to callus induction varied from 48 (H4) to 87 (H2)

4.1.2.8 Effect of Proline on callus induction

The results of ANOVA of the effect of proline on callus induction showed that there was no significant difference between the treatments on callus induction (Table 26). The treatment P2 has given the highest callus induction of 5.77% followed by P3 at 5.00% which were on par with each other (Table 27). The least callus induction was recorded for the treatment P5 which recorded a callus induction of 0.61% while the treatments P1 and P4 recorded treatments of 1.27%. The treatments P1 (1.27%), P3 (5.0%), P4 (1.27%) and P5 (0.61%) were on par with each other.

4.1.2.9 Effect of activated charcoal on callus induction

The ANOVA of the different treatments of activated charcoal showed that there was no significant difference among the treatments (Table 28). Most of the treatments had given nil response for callus induction which include the treatments AC3, AC4, AC5.The treatments AC1 and AC2 gave a response of 0.56% (Table 39).

4.1.2.10 Effect of Yeast extract on callus induction

There was no significant difference in the effects of various levels of yeast extract on callus induction (Table 30). The treatment Y5 recorded the highest callus induction of 1.3% while Y1 and Y3 recorded callus induction of 0.67% each. The treatments Y2 and Y4 recorded no callus response (Table 31).

Table 32. ANOVA for effect of different hybrids and maltose levels on callus induction

Source of Variation	df	SS	MSS	F -value
Treatment	5	19.328	3.866	3.135*
Hybrid	1	16.531	16.531	13.407*
Maltose	2	1.404	0.702	0.569NS
Hybrid x Maltose	2	1.393	0.697	0.565NS
Error	12	14.796	1.233	
Total	17	34.124		

*-Significant at 5 per cent level, NS-Non significant

Table 33. Mean callus induction at various levels of maltose in hybrids

Treatment No.	Hybrid	Maltose	Total		
			AI	CI	Per cent CI
T1	**H1**	**3%**	186	7	3.76[a]
T2	**H1**	**4%**	192	5	2.60[ab]
T3	**H1**	**5%**	195	5	2.56 [ab]
T4	**H2**	**3%**	191	2	1.05 [b]
T5	**H2**	**4%**	191	2	1.05 [b]
T6	**H2**	**5%**	191	2	1.05 [b]

Table 34. ANOVA for the effects of cold pre-treatments on callus induction (Hybrids)

Source of Variation	df	SS	MSS	F -value
Treatments	17	13.985	0.823	3.572**
Cold pre-treatment	5	5.238	1.048	4.549**
Hybrid	2	2.957	1.479	6.421**
Cold pre-treatment x hybrid	10	5.789	0.579	2.514*
Error	18	4.145	0.23	
Total	35			

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

Table 35. Cold pre-treatment on callus induction in hybrids

Treatment No.	Hybrid	Cold pre-treatment days	Total		
			AI	CI	Per cent CI
T7	**H1**	**0**	185	0	0.00[c]
T8	**H1**	**3**	184	1	0.54 [bc]
T9	**H1**	**6**	184	2	1.09 [abc]
T10	**H1**	**9**	184	3	1.63 [ab]
T11	**H1**	**12**	191	2	1.05 [abc]
T12	**H1**	**15**	186	1	0.54 [bc]
T13	**H3**	**0**	185	1	0.54 [bc]
T14	**H3**	**3**	189	0	0.00 [c]
T15	**H3**	**6**	191	3	1.57 [ab]
T16	**H3**	**9**	193	2	1.04 [abc]
T17	**H3**	**12**	192	2	1.04 [bc]
T18	**H3**	**15**	186	4	2.15[a]
T19	**H4**	**0**	192	0	0.00 [c]
T20	**H4**	**3**	185	1	0.54 [bc]
T21	**H4**	**6**	188	1	0.53 [bc]
T22	**H4**	**9**	182	2	1.10 [abc]
T23	**H4**	**12**	184	0	0.00 [c]
T24	**H4**	**15**	186	0	0.00 [c]

Table 36. Main and interaction effects of cold treatment on hybrids callus induction

Cold pre-treatment (days) / (10°C)	H1	H3	H4	Mean
0	0.00	0.54	0.00	0.18
3	0.54	0.00	0.54	0.36
6	1.09	1.57	0.53	1.06
9	1.63	1.04	1.10	1.26
12	1.05	1.04	0.00	0.70
15	0.54	2.15	0.00	0.90
Mean	0.81	1.06	0.36	0.74

4.2.2 Inoculation of F_1 anthers

After the preliminary studies, the F_1 anthers were inoculated on the media. The results were as follows:

4.2.2.1 Effect of maltose on callus induction

The anthers of hybrids H1 and H2 were inoculated on media containing varying levels of maltose and the results indicated significant differences in the effect of hybrids on callus induction and no significant difference between levels of maltose on callus induction (Table 32). Also, there was no interaction effect of hybrid and maltose level on callus induction. The hybrid H1 recorded a callus induction of 3.76% at T1, 2.6% at T2 and 2.56% at T3 treatment levels (Table 33). The callus induction of H1 hybrid at all the levels were on par with each other while the treatment T1 has recorded more callus induction than the T2 and T3 levels. The hybrid H2 recorded a callus induction of 1.05% irrespective of the levels of maltose.

4.2.2.2 Effects of pre-cold treatment on callus induction of hybrids

The three hybrids subjected to cold pre-treatment at various levels registered a significant difference in their response to cold pre-treatment (Table 34). A considerable difference between the number of days of cold pre-treatment, interaction between the hybrids and days of pre-cold treatment was observed. The treatment T18 (2.15%) recorded the highest percentage of callus induction while many treatments like T7, T14, T19, T23, T24 did not respond (Table 35). Among the hybrids, response ranged from 1.06% (H3) to 0.36% (H4). With regard to the response for duration of cold pre-treatment, 9 days of cold pre-treatment has given the highest mean response of 1.26% while 0 days (control) gave 0.18% response which is the least among the cold treatments (Table 36). The callus induction range for the hybrids were H1 - 1.63 (9 days) to 0% (0 days), H3 - 0% (3 days) to 2.15% (15 days) and hybrid H4 - 0 % (0,12,15 days) to 1.1% (9 days).

4.2.2.3 Effects of media and growth hormone combinations on different hybrids

The analysis of variance (Table 37) of the effects of different media and growth hormone combinations reveals significant differences between treatments and interaction between the factors on callus induction. Among the treatments, the highest callus induction was recorded by treatment T33 (9.75%) followed by T25 (7.35%) (Table 38). Out of the 32 combinations, callus induction ranged from 0.75% (T47) to 9.75% (T33) excluding the treatments which have yielded nil response as shown in

Table 38. The four hybrids have shown significant difference in their callusing ability. H3 recorded the highest mean callus induction of 2.16% followed by H1 (1.55%). The least callus induction was recorded by H2 (0.43%). Among the media, N_6 media recorded a high callus induction of 1.58% than B_5 media (0.74%). Out of the four growth hormone combinations, the combination with 2,4-D (2mg/L) and Kn (0.5mg/L) recorded highest mean callus induction of 2.79% and the least observed in the combination of 2,4-D (2mg/L) + Kn (0.5mg/L) + NAA (0.5mg/L) at 0.19%.

H3 obtained the highest callus induction of 3.05% in N_6 and H2 obtained the least callus induction of 0.21% in B_5 media revealing a considerable interaction between the media and hybrid (Table 39). While all the genotypes obtained high callus induction when the media was N_6, H4 obtained less callus induction when the media was N_6 compared to B_5. There was significant interaction between the hybrids and growth hormones too. All the four hybrids obtained the highest callus induction when the combination was 2,4-D (2mg/L) + Kn (0.5mg/L) and the least callus induction was obtained by all the hybrids at the hormone combination 2,4-D (2mg/L) + Kn (0.5mg/L)+NAA (0.5mg/L) except H4 which obtained nil callus induction (least) when the combination was 2,4-D (2mg/L) + BAP (0.5mg/L).When the media and growth hormone combination was observed, the highest callus induction of 4.9% was obtained when the N_6 is combined with 2,4-D (2mg/L) + Kn (0.5mg/L).The least callus induction of 0.19% was obtained when B_5 was combined with 2,4-D (2mg/L) + Kn (0.5mg/L)+NAA (0.5mg/L)

4.2.3 Callus regeneration

The calli obtained from the preliminary studies on effects of silver nitrate, casein hydrolysate and proline were inoculated in the R1 media as suggested by Chandrahasan (2004). The number of embryogenic calli and non-embryogenic calli from the treatments is given in the Table 40. The total number of calli obtained was 73, out of which 22 were embryogenic and 51 non-embryogenic. When these calli were plated, there was no plantlet regeneration (Table 41). The number of embryogenic and non-embryogenic calli obtained from experiment No.4.2.2.1 were given in the Table 42. Out of 23 calli obtained in those treatments, 9 were embryogenic and 14 non-embryogenic. From 9 embryogenic calli, 2 plantlets had regenerated which were albinos. No plants were regenerated from non-embryogenic calli (Table 43).

Table 37. ANOVA for the effects of media and growth hormones on hybrids

Source	**df**	**SS**	**MSS**	**F value**
Treament	31	278.8	8.994	12.442**
Hybrid	3	33.536	11.179	15.466**
Media	1	11.223	11.223	15.526**
GH	3	62.383	20.794	28.768**
HybridxMedia	3	10.069	3.356	4.643**
HybridxGH	9	50.136	5.571	7.707**
MediaxGH	3	62.57	20.857	28.855**
HybridxMediaxGH	9	48.884	5.432	7.514**
Error	32	23.13	0.723	
Total	63	301.93	4.79254	

Table 38. Mean Performance of different treatments comprising different hybrids, media and growth hormone combinations on callus induction

Hybrids	**H1**		**H3**		**H4**		**H2**	
Media and growth hormone combinations	**Trt. No**	**CI %**	**Trt. No**	**CI %**	**Trt. No**	**CI %**	**Trt. No**	**CI %**
N6+2,4-D (2) + Kn (0.5)	T25	7.35^{b}	T33	9.75^{a}	T41	0.8^{cd}	T49	1.7^{cd}
N6+2,4-D (2) + BAP (0.5)	T26	1.6^{cd}	T34	2.45^{c}	T42	0^{d}	T50	0^{d}
N6+2,4-D (2) + Kn (0.5) + NAA (0.5)	T27	0^{d}	T35	0^{d}	T43	0.8^{cd}	T51	0^{d}
N6+2,4-D (2) + Kn (0.5) + NAA (1)	T28	0^{d}	T36	0^{d}	T44	0^{d}	T52	0.85^{cd}
B5+2,4-D (2) + Kn (0.5)	T29	0^{d}	T37	1.8^{cd}	T45	0.95^{cd}	T53	0^{d}
B5+2,4-D (2) + BAP (0.5)	T30	2.55^{c}	T38	0.8cd	T46	0^{d}	T54	0.85^{cd}
B5+2,4-D (2) + Kn (0.5) + NAA (0.5)	T31	0^{d}	T39	0^{d}	T47	0.75^{cd}	T55	0^{d}
B5+2,4-D (2) + Kn (0.5) + NAA (1)	T32	0.9^{cd}	T40	2.45^{c}	T48	0.85^{cd}	T56	0^{d}

Table 39. Main and interaction effects of different hybrids in different media and growth hormone combinations on callus induction

Growth hormones(mg/L)				H1			H3			H4			H2			GH Mean	N6 mean	B5 mean
2,4-D	Kn	NAA	BAP	N6	B5	Mean	N6	B5	Mean	N6	B5	Mean	N6	B5	Mean			
2	**0.5**	**0**	**0**	7.35	0.00	3.68	9.75	1.80	5.78	0.80	0.95	0.88	1.70	0.00	0.85	2.79	4.90	0.69
2	**0**	**0**	**0.5**	1.60	2.55	2.08	2.45	0.80	1.63	0.00	0.00	0.00	0.00	0.85	0.43	1.03	1.01	1.05
2	**0.5**	**0.5**	**0**	0.00	0.00	0.00	0.00	0.00	0.00	0.80	0.75	0.78	0.00	0.00	0.00	0.19	0.20	0.19
2	**0.5**	**1**	**0**	0.00	0.90	0.45	0.00	2.45	1.23	0.00	0.85	0.43	0.85	0.00	0.43	0.63	0.21	1.05
Mean				2.24	0.86	1.55	3.05	1.26	2.16	0.40	0.64	0.52	0.64	0.21	0.43	1.16	1.58	0.74

Table 40. Percent embryogenic and non-embryogenic calli induced

Treatment	No of calli produced	No. of embryogenic calli	Per cent embryogenic calli	No. of non-embryogenic calli	Per cent Non-embryogenic calli
P1	2	0	0.0	2	100.00
P2	9	1	11.1	8	88.89
P3	8	1	12.5	7	87.50
P4	2	0	0.0	2	100.00
P5	1	0	0.0	1	100.00
A1	2	1	50.0	1	50.00
A2	3	1	33.3	2	66.67
A4	3	2	66.7	1	33.33
A5	3	1	33.3	2	66.67
A6	5	1	20.0	4	80.00
A7	5	2	40.0	3	60.00
A8	4	1	25.0	3	75.00
A9	1	1	100.0	0	0.00
A10	5	2	40.0	3	60.00
A11	1	0	0.0	1	100.00
H1	3	1	33.3	2	66.67
H2	2	1	50.0	1	50.00
H4	6	2	33.3	4	66.67
H7	1	0	0.0	1	100.00
H8	2	1	50.0	1	50.00
H9	1	1	100.0	0	0.00
H10	2	1	50.0	1	50.00
H13	1	0	0.0	1	100.00
H14	1	1	100.0	0	0.00
Total	73	22	30.1	51	69.86

Table 41. Plant regeneration from various treatments on callus induction

Treatment	Type of calli	No. of calli plated	No. of regenerated plants	No. of green plants	No. of albino plants
R1	Embryogenic calli	22(30.13)	0	0	0
	Non-embryogenic calli	51(69.86)	0	0	0
	Total	73(100)	0	0	0

Values in the parentheses are percentages

Table 42. Percent embryogenic and non-embryogenic calli obtained from hybrids at various maltose levels

Treatment	No of calli produced	No. of embryogenic calli	Per cent embryogenic calli	No of non-embryogenic calli	Per cent Non-embryogenic calli
T1	7	4	57.1	3	42.9
T2	5	2	40.0	3	60.0
T3	5	2	40.0	3	60.0
T4	2	1	50.0	1	50.0
T5	2	0	0.0	2	100.0
T6	2	0	0.0	2	100.0
Total	23	9	39.1	14	60.9

Table 43. Plant regeneration from the callus induced from hybrids subjected to various maltose levels for callus induction

Treatment	Type of calli	No. of calli plated	No. of regenerated plants	No. of green plants	No. of albino plants
R1	Embryogenic calli	9 (39.13)	2 (22.22)	0	2 (100)
	Non-embryogenic calli	14 (60.86)	0	0	0
	Total	23 (100)	2 (8.69)	0	2 (100)

Values in the parentheses are percentages

Table 44. Percent embryogenic and non-embryogenic calli obtained from hybrids subjected to various cold pre-treatments and media combinations during callus induction

Treatment	No of calli produced	No. of embryogenic calli	Per cent embryogenic calli	No of non-embryogenic calli	Per cent Non-embryogenic calli
T8	1	1	100.0	0	0.0
T9	2	1	50.0	1	50.0
T10	3	1	33.3	2	66.7
T11	2	2	100.0	0	0.0
T12	1	1	100.0	0	0.0
T13	1	1	100.0	0	0.0
T15	3	2	66.7	1	33.3
T16	2	0	0.0	2	100.0
T17	2	0	0.0	2	100.0
T18	4	2	50.0	2	50.0
T20	1	0	0.0	1	100.0
T21	1	0	0.0	1	100.0
T22	2	1	50.0	1	50.0
T25	9	3	33.3	6	66.7
T26	2	0	0.0	2	100.0
T27	3	1	33.3	2	66.7
T30	3	2	66.7	1	33.3
T32	1	0	0.0	1	100.0
T33	12	5	41.7	7	58.3
T34	3	1	33.3	2	66.7
T37	2	1	50.0	1	50.0
T38	1	1	100.0	0	0.0
T40	3	2	66.7	1	33.3
T41	1	1	100.0	0	0.0
T43	1	0	0.0	1	100.0
T45	1	0	0.0	1	100.0
T47	1	0	0.0	1	100.0
T48	1	1	100.0	0	0.0
T49	2	0	0.0	2	100.0
T52	1	0	0.0	1	100.0
T54	1	0	0.0	1	100.0
Total	73	30	41.1	43	58.9

Table 45. Plant regeneration of calli obtained from various induction treatments on hybrids

Treatment	Type of calli	No. of calli plated	No. of regenerated plants	No. of green plants	No. of albino plants
R2	Embryogenic calli	30 (41.1)	4 (13.33)	0	4(100)
	Non-embryogenic calli	43 (58.95)	3 (6.97)	0	3(100)
	Total	73 (100)	7 (9.58)	0	7(100)

Values in the parentheses are percentages

The number of embryogenic and non-embryogenic calli obtained from the hybrids studied for cold pre-treatment and different media and growth hormone combination (Expt. no 4.2.2.2 and 4.2.2.3) were given in the Table 44. Out of 73 calli obtained from these experiments, 30 were embryogenic whereas 43 were non-embryogenic. Out of the 30 embryogenic calli, 4 plants regenerated all of which were albinos and out of 43 non-embryogenic calli, 3 plants were regenerated all of which are albinos. Out of 73 calli, 7 plants had regenerated all of which were albinos (Table 45).

Discussion

5. DISCUSSION

Iron toxicity is one of the major abiotic stress in soils of Kerala. Developing varieties with iron toxicity tolerance within a short span of time is the need of the hour. The present study "Development of doubled haploids for iron toxicity tolerance in rice (*Oryza sativa* L.)" was taken up with the objective of developing doubled haploids using anther culture technique to develop mapping populations and also varieties tolerant to iron toxicity, if possible, as stable homozygous recombinants can be obtained using this technique within a short span of time (Devaux and Pickering, 2005; Germana, 2011). The results obtained are discussed below:

5.1 Evaluation of Parents and hybrids

All the genotypes studied in this investigation differed significantly for the characters studied. Generally, dwarf and early duration varieties and hybrids are desirable. For all the other characters studied like panicles per plant, panicle length, grains per panicle, test weight, spikelet fertility, grain yield per plant, a higher value is desirable to realise increased yields.

For days to flowering, all the hybrids except H3 exhibited positive relative heterosis as well as heterobeltiosis. In case of days to flowering, negative heterosis is desired as it increases the productivity per day per unit area (Tirkey *et al.*, 2006). H3 recorded -1.43 and 2.81 for relative heterosis and heterobeltiosis respectively. Similar results were obtained by Tirkey *et al.* (2006), Palaniraja *et al.* (2010), Nayak *et al.* (2012), Latha *et al.* (2013). For plant height, Hybrid H4 recorded a significant negative relative heterosis (-7.19) as well as heterobeltiosis (-8.10). The hybrid H2 recorded a significant and negative heterobeltiosis (-6.68). The findings are in concurrence with the results obtained by Palaniraja *et al.* (2010), Latha *et al.* (2013).

All the hybrids have recorded a significant positive relative heterosis as well as heterobeltiosis for panicles per plant which is a desirable character. The values for relative heterosis ranged from 45.10 (H2) to 73.00 (H1). The heterobeltiosis ranged from 13.90 (H2) to 62.70 (H4). Similar results were obtained by Biswas and Julfiquar (2006), Nayak *et al.* (2012). In case of panicle length, the relative heterosis ranged from -21.31 (H4) to 13.61 (H1) while the heterobeltiosis ranged from -23.97 (H4) to 10.75 (H1). For panicle length, positive heterosis is desirable (Tirkey *et al.,* 2006). Similar

values were obtained by Biswas and Julfiquar (2006), Tirkey *et al* (2006), Palaniraja *et al.* (2010), Latha *et al.* (2013).

The relative heterosis for grains per panicle ranged from -14.58 (H4) to 8.72 (H2). Only the hybrid H2 recorded a positive relative heterosis. The heterobeltiosis for this character ranged from -25.57 (H4) to -2.41 (H2). Biswas and Julfiquar (2006), Palaniraja *et al.* (2010) have obtained similar results for relative heterosis. A positive heterosis for grains per panicle is desirable as it increases the yield.

The relative heterosis for the hybrids ranged from 3.23 (H4) to 13.75 (H2) for test weight. The heterobeltiosis for test weight varied from -11.11 (H4) to 9.69 (H2). All the hybrids registered a positive relative heterosis as well as heterobeltiosis for test weight which contributes to increase in yield except H4 which recorded a negative heterobeltiosis value which is in concordance with the results of Tirkey *et al.* (2006) and Latha *et al.* (2013).

All the hybrids showed a negative heterosis for spikelet fertility which is undesirable as increase in spikelet fertility increases the number of filled grains which considerably increases the yield. The hybrids recorded relative heterosis values ranging from -12.27 (H3) to -1.47 (H4). The heterobeltiosis values ranged from -14.25 (H2) to -6.19 (H4). Biswas and Julfiquar (2006) observed a relative heterosis ranging from -50.20 to 273.70 and heterobeltiosis from -55.70 to 203.00. Latha *et al.* (2013) have obtained a relative heterosis range from -98.92 to -6.27 and heterobeltiosis from -98.99 to -10.04 which are similar to the results obtained in this study.

All the hybrids registered a highly significant and positive heterosis for grain yield, which is a desirable trait. The relative heterosis for the hybrids ranged from 27.86 (H4) to 76.41 (H2). The heterobeltiosis ranged from 16.91 (H1) to 52.13 (H3). Despite a negative heterosis for some characters like panicle length, grains per panicle and spikelet fertility, the hybrids recorded significant yield because of positive heterosis for some characters like panicles per plant, test weight which might have contributed to the overall grain yield per plant. Similar results were obtained by Sateesh kumar and Saravanan (2011) and Nayak *et al.* (2012).

5.2 Anther culture

The anthers required for culture were obtained in this study from panicles when the distance between the auricles of flag leaf and penultimate leaf are between 6 and 10 cm as suggested by Reiffers and Freire (1990), Afza *et al* (2000) and Chandrahasan (2004). Most of the anthers at the middle of the panicle, when stained for identifying the stage of the anthers were in mid to late uninucleate stage which is the stage optimum for callus induction as advocated by many scientists. Since identifying the stage of every anther is difficult, the anthers which were 1/3 to 1/2 in length of the spikelet are used for inoculation as suggested by Lapitan *et al.* (2009).

The results of the preliminary studies on callus induction, inoculation of F_1 anthers are discussed as under

5.2.1 Effect of cold pre-treatment on callus induction

Matsusmisha *et al.* (1998) opined that cold pre-treatment induces the sporophytic way of development in microspores rather than towards gametophytic pathway to develop into gametes. It also induces synchronisation of cell division and maintains viability of the microspores. Cold treatment promotes the separation of the pollen from anther wall through the degeneration of tapetum and therefore, the pollen is triggered to enter in the sporophytic phase.

Cold pre-treatment in the preliminary studies ~~i~~ndicated that callus induction was high when the duration of cold pre-treatment was 9 days (7.5%) followed by 6 days (2.7%). The callus induction per cent was low when 0 (1.1%), 3 (0%), 12 days (0.5%) and 15 days (0.6%) days pre-treatment was given. Similar results were obtained by Lentini *et al.* (1995), Lapitan *et al.* (2009), Naik *et al.* (2016), Solanki (2016).

The results varied with the hybrids. The hybrids H1 and H4 have responded favourably for 9 days cold pre-treatment while hybrid H3 responded better to 15 days of cold pre-treatment (2.15%). H1 has shown gradual increase in callus induction up to 9^{th} day after which the response decreased. The overall callus induction response in case of H4 was less than H1 and H3.

Out of the three genotypes, H3 recorded a callus induction response for all cold pre-treatments from 1- 15^{th} day which showed that both genotype and cold pre-

treatment have an effect on callus induction. In the case of H1, which is a moderately responsive genotype among the three, there was callus response even after 9 days of treatment. H4 was a less responsive genotype compared to others where there was no response after 9 days. All these results indicate that the number of cold pre-treatment days varies according to the genotypes (Swapna, 2000; Trejo-Tapia *et al.*,2002; Datta, 2005; Herath *et al.*,2009).

Rout *et al.* (2016) have observed that out of the temperatures 4ºC, 8ºC, 10ºC and 12ºC, 10ºC is better for cold pre-treatment. They observed that cold pre-treatment at 10ºC for 2 days is the best followed by 8 days which is in contrast to the result obtained in this study as we have found that less number of days of callus induction like one and four days results in poor callus induction. Herath *et al.* (2009) favoured cold treatment at 8ºC for 14 days which is reflected in the hybrid H3 of the present study. These results indicate that the optimum temperature and duration of the cold pre-treatment depends on the species or variety (Datta,2005).

Genovesi and Magill (1979) opined that the positive effect of cold pre-treatment on callus induction is because at low temperatures most of the microspores slowly develop in to normal pollen whereas some microspores develop into embryos. Herath *et al.* (2009) cautioned against going for treatments with extended duration (more than 20 days) as it leads to an increase in the number of albinos

5.2.2 Explant sterilisation

Various explant sterilisation methods were attempted such as use of 70% Ethanol, 0.1% $HgCl_2$ and sodium hypochlorite. The least contamination of 4.17% was observed when the explant was exposed to 5.25% sodium hypochlorite for 5 mins (S6) and the highest contamination of 19.17% when exposed to 70% Ethanol for 30s (S1). 0.1% $HgCl_2$ (Mercuric Chloride) alone or in combination with Ethanol (S4 and S5) has reduced the contamination compared to 70% Ethanol alone. 70% Ethanol with 0.5% sodium hypochlorite (S3) reduced the contamination to 11.67% but 5.25 % sodium hypochlorite (S6 and S7) alone for various durations had reduced the contamination considerably to 4.17% (S6) and 5.83% (S7). Both the treatments S6 and S7 were on par with each other. Results indicate that sodium hypochlorite is a better sterilant for rice anther culture. Results obtained in the present study are in concurrence with the reports of Serrat *et al.* (2014) in that the contamination is less with 5.25% sodium hypochlorite

treatment along with normal callus induction compared to 70 % Ethanol. Mishra *et al.* (2015) Trejo-Tapia *et al.* (2002) and Lapitan *et al.* (2009) too have reported success with the same level of treatments.

5.2.3 Effect of genotypes, media, growth hormones and carbon sources on callus induction

Different studies on the best combinations of media, growth hormones and carbon sources for different genotypes has been studied.

5.2.3.1 Media and Growth hormones

Media plays a vital role in the callus induction as well as green plantlet regeneration in anther culture. In rice N_6 (Chu *et al.*, 1975) and B_5 (Gamborg *et al.*, 1968) are generally used in rice anther culture. These two media contain less NH_4^+ nitrogen compared to NO_3^- which is required to induce callus in rice (Grimes and Hodges, 1990; Silva, 2010).

In this study, the two hormones 2,4-D and Kinetin were studied at two levels and it has been observed that there was a significant interaction between the levels of these two hormones. The combination 2,4-D at 2mg/L and Kn at 0.5mg/L responded better to callus induction when compared to 2,4-D at 1mg/L and Kn at 1mg/L. This proves that a high ratio of 2,4-D and Kn is crucial for callus induction. The result obtained in this study is in agreement with the results obtained by Chandrahasan (2004) and Mishra *et al.* (2015). Scientists like Shimada *et al.* (1999), Islam *et al.* (2004) and Serrat *et al.* (2014) have obtained best callus induction when 2,4-D (2mg/L) and Kn (1mg/L) is used which is in contrast to the results obtained in this study.

Among the two media used, the media N_6 (9.1%) induced more callus induction response than B_5 (3.2%) media at various levels of growth hormone combination.

There is considerable interaction between growth hormone combinations and media. The highest mean callus induction for the media N_6 was obtained when the combination 2,4-D (2mg/L) + Kn (0.5mg/L) was used whereas it was 2,4-D (1mg/L) + Kn (0.5mg/L) for B_5. The least mean callus induction for the two media N_6 and B_5 were obtained at the combinations 2,4-D (1mg/L) + Kn (1mg/L) and 2,4-D (2mg/L) + Kn (1mg/L) respectively. Differences in media response to different growth hormone

combinations is reported by Rout *et al.* (2016) between N_6, MS and SK-1 media with different growth hormone combinations.

5.2.3.2 Carbon sources and Growth hormone

The analysis of variance to study the effect of the two carbon sources namely maltose and sucrose at various levels (30,40,50g/L) and different combinations of 2,4-D (1mg/L and 2mg/L) and Kn (0.5mg/L and 1mg/L) revealed significant differences between the levels of carbon sources, different growth hormone combination on callus induction and also a significant interaction. The results reveal that maltose is better for callus induction than sucrose. Maltose gave highest callus induction of 8.60% at 30g/L whereas the least induction for maltose was 1.32% at 50g/L. In case of sucrose, highest callus induction of 1.34% was obtained at both 40g/L and 50g/L whereas 30g/L of sucrose yielded only a low callus induction of 0.65%.

With regard to growth hormone combination, the combination 2,4-D at 1mg/L and Kinetin at 0.5mg/L yielded lower callus induction of 1.31% while the highest callus induction of 11.93% was obtained when the combination is 2,4-D (2mg/L) and Kn (0.5mg/L). For maltose, the highest callus induction was obtained for the combination 2,4-D (2mg/L) and Kn (0.5mg/L) and the least was obtained when the combination of growth hormones is 2,4-D (1mg/L) and Kn (1mg/L). For sucrose, the highest callus induction of 0.67% was obtained at 2,4-D (1mg/L) and Kn (1mg/L). This shows that there is a significant interaction between carbon source and growth hormone combinations. The observation that maltose is a better source than sucrose for callus induction has been reported by several workers like Last and Bretell (1990), Xie *et al*. (1995), Bishnoi *et al.* (2000), Naik *et al.* (2016). Maltose is considered to be a better carbon source than sucrose because of its ability to stabilize the osmolality of the medium and its ability to degrade slowly than sucrose (Kuhlmann and Foroughi-Wehr, 1989),

5.2.3.3 Growth hormones

The results of callus induction response at various growth hormone combination shows that 2,4-D is a better auxin than NAA for callus induction and Kn is better than BAP for callus induction. The highest callus induction was obtained for treatment G2 (2,4-D (2mg/L) + Kn (0.5mg/L)) at 5.9% followed by G6 (NAA (2mg/L) and Kn (0.5mg/L)) at 1.6%. This shows that irrespective of the type of auxin, higher

auxin/cytokinin ratio is important (Raina, 1989; Trejo-Tapia *et al.,* 2002) and also 2,4-D is better than NAA for callus induction response. Chen *et al.* (2001) observed similar results and opined that 2,4-D is a good callus inducing auxin at an optimum concentration of 2-2.5mg/L above or below of which it will cause less calli. Niroula and Bimb (2009) also observed that 2,4-D is a better callus inducer than NAA but from plant regeneration perspective they opined that more calli can be regenerated into green plants when NAA is used as an auxin than 2,4-D. According to them, balance should be made by using the concentration of 2,4-D at a concentration such that more calli are produced and most of them are converted into green plantlets. Lee *et al.* (2003), Swapna (2000) and Min *et al.* (2015) opined NAA to be comparatively superior to 2,4-D for induction of callus which is in contrast to this study. Iyer and Raina (1972) opined that both 2,4-D and NAA are equally good for inducing callus response. In the present study, Kinetin responded better than BAP. But BAP gave better callus induction than Kn for Naik *et al.* (2016) and Rout *et al.* (2016). However, the combination of 2,4-D and Kn at optimum concentrations was reported to be best for callus induction by majority of the studies (Chandrahasan, 2004; Grewal *et al.*, 2006).

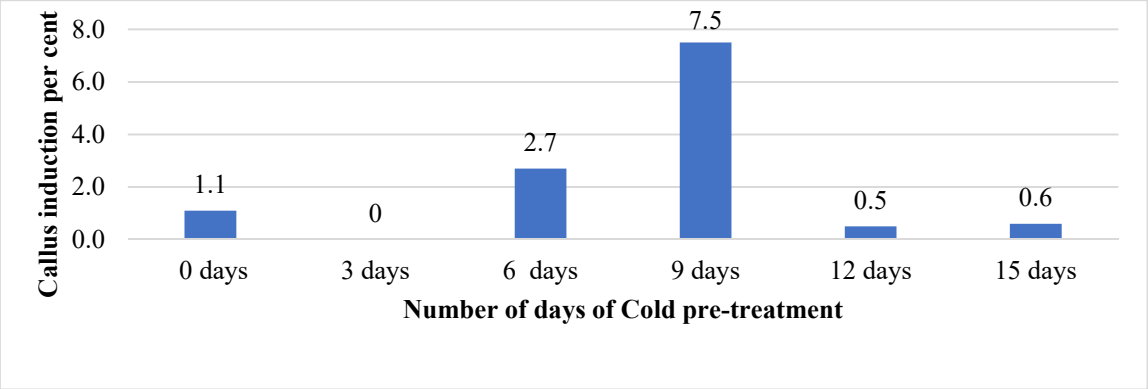

Figure 1. Effect of number of cold pre-treatment days on callus induction

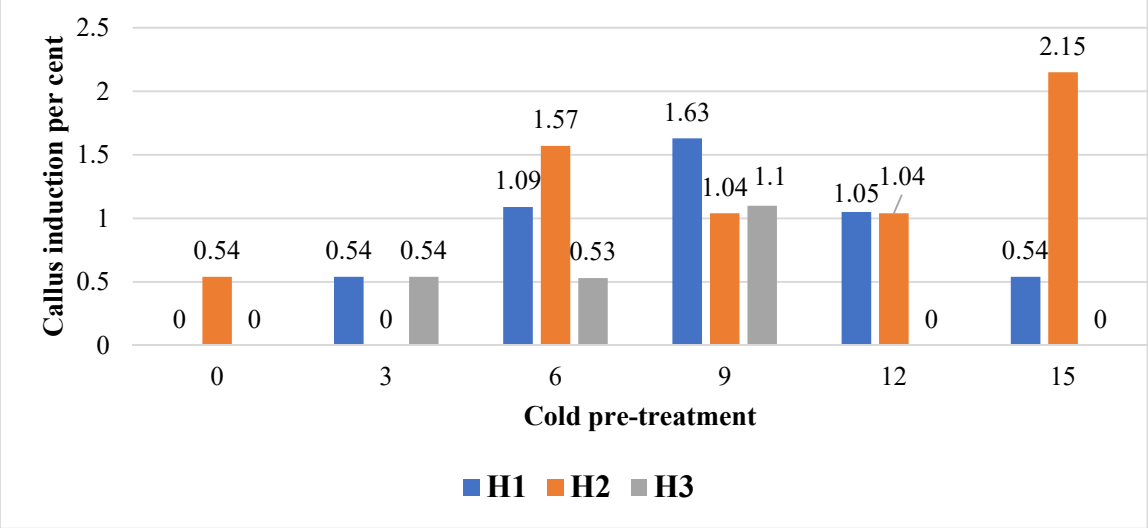

Figure 2. Effect of cold pre-treatment on callus induction

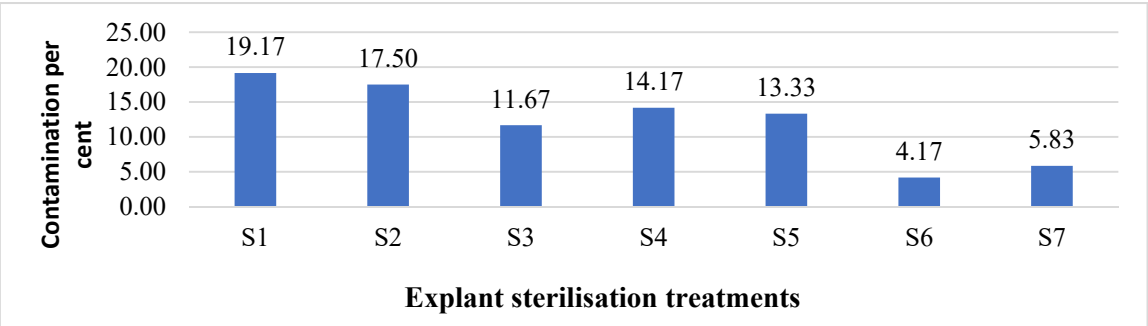

Figure 3. Effect of Different sterilisation treatments on contamination

S1	**70 % Ethanol for 30s**	**S5**	**0.1 % HgCl2 for 5 mins**
S2	**70 % Ethanol for 5 mins**	**S6**	**5.25 % Sodium hypochlorite for 5 minutes**
S3	**70 % Ethanol for 20s+0.5% Sodium Hypochlorite for 10 mins**	**S7**	**5.25 % Sodium hypochlorite for 20 minutes**
S4	**70 % Ethanol for 2 to 3 mins + 0.1 % HgCl2 8 to 10 mins**		

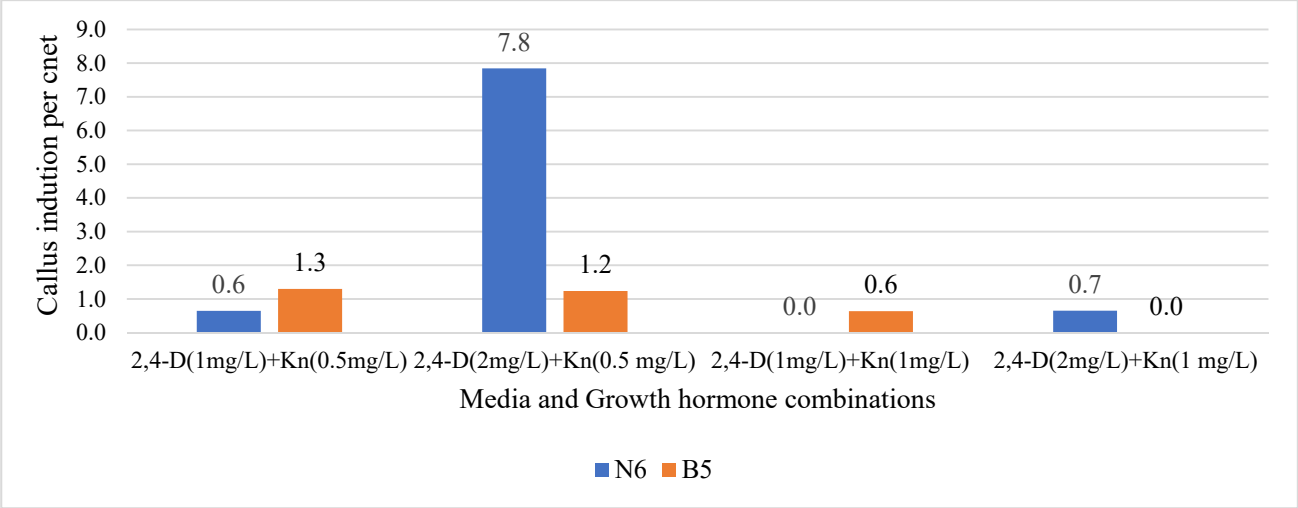

Figure 4. Effect of media and growth hormones on callus induction

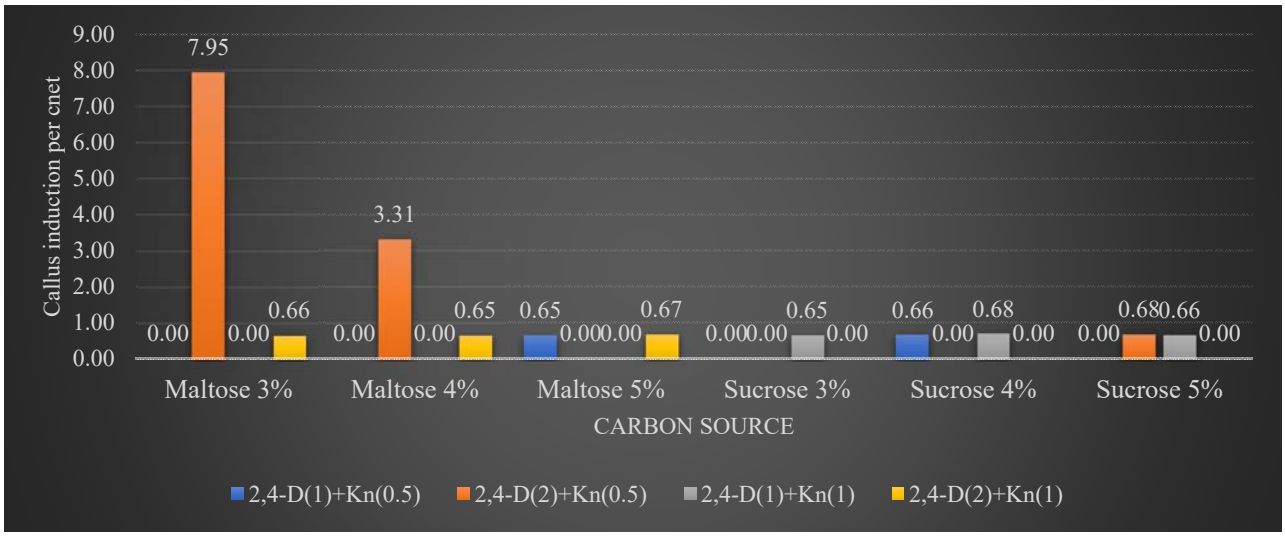

Figure 5. Effect of carbon source and growth hormone on callus induction

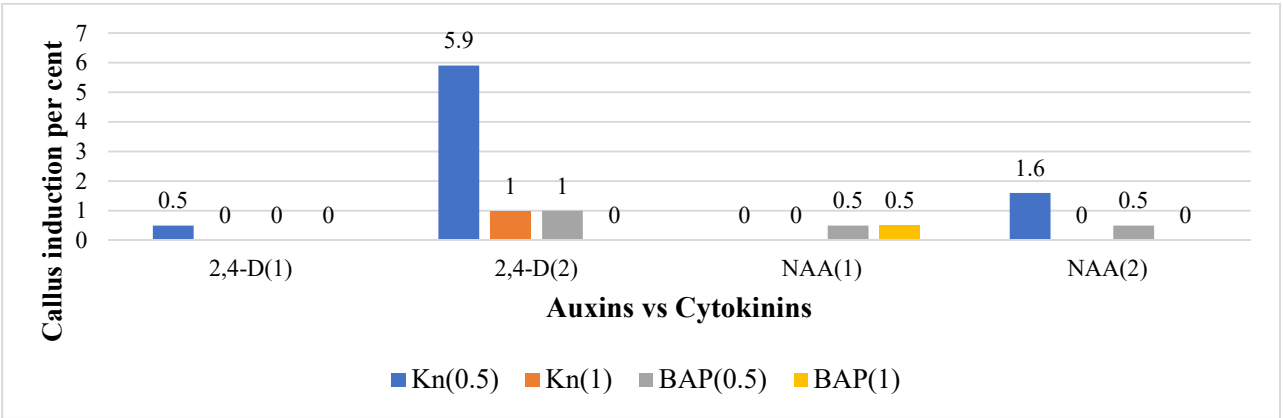

Figure 6. Effect of growth hormone combinations on callus induction

5.2.3.4 Silver nitrate ($AgNO_3$)

All the treatments with 0.1N of $AgNO_3$, except 0.2ml of 0.1N $AgNO_3$ which amounts to 3.4mg/L of $AgNO_3$ have yielded a response although the response to callus induction is lower when the volume of silver nitrate used is 0.8ml (13.mg/L) and 1ml (16.9mg/L) at 0.66 and 0.67% respectively compared to the control which yielded a response of 1.36%. There is good callus induction ranging from 1.95 to 3.36% for the treatments from A4 to A8 when the concentration of silver nitrate used is 0.3 to 0.7ml (5.1 to 11.9mg/L). This result is consistent with the result obtained by Lentini *et al.* (1995); Niroula and Bimb (2009); Kaushal *et al.* (2014). Lentini *et al.* (1995) opined that the positive effect of $AgNO_3$ may be due to its ability to inhibit the synthesis of ethylene in cultures which has an inhibitory effect on anthers and calli.

5.2.3.5 Casein hydrolysate and maltose

The effect of casein hydrolysate at different levels of maltose indicated that there is no significant main effect of casein hydrolysate (CH) but its interaction with maltose level is important. Callus induction was observed at all levels of CH when the maltose level was maintained at 30g/L. The highest callus induction was obtained at 250 mg /L of CH when maltose was used at 30g/L. The callus induction was obtained at 500mg/L of CH at all the levels of maltose. Raina and Zapata (1997) too obtained similar results when casein hydrolysate at 500mg/L was used in their studies on modification of media for anther culture and found it to be an optimum concentration for callus induction. Nguyen *et al.* (2016) and Bishnoi *et al. (*2000) have obtained callus induction response when they utilised CH at 300mg/L which is also consistent with the result obtained in our study. Roy and Mandal (2005) found no correlation between callus induction and concentration of casein hydrolysate. They also opined that the beneficial effect of casein hydrolysate may be due to the presence of undefined nitrogenous compounds which favours embryogenic callus induction. Lee *et al.* (2003) included casein hydrolysate (1g/L) supplemented media and obtained good callus induction. Javed *et al.* (2007) used 2g/L of casein hydrolysate in their media and obtained good response. This shows that addition of casein hydrolysate is beneficial for increasing the callus induction in anther culture.

5.2.3.6 Proline

The table 25 shows that there is no significant difference in callus induction at different concentrations of proline ranging from 0 to 1000mg/L. But the mean value of callus induction is high at 5.77% and 5.0% at 250mg/L and 500mg/L respectively. Even though there is callus induction even without proline application, the callus induction increased considerably when the concentration is 250mg/L and 500mg/L and then the callus response decreased from 750mg/L to 1000mg/L. This shows that proline application from 250 to 500mg/L is beneficial in increasing the callus induction response. Similar results were obtained by Cho and Zapata (1988); Serrat *et al.* (2014).

5.2.3.7 Activated charcoal

The callus response at 0 and 25mg/L activated charcoal was 0.56%. There was no response in callus induction when activated charcoal was applied at higher doses. Therefore, it can be concluded that there is no profound effect of activated charcoal on callus induction. Activated charcoal is said to promote callus induction by way of pH balancing as well as adsorption of inhibitors and growth preventers (Anagostakis,1974). It is said to be capable of trapping gases like ethylene released from the culture tissues, thereby increasing the formation of embryos (Johansson *et al.*,1982). Premvaranon *et al.* (2011) found a positive effect of activated charcoal on callus induction when it is added at 1mg/L in the media.

5.2.3.8. Yeast extract

The callus induction when media was supplemented with yeast extract at 0 mg/L (control) and 500 mg/L was 0.67% and it was 1.3% at 1000 mg/L. The treatments were on par with each other (Table 31). Therefore, it can be concluded that there was no significant effect of yeast extract on callus induction which is in contradiction to the reports of Roy and Mandal (2005) who recorded good callus induction at 200mg/L compared to 400mg/L and 1000mg/L.

5.2.3.9. Maltose

This study revealed that there was significant difference between genotypes for callus induction and there was no significant difference between the levels of maltose on callus induction and their interaction. The hybrid H1 was more responsive than H2 for callus induction. All the levels of maltose were on par with each other in

both hybrids. They have performed constantly at all the levels of maltose indicating that there is no significant interaction between the level of maltose and hybrids. Therefore, we can infer that a genotype which is responsive to callus induction performs consistently irrespective of the maltose levels.

5.2.3.11. Media and growth hormone combinations

The Analysis of variance of all the treatments indicates that there is a strong interaction between all the factors involved in this study i.e.; hybrid, media and GH combination and also all the factors differed significantly among themselves (Table 37). Among the hybrids involved, H3 (2.16%) is the most responsive hybrid and H2 is the least responsive to callus induction. The response of different hybrids is genotype dependent. N_6 yields more average response than B_5 medium. Among the growth hormone combinations, 2,4-D (2mg/L) and Kn (0.5mg/L) (2.79%) is the best. All the hybrids recorded higher callus induction in N_6 media compared to B_5 except H3. All the hybrids obtained highest callus induction when the growth hormone combination was 2,4-D (2mg/L) and Kn (0.5mg/L). The least callus induction was obtained for all the hybrids when 2,4-D (2mg/L) + Kn (0.5mg/L) + NAA (0.5mg/L) was the combination except H4 which obtained the nil response when 2,4-D (2mg/L) + Kn (1mg/L) is the growth hormone combination. When N_6 was the media, the highest callus response was obtained when 2,4-D (2mg/L) and Kn (0.5mg/L) was the growth hormone combination and least callus induction was obtained when 2,4-D(2mg/L) + Kn(0.5mg/L) + NAA(0.5mg/L) was the growth hormone combination. This is in contrast to Raina and Irfan (1998) who got better induction when NAA was combined with 2,4-D. When B_5 is the media, the highest callus induction was obtained at both 2,4-D (2mg/L) and Kn (1mg/L) and 2,4-D (2mg/L) + Kn (0.5mg/L) + NAA (1mg/L) combinations and the least callus induction is obtained when 2,4-D (2mg/L) + Kn (0.5mg/L) + NAA (0.5mg/L) was the growth hormone combination. Similar result was obtained by Kaushal *et al.* (2014). Islam *et al.* (2004) have obtained higher callus induction when NAA (6.0%) alone was used with Kn compared to 2,4-D (1.2-4.5%) when it is used alone. But they obtained higher callus induction when NAA and 2,4-D are combined (13.1-35.5%) compared to either of them alone. This shows that a combination of NAA and 2,4-D is better than application of either of the auxins alone.

5.2.4 Callus regeneration studies

The calli from the preliminary studies on effect of proline (3.2.3.11.6), silver nitrate (3.2.3.11.4) and casein hydrolysate (3.2.3.11.5) were inoculated on R1 treatment consisting of MS + NAA (1mg/L) + Kn (2mg/L) + IAA (0.5mg/L) + CW (5%). There was no proper callus growth and plantlet regeneration in this media. The development of callus into plantlet also depends on the callus induction media and genotype among many other factors. All the genotypes used in this experiment were from Swetha variety. There was browning in the calli. When the same media was used, Chandrahasan (2004) reported good regeneration ranging from 22.22 per cent in case of non-embryogenic calli to 29.82 per cent for embryogenic calli.

The calli from the experiment on effect of maltose levels on callus induction in hybrids H1 and H2 were also inoculated on the R1 medium (3.2.3.12.1.1). Out of the 23 calli obtained from this experiment 9 are embryogenic calli whereas 14 are non-embryogenic calli. Out of the 9 embryogenic calli 2 (22.22%) plants regenerated which are albinos and from the remaining 14 non-embryogenic calli, no plantlets regenerated.

The calli obtained from the experiment on effect of various cold pre-treatments (3.2.3.12.1.2) and media combinations (3.2.3.12.1.3) on the hybrids were inoculated on the R2 medium as suggested by Mishra *et al.* (2015). 30 calli (41.1%) obtained from these experiments were embryogenic calli whereas 43 calli (58.9%) obtained from these experiments were non-embryogenic. Out of the 30 embryogenic calli, 4 (13.33%) plants regenerated, all of which were albinos and out of 43 non-embryogenic calli, 3 (6.97%) plants regenerated which were also albinos. No green plantlets have regenerated from all the experiments. These results were consistent with the observation by Talebi *et al.* (2007) that albino production frequency is very high in rice anther culture ranging from 5 to 100 %. Silva (2010) opined that this problem is especially high in indica varieties than japonica varieties. Albino production is because of both genotypic as well as environmental factors. Yamagishi (2002) observed that albinos have large-scale deletions in plastid genome which were not present in green regenerants. Silva (2010) opined that albinos can also be reduced by giving appropriate starvation and cold pre-treatments simultaneously. Therefore,

studies on appropriate pre-treatments and culture conditions should be taken up to reduce the albino production.

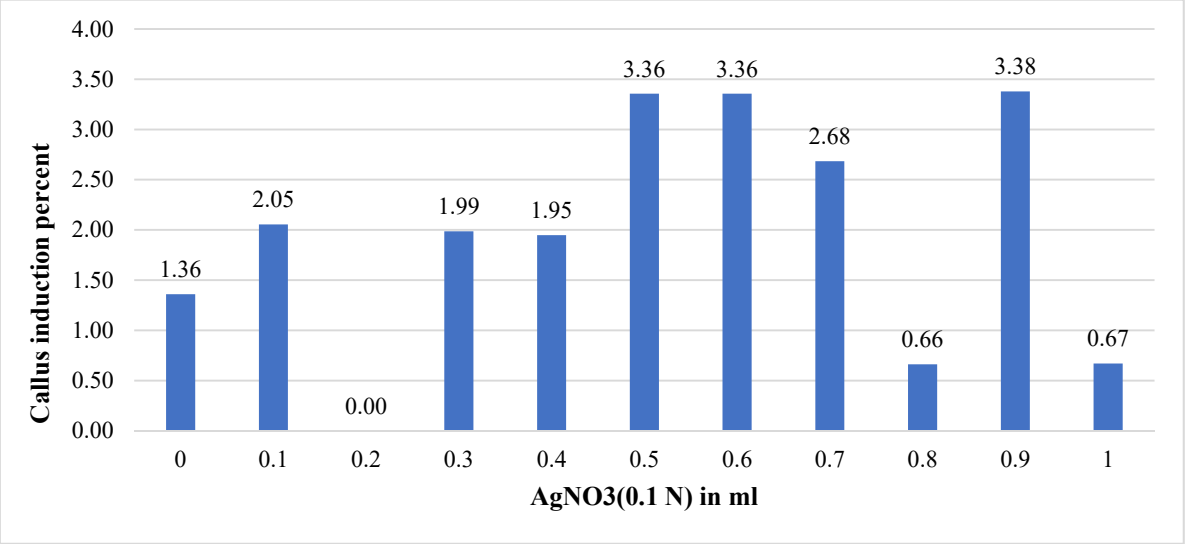

Figure 7. Effect of silver nitrate($AgNO_3$) on callus induction

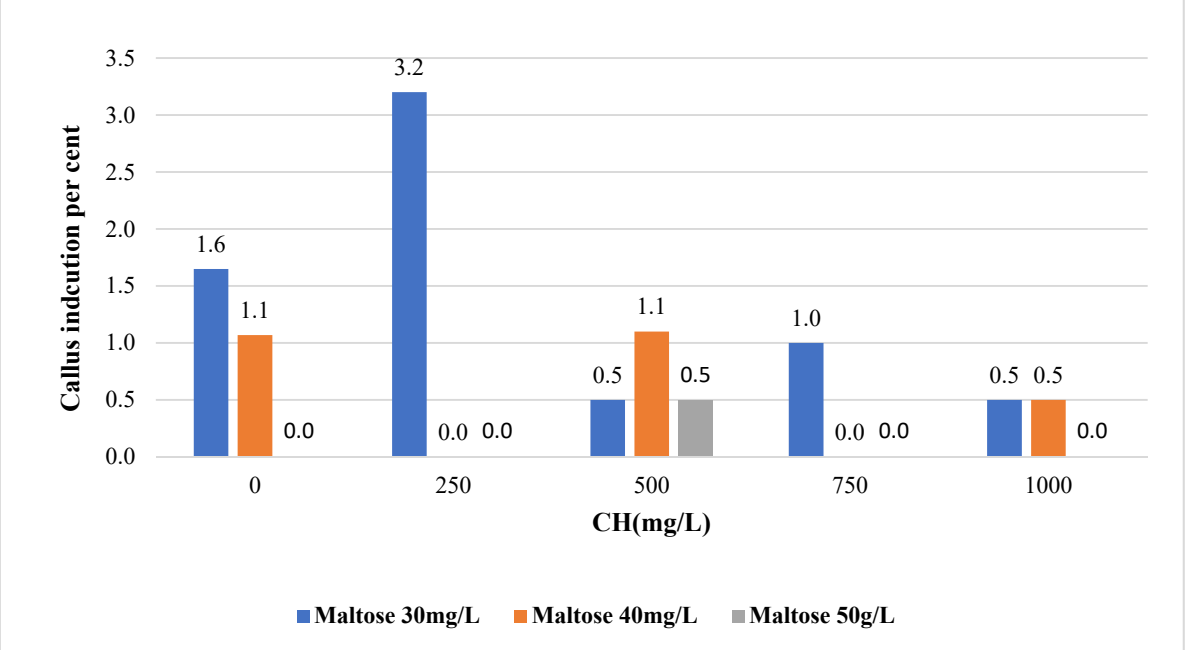

Figure 8. Effect of casein hydrolysate and maltose on callus induction

Anther culture is a very useful tool to generate stable DH population within a very short span of time but it has many hurdles to become successful to be implemented in a wide range of species and varieties. Therefore, the following recommendations are made based on this study:

1.) The genetic and molecular basis for the high callus induction and regeneration in highly anther culture responsive genotypes has to be elucidated to know the genes and molecular mechanism underlying the anther culturability.
2.) The genes responsible should be characterised and markers if any, for these genes should be identified so as to know the anther culture potential of the genotype in question, thereby saving the time and resources of workers in this field as anther culturability is a highly genotype dependent factor.
3.) Extensive studies on media with various combinations of salts, vitamins, growth hormones and organic supplements should be done so as to develop a robust protocol which can induce callus response in most of the indica varieties.

Plate 1. Hybridisation

Clipping

Emasculation

Pollen collection

Pollination

Bagging, Tagging and Labelling

Plate 2. Raising of F_1s

Swetha x Tulasi
(H1)

Mangala Mahsuri x Tulasi
(H2)

Cul-90-03 x Tulasi
(H3)

Cul 8709 x Tulasi
(H4)

Plate 3. Callus regeneration

Albinos

Albino Shootlet

Summary

6. SUMMARY

The present study **"Development of doubled haploids for iron toxicity tolerance in rice (*Oryza sativa* L.)"** was taken up in the Department of Plant breeding and Genetics, College of Horticulture, Vellanikkara. Four female parents Mangala Mahsuri (PTB-53), Swetha (PTB-57), Cul-90-03 and Cul 8709 were raised and crossed with male parent, Tulasi (Experiment I) to produce F_1 hybrids. The hybrids obtained from such crosses were raised (Experiment II) and the anthers obtained from such hybrids were used for anther culture studies (Experiment III).

The salient findings of the study are summarized below:

Evaluation of parents and hybrids

1. Significant variability existed among all the genotypes for yield and its attributes. The hybrids varied for all characters under study.
2. Hybrids in this study recorded both positive and negative relative heterosis and heterobeltiosis for the characters days to flowering, plant height and panicle length. In case of grains per panicle and spikelet fertility all the hybrids recorded negative relative heterosis and heterobeltiosis.
3. All the hybrids recorded significant and positive relative heterosis and heterobeltiosis for panicles per plant and test weight over mid and better parent respectively.
4. The hybrids recorded significant positive relative heterosis as well as heterobeltiosis for grain yield per plant

Anther culture studies

- **Callus induction**

1. Cold pre-treatment at 10°C for a period of 9 days resulted in better callus induction
2. Sterilization of explants with 5.25% Sodium hypochlorite alone or in combination with 70 % Ethanol or 0.1% Mercuric chloride resulted in less contamination and adequate callus response. Good control of contamination was obtained when 5.25% Sodium hypochlorite was used for 5 to 20 min.
3. Out of the two media used in the study, namely N_6 and B_5, N_6 responded better for callus induction.

4. Among the two carbon source included in the study maltose (30g/L) and sucrose (40g/L and 50g/L) could generate a higher response for callus induction. Overall, maltose could induce more callus induction than sucrose.
5. Auxin 2,4-D and cytokinin Kinetin were more responsive in inducing callusing. The auxin/cytokinin ratio plays a crucial role in callus induction.
6. Addition of silver nitrate reduces the days to induction of callus considerably and 0.5 to 0.6 ml of 0.1N silver nitrate ($AgNO_3$) per litre of media is the best concentration for callus induction
7. Casein hydrolysate at a concentration of 250-500mg/L was optimum for callus induction.
8. Media supplemented with proline at concentration of 250mg/L can be used to increase per cent callus induction.
9. Activated charcoal added to the media at concentration from 0 to 100mg/L did not have any favourable effect on callus induction
10. Addition of yeast extract (at concentrations of 0,250,500,750,1000mg/L) to the callus induction media did not result in considerable callus induction.
11. Irrespective of the maltose level, H1 hybrid is more responsive to callus induction than H2 showing that genotype is more important than maltose levels for callus induction.
12. Among the hybrids used for callus induction study, H4 responded better for callus induction.
13. The optimum media and growth hormone combinations for callus induction depends on the genotype.

- **Callus regeneration**

14. In the calli generated in this study, the number of embryogenic calli was less than non-embryogenic calli, which may the reason for poor regeneration response.
15. Callus regeneration studies with MS+ NAA (0.25mg/L) +BAP (0.75mg/L) + Kn (0.25mg/L) led to the development of albinos.

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*Original not seen

DEVELOPMENT OF DOUBLED HAPLOIDS FOR IRON TOXICITY TOLERANCE IN RICE (*Oryza sativa* L.)

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ABSTRACT OF THE THESIS

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Abstract

Rice is the staple food crop of the people of Kerala. The production of rice in Kerala is impeded by many biotic and abiotic stresses. Iron toxicity is one of the major abiotic stresses of acidic soils in Kerala. The present study **"Development of doubled haploids for iron toxicity tolerance in rice (*Oryza sativa* L.)"** was taken up with the objective of developing doubled haploids with tolerance to iron toxicity through *in vitro* anther culture. Doubled haploids being homozygous stable lines, can be used both as immortal populations for QTL mapping as well as varieties, if suitable agronomic traits are present in them. Therefore, this study was taken up which comprised of three experiments.

In experiment I, two tolerant genotypes (Swetha and Mangala Mahsuri) and two susceptible genotypes (Cul-90-03 and Cul 8709) were crossed with Tulasi. In the second experiment the parents along with their hybrids were evaluated for biometric characters and yield. The hybrids obtained from the crosses were significantly different for all the characters observed. Panicles per plant and grain yield per plant recorded highly significant and positive relative heterosis and heterobeltiosis.

The anthers from the F_1s produced in the previous experiment were used for anther culture studies in the third experiment. Sterilisation with various sterilants like 70 % ethanol, 0.1 % mercuric chloride, 5.25% sodium hypochlorite and their combinations were done. Explant sterilisation with 5.25% sodium hypochlorite for 5 to 20 minutes was effective in controlling contamination in the *in vitro* cultures.

Study to know the best number of days of cold pre-treatment at 10^{o}C was done (0, 3, 6, 9, 12, 15 days). Cold pre-treatment of anthers at 10^{o}C for 9 days was found to be optimum for most of the genotypes studied. Two different media *viz.,* N_6 and B_5 were tried with different combinations of 2,4 - D and Kn. N_6 media responded better than B_5 media for callus induction in all the growth hormone combinations.

The effect of carbon source on callusing was studied using maltose and sucrose at various levels (30,40,50 mg/L) and it was found that maltose at 30g/L gave the best callus induction (7.95%). Among the auxins used in the present study,2,4-D was found to be better than NAA for callus induction while among cytokinins, Kinetin

responded better than BAP for callus induction. The growth hormone combination 2,4-D (2mg/L) + Kn (0.5mg/L) was adjudged the best for callus induction.

Additives like silver nitrate ($AgNO_3$), casein hydrolysate (CH), yeast extract (YE), proline and activated charcoal were added to the basal media to improve callusing. when $AgNO_3$ is applied from 0 to 1ml with 0.1 ml gradation, 0.5-0.6ml of 0.1N $AgNO_3$ was found to be better in callus induction as well as the days to callus induction was reduced at these concentrations. 250 – 500 mg/L of CH and 250mg/L proline were found to induce significant response for callusing while activated charcoal and yeast extract did not have any considerable effect on callus induction.

Hybrid H1 recorded good callus response when maltose at three levels was tried in the media (30, 40, 50g/L) whereas hybrid H2 gave uniform response at all the levels of maltose.

There was significant variation among the genotypes in their response to all the factors studied. The best responses were as follows: -

H1(Swetha x Tulasi) - N_6+2,4-D (2mg/L) + Kn (0.5mg/L) + 30g/L maltose +0.5ml (0.1 N $AgNO_3$) + 250mg/L proline + 250mg/L CH +2.5g/L gelrite gellan gum.

H2(Mangala Mahsuri x Tulasi) - N_6+2,4-D (2mg/L) +Kn (0.5mg/L) + 30g/L maltose +0.5ml (0.1N $AgNO_3$) +250mg/L proline+250mg/L CH +2.5g/L gelrite gellan gum.

H3(Cul-90-03 x Tulasi) - N6+2,4-D (2mg/L) +Kn (0.5mg/L) + 30g/L maltose + 0.5ml ($AgNO_3$) + 250mg/L proline + 250mg/L CH + 2.5g/L gelrite gellan gum.

H4(Cul 8709 x Tulasi)- B5 + 2,4-D (2mg/L) + Kn (0.5mg/L) + 30g/L maltose + 0.5ml (0.1N $AgNO_3$) + 250mg/L proline + 250mg/L CH + 2.5g/L gelrite gellan gum.

The calli obtained from the different genotypes were plated on two different callus regeneration media R1 (MS+ NAA(1mg/L) + Kn (2mg/L) + IAA (0.5mg/L) + CW (5%)) and R2 (MS+NAA (0.25mg/L) + BAP (0.75mg/L) + Kn(0.25mg/L)). There was no response in R1 media. Callus regenerated into plantlets in R2 but all the plantlets obtained were albinos leading to mortality and hence plantlet hardening and field planting could not be undertaken.