“RICE DOUBLED HAPLOID PRODUCTION AND HOMOZYGOSITY TESTING USING PHENOTYPIC AND MOLECULAR APPROACHES”

M. Sc. (Ag.) THESIS

by

Prem Narayan Patel

DEPARTMENT OF PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
INDIRA GANDHI KRISHIVISHWA VIDYALAYA,
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THESIS

Submitted to the

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by

Prem Narayan Patel

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CERTIFICATE-I

This is to certify that the thesis entitled **"Rice doubled haploid production and homozygosity testing using phenotypic and molecular approaches"** submitted in partial fulfillment of the requirement for the degree of **"Master of Science in Agriculture (Plant Molecular Biology and Biotechnology)"** of the Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.), is a record of the bonafide research work carried out by **Prem Narayan Patel** under our guidance and supervision. The subject of the thesis has been approved by Student's Advisory Committee and the Director of Instructions.No part of the thesis has been submitted for any other degree or diploma (certificate awarded etc.) or has been published / published part has been fully acknowledged. All the assistance and help received during the course of the investigation have been duly acknowledged by him.

Date: 21/07/17

Chairman

THESIS APPROVED BY THE STUDENT'S ADVISORY COMMITTEE

Chairman: DR. (Smt) Zenu Jha

Member: Dr. Girish Chandel

Member: Dr. N.K. Rastogi

Member: Dr. R. R. Saxena

CERTIFICATE-II

This is to certify that the thesis entitled **"Rice doubled haploid production and homozygosity testing using phenotypic and molecular approaches"** submitted by **Prem Narayan Patel** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur in partial fulfillment of the requirements for the degree of **Master of Science** in the Department of Plant Molecular Biology and Biotechnology has been approved by the external examiner and Student's Advisory Committee after oral examination.

Date: 09/09/17

Signature External Examiner 09/09/17

(Name: Dr. B.N. Tiwari **)**

Major Advisor

Head of the Department/Section

Dean/ Dean Faculty

Approved / Not approved

Director of Instructions

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Department of Plant Molecular Biology & Biotechnology,

College of Agriculture, IGKV, Raipur (C.G.)

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Prem Patel

Prem Narayan Patel

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LIST OF NOTATIONS/SYMBOLS

%	Percentage
1N	One normality
2,4-D	2, 4-dichlorophenoxyacetic acid
BAP	6-benzylamino purine
ABA	Abscisic acid
CIM	Callus induction media
Est	(Latin; for instance)
G	Gram
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indol buteric acid
KIN	Kinetin
Ls	Linsmaier and Skoog (1965) basal medium
Lux	Unit of illumination
MO19	Raina and Zapata (1997) callus induction media
MS	Murashige and Skoog (1962) basal medium
N6	Chu (1978) callus induction media
NAA	a-naphthalene acetic acid
NaOCl	Sodium hypochloride
M*m*	Micro meter
Cm	Centimeter
pH	Negative logarithm of hydrogen ion concentration [-log(H+)]

LIST OF ABBREVIATIONS

µl	Microliter
Al	Aluminium
Calli	More than one callus
Cat	Catalase
Cult	Culture
cv.	Cultivar
DH	Double haploid
Est	Esterase
Fig.	Figure
g/L	gram per liter
GDP	Gross domestic products
L	Litre(s)
m,	Metre
Mg	Milligram
Min	Minute
Ml	Millilitre
No.	Number
Org	Organisation
Psi	pound per square inch
QTLs	Quality trait loci
Tiss	Tissue
Uv	Ultraviolet light
v/v	Volume by volume
w/v	Weight by volume

THESIS ABSTRACT

Title of the Thesis:	Rice doubled haploid production and homozygosity testing using phenotypic and molecular approaches
Full Name of the Student:	Prem Narayan Patel
Major Subject:	Plant Molecular Biology and Biotechnology
Major Advisor:	Dr. (Smt.) Zenu Jha (*Associate Professor*)
	Department of Plant Molecular Biology and Biotechnology
Degree to be awarded:	Master of Science

Prem Patel
Signature of the Student

Signature of major Advisor

Signature of Head of the Department

Date;- 21/07/2017

ABSTRACT

Anther culture based double haploid (DH) production is a technology which, can significantly reduce the time period require for development of new crop variety. In the present investigation, on attempt is made to develop DHs using anther culture in rice (Oryza sativa L.), two crosses Swarna sub 1 x IR 90019-17-159-B and MTU1010 x Dagaddeshi were subject for the study. The anther are excised and plated on to N6 (Chu. 1978) media supplemented with 3% maltose 0.8% agar and the pH was maintained of 5.8 and 2 ml/l 2,4-D. In cross Swarna sub 1 x IR 90019-17-159-B highest number of callus was induce 23.44% and cross MTU1010 x Dagaddeshi 12.19%.The induce callus was transfer to 2 different media T11, T15 along with control for regeneration.T15 was found to be best as it has produce 254 number of green plant were in Swarna sub 1 x IR 90019-17-159-B and 5 number of green plant MTU1010 x Dagaddeshi.(Rukmini*et al.* 2013)

Morphological charactrization and molecular markers were used to differentiate between diploid and DHs plants. DUS assay and SSR marker are used for differentiate DHs. The line selected are DHs line of kharif 2015 developed through anther culture at PMBB, Raipur . 60 plants randomly selected from all 6 DH lines S-17 x RYT-3275(185) S-17 x PB(717), S-17 x IR-64(78), S-17 x IR-64(600), S-17 x IR-64(114), S17 x RP-BIO(1246). All the line are individually assessed for their genetic stability and homozygosity using SSR markers. In DUS assay character like plant height , Panicle length, Number of tiller , Number of panicle, Flag leaf length ,Flag leaf width, Grain length, Grain width, 1000 seed weight , Seed weight per/plant has showen less degree of standard error (0.83-0.018) in all characters that confirms homozygosity in these lines.(Rasoazanakolona *et al.*2017).Molecular analysis , we have use 130 no of SSRs marker of different locus of chromosome to check for homozygosity and uniformity of the 6 DHs line out 130 SSR marker 70 SSR marker has amplified and 6 has shown monomorphic bands. Which confirms that the generated lines were complete DHs lines. These lines were at trail under MLT for there evaluation.(Naik *et al.*(2016)

शोध साारांश

अ)	शोध का शीर्षक	:	धान, द्विगुणित उत्पादन और समरूपता परीक्षण बाह्य अकार्यकी और आण्विक पहुंच
ब)	छात्र का पूरा नाम	:	प्रेम नारायण पटेल
स)	प्रमुख विषय	:	पादप आण्विक जीव विज्ञान एवं जैव प्रौद्योगिकी
द)	प्रमुख सलाहकार का नाम एवं पता	:	डॉ. जेनू झा सहा प्राध्यापक, पादप आण्विक जीव विज्ञान एवं जैव प्रौद्योगिकी विभाग, कृषि महाविद्यालय, इं. गा. कृ. वि., रायपुर (छ.ग.), 492012
इ)	डिग्री से सम्मानित किया जाना है	:	एम. एससी. (कृषि)

प्रमुख सलाहकार के हस्ताक्षर

Prem Patel
छात्र के हस्ताक्षर

दिनांकः 21/07/17

विभागाध्यक्ष के हस्ताक्षर

शोध सरांश

परागकोष संवर्धन उपयुक्त माध्यम पर प्रयोंगषला अगुणित पौधें उत्पादन की एक तकनीक है। जो नई फसल किस्म के लिए समय की अवधि को काफी कम कर सकती है। स्वर्णा सब1Xआई आर 90019–17–159 बी और एम टी यू–1010 X दगडदेषी को अध्ययन के अधीन थे। परागकोष उत्तेजन लिए एन6 माध्यम का उपयोग किया गया है माध्यम पर 3 प्रतिषत माल्टोज 0.8 प्रतिषत अगर से पूरक बनाया गया हैं 2मिमी लीटर 2,4–डी मिलाया गया और पी एच को 5.8 बनाया रखा गया । संकरण स्वर्णा सब 1 X आई आर 90019–17–159 बी सबसे अधिक संख्या (23.44प्रतिषत) में कैलस उत्तप्रेरित हुआ । और एम टी यू–1010Xदगडदेषी में 12.19 प्रतिषत कैलस उत्तप्रेरित हुआ ।उत्तप्रेरित कैलस को दो अलग–अलग माध्यम टी11 टी 15 में स्थानांतरित किया गया जिसमें से टी 15 सबसे अच्छे हरे कैलस उत्तप्रेरित हुए जिसमूं से 254 हरे पौधे उत्तपादित हुए और एम टी यू–1010 में X दगडदेषी में 5 हरे पौधे प्राप्त हुए द्विपादीय और द्विगुणित पौधे` बीच अन्तर करनें के लिए आकारिकी लक्षण और आणविक मार्करों का इस्तेमाल किया गया डी यू एस लक्षण और एस एस आर मार्कर का इस्तेमाल द्विगुणित के अन्तर के लिए किया जाता है। चुनी गई लाईन पी एम बी बी रायपुर में उत्तपादित की गई ।

परागकोष संवर्धन के माध्यम से खरीफ 2015 की द्विगुणित लाईन विकसित की गई सभी 6 द्विगुणित लाईनों एस 17Xआर वाई टी–3275 , एस 17Xपी बी 717 एस 17Xआई आर 64 (78) एस 17X आई आर 64 (600) एस 17 आई आर 64(114) एस 17 X आर पी बायो 1246 से अनियमित रुप से 60 पौधों को चयन किया गया । और उसमें एस एस आर मार्कर का उपयोग करते हुए सभी लाईनों को व्यक्तिगत रुप से उनके अनुवांषिक स्थिरता और समरुपता के लिए मूल्यांकन किया गया । पौधों की उॅचाई और पुष्प गुच्छ की लम्बाई, पौधे की लम्बाई, झंडा पत्ती की लम्बाई और चौडाई , कन्सें की संख्या, दानों की लम्बाई, चौडाई व 1000 दानों का बजन बीज वनज प्रति पौधा जैसे डि यू एस परख चरित्र में कम मानक त्रुटि दिखाई देते है (0.83–0.018) सभी मानक में जों इन पंक्तियों में समरुपता की पुष्टि करता है आणविक विष्लेषण में हमनें 130 एस एस आर मार्कर उपयोग किया जिसमें 70 एस एस आर मार्कर हि समरुपता व समानता की पुष्टि करते है और उसमें स 6 मोनोमार्फिक बैंड दिखाते है जो पुष्ट करता है कि उतपन्न लाईने पूरी द्विगुणित लाईन थी इन लाईनों के मूल्यांकनों के लिए एम एल टी के तहत परिक्षण के लिए किया गया।

CHAPTER-I
INTRODUCTION

Rice (*Oryza Sativa* L.) is the most important food crop of Southeast Asia where almost half the world's population lives. Asian countries cultivate around 137 million hectare of paddy and Indian contribution to 45 million hectare. Rice plays an important role in the growth of annual GDP of India by 15% and provides essential nutrient with 43% calorie to more than 70% of the total population. The population of rice eaters is increasing day by day and the number of rice consumers will probably two fold by the year of 2020.

In Chhattisgarh, rice occupies average of 3.6 million hectare. with the productivity of the State ranging between 1.2 to 1.6 tone/hectare depending upon the rainfall. The state is comprised with three agro-ecological zones i.e. Chhattisgarh plain. Bastar plateau and northern hill region of surguja. These zones have huge variations in terms of soil topography, rainfall intensity and distribution, irrigation and adoption of agricultural production system and thus varies in the productivity of rice in these regions. Attack of insect-pest are the major contrasts in rice productivity in Chhattisgarh.So to develop new rice variety for different traits by conventional method needs 7-8 year. Anther culture and addrent this production by accelerating the breeding cycle by reducing the generation needed to fix a homozygosity (above F7-F9 generations) in a shorter period of time and also reduce the time required for new variety development (F1 or advanced breeding lines). Anther culture, an unconventional approach, could be a complementary technique along with conventional breeding for rice improvement. This technique also contributes to the development of doubled-haploid or dihaploid lines for rapid development of new varieties, which are useful for developing mapping populations for molecular analysis. Several superior DHs (CRHR5 and CRHR7) have been selected for yield and also are now being used as parents in breeding programme . Anther culture technique also offers great opportunities for improving grain quality of rice and production of female plant in Rice. Albino plant regeneration from anther culture is a crucial problem that interferes in generating a large number of DHs. Therefore, high

rate of green plant regeneration along with better agronomic performance is a prerequisite in anther culture for varietal development. Development of rice varieties using anther culture techniques has been reported in several countries. Most of the anther culture derived varieties are of the *Japonica* type because *Japonica* cultivars are generally easier to culture than *indica* ones. Early anther necrosis, poor callus proliferation and albino-plant regeneration are currently recognized as the major problems in *indica* rice varieties (Chen et al. 1991). the *indica* type rice is generally recalcitrant to culturability compare to *japonica* and needs improvement through basic research of culturability. Therefore, overcoming recalcitrant in culturability of *Indica* rice preferred by South East Asian countries is essential towards varietal development.

DH is a genotype formed when haploid cells undergo chromosome doubling. Following regeneration, haploid plants obtained from either anther or ovule culture may grow normally under *in vitro* conditions or can even be acclimatized to form vital mature plants. Such plants often express reduced vigor but in some crops such as onion, even haploid plants might grow vigorously. At the flowering stage, haploid plants form inflorescences with evident malformations. Due to the absence of one set of homologous chromosomes, meiosis cannot occur, so there is no seed set. Duplication of the chromosome complement is therefore necessary. Various methods have been applied over several decades and are still in development. The most frequently used application is treatment with anti-microtubule drugs, such as colchicine (originally extracted from autumn crocus *Colchicum autumnale*), which inhibits microtubule polymerization by binding to tubulin. Although colchicine is highly toxic, used at a millimolar concentration and known to be more efficient in animal than in plant tissues, it is still the most widely used doubling agent. Other options are oryzalin, amiprophosmethyl (APM), trifluralin and pronamide, all of which are used as herbicides and are effective in micromolar concentrations. Colchicine application on anther culture medium, for instance, showed a significant increase in embryo formation and green plant regeneration in wheat (Islam, 2010). DHs systems have the unique genetic property of producing completely homozygous lines from heterozygous parents in a single generation (Snape, 1989). (DH) breeding

not only helps in accelerating conventional plant breeding programmes and make early release of cultivars with superior and desirable traits possible but it has greater utility in other research aspects of plant breeding, genetics and genetic engineering. DHs are important constituent of germplasm. These also helps in complementing back cross breeding by transferring genes of interest between wild relatives thus breaking genetic barriers. On the other hand unique complete homozygous nature of DHs, less time requirement to produce a large number of DHs, absence of heterozygosity, efficiency over conventional systems and absence of gametoclonal variation in DHs make them very valuable material for very important genetic and molecular studies. So, DHs are extensively used for genetic studies like studying inheritance of quantitative traits, quantitative trait loci (QTL) mapping, genomics, gene identification, whole genome mapping and production of stable transgenic plants. One of advantages of tissue culture techniques that the protocols may permit the recovery of variants not easily obtained by conventional breeding practice. The probability for the *in vitro* recovery of benefit homozygous genotype reasonably high when anther culture, which provides the benefits of haploid event, is used to provide cells for biochemical selections. However, variation may be benefit or deleterious. Generally green plant regeneration from androgenic calli is very low irrespective of varieties. The low anther culture response, high percentage of albino plantlet generation and abundance of haploids are the main constraints in establishing a successful anther culture in rice. However, rice anther culture response is genotype-specific and successful use of anther culture technology in varietal development depends on the efficient production of adequate numbers of DHs for field evaluation and selection (Chu et al., 2001; Jiang et al., 2002). In C.G. State insect pest attacks is one of the major causes of decrease in rice productivity. Other being drought which severity effect rice productivity. MTU1010 and Swarna sub 1 are one of the most papular variety of C.G. State but MTU1010 and Swarna sub 1 are drought susceptible they have been crossed with dagaddeshi and IR 90019-17-159-B where are drought resistant so that to increase rice productivity of popular variety. If we developed conventional Plant breeding method it will require at few 7-8 year to develop new variety hence subjected to anther culture. So MTU1010 x dagaddeshi

and Swarna sub 1 x IR 90019-17-159-B have been subjected to anther culture for development DHs. In anther culture, some time the anther walls, which is diploid, can regenerated into a diploid plant. These diploid is needed to be detected and rejected before development of DHs. To differentiate between diploid and DH plants morphological characters and molecular marker can be used. Morphological DUS assay and SSR marker are used for differentiaty DHs from diploid were selected (DHs line of kharif 2015). 60 plants randomly selected from all 6 DH lines (S-17 x RYT-3275(185) , S-17 x PB(717), S-17 x IR-64(78), S-17 x IR-64(600), S-17 x IR-64(114), S17 x RP-BIO(1246) individually were assessed for their genetic stability and homozygosity using SSR markers with therefore analyzing information we have formulated the research object as under:-

- Production of doubled haploid rice through anther culture.
- Morphological characterization of doubled haploid rice progenesis to establish the homozygosity.
- Molecular characterization of doubled haploid rice.

CHAPTRE II
REVIEW OF LITRATURE

Work done in India and elsewhere in recent year on anther culture for generation of double haploid population in rice (oryza sativa L) have been reviewed and presented in this chapter under following heads

2.1 Production of doubled haploid rice through anther culture.

A doubled haploid (DH) is a genotype formed when haploid cells undergo chromosome doubling. Haploid cells are produced from pollen or egg cells or from other cells of the gametophyte, then by induced or spontaneous chromosome doubling, a doubled haploid cell is produced, which can be grown into a doubled haploid plant.

Ishra *et al*. (2015) initiated a doubled haploid (DH) breeding approach to develop new lines from two elite indica rice hybrids(CRHR5 and CRHR7) through rapid fixation of homozygosity in the recombinants. *In vitro* culture of the rice anthers resulted in 243 and 186 fertile DH lines of CRHR5 and CRHR7,respectively.

Javed *et al*. (2007) reported Indica rice is recalcitrant to in-vitro techniques including anther culture. Reported maltose media enhanced RA 1.4 and 1.1 times, and CP 1.8 and 1.5 times in Nona Bokra and Pokkali, respectively. Maltose and alternate culture temperatures could be beneficial factors to develop an appropriate anther culture protocol for other recalcitrant indica rice cultivars.

Khatun *et al*. (2003) reported anthers of five rice varieties viz. BR-5, BR-31, BR-34, BR-37 and BR-38 were cultured for callus induction and plant regeneration. Anthers were cultured on N6, Z2 and R2 media containing the same hormonal combination 2.5mg/l NAA, 0.5mg/l KN and 0.5mg/l 2.4-D and incubated at 25 ± 1˚C in dark for callus induction. All the varieties in Z2 medium, two varieties in N6 medium and only one variety in R medium produced callus. Out of all responding varieties BR-38 produced highest percentage of callus.

Maria *et al*. (2010) study that Eight genotypes of two rice species (*Oryza sativa* and *Oryza glaberrima*) were studied for their response to anther culture in terms of callus induction and frequency of plant regeneration. N6 medium (Chu et al.,

1975) was used for callus induction, and MS medium (Murashige and Skoog, 1962) with 1 mg/l BAP and 0.5 mg/l NAA for plant regeneration. Generally, *O. glaberrima* genotypes produced more callus than *O. sativa* genotypes. All *O. glaberrima* genotypes regenerated plants. Among *O. sativa* genotypes only japonica variety IKP produced plants. Other *O. sativa* genotypes showed low frequency of callus induction without plant regeneration. Many albino plants were obtained from the culture. Only one *O. glaberrima* genotype (6202 Tog) produced green plants. In this experiment, a total of 93 plants were regenerated with 14 green plants and 79 albino plants. Anther culture response is largely species and genotype dependent.

Premvaranon *et al*. (2011) the aim of this investigation was to improve in vitro technique of production of double haploid in Indica hybrid rice by combining anther culture, hormone shock and doubling chromosome. It was discussed how to avoid Somaclonal variation during culturing and to reduce the time of this process. The anthers of KDML 105 × SPR 1 (Indica × Indica) were cultured in Linsmaier and Skoog (LS) medium, which contained nutrients, growth regulators [(2,4,-dichlorophenoxy acetic acid (2,4-D) and naphthalene acetic acid (NAA)] and organic compounds, and then subcultured by inducing embryo-like structure (ELS) LS media. During 4 weeks used LS media supplemented with 10 μM KNO3 + 2 mg/L 2,4-D + 2 mg/L NAA + 20% coconut water + 1 mg/L of activated charcoal had induced high embryogenic frequent callus with length of 4–5 mm. The supplementation of 0.2 g/L colchicine and 100 μM 2,4-D was the most efficient in LS media. Over 70% of viable double haploid ELS were produced in 8 weeks and subcultured only twice compared with conventional anther which takes more than 12 weeks. This new technique can therefore be applied to rice in order in shorten time to produce higher number of double haploid plantlets.

Romana Siddiqui. (2014) reported that In vitro production of doubled haploid (DH) plants through anther-culture provides an efficient method for rapid production of homozygous lines. For this purpose, two different media MS and N6 supplemented with 1mg/L BAP +1mg/L 2,4-D+0.5mg/L NAA were evaluated for callus induction and green plant regeneration. Results showed that the highest percentages of calluses and green plants as a continuous process were obtained by N6 compared to MS. The

best response to callus formation was obtained by BR48 and and minimum green plant obtained from OM576.

Rukmini *et al.* (2013) reported that application of anther culture techniques for improvement of *indica* rices is a formidable task as they are known to be recalcitrant to culture unlike *japonica* rices. An effort was made to assess the influence of cold pretreatment and phyto hormones on the anther culture response of Rajalaxmi (CRHR 5) and Ajay (CRHR 7), two elite and popular *indica* rice hybrids. Cold pretreatment for 7-9 days at 10oC was found to have a positive influence on the callus induction frequency and a ratio of 1:4 for 2,4-D and NAA and 1:3:1 ratio of Kinetin: BAP: NAA ratio proved to be optimal for callus induction and green plant regeneration respectively.

Sahu *et al*. (2015) studied one cross Safri-17xIR-64 and one variety MTU1010. Parameter like media composition, hormonal treatment etc was standardized for efficient development of DH. The anther were excised and plated on to N6 (Chu.,1978) media supplemented with 3% maltose 0.8% agar , 2 ml/l 2,4-D and the pH was maintained of 5.8. In cross Safri-17xIR64 the callus induction percent was 0.49% and in variety MTU1010 callus induction percent is 0.40%. The induce callus was transfer in 16 different media (T1 to T16) for regeneration. Then green callus was transfer to green plant regeneration media. Treatment no.T15 was found to be best as it has produce 106 number of green plant.

Shahnewaz and Bari. (2004) investigated Effect of various concentrations of sucrose in culture media on the frequency of callus induction and regeneration of green plantlets from anther culture of rice .Results revealed that 4% sucrose was suitable for high green plant regeneration (65%). Highest concentration of sucrose (6% and above) in the culture medium also prompted the regeneration of albino plants.

Talebi R *et al.* (2007) reported among seven varieties upon transfer to SK 11 medium, highest percentage (40%) of green plants were produced in Hassani and in N 19 medium the highest percentage (15.78) of green plants and albino plants (21.05) were produced in Anbarbo. also showed embryogenesis and green plant regeneration in rice anther culture dependent on medium culture components.

Tran Dinh Gioi, and Vuong Dinh Tuan. (2002) observed the calli were sub cultured in N6 medium supplemented with NAA (0.5 mg/L) + BAP (2 mg/L) for plant regeneration. Reported calli from the cross of IR64/IR68530 showed the highest response in plant regeneration (1.12% in total of inoculated anthers), and none green plant obtained in cross of IR64/IR70441.

Usenbekov *et al.* (2013) has reported that Rice anthers were cultured in liquid N6 medium containing various concentration of growth harmones. Haploid plants were treated with different doses of colchicine to develop doubled haploid (DH) plants. The DH plants were transferred to soil and seeds were harvested from mature plants. The DH plants would be suitable for genetics and molecular breeding activities.

2.2 Morphological characterization of doubled haploid rice progenesis to establish the homozygosity.

The plant materials were planted in the field for recording the morphological observations during the wet season 2016.The following observations were recorded based on the procedures described in following National guideline for the conduct of tests for distinctness, uniformity and stability of Rice.

Barakat, *et al*., (2013) in this study compare the agronomic performance of wheat doubled haploid (DH) lines derived via microspore culture against their corresponding parental lines under field conditions. They have estimated the genetic diversity using molecular markers and agronomic performance to find an association between molecular markers and agronomic traits.

Chakrabarty et al. (2012) ninety one farmers grown varieties collected from Southern part of West Bengal were evaluated for 52 plant morphological and grain characterization for two years and concluded that many varieties with distinct and distinguishable characteristics and better economic and genetic values can be used in breeding programs.

Chu *et al.* (2000) discussed the use of anther culture to produce double haploid (DH) lines that are beginning to meet the requirement of commercial varieties. The results of phenotypic evaluation of agronomic characteristics of a large number of DH2 lines are described.

Das and Ghosh. (2011) characterize thirty one qualitative traits of four hundred thirty one traditional rice cultivars from germplasm collection of Rice Research Station, Chinsurch. Among the qualitative traits considerable variability were recorded for basal leaf sheath color, awning and auricle color. Maximum variability was observed for grains per panicle followed by spikelet per panicle.

Liangyong *et al.* (2009) the present study, a doubled haploid (DH) population, derived from a cross between japonica rice Chunjiang 06 and indica rice TN1 was used to analyze the quantitative trait locus (QTL) for three related traits of panicle layer- uniformity; that is, the tallest panicle height, the lowest panicle height and panicle layer disuniformity in two locations. A total of 16 QTLs for three traits distributed on eight chromosomes were detected in two different environments. almost all other QTLs for the same trait were detected only in one environment, indicating that these three traits were dramatically affected by environmental factors. The results may be useful for elucidation of the molecular mechanism of panicle-layer-uniformity and marker assisted breeding for super-rice.

Naik *et al.* (2016) the generation of doubled haploids in anther culture aims to accelerate the acquisition of pure lines within a short span of time. Selection of the desired traits can be done directly from anther culture resulted progeny at early generations. However, there is a possibility of segregation in doubled haploids lines in the future for which this experiment was taken up to determine the agronomic traits, uniformity and stability of the DH lines derived from a popular rice hybrid, BS6444G. The first experiment used 200 DH lines of the first generation of anther culture results (A1) following completely randomized design in two years showed each line has uniform agronomic traits; variation was observed in all 200 DHs. However, based on the better performance, 13 DH lines were selected for further evaluation along with the parent hybrid and two better checks in split plot for four seasons. The results showed that the progenies of individuals of 13 DH lines derived from the second to fifth generations showed no difference between generations for each agronomic trait of the same lines which were stable from generation to generation. Simultaneously, SSR markers also confirmed the stability of all the 13 selected DH lines showing homozygosity in all individual DH populations. Therefore,

the selected DH lines could be efficiently used for the successful products in future. The 6 selected DH lines could reach the heterotic level over-yielding (5.4%- 41.9%) than the hybrid and better checks.

Rukmini *et al.* (2015) study morpho-agronomic evaluation revealed 100% uniformity and stability for all the characters in the DH lines of both hybrids. Nineteen promising DH lines of each hybrid were advanced to A2 generation for yield evaluation. The yield levels of the DH lines ranged from 5097–6965kg/hm2 for CRHR5 and 5141–7 235kg/hm2 for CRHR7,which were at par or higher than the parental hybrids. Physico-chemical characterization and cooking quality analyses revealed significant and acceptable values for grain length and width, alkali spreading value, amylose content and water uptake ratio of the selected DH lines. Three DH lines, CR5-10, CR5-49, CR5-61 from CRHR5,and four DH lines, CR7-5, CR7-7, CR7-12 and CR7-52 from CRHR7,showed significant grain yield and quality characteristics and have been recommended for multi-location trials for subsequent release as new indica doubled haploid rice varieties.

Sah *et al.* (2000) recorded a total of 21 green and 31 albino plants were obtained from 1904 anthers of O. sativa cv. Himali x O. rufipogon. Six types of androclonal variants were identified based on morphology and panicle characteristics of regenerated plants. Among 21 plants, only six were found diploid with partially fertility. Quimio and Zapata. (1990) reported the inheritance of callus induction and plant regeneration in rice (***Oryza sativa*** L.) anther culture. Sixteen genotypes derived from a four-parent diallel mating set of two Japonica cultivars (Taipei 309 and Taipei 177) and two indica cultivars (IR36 and Basmati 370) were for their callus induction and plant regeneration abilities.

Syafii *et al.* (2011; 2013) this study was aimed to investigate the agronomic characters of double haploid lines developed from anther culture, evaluate genetic diversity, phenotypic variations and broad sense heritability of double haploid lines developed from anther culture, and to obtain rice genotypes potential as superior lines.

Rasoazanakolona *et al.* (2017) has studed that Anther/pollen culture technology is being efficiently used for accelerating rice breeding progress and

improving grain quality characters. Using pollen culture technique, we obtained elite rice (Oryza sativa L.) line DHP6, which is well adapted to the highlands of Madagascar. Here we show that certain grain, nutritional, and culinary quality characteristics have been significantly improved in line DHP6. Morphometric and physico-chemical analysis demonstrated that physical (colour, transluscency, and chalkiness), chemical (amylose content, alkali spreading value, and gel consistency) and cooking (volume expansion, elongation ratio) features were greatly improved compared to the parental line.

2.3. Molecular characterization of doubled haploid rice.

Molecular and morphological analyses are among the most used tools for the estimation of genetic distances within a group of genotypes. Morphological markers reflect variation of expressed regions of genome while molecular markers indicate variation of all genome including expressed and non expressed regions in chromosomes it provide an excellent tool for obtaining genetic information.

Araújo *et al*. (2010) study that a doubled-haploid (DH) population, obtained by anther culture of F1 plants from a cross between a highly susceptible rice cultivar Lijiangxintuanheigu and the resistant somaclone (SC09), of the cultivar Araguaia, was used to identify RAPD markers linked to the blast resistance gene Pi-ar. The 86 DH plants, inoculated with the race IB-9 of Magnaporthe oryzae, segregated in 1:1 ratio of resistant and susceptible plants. The 67 primers used 31 produced DNA profiles that differentiated resistant and susceptible bulks as well as the parental cultivars. The resistance gene was found linked to the primer OPS162072 (‘AGGGGGTTCC’) at a distance of 3.6 cm. The selection efficiency of this primer was assessed in a BC3 F1 population derived from another cross between a susceptible cultivar IAC 201 and SC09. The marker OPS16 showed efficiency of 86.9%, when six resistant and two susceptible plants were considered as negatives in RAPD analysis.

Bindeshwar *et al.* (2015) study that a doubled haploid regenerated through anther culture of wide cross rice hybrids is one of the potential tools to produce various useful genetic and cytogenetic stocks for molecular studies. In this study, an interspecific hybrid derived from *Oryza sativa* and *O. rufipogon* was subjected to

anther culture with the aim to regenerate doubled haploid population for their characterization. A total of 21 green and 31 albino plants were obtained from 1904 anthers of *O. sativa* cv. Himali x *O. rufipogon*. Six types of androclonal variants were identified based on morphology and panicle characteristics of regenerated plants. Among 21 plants, only six were found diploid with partially fertility. The genotyping study using SSR markers also revealed the substantial variation among regenerants.The regenerated doubled haploid plants showed large amount of useful variation both at phenotype and molecular level and they can be exploited to enrich the current rice gene pool.

Gyulai *et al*. (2013) aimed to investigate the Pollen grains of anther cultures showed a high decrease in viability during the first two weeks of incubation time. The DNA samples of the 47 DH-individuals were bulked in nine groups and subjected to PCR analysis for estimating the level of homogeneity versus heterogeneity in comparison to the control of anther donor bulk of five plants of F1 hybrid pepper cv.Mazurka (DH-Ro). In total 26 primers were applied, 22 of them revealed scorable PCR profile with 54 scorable PCR bands. Of these, 19 (35.2%) bands generated by 7 primers were polymorphic. All of the nine bulks of DH-R2 plants were discriminated at least by one primer.

Hemaprabha *et al*. (2013) this study random amplified polymorphic DNA (RAPD) analysis and inter-simple sequence repeats (ISSR) primers were used to determine the occurrence and extent of variation in rice (*Oryza sativa* L.) plants regenerated from anther culture. Genetic diversity among 27 regenerants of the cross CO43/Nootripathu and their parents were assessed using 25 RAPD primers and 19 ISSR primers. RAPD primers used in the study produced 285 polymorphic markers (81.65% polymorphism) and ISSR primers produced 201 polymorphic markers (79.37% polymorphism). The number of markers produced per primer ranged from 4-27 in case of RAPD with a mean of 14.04 and 7-23 in case of ISSR with a mean of 13.05. The Polymorphism information content (PIC) values ranged from 0.702 to 0.952 with a mean of 0.887 for RAPD markers. In ISSR analysis, the mean PIC value was 0.891 and the highest and lowest PIC values were 0.940 and 0.832 respectively.

In the present study, RAPD markers were able to reveal greater genetic diversity among the regenerants screened than ISSR markers.

Hung *et al*. (2001) in the present study, we produced a DH population from the hybrid of *japonica* 668B and wide compatible *indica* T23. Genotyping of the *S5* locus with allele-specific markers for *ORF3*, *ORF4* and *ORF5* revealed a potential recombination investigate hot spot in the *ORF3-ORF4* region. Haplotyping analysis revealed that 21/34 subspecies specific Indel markers segregated in distortion in the DH population, with a few lines having *indica* alleles either extremely low (1.7%) or high (98.3%), with little effect of the *S5* allele. While DH lines with the *S5* allele had higher frequency of *indica* alleles, no effect of the *S5n* allele was observed on all agronomic traits but flowering time. Taken together, the present study advanced understanding of the genetics of wide crosses in general, and DH production in particular between the two rice subspecies, and the new DH population generated will become a useful resource for rice genetic study and breeding in the future.

Zhang *et al*. (2001) here, we report a comprehensive study of mapping the drought resistance components in a doubled-haploid rice (*Oryza sativa* L.) population of 154 lines. A genetic linkage map consisting of 315 DNA markers was constructed. A total of 41 quantitative trait loci (QTLs) were identified for osmotic adjustment and root traits, and individually explained 8–38% of the phenotypic variance. A region on chromosome 4 harbored major QTLs for several root traits. Consistent QTLs for drought responses across genetic backgrounds were detected and should be useful for marker-assisted selection towards the incorporation of a trait of interest into an elite line. Comparative mapping identified three conserved genomic regions associated with various physiological responses to drought in several grass species. These results suggest that these regions conferring drought adaptation have been conserved across grass species during genome evolution and might be directly applied across species for the improvement of drought resistance in cereal crops.

CHAPTER – III
MATERIALS AND METHODS

This chapter deals with the investigation methodologies implemented to satisfying the objective of "Rice doubled haploid production and homozygosity testing using phenotypic and molecular approaches". All the experiments were conducted in tissue culture lab, green house and field of Department of Plant Molecular Biology and Biotechnology, and field of Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.).

3. Production of doubled haploid rice through anther culture.

3.1.1 Experiment Materials

Two Indica Rice (*Oryza sativa* L.) Swarna sub 1 x IR 90019-17-159-B (F5) [Drought resistant] and MTU1010 x Dagaddeshi (F3) [Drought resistant] maintained at Research institute farm, I.G.K.V. Raipur Department of Plant Molecular Biology and Biotechnology, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur provided by Dr. S.B. Verulkar (Professor and Head) has been used for the current experiment.

Experimental Conditions

All *invitro* culturing experiment was conducted in tissue culture lab and common laboratory of Department of Plant Molecular Biology and Biotechnology under well sterilized and controlled condition in the kharif season crop 2016-17. During the experiment, the cultural conditions were well maintained. The average temperature of culture growth room was 25^0 C $\pm$ 2^0C, 1000 lux and humidity was 60% maintained. Before starting the experiment, it is necessary to ensure all the material and tools used are sterile. The cultural media, distill water, forceps, scalpel, bottle, are sterile for 20 minutes 15 psi. Metal container used for that. On switch and starter button left it. On when complete the 15 psi in autoclave. The media after sterilization was poured into Petri plate after adding growth regulators and all other material kept in the oven. Filter sterilization was done for hormone, syringe filter of

sine was used to filter hormone, and preparation of hormone was elaborated on (paragraph no.3.2.2) Fumigation of laboratory must to avoid contamination during experiment. Fumigation was done with KMnO4 and liquid formalin. After adding of both in a culture bottle fumes will be released to decontamination the laboratory area. As the fumes are bit dancers hence fumigation was done during every hours of Saturday the laboratory was close fumigation Sunday to make fumigation process more effective. Wrapped panicle were firstly treated panicle boots with 0.1% HgCl2 for 5 minute then after wash it with autoclaved distil water ,5 times and kept in tissue paper for drying then after anther dusted in media petriplate full N6 media, maltose, agar/l with 2-4-D hormone 2mg/l. dusted media plate keep in the incubator 25±2□c for callus induction.

3.1.2 Methodology:-

Wrapped rice panicle (boot) anther are collected from the field , and inoculates at 10□C for 10 days. the boots were opened stained of anther were excised from the spikelet's and dusted on to the containing N6 media +maltose+ agar with 2-4-D hormone inside laminar air hood. After during the petriplate are kept inside BOD incubation at 25±2□C. Under dark condition callus induction.

3.1.3 Explants collection and cold pretreatment.

Boots were taken which has flag leaf to second leaf distance 5-8 cm and totally packed panicles with leaf, stage effective to induce callus. The boots (panicles along with the boot leaves in which panicles are still enclosed) were pulled out of the tillers in the morning hr from a healthy crop of the Swarna sub 1 x IR 90019-17-159-B (F5) and MTU1010 x Dagaddeshi (F3) were collected. Removed the extra leaves by cutting out. The boots were wiped and clean 2-3 times, with a clean muslin cloth moistened with 70% alcohol. After drying for while. Processed boots were wrapped with whattmen paper marked well the name and date for pretreatment. The wrapped boots were placed in a freezer that was maintained at 10□C for 10-14 days for cold pretreatment. After completion of the cold treatment. The middle portion of the panicle from the pretreated spikelets was used for anther culturing. Spikelets must not be over matured or less matured. The spikelets were surface treated to remove of

bacteria and fungus of panicles using 0.1% HgCL2 for 5 minutes and rinsed 3-5 times with distilled autoclaved water.

3.1.4 Anther Inoculation for callus induction

For Callus induction required N6 media (Chu 1978) was used. N6 media is required for callus induction .N6 media composition is given in table 3.2. Anther is dusted in the N6 media plated and kept it into BOD incubate which were programmed for 25+_2˚C and dark condition.

Stock solution of hormone

2, 4-D is not directly dissolved in water so it was first made soluble in water miscible solvents and then water was added to get final volume. First 50 mg 2-,4-D was dissolved in 1 ml 70% alcohol and final volume was made up to 50 ml with autoclaved double distilled water to prepare stock solution of 1 mg/ml 2,4-D. Precaution taken always prepare 2,4-D hormone in amber color bottle or wrapped the bottle with Aluminium foil.

Preparation of hormone concentration:-

1. Weight the 2, 4-D 50 mg and dissolve fist 70% alcohol in 1 ml and then after dissolve 49 ml autoclave distil water.
2. Then in laminar (HEPA) filter for sterilization of hormone from bacteria and fungus.
3. Preserve the hormones in freeze.
4. Then taken 2 ml/l hormone per 1litter media.

Process for preparation of N6 media:-

1. 1 litter autoclaved Borosil bottle was taken.
2. 750 ml of autoclaved distilled water measured with measuring cylinder.
3. Weight the N6 media 3.97 gm/l and dissolve in distil water then weight the maltose 30 gm/l then dissolve distilled water.
4. Then magnetic stirrer was used to dissolve solution. When the solute was completely the dissolved the pH measured.
5. Solutions pH maintained 5.8 1N NaOH was used to increase the pH and 1N HCl used for lowering value of solution.

6. After taking pH, the volume of the solution was making up by adding double distil. Water to 1 liter.
7. Then weight the agar-agar 8 gm/l and dissolve the solution.
8. Prepared media was autoclaved at 121^0C, 15 psi for 20 minutes.
9. The media kept in growth chamber for cold at list 1 hour.
10. Then pouring the media in petriplate
11. Before pouring media in plate hormone mixed in media bottle then pouring the plate and kept in growth chamber.

Table 3.1 Pros for preparation of N6 media for 1 litter

S/NO.	Composition	Required amount/l
1	N6 media	3.97 gm/l
2	Maltose	30 gm/l
3	Agar- Agar	8 gm/l
4	pH	5.8
5	Hormone 2,4- D	2 ml/l

3.1.5 Process of anther dusting -

Check the date of wrapped panicle in freeze which has 10 days pretreated period. In laminar air flow we dusted the panicle boots. Removed the leaf from the panicles and selected the middle portion of panicle. Middle portion take in the bottle and treated with .1% $HgCl_2$ surface sterilized for 5 minutes. Then washed the panicle 5 times with the double distilled water. Keep the panicle in tissue paper for drying. With the help forceps and scalpel the seeds were cut, pulled out anther and placed in the media containing petriplate. Mention on above table then marked the dada of inoculation and cross name kept the plate for incubation 27□C in dark condition. The dusted plate was checked every day for contamination and callus regeneration observation was taken for any contamination (bacteria, fungus) and callus induction. Noted the callus induction date and contaminated plate sub culture contaminated items. Transferred items to in fresh plates. The Callus was allowed to grow up to 2mm size.

Table 3.2 Composition of N6 medium

Composition of N6 medium	Amount (mg/L) Present in Standard medium
Macro (mg/l)	
KNO_3	2830
$(NH_4)\ 2SO_4$	463
$MgSo_4.7H_2O$	185
KH_2PO_4	400
$CaCl_2.2H_2O$	166
Micro (mg/l)	
KI	0.8
H_3BO_3	1.6
$ZnSO_4.7H_2O$	1.5
$Na_2MoO_4.2_{H2}O$	0.250
$Na_2EDTA.2H_2O$	37.25
$FeSO_4.7H_2O$	27.85
Organics	
Nicotinic acid	0.5
Pyridoxine-HCL	0.5
Thymine-HCL	1.0
Carbohydrate	
Sucrose	30 (mg/l)

3.1.6 Regeneration of green callus from white callus.

Preparation of chemicals and solutions for rice callus regeneration plant.

The formulation of Murashige and Skoogs (MS) (1962) basal medium was used in the present experiments, as it is the most widely used media in contemporary for tissue culture work. A general composition of basal MS medium is given in the **(Table3.3).** All high quality chemicals were used for current research purchased from Himedia Company. For double haploid regeneration we use ½ MS media for greening of callus also contain maltose, sucrose as a carbon sources and agar was used for the solidifiers of media. Composition of growth hormone NAA, KINETINE, BAP was used as treatment in table 3.4. Transferred callus were kept in the growth chamber maintained 25+_2˚Cand 1000 lux light intensity for regeneration. Growth chamber well control.

3.1.7 The procedure for the preparation of stock Hormone.

Stock solution of hormone

a) NAA is not directly dissolved in water so it was first made soluble in water miscible solvents and then water was added to get final volume. First 50 mg NAA was dissolved in 1 ml 1N NaOH and final volume was made up to 50 ml with autoclaved double distilled water to prepare stock solution of 1 mg/ml NAA.

b) Kinetin is not directly dissolved in water so it was first made soluble in water miscible solvents and then water was added to get final volume. First 50 mg kinetin was dissolved in 1 ml 1N NaOH and final volume was made up to 50 ml with autoclaved double distilled water to prepare stock solution of 1 mg/ml kinetin.

c) BAP is not directly dissolved in water so it was first made soluble in water miscible solvents and then water was added to get final volume. First 50 mg BAP was dissolved in 1 ml 1N NaOH and final volume was made up to 50 ml with autoclaved double distilled water to prepare stock solution of 1 mg/ml BAP.

Table 3.3 Chemical composition of Murashige and Skoogs (1962) Media

Constituents	Amount (mg/L) Present in Standard mediun
Inorganic compound	
Macro nutrients	
KNO_3	1900
NH_4NO_3	1650
$CaCl_2 .2H_2O$	440
$MgSO_4. 7H_2O$	370
KH_2PO_4	170
Micronutrient	
$MnSO_4. 4H_2O$	22.3
$ZnSO_4. 7H_2O$	8.6
H_3BO_3	6.2
KI	0.86
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
$CuSO_4. 5H_2O$	0.025
Iron Source	
Na_2.EDTA	37.25
$FeSO_4.7H_2O$	27.85
II. Organic Compounds	
Vitamins	
Myoinositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Amino acid	
Glycine	2
Carbon source	
Sucrose	30,000
Gelling agent	
Agar	8,000

Preparation of culture media

In this experiment, for androgenesis for production of double haploid population in rice (MS, (1962) media was used as basal nutrient medium. Procedure for preparing one liter (1000 ml) of MS media was as follows-

1) Autoclaved 1 little bottle and distil water taken.

2) Weight 1/2ms media 2.2 gm/l and dissolved in distil water.

3) Weight maltose/sucrose 1.5 and 3 % concentration, and dissolved in distil water.

4) Then magnetic stirrered for uniform dissolving solution, after completion of the dissolving process pH maintained 5.8.

5) 1N NaOH used for increase the pH of solution.

6) 1N HCL used for decrease the pH.

7) After adjustment pH, make up the volume of 1 litter.

8) Then weight the agar-agar 8 gm/l and dissolve the solution.

9) Prepared media was autoclaved at 15 psi for 20 minutes.

10) Then kept it in growth chamber for cooling the media.

11) Before pouring the media in plates add the hormone they are NAA Kinetin BAP, as required amount according to treatment given in the table 3.4.

12) Then pouring the media in the laminar air flow in controlled condition , media are pouring in the petriplate

13) Then keep in the growth chamber in controlled condition

Hormone added in before for poring time

NAA +KINETIN+BAP

a) 0.25 mg/l: 0.25 mg/l: 0.75 mg/l

b) 1 mg/l: 1 mg/l: 2 mg/l

c) 0.5mg/l: 0.5mg/l: 1.5mg/l

Callus transfer process as follows

1) Clean the laminar air flow by 70% ethanol and give the UV light for 15 minute
2) Callus size of 2 mm transferred in 1/2MS media with various combinations of hormones demarked as treatments.
3) Used forceps and transferred 2mm size callus in the media plates

4) Inoculated plate were marked the name of cross treatment no, inoculation date of callus induction, and date of callus.
5) Kept in the growth chamber for regeneration.
6) Then taken observation of plate every day and note the record.

Table 3.4 Constituents of green regenerative callus media.

SN.	TREATMENT
T11	1/2 MS + 3M + 0.8 AGAR{01:01:02 (NAA:KIN:BAP) mg/l}
T15	1/2 MS + 1.5S + 0.8 AGAR {0.5 : 0.5: 1.5 (NAA:KIN:BAP) mg/l}
Control	1/2 MS + 3S + 0.8 AGAR{0 : 0: 0 (NAA:KIN:BAP) mg/l}

3.1.8 Shooting media preparation.

The entire responding callus (greening or albinism) were subjected to shooting media. That media was for further differentiation into seedlings. Some of the treatment only develops albinos or green or both were transferred separately. Culture bottles and test tubes were used. Placed it in growth chamber.

Procedure of shooting media preparation.

1) Initially weight 4.41 g ms media powder and dissolved in 500 ml of double distilled water with the help of magnetic stirrer.
2) Weight 30 gm (3%) sucrose and add in media.
3) Add adenine sulphate 0.18 gm/l (ADS) ads is heated separately before adding.
4) Then add Inositol 100 mg/l.
5) Add BAP 4 ml /l and IAA 1 ml/1.
6) Make up final volume by double distilled water and adjust ph 5.8 by adding 1 N – NaOH /L, 1 N HCL.
7) Add 8 gm /l (0.8%) agar agar and heat it under gas flame.
8) Poured media in culture bottle about 30 ml cover it with paper and autoclaved at 121˚C at 15 psi and stored.

3. 5 Table shooting media composition.

S. No.	Composition for 1 lit.	For 1 lit.
1	MS media	4.41g
2	Sucrose	30 g
3	Inositol	100 mg
4	Adenine sulphate	0.18 g
5	BAP	4 mg
6	IAA	1 mg
7	pH	5.8
8	Agar- agar	8 gm

3.1.9 Rooting media preparation:-

When come shooting done plants were transferred to rooting media. Culture bottle were used for pouring media .that allows good root growth make able to survive in green house condition. MSO was used as table describes no hormones used.

Process of rooting media preparation

1) 1 lit autoclaved borosil bottle was taken.
2) Measured autoclaved distil water 500 ml in measuring cylinder.
3) Weight the MS media 4.41 gm/l and dissolved in distil water.
4) Weight the Sucrose 30 gm/l then dissolve distils water.
5) Magnetic starrier the solution when complete the dissolved went for pH. Adjustment pH value 5.8.
6) NaOH for increase the pH of solution HCL for decrease the pH.
7) After pH adjustment, make up the volume of 1 litter.
8) Then weight the agar-agar 8 gm/l and dissolve the solution.
9) Prepared media was autoclaved at 15 psi for 20 minutes.

Table 3.6 Composition rooting media green and albino plant rooting.

S. No..	Composition rooting media	Amount /l
1	MS(media)	4.41 gm/l
2	Sucrose	30 gm/l
3	Agar	8 gm/l
4	pH	5.8

3.1.10 Chromosome doubling

3.1.11 Colchicine treatment of plants

Plants are treated with colchicine at two stages of plants seedling & tillering stages,at seedling stage plants are treated with two types, 1st is cutting of root tips (show in fig.) and plants are dipped in colchicine solution (0.1% or 0.05 %) for 12 / 24 hrs after that plants are transfer in pot and shifted in greenhouse for hardening. The 2nd method is pinching of leaf (at the portion where new leaves are emersed show in fig. ,than plants are dipped in colchicine solution (0.1% or 0.05 %) for 24 hrs.

1.) We have to taken out rooted plants.

2.) Washing of roots with distill water than tab water

3.) We have done cutting root tips with the help of scalpel blade / done pinching for proper entering of colchine inside stem cell.

4.) We have done dipping of plants in colchicine for 24 hours.

5.) We have done washing of plant properly.

6.) Transfer in to pots.

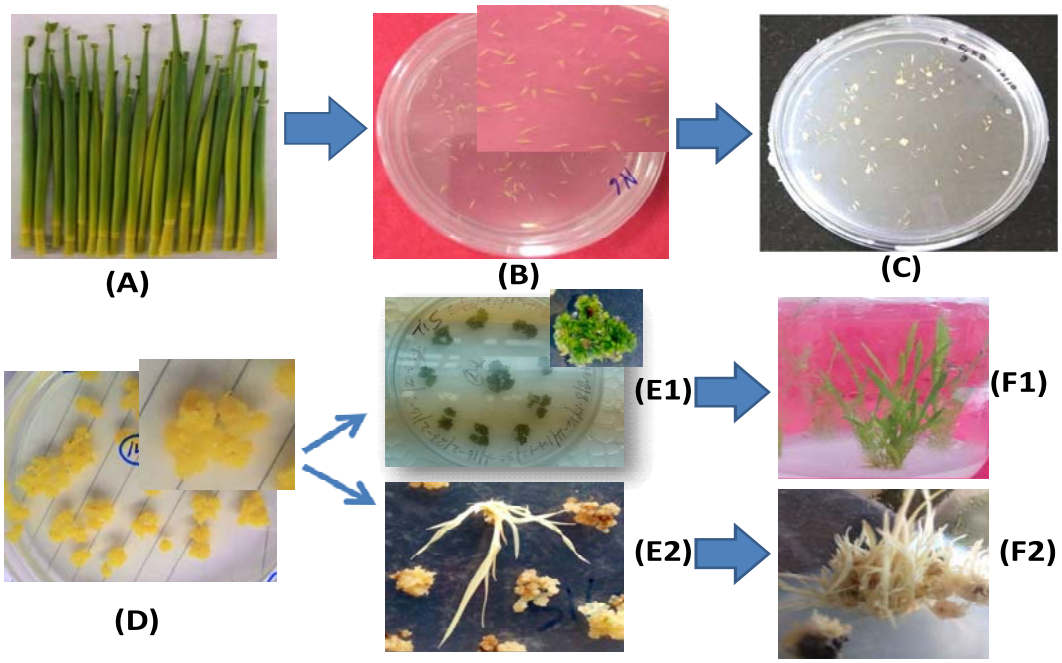


Fig 3.1(A)Explant (B) Inoculated plates (C) Callus initiation from dusted anther plates (D)Callus (E1 /F1) Green Callus (E2/F2) Albino plants

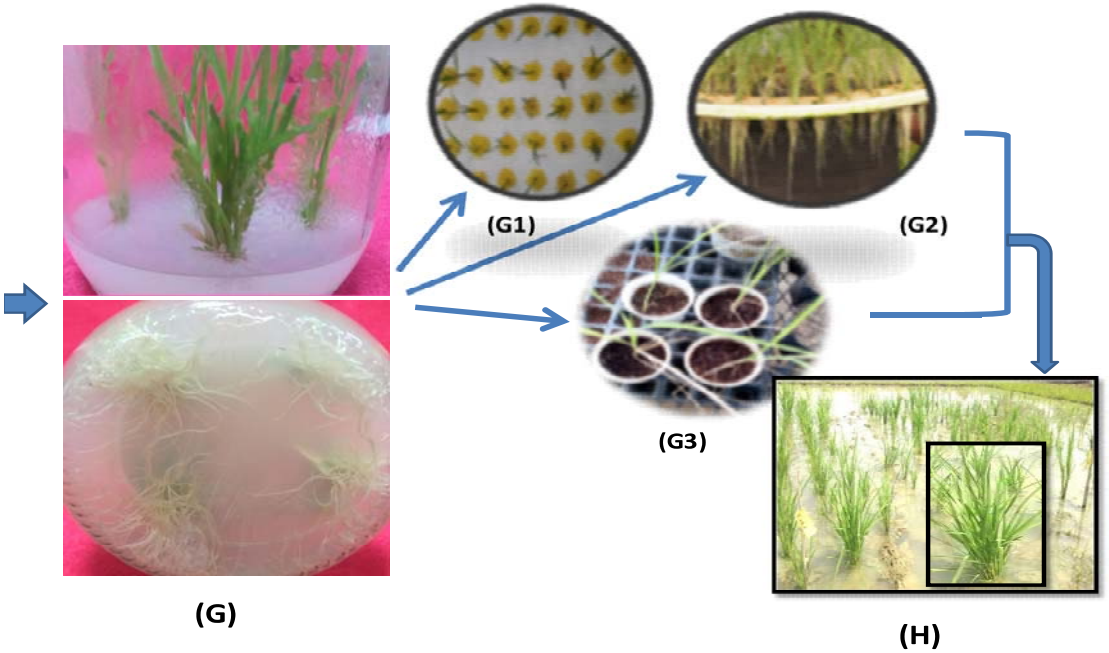


Fig 3.2(G) Green plants in rooting media, (G1,G2,G3) Plants in green house ,(H) Plants in field

3.2. Morphological characterization of doubled haploid rice progenesis to establish the homozygosity.

Uniformity in DUS charactared is on indicator that the generated from anther culture plants are homozygosity or hetrozygosity. There for DUS assy of different anther culture plant given in (tab.no. 3.10)

Table 3.7 Cross name of doubled haploid line use in DUS assyand molecular assy.

Code.	Cross name
A	S-17 x RYT-3275(185)
B	S-17 x PB(717)
D	S-17 x IR-64(78)
E	S-17 x IR-64(600)
F	S-17 x IR-64(114)
G	S17 x RP-BIO(1246)

The plant materials were planted in the field for recording the morphological observations during the wet season 2016. The seed were sown on date 8th July 2016 and transplanted on 27 July. The plant to plant and row to row spacing was 20x20 cm**.** The NPK fertilizer was applied @ 100-60-40 kg per hectare. All normal packages of practices were followed to raise a crop. The following observations were recorded based on the procedures described in following National guideline for the conduct of tests for distinctness, uniformity and stability of Rice (IIRR, 2006).

3.2.1 Leaf: Length of blade

The length of the leaf blade was measured in centimeter and categorized in to short, medium and long leaves.

3.2.2 Leaf: Width of blade

The width of the leaf blade was measured in centimeter and categorized in to narrow, medium and broad leaves.

3.2.3 Plant height (cm)

Pant height was measured at the time of maturity from ground level to the tip of the panicle.

3.2.4 Panicle: Length of main axis (cm)

Panicle length was measured at the time of maturity from the base of panicle to the tip of last spikelet prior to harvesting. The categories under this class are very short (<16 cm), short (16-20 cm), medium (21-25 cm), long (26-30 cm) and very long (>30cm).

3.2.5 Number of total tiller per plant

Number of total tiller per plant was counted from randomly selected plant.

3.2.6 Panicle: Number of effective tillers per plant

Number of panicle per plant was recorded from dough development (spikelets become hard) to ripening terminal spikelets ripened through visual observation and classified into few, medium and many by observation of individual plants.

3.2.7 Seed weight of 1000 seed (gm)

The 1000 seed were taken randomly and average weight of 1000 seed was measured in gram. These were classified in to very low, low, medium, high and very high classes.

3.2.8 Grain: length

Ten grains (with husk) were taken randomly and average length was measured in centimeter. These were classified in to very short, short, medium, long and very long classes.

3.2.9 Grain: width

Ten grains (with husk) were taken randomly and average width was measured in centimeters. These were classified in to very narrow, narrow, medium, broad and very broad classes.

3.2.10 Seed weight of per plant

The seed weight of per plant was taken in gram from randomly selected ten plant.

S-17 x PB(717)

S-17 x RYT-3275(185)

[redacted]**S-17 x IR-64(78)**

S-17 x IR-64(600)

S-17 x IR-64(114)

S17 x RP-BIO(1246)

Fig.3.3 Morphological observations of different crosses of dubbled haploid pappulation.

3.3.Molecular characterization of DHs rice.

Early identification of haploid / double haploid plants can be done by using molecular marker. For identification of anther culture derived haploid/double haploid plant,the DNA of donor lines S-17 x RYT-3275(185) , S-17 x PB(717)), S-17 x IR-64(78), S-17 x IR-64(600), S-17 x IR-64(114), S17 x RP-BIO(1246) for anther culture and the DNA of anther culture derived plants were subjected to SSR analysis. The list of SSR marker is given in the table 3.8

Table 3.7 List of SSR markers

Marker	Ch.	Forward primer sequence Reverse primer sequence	At	Ps (bp)	Pic	Position in cM
RM1	1	F – GCGAAAACACAATGCAAAAA R – GCGTTGGTTGGACCTGAC	55	113	0.465	29.7
RM19	12	F – CAAAAACAGAGCAGATGAC R - CTCAAGATGGACGCCAAGA	55	226	0.266	20.9
RM5	1	F- TGCAACTTCTAGCTGCTCGA R- GCATCCGATCTTGATGGG	55	113		94.9
RM 408	8	F – CAACGAGCTAACTTCCGTCC R – ACTGCTACTTGGGTAGCTGACC	55	128		1.1
RM85	3	F - AACGCGAGGACACGTACTTAC R – ACGAGATACGTACGCCTTTG	61	231		92.8
XA21						

3.3.1.Genomic DNA isolation

Total rice genomic DNA was extracted from four-week old plants of the dubbled haploid line by modified CTAB (Pervaiz *et al*., 2011) protocol. Before starting, add β-merceptaethanol to CTAB extraction buffer @ 20 µl/20 ml. then follow the stepwise protocol given below: About 100 mg of young leaf was grinded in 1000 µl 2X CTAB extraction buffer with the help of mortar and pestle.

1. Cut leaves to small pieces in a mortar and pestle; add 1 ml of CTAB buffer (Add 500 ul more CTAB if required) to CTAB add 1% PVP (1g in 100ml) and Beta-mecaptoethanol 1 ul/ml of buffer. Then transfer homogenate to 2 ml tubes

2. Add 5 ul of proteinase K (10 mg/ml) and incubate at 65 °C on water bath for 15-20 minutes. Mix the sample 3 times (by inversion) during incubation.

3. Take out and add 5 ul of RNaseA (10 mg/ml) mix well and incubate at 37 °C at room temperature for 15 minutes

4. Allow to cool & then add 700 ul of Chloroform: Isoamyl Alcohol (CIA-24:1) shake vigorously to mixed and leave for another 10 minutes.

5. The contents were shaken by hands intermittently and kept at room temperature for 15minutes. Then tubes were centrifuge at 14,000 rpm for 5 minutes.

6. Collect supernatant in fresh 1.5 ml Eppendorf tube.

7. Add double volume of 100% chilled Iso-Propanol and mix it by inverting the tube & Incubate for 1 hr at -20 °C or 4 °C over night.

8. The sample was centrifuge for 10 min at 14000 rpm at 5 ^{0}C. & Wash the pellet with 70% ethanol & Centrifuge it for 3 min (14000 rpm), decant the ethanol.

9. Pallets were dissolved in 200 ul of TE buffer and add 1 ml of absolute ethanol.

10. Then again centrifuge at 14000 rpm at 5 ^{0}C for 3 minutes.

11. DNA pellet was air dried for 30 minutes then dissolved in 50-100 ul of TE buffer.

12. Proceed for DNA quantification.

Quantification and PCR amplification of DNA

The DNA samples were quantified on Nano Drop Spectrophotoscopy (*NANODROP 2000c)*. 1.0 µl of sample was taken and placed on the Nano Drop followed by closing the lid, enter 1st blank (TE) whose range should be 0.0 to 0.1. After quantification,

the DNA was diluted with TE or sterile water such that the final concentration of DNA was approximately 40-50 ηg/µl for PCR amplification.

2 µl of diluted template DNA of each genotype was dispensed at the bottom of 96 well PCR plate (AXYGEN). Separate cocktail was prepared in an Eppendorf tube as described in Table….. About 18µl of cocktail was added to each sample.

Table 3.9. PCR components with their quantity for microsatellite analysis

S. No	Components	Concentration	Quantity
1	PCR buffer with $MgCl_2$	10X	2.0 µl
2	DNTPs	2 mM	2.0 µl
3	Primer (Forward)	10 µM	1 µl
4	Primer (Reverse)	10 Mm	1 µl
5	*Taq* DNA Polymerase	5 U	1 µl
6	Sterile water	-	11 µl
7	Template DNA	50 ηg/µl	2.0 µl
	Total		**20.0 µl**

Table 3.10 Temperature profile used for PCR amplification using microsatellite markers

Steps	Temperature	Duration	Cycle	Activity
1	94	4min.	1	Denaturation
2	94	45sec.		Denaturation
3	55	30sec.	35	Annealing
4	72	45sec.		Extension
5	72	7min.	1	Final Extension
6	4	∞	1	Storage

Visualization of amplified products in Agarose gel electrophoresis

3.5 per cent agarose gels were used for separation and visualization of PCR amplified products of SSR markers. Gels were casted in electrophoresis unit.

Assembling and pouring the gel

Gasket was fixed to the three sides of the outer plate (without notches). Spacers of 1.5mm thickness were placed along the sides by just attaching the gasket of outer plate.

Later, notch plate was kept on the outer plate so that spacers were between the two plates. Clamps were put on the three sides of plates leaving notch side of unit. It was checked with water to found any leakages.

For casting each gel, 65 ml of acrylamide gel (5%) solution was prepared just prior to pouring. For each 65 ml of solution, 70 µl of TEMED (N-N-N-N-Tetramethylethylene diamine) and 700 µl of (freshly prepared) ammonium per sulphate (APS, 10%) were added to initiate the polymerization process.

The contents were mixed gently by swirling, but bubbles were avoided. Before pouring, assembly was kept on the bench top so that it made 45 degree angle with bench top.

Then gel solution was poured from notch side with maximum care to avoid air bubbles. Comb of 1.5 mm thickness (63 wells) was inserted with tooth side in the gel.

Later, the assembly was kept for polymerization for 20-30 min.

Electrophoresis

- After polymerization process, gasket was removed and assembly was kept in the electrophoresis unit with electrophoresis unit clamps so that notch side facing inner side of the unit and facing other plate without notch to outer side.
- TBE (1x) was poured in upper tank in the unit and the rest was poured in bottom chamber.
- Comb was removed with care so that it does not disturb the wells formed in the gel.
- At last, 4 µl loading dye (10x) was added to PCR products.
- Finally, 5 µl of each sample were loaded into the wells for facilitating the sizing of the various alleles. Ladder (100bp, Bangalore GeNei, Mereck Bio Science) was loaded in the first well.
- Gel was run at 180 volts till the dye reached bottom of the gel.

- After electrophoresis, gels were stained with Ethidium bromide (10µl/ 100ml) and visualized in BIORAD Gel Doc XR^+.

Visualization of bands

After electrophoresis, clamps were removed and glass plates were separated without damaging the gel.

a) Gel was taken out from plate into staining box with care by flipping the gel with help of spatula and by pouring little amount of water for easy removal.
b) Ethidium bromide solution (prepared by adding 10 µl to 100 ml double distilled water) was poured into the staining box to stain the gel.
c) It was agitated for about five minutes to stain the gel.
d) Gel stained with Ethidium Bromide was washed two times with double distilled water to have clear images.
e) The gels were scanned with the help of *BIO-RAD* gel doc XR^+.
f) Care was taken while using TEMED and staining with Ethidium bromide solution as they are carcinogenic and mutagenic agents, respectively.

3.3.2.Reagents and solutions

Reagents for PCR

a. Primers

Highly variable microsatellite markers from ILS/SIGMA-SviBiosolutions Private limited. Primer (Farword and Reverse) are diluted according to their ηmol concentration, for stock preparation SIGMA water is added 10 times more to its ηmol concentration and then 5µl of each primer (F & R) was taken in Eppendorf tube and added 90 µl of SIGMA water to makes 100 µl.

b. dNTPs: (dATP/dCTP/dGTP/dTTP)

10 mM stock of dNTP (GeNeiTM) was used. Stock preparation- takes 10µl of each dNTPs (i.e. dATP/dCTP/dGTP/dTTPs) in 1.5 ml of Eppendorf tube, mix well by vortexing, final volume is 40µl having 100 mM dNTPs stock concentration. For dilution 10 µl dNTPs of stock solution was taken in 1.5 ml Eppendorf tube and add 990 µl SIGMA water to the tube, so the total volume became 1000 µl. This makes 1mM dNTPs is ready to use for PCR.

c. PCR buffer (10X)

GeNeiTMmake was used.

d. *Taq* DNA polymerase

3U/µl of 1000 u *taq* (GeNeiTM) was used for PCR.

Stock solutions

A. DNA extraction buffer

Tris HCl (1M; pH-8)	5	5ml
EDTA (0.5M; pH-8)	1	10ml
NaCl (4M)	7.	7.5 ml
SDS (20% W/V)	5	5ml

Final volume was adjusted to 100 ml with distilled water.

B. TE buffer

1M Tris-Hcl (pH-8)	1 ml
0.25 EDTA	0.4 ml

Final volume was adjusted to 100 ml and autoclaved.

C. EDTA (0.5M; pH-8)

186.12 g of EDTA was dissolved in 700 ml of distilled water. The pH was set to 8 using NaOH. Final volume was adjusted to 1000 ml with distilled water and sterilized by autoclaving.

D. 4M NaCl

23.36 g of NaCl was dissolved in 80 ml of distilled water. Final volume was adjusted to 100 ml and sterilized by autoclaving.

E. 1M Tris HCl (pH 8.3 at 25°C)

30.28 g of Trizma base was dissolved in 200 ml of distilled water. The pH was set to 8.3 using concentrated HCl. The final volume was adjusted to 250 ml with distilled water and sterilized by autoclaving.

F. Iso propanol (pre chilled)

G. Absolute alcohol (pre chilled)

H.70% Ethanol (pre chilled)

Reagents for PCR

A. Primers: Highly variable microsatellite markers from ILS, USA.

B. dNTPs:(dATP/dCTP/dGTP/dTTP) 2 mM stock of dNTP (Thermo) was used.

C. PCR buffer (10X): 10X Genaxy buffer was used

D. *Taq* polymeras: 5 unit/µl, *Taq* polymerase (Genaxy) was used for PCR.

E. Tank buffer (1X TAE): 20 ml 50X TAE + 980 ml of distilled water.

F. Orange loading dye

Solutions for electrophoresis

a. 10X TBE buffer

Tris base 104 g

Boric Acid 55g

EDTA (0.5M) 40 ml

Distilled water 500 ml

Final volume was adjusted to 1 liter with distilled water.

b. Tank buffer (1X TBE)

100 ml of 10X TBE was taken and 900 ml of distilled water was added.

c. 10X loading dye

Bromophenol Blue (HIMEDIA) 0.25 g

Glycerol 40ml

Final volume (100ml) adjusted by distilled water.

d. 100bp DNA Ladder (GeNeiTM Merck Specialities Private Limited)

1.5µl ladder per well is used.

Step Up 100bp ladder 0.1ml

Gel loading buffer (6x) 0.2ml

Water 0.4ml

3.3.3. Instruments used in the laboratory

- Veriti 96 well thermal cycler (Applied Biosystems)
- Refrigerated centrifuge
- Microwave oven
- Transilluminator and Bio Rad Gel documentation system
- Micropipettes
- Eppendorf tubes
- Electronic balance

CHAPTER IV
RESULT AND DISSCUSSION

In the present study, emphasis is given to produce haploid develop through anther culture. *In vitro* androgenesis *via* anther-culture is most preferred techniques for obtaining haploids plants. The production of haploids and Double haploids (DHs) provides a particularly attractive biotechnological tool to produce homozygous plants which help the plants breeder to develop new variety within a short span of time. Research efforts on the enhancement of response to anther culture have been confined mostly on manipulation of callus induction and plant regeneration protocols (Munesh *et al*., 2015). In anther culture, some time the anther walls, which is diploid, can regenerated into a diploid plant. These diploid is needed to be detected and rejected before development of DHs. To differentiate between diploid and DH plants morphological characters and molecular marker can be used. Morphological DUS assay and SSR marker are used for differentiaty DHs from diploid were selected (DHs line of kharif 2015).

4.1. Production of doubled haploid rice through anther culture.

In *indica* rice varieties, callus induction and plant regeneration are affected by genotypes and types of cultural medium (Sen and Singh,2011). Cross Swarna sub 1 x IR 90019-17-159-B (F5) and MTU1010 x Dagaddeshi (F3) were use for anther culture. The collected panicle of both of the crosses were sterilized with clean water then lower leaves were removed and washed with 70 % ethanol and kept in 10 ˚C for 10 days to fix the meiotic division in pollen mid microspore and late uninuclear stage ensuring haploid plant generation.

4.1.1 Anther inoculation

After pretreatment of panicle, the panicles were take out and sterilized with 0.1 % HgCl2 for five minutes then wash for five times with distilled water. The anther were dusted on petri plate containing solidified N6 media supplemented with maltose 3 %,agar 0.8 % and 2mg/l 2,4-D.Each petri plate were dusted with excised

anthers as shown in (fig 4.1) Anthers were dusted on each petri plate. Then the petri plates were incubated in 25±2˚C inside a BOD incubator for callus induction.

4.1.2 Callus induction

In Swarna sub 1 x IR 90019-17-159-B (F5), in total 6981 anther were dusted in 159 Petri plate, (N6 media supplemented with 2mg/l 2,4-D).Out of which 1647 callus were induced. The average Callus induction percentage in Swarna sub 1 x IR 90019-17-159-B (F5) was 23.44%.

Table 4.1: Callus induction in Swarna sub 1 x IR 90019-17-159-B (F5).

SL NO.	Day required for callus induction	Avg day for callus induction	Total no. of anther dusted/plat	Avg no. of anther dusted/plate	Total no of callus induction	Callus inductionso%
1	20-22	21	725	45.31	95	13.18
2	25-31	28	793	41.73	131	16.51
3	20-26	23	1371	50.77	280	20.42
4	22-26	24	1752	46.1	523	29.85
5	27-31	29	274	40.93	90	32.84
6	31-35	33	1012	43.31	289	28.55
7	30-36	33	1054	52.68	239	22.77
Total		**27.28(Av.)**	**6981**	**45.83(Av.)**	**1647**	**23.44(Av)**

In MTU1010 x Dagaddeshi (F3), in total 4282 anther were dusted in 130 Petri plate, (N6 media supplemented with 2mg/l 2,4-D).Out of which 481 callus were induced. The average Callus induction percentage in MTU1010 x Dagaddeshi (F3) was 12.19%.

Table 4.2: Callus induction in MTU1010 x Dagaddeshi (F3)

SL NO.	Day required for callus induction	Avg day for callus induction	Total no.of anther dusted/plate	Avg no. of anther dusted/plate	Total no of callus induction	Callus induction%
1	20-24	22	343	49	42	12.24
2	25-31	28	505	45.9	74	14.7
3	20-26	23	358	44.75	73	18.34
4	22-26	24	589	43.28	77	13.07
5	27-33	30	701	48.78	100	14.27
6	31-35	33	932	49.09	70	7.5
7	32-36	34	854	42.68	45	5.27
	Total	**27.71(Av.)**	**4282**	**46.21(Av.)**	**481**	**12.19(Av)**

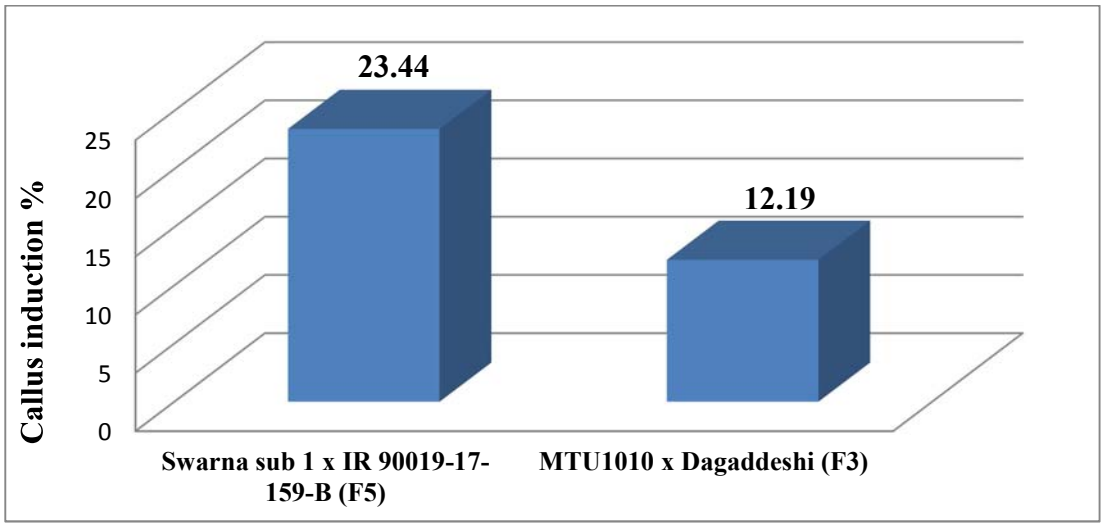


Fig -4.1 Graphical representation of callus induction in both the crosses.

In Swarna sub 1 x IR 90019-17-159-B (F5), callus induction percentage was higher compare to MTU1010 x Dagaddeshi (F3). The callus induction % was moderate in both the crosses. Several researchers have reported the callus induction % in anther culture were very low. Tran and Vuong (2002) obtained 3.53 % callus induction in N6 medium and 1.12% in plant regeneration. (Munesh *et al*., 2015) reported In cross Safri-17x IR64 the callus induction percent was 0.49% and in variety MTU1010 callus induction percent is 0.40%. However some work report high

callus induction % (Rukmini *et al.,* 2013) reported callus induction was 32.11 % in CRHR-5 and CRHR-7.

4.1.3 Green callus regeneration

T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, T13, T14 and T15, has been checked for *indica* rice variety for best callus regeneration at PMBB, IGKV in previous research in anther culture out of which (T11, T15) was found good compare to other treatments .These 2 treatments along with control was taken in the current experiment for both of the crosses (Swarna sub 1 x IR 90019-17-159-B and MTU1010 x Dagaddeshi for callus regeneration.(tab 4.3) In Swarna sub 1 x IR 90019-17-159-B (F5) T11,T15 media callus greening percent was 12.91% and 21.21% observed after 15 days of callus transfer. Highest callus greening was T15 (21.21%) followed by T11 (12.91%). There was no callus greening observed in control (without hormone).

In MTU1010 x Dagaddeshi on an average 461 callus was subjected to 2 different treatments T11, T15 along with control (without hormone) shown in (table 4.4). These 2 treatments along with control, T11 (01: 01: 02 (NAA:KIN:BAP) mg/l, (1/2 MS + 1.5S+0.8AGAR) and T15 (0.5 : 0.5 : 1.5 (NAA:KIN:BAP) mg/l ,1/2 MS + 1.5S + 0.8AGAR) has responded for greening of the callus. In T11 and T15 callus greening percent was 2.47% and 3.15% observed after 15 days of callus transfer. Highest callus greening was with T15 (3.15%) followed by T11 (2.47%) . There was no callus greening observed in treatment without hormone.

In Swarna sub 1 x IR 90019-17-159-B (F5), callus regeneration percentage was higher compare to MTU1010 x Dagaddeshi (F3). The callus regeneration % was moderate in both the crosses. Several works has report the callus regeneration % in anther culture to be very low. (Munesh *et al*., 2015) reported that T15 was found to be the best with 8.33 % of green callus regeneration and 32 green plant regeneration. However some work report high callus induction %.(Herath *et al* 2007) reported that callus regeneration percent was 41.0% in F1 hybrids. Reiffers, *et al.* (1990) obtained frequency 29% in plant regeneration. (Rukmini *et al* 2013) reported callus regeneration was 36.22 % in CRHR-5 and CRHR-7.

Table no 4.3: Green callus Swarna sub 1 x IR 90019-17-159-B (F5).

Treatment	Composition	No. Of callus Inoculated	Callus Greeing	Greening %
T11	1/2 MS+ 1.5S +0.8AGAR (01:01:02) (NAA:KIN:BAI mg/l	240	31	12.91
T15	1/2 MS + 1.5S +0.8AGAR (0.5 : 0.5: 1.5) (NAA:KIN:BAP)mg/l	1362	289	21.21
controlled	1/2 MS + 1.5S +0.8AGAR	45	0	0
Total		**1647**		**11.37**

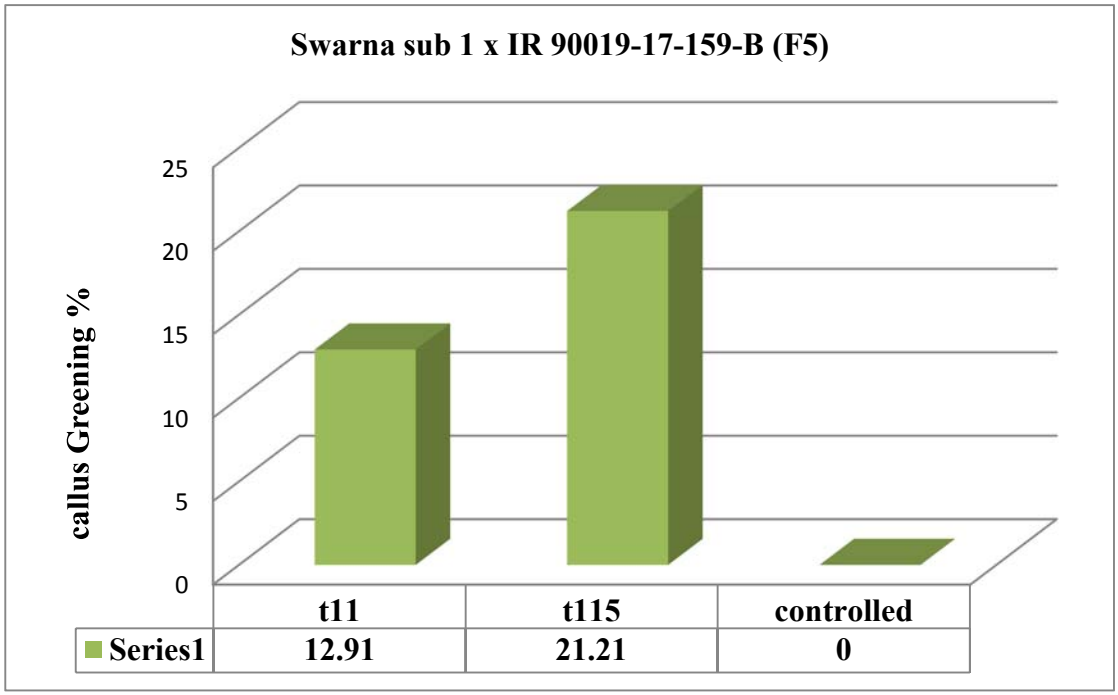


Fig 4.2: Graphical representation of Relation between treatment and greening % of Swarna sub 1 x IR 90019-17-159-B (F5)

Table no 4.4: Green callus regeneration in MTU1010 x Dagaddeshi.

Treatment	Composition	No. Of callu Inoculated	Callus Greeing	Greening %
T11	1/2 MS + 1.5S +0.8AGAR (01:01:02) (NAA:KIN:BAP) mg/l	81	2	2.47
T15	1/2 MS + 1.5S +0.8AGAR (0.5 : 0.5: 1.5) (NAA:KIN:BAP)mg/l	380	12	3.15
controlled	1/2 MS + 1.5S +0.8AGAR	20	0	0
Total		**461**		**1.87**

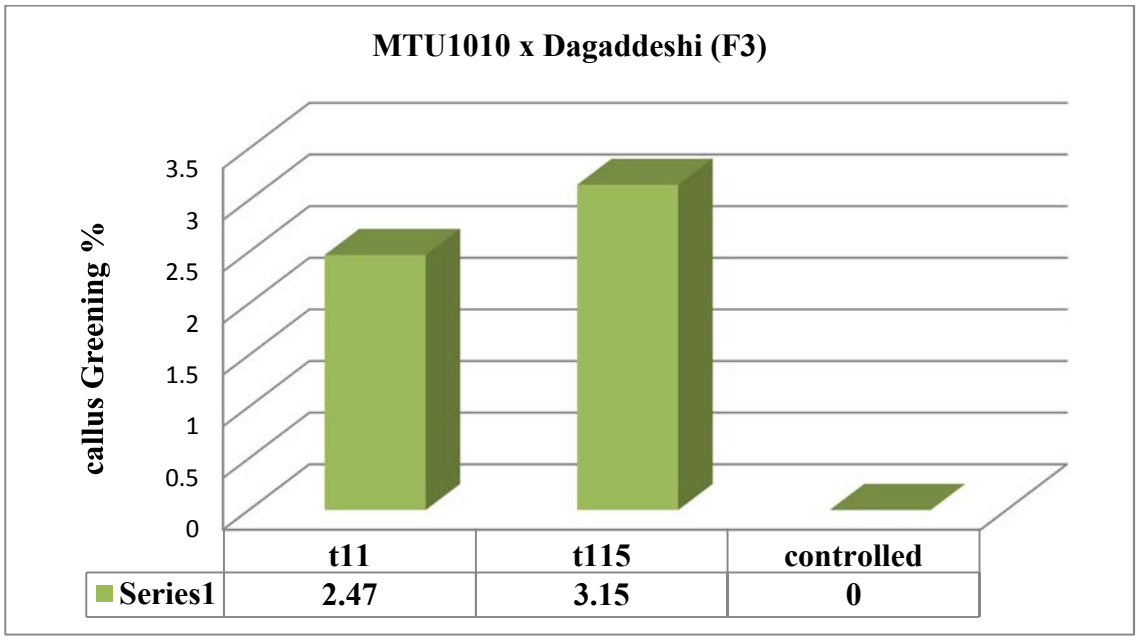


Fig 4.3: Graphical representation of relation between treatment and greening % of MTU1010 x Dagaddeshi (F3).

4.1.4 Green plant regeneration:

After callus greening, the callus were transferred to green plant regeneration media (shooting media). All the callus were transfer to the same media for green plant regeneration. In cross Swarna sub-1 x IR 90019-17-159-B , total 1647 number of callus were inoculated ,out of which 320 callus has became green which was further transferred to shooting media.T15 has generated highest 289 green callus. Out of which 25 contaminated by the time of subculture or incubation at growth chamber. And 254 total number of green plant were produced.(Tab.4.5)

In cross MTU1010 x Dagaddeshi , total 461 number of callus was inoculated out of which 14 callus became green which was further transferred to shooting media.T15 has generated highest 12 green callus. Out of which 5 green callus became contaminated by the time of subculture or incubation at growth chamber. 7 total number of green plant were produced (Munesh *et al.,* 2015). T15 was found to be best as it has produce 261 numbers of green plants (tab.no.4.5 and 4.6). The green plant are planted in hydroponics (greenhouse) and field. This is the result recorded till the submission of the thesis, observation for DHs production is still continued.

Table no 4.5: Green plant regeneration in Swarna sub 1 x IR 90019-17-159-B.

Treatment	No. Of callus Inoculated	Callus Greening	Green plant regeneration
T11	81	31	20
T15	380	289	254
controlled	20	0	0
Total	**461**		**274**

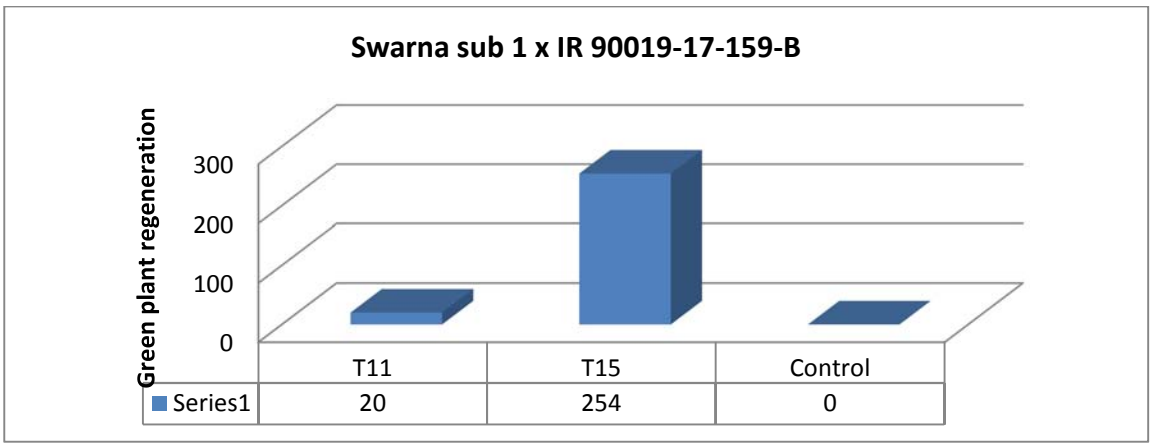


Fig 4.4: Graphical representation of relation between treatment and green plant regeneration% of Swarna sub 1 x IR 90019-17-159-B.

Table no 4.6: Green plant regeneration in MTU1010 x Dagaddeshi.

Treatment	No. Of callus Inoculated	Callus Greening	Green plant regeneration
T11	81	2	2
T15	380	12	7
controlled	20	0	0
Total	**461**	**14**	**9**

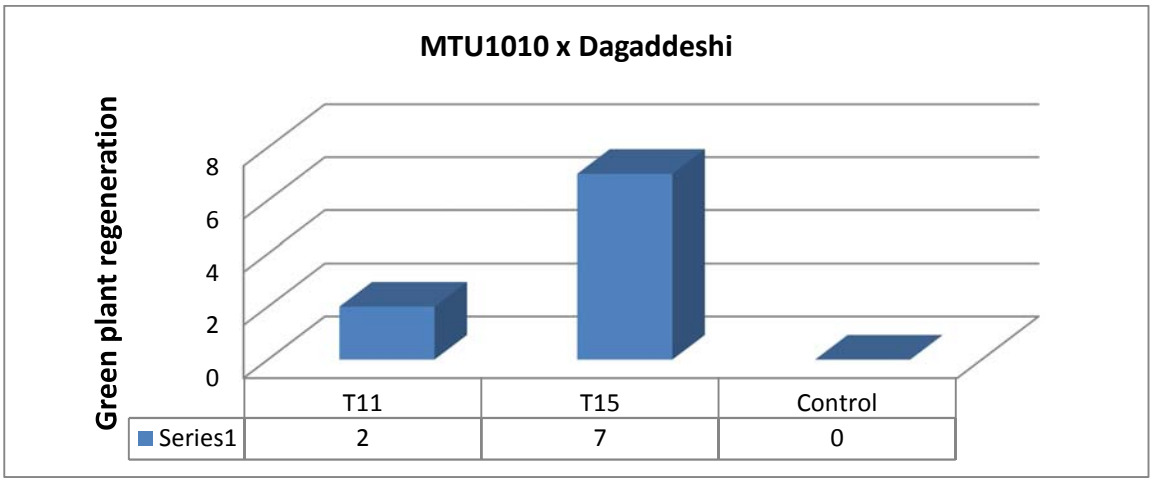


Fig 4.5: Graphical representation of Relation between treatment and green plant regeneration% of MTU1010 x Dagaddeshi (F3).

4.2.Morphological characterization of doubled haploid rice progenesis to establish the homozygosity.

The observation for morphological traits were used for calculating the mean performance. The observations were recorded for every lines of mapping population with ten randomly selected plants from each DHs line. The mean performance, standard deviation (SD) and standard error (SE) for all the traits studied in 6 DHs line this are presented in table 4.7 .

4.2.1. Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line (S-17 x RYT-3275(185)

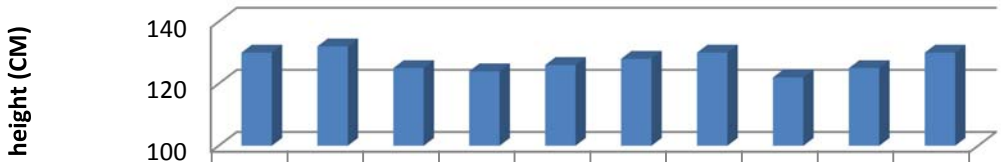
The S-17 x RYT-3275(185) showed mean performance of different character like plant height 127.2 (cm), Panicle length 28.9 (cm), Number of tiller 18.4, Number of panicle 18.4, Flag leaf length 38.9 (cm), Flag leaf width 1.48 (cm), Grain length (mm) 8.81 (mm), Grain width 2.38 (mm), 1000 seed weight 25.27(gm), Seed weight per/plant 49.31(gm) and also showed less degree of standard error of all character that can be confirm the DHs line should be homozygous and uniform data given in tab-4.7.

Table no 4.7: Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line (S-17 x RYT-3275(185)

S-17 x RYT-3275(185)				
S.N.	**Character**	**Mean**	**SD**	**SE**
1	Plant height (cm)	127.2	3.25	1.02
2	Panicle length	28.9	2.46	0.77
3	Number of tiller	19.3	3.8	1.2
4	Number of panicle	18.4	3.56	1.12
5	Flag leaf length (cm)	38.9	3.69	1.16
6	Flag leaf width(cm)	1.48	0.27	0.08
7	Grain length (mm)	8.81	0.07	0.02
8	Grain width (mm)	2.38	0.06	0.01
9	1000 seed weight	25.27	0.92	0.29
10	Seed weight per/plant	49.31	9.08	2.87

SE-1.02

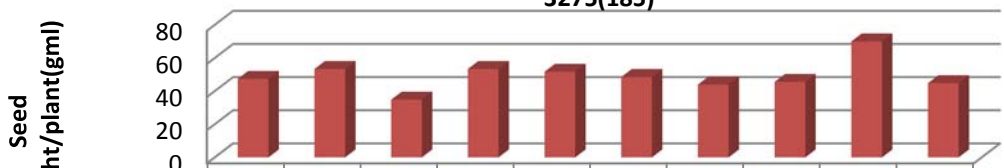
Fig 4.6 Plant height of ten random selected plant of DHs line(S-17 x RYT-3275(185)



	1	2	3	4	5	6	7	8	9	10
PH	130	132	125	124	126	128	130	122	125	130

SE-2.87

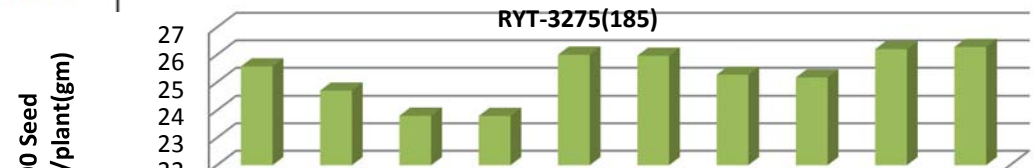
Fig 4.7 Seed weight/plant of ten random selected plant of DHs line(S-17 x RYT-3275(185)



	1	2	3	4	5	6	7	8	9	10
SW	47.38	53.32	34.8	53.32	51.66	48.46	43.94	45.51	69.95	44.8

SE-0.29

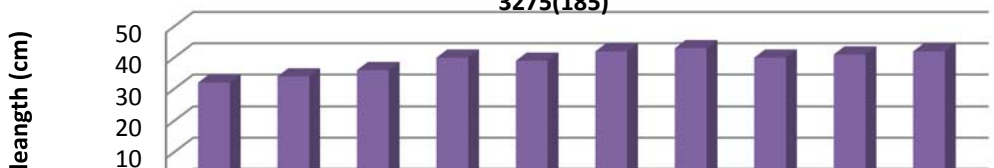
Fig 4.8 1000 Seed weight/plant of ten random selected plant of DHs line(S-17 x RYT-3275(185)



	1	2	3	4	5	6	7	8	9	10
1000SW	25.57	24.69	23.8	23.79	26	25.96	25.27	25.17	26.2	26.28

SE-1.16

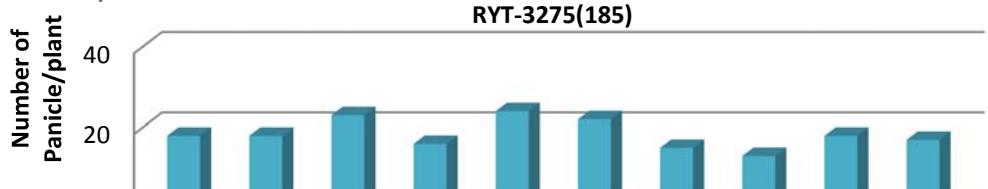
Fig 4.9 Flag leaf leangth of ten random selected plant of DHs line(S-17 x RYT-3275(185)



	1	2	3	4	5	6	7	8	9	10
FLL	32	34	36	40	39	42	43	40	41	42

SE-1.12

Fig 4.10 Number of Panicle/plant of ten random selected plant of DHs line (S-17 x RYT-3275(185)



	1	2	3	4	5	6	7	8	9	10
PN	18	18	23	16	24	22	15	13	18	17

4.2.2. Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x PB(717)

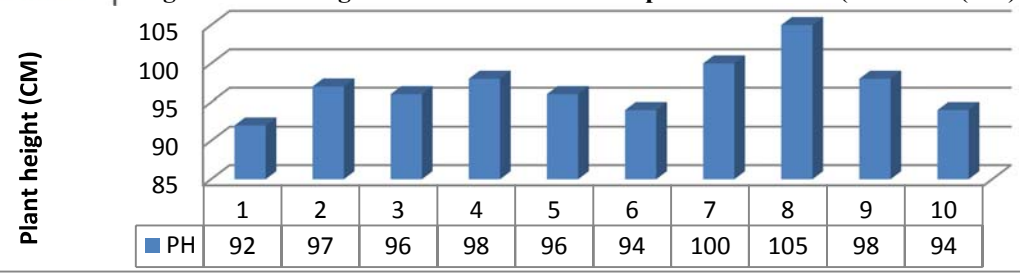
In DHs line S-17 x PB(717) showed mean performance of different character like plant height 97 (cm), Panicle length 26.7 (cm), Number of tiller 17.5, Number of panicle 16.5, Flag leaf length 33.2 (cm), Flag leaf width 1.47 (cm), Grain length (mm) 9.23 (mm), Grain width 2.68 (mm), 1000 seed weight 29.47(gm), Seed weight per/plant 41.05(gm) and also showed less degree of standard error of all character that can be confirm the DHs line should be homozygous and uniform data given in tab.4.8

Table no 4.8: Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x PB(717)

S-17 x PB(717))				
S.N.	**Character**	**Mean**	**SD**	**SE**
1	Plant height (cm)	97	3.46	1.09
2	Panicle length	26.7	1.55	0.49
3	Number of tiller	17.5	1.5	0.47
4	Number of panicle	16.5	1.8	0.56
5	Flag leaf length (cm)	33.2	2.35	0.74
6	Flag leaf width(cm)	1.47	0.29	0.09
7	Grain length (mm)	9.23	2.68	0.84
8	Grain width (mm)	2.68	0.074	0.023
9	1000 seed weight	29.47	0.67	0.21
10	Seed weight per/plant	41.056	8.75	2.76

SE-1.09

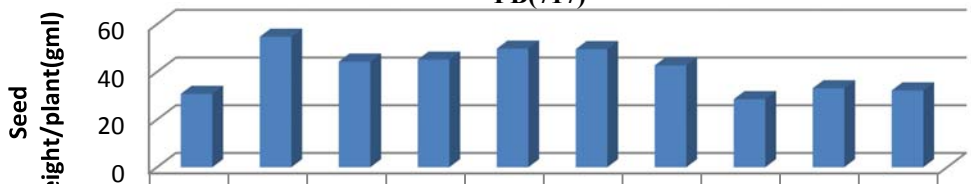
Fig 4.11 Plant height of ten random selected plant of DHs line(S-17 x PB(717)



	1	2	3	4	5	6	7	8	9	10
PH	92	97	96	98	96	94	100	105	98	94

SE-2.76

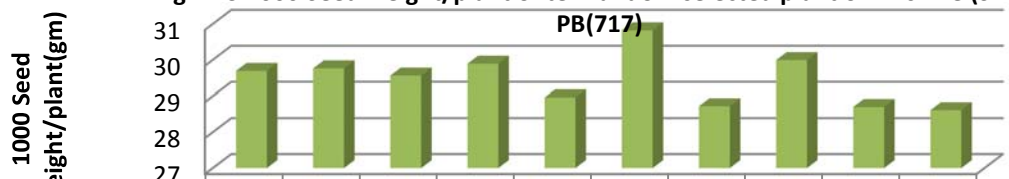
Fig 4.12 Seed weight/plant of ten random selected plant of DHs line(S-17 x PB(717)



	1	2	3	4	5	6	7	8	9	10
SW	30.76	54.7	44.3	45.1	49.7	49.5	42.7	28.5	33.1	32.2

SE-0.21

Fig 4.13 1000 Seed weight/plant of ten random selected plant of DHs line (S-17 x PB(717)



	1	2	3	4	5	6	7	8	9	10
1000SW	29.69	29.76	29.56	29.89	28.96	30.82	28.72	29.99	28.7	28.61

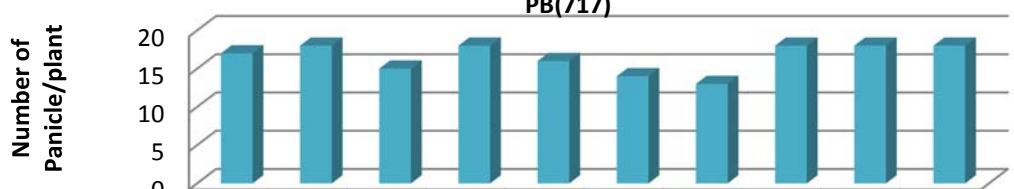
SE-0.74

4.14 Flag leaf leangth of ten random selected plant of DHs line (S-17 x PB(717)

	1	2	3	4	5	6	7	8	9	10
FLL	33	30	32	33	34	32	34	36	38	30

SE-0.56

Fig 4.15 Number of Panicle/plant of ten random selected plant of DHs line (S-17 x PB(717)



	1	2	3	4	5	6	7	8	9	10
PN	17	18	15	18	16	14	13	18	18	18

4.2.3 Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x IR-64(78)

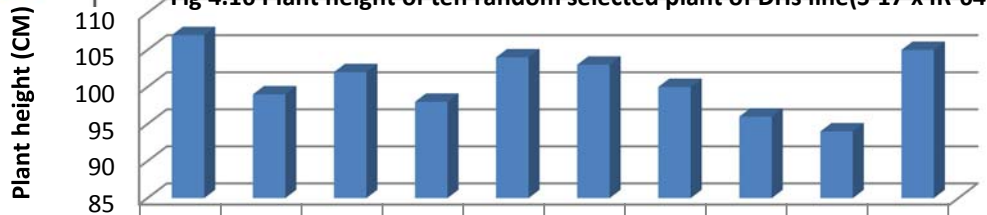
The S-17 x IR-64(78) showed mean performance of different character like plant height 100.8 (cm), Panicle length 21.2 (cm), Number of tiller 21.3, Number of panicle 19, Flag leaf length 39.9(cm), Flag leaf width 1.47 (cm), Grain length (mm) 8.7 (mm), Grain width 2.91 (mm), 1000 seed weight 25.98(gm), Seed weight per/plant 49.48(gm) and also showed less degree of standard error of all character that can be confirm the DHs line should be homozygous and uniform data given in tab

Table no 4.9: Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x IR-64(78)

S-17 x IR-64(78)				
S.N.	**Character**	**Mean**	**SD**	**SE**
1	Plant height (cm)	100.8	4.13	1.3
2	Panicle length	21.2	2.57	0.81
3	Number of tiller	21.3	4	1.15
4	Number of panicle	19	2.98	0.94
5	Flag leaf length (cm)	39.9	3.98	1.25
6	Flag leaf width(cm)	1.47	1.47	0.46
7	Grain length (mm)	8.7	8.7	2.75
8	Grain width (mm)	2.91	2.91	0.92
9	1000 seed weight	25.98	1.57	0.49
10	Seed weight per/plant	49.48	9.08	2.87

SE-1.3

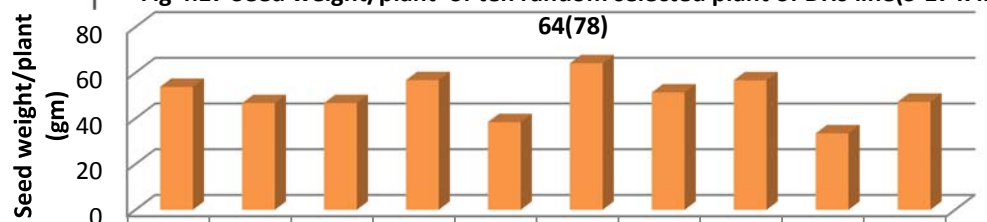
Fig 4.16 Plant height of ten random selected plant of DHs line(S-17 x IR-64(78)



	1	2	3	4	5	6	7	8	9	10
PH	107	99	102	98	104	103	100	96	94	105

SE-2.87

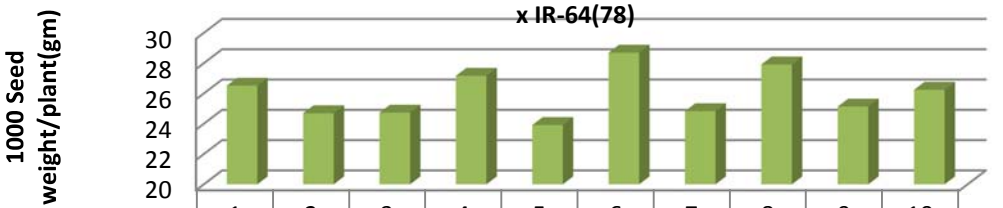
Fig 4.17 Seed weight/plant of ten random selected plant of DHs line(S-17 x IR-64(78)



	1	2	3	4	5	6	7	8	9	10
SW	53.77	46.65	46.66	56.7	38.35	64.11	51.31	56.65	33.42	47.23

SE-0.49

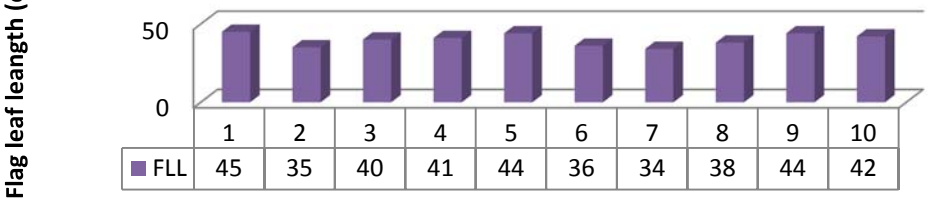
Fig 4.18 1000 Seed weight/plant of ten random selected plant of DHs line(S-17 x IR-64(78)



	1	2	3	4	5	6	7	8	9	10
1000SW	26.51	24.69	24.73	27.16	23.91	28.69	24.83	27.91	25.13	26.24

SE-1.25

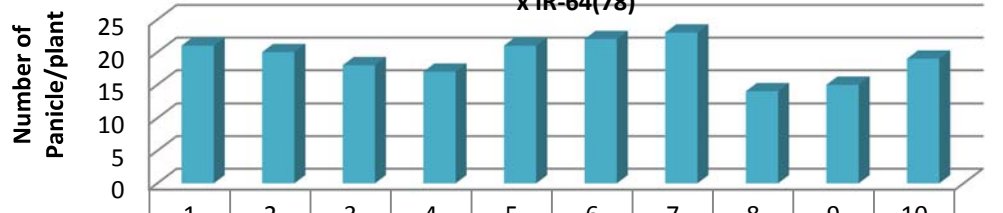
Fig 4.19 Flag leaf leangth of ten random selected plant of DHs line(S-17 x IR-64(78)



	1	2	3	4	5	6	7	8	9	10
FLL	45	35	40	41	44	36	34	38	44	42

SE-0.94

Fig 4.20 Number of Panicle/plant of ten random selected plant of DHs line(S-17 x IR-64(78)



	1	2	3	4	5	6	7	8	9	10
PN	21	20	18	17	21	22	23	14	15	19

4.2.4. Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x IR-64(600)

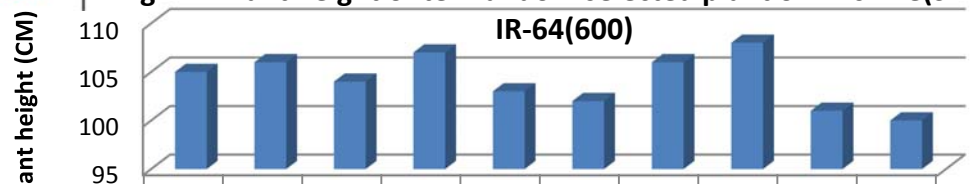
The DHs line S-17 x IR-64(600) showed mean performance of different character like plant height 104.2 (cm), Panicle length 26.4 (cm), Number of tiller 21.4, Number of panicle 19.9, Flag leaf length 33.2 (cm), Flag leaf width 1.49 (cm), Grain length (mm) 9.06 (mm), Grain width 2.43 (mm), 1000 seed weight 25.47(gm), Seed weight per/plant 47.07(gm) and also showed less degree of standard error of all character that can be confirm the DHs line should be homozygous and uniform data given in tab

Table no 4.10: Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x IR-64(600)

S-17 x IR-64(600)				
S.N.	**Character**	**Mean**	**SD**	**SE**
1	Plant height (cm)	104.2	2.65	0.83
2	Panicle length	26.4	2.83	0.89
3	Number of tiller	21.4	2.31	0.73
4	Number of panicle	19.9	2.55	0.8
5	Flag leaf length (cm)	33.2	2.09	0.66
6	Flag leaf width(cm)	1.49	0.24	0.075
7	Grain length (mm)	9.06	0.06	0.018
8	Grain width (mm)	2.43	0.1	0.031
9	1000 seed weight	25.47	0.36	0.11
10	Seed weight per/plant	47.7	4.28	1.35

SE-0.83

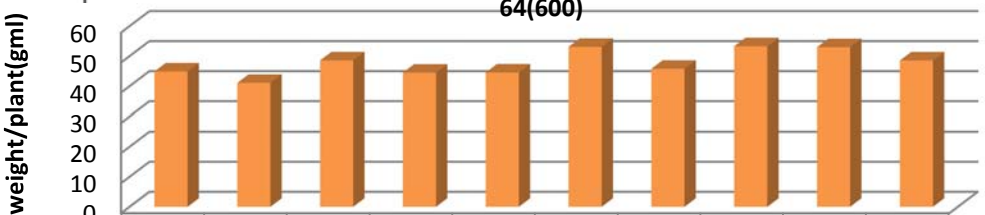
Fig 4.21Plant height of ten random selected plant of DHs line(S-17 x IR-64(600)



	1	2	3	4	5	6	7	8	9	10
PH	105	106	104	107	103	102	106	108	101	100

SE-1.35

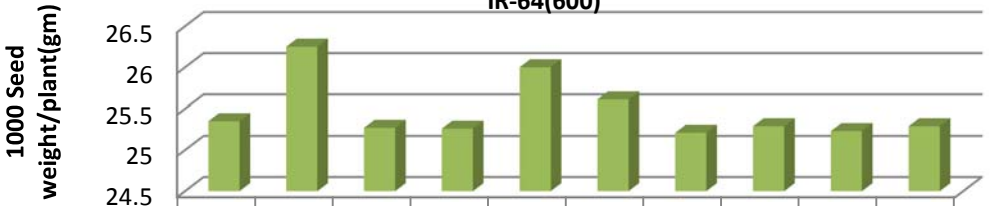
Fig 4.22 Seed weight/plant of ten random selected plant of DHs line(S-17 x IR-64(600)



	1	2	3	4	5	6	7	8	9	10
SW	44.88	41.06	48.55	44.49	44.53	53.06	45.77	53.27	52.96	48.52

SE-0.11

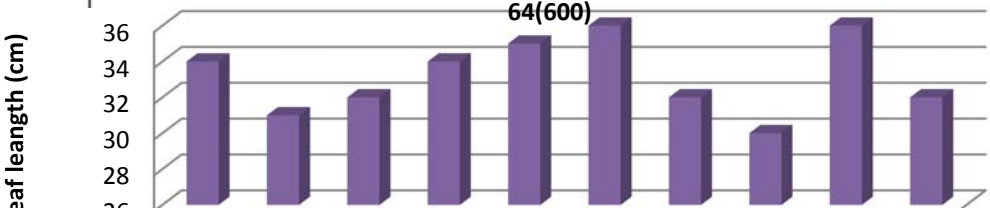
Fig 4.23 1000 Seed weight/plant of ten random selected plant of DHs line(S-17 x IR-64(600)



	1	2	3	4	5	6	7	8	9	10
1000SW	25.35	26.25	25.27	25.26	26	25.61	25.21	25.29	25.23	25.29

SE-0.66

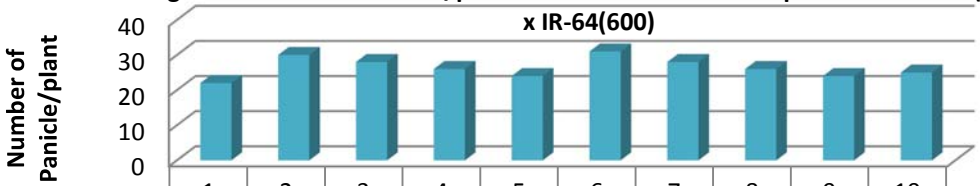
Fig 4.24 Flag leaf leangth of ten random selected plant of DHs line (S-17 x IR-64(600)



	1	2	3	4	5	6	7	8	9	10
FLL	34	31	32	34	35	36	32	30	36	32

SE-0.8

Fig 4.25 Number of Panicle/plant of ten random selected plant of DHs line (S-17 x IR-64(600)



	1	2	3	4	5	6	7	8	9	10
PN	22	30	28	26	24	31	28	26	24	25

4.2.5. Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x IR-64(114)

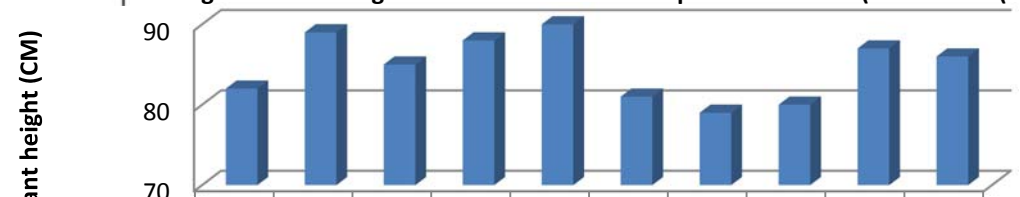
The DHs line S-17 x IR-64(114) showed mean performance of different character like plant height 84.7 (cm), Panicle length 21.4 (cm), Number of tiller 29.7, Number of panicle 28.7, Flag leaf length 27.6 (cm), Flag leaf width 1.36 (cm), Grain length (mm) 8.71 (mm), Grain width 2.36 (mm), 1000 seed weight 21.4(gm), Seed weight per/plant 49.51(gm) and also showed less degree of standard error of all character that can be confirm the DHs line should be homozygous and uniform data given in (tab.4.11).

Table no 4.11: Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x IR-64(114)

S-17 x IR-64(114)				
S.N.	**character**	**Mean**	**SD**	**SE**
1	Plant height (cm)	84.7	3.74	1.18
2	Panicle length	21.4	1.56	0.49
3	Number of tiller	29.7	3.92	1.24
4	Number of panicle	28.7	3.46	1.09
5	Flag leaf length (cm)	27.6	2.05	0.4
6	Flag leaf width(cm)	1.36	0.24	0.07
7	Grain length (mm)	8.71	0.16	0.05
8	Grain width (mm)	2.36	0.04	0.01
9	1000 seed weight	21.4	0.41	0.12
10	Seed weight per/plant	49.51	7.04	2.22

SE-1.18

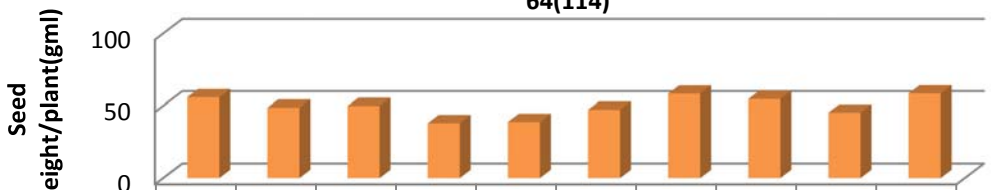
Fig 4.26 Plant height of ten random selected plant of DHs line(S-17 x IR-64(114)



	1	2	3	4	5	6	7	8	9	10
PH	82	89	85	88	90	81	79	80	87	86

SE-2.22

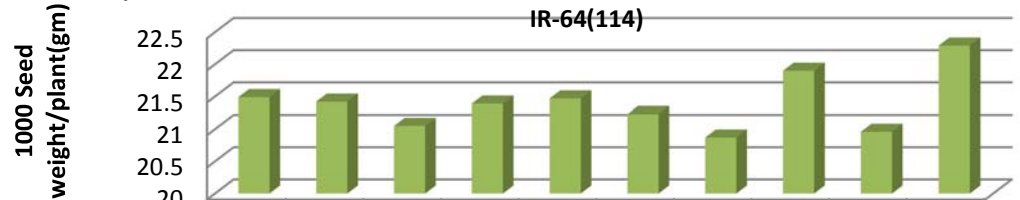
Fig 4.27 Seed weight/plant of ten random selected plant of DHs line(S-17 x IR-64(114)



	1	2	3	4	5	6	7	8	9	10
SW	55.86	48.72	49.85	37.94	38.78	47.24	58.34	54.66	45.32	58.44

SE-0.12

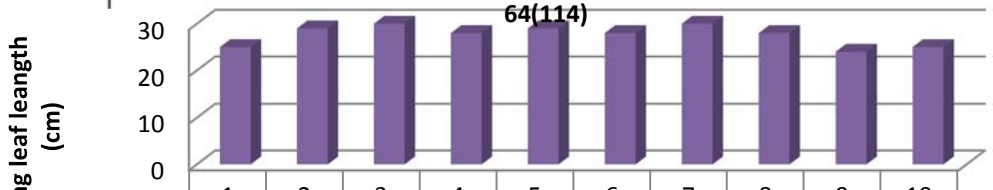
Fig 4.28 1000 Seed weight/plant of ten random selected plant of DHs line(S-17 x IR-64(114)



	1	2	3	4	5	6	7	8	9	10
1000SW	21.49	21.42	21.05	21.39	21.47	21.23	20.87	21.9	20.96	22.29

SE-0.4

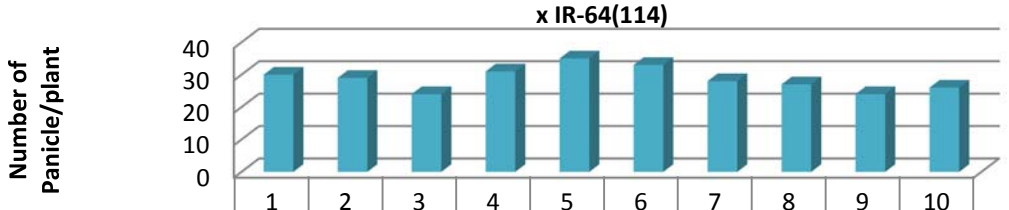
Fig 4.29 Flag leaf leangth of ten random selected plant of DHs line (S-17 x IR-64(114)



	1	2	3	4	5	6	7	8	9	10
FLL	25	29	30	28	29	28	30	28	24	25

SE-1.09

Fig 4.30 Number of Panicle/plant of ten random selected plant of DHs line (S-17 x IR-64(114)



	1	2	3	4	5	6	7	8	9	10
PN	30	29	24	31	35	33	28	27	24	26

4.2.6. Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S17 x RP-BIO(1246)

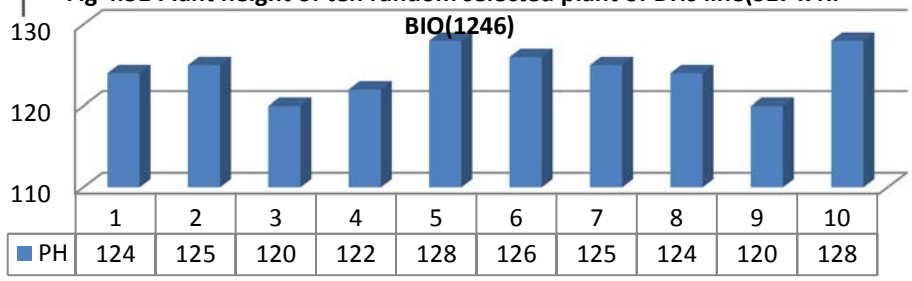
S17 x RP-BIO(1246) showed mean performance of different character like plant height 124.2 (cm), Panicle length 24 (cm), Number of tiller 17.1, Number of panicle 16.5, Flag leaf length 29.7 (cm), Flag leaf width 1.52 (cm), Grain length (mm) 9.23 (mm), Grain width 2.33 (mm), 1000 seed weight 25.39 (gm), Seed weight per/plant 49.67 (gm) and also showed less degree of standard error of all character that can be confirm the DHs line should be homozygous and uniform data given in tab.4.12

Table no 4.12: Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S17 x RP-BIO(1246)

S17 x RP-BIO(1246)				
S.N.	**Character**	**Mean**	**SD**	**SE**
1	Plant height (cm)	124.2	2.71	0.85
2	Panicle length	24	1.34	0.42
3	Number of tiller	17.1	1.7	0.53
4	Number of panicle	16.5	1.6	0.56
5	Flag leaf length (cm)	29.7	1.79	0.56
6	Flag leaf width(cm)	1.52	0.153	0.04
7	Grain length (mm)	9.23	0.45	0.14
8	Grain width (mm)	2.33	0.06	0.01
9	1000 seed weight	25.39	1.44	0.45
10	Seed weight per/plant	49.67	7.44	2.35

SE-0.85

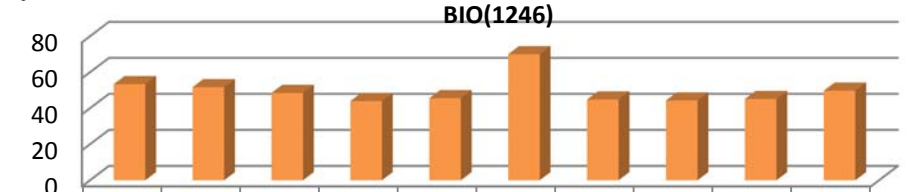
Fig 4.31 Plant height of ten random selected plant of DHs line(S17 x RP-BIO(1246)



	1	2	3	4	5	6	7	8	9	10
PH	124	125	120	122	128	126	125	124	120	128

SE-2.35

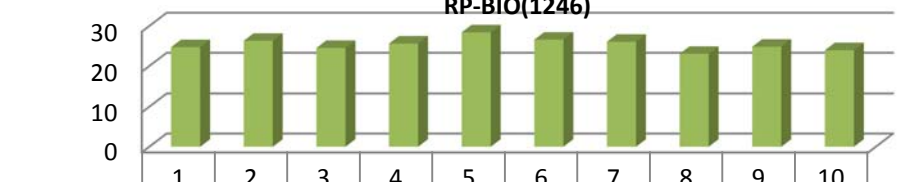
Fig 4.32 Seed weight/plant of ten random selected plant of DHs line(S17 x RP-BIO(1246)



	1	2	3	4	5	6	7	8	9	10
SW	53.32	51.66	48.46	43.94	45.51	69.95	44.8	44.3	45.1	49.7

SE-0.45

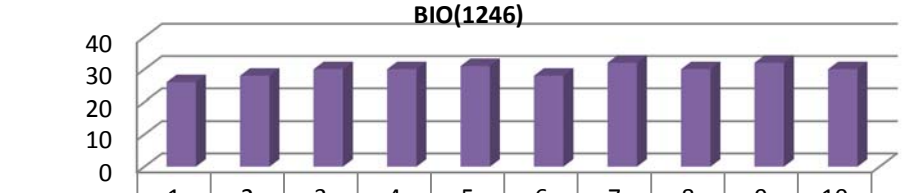
Fig 4.33 1000 Seed weight/plant of ten random selected plant of DHs line (S17 x RP-BIO(1246)



	1	2	3	4	5	6	7	8	9	10
1000SW	24.7	26.3	24.5	25.6	28.4	26.58	26.05	23.04	24.81	23.94

SE-0.56

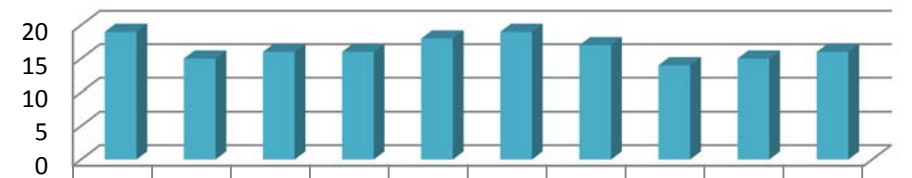
Fig 4.34 Flag leaf leangth of ten random selected plant of DHs line (S17 x RP-BIO(1246)



	1	2	3	4	5	6	7	8	9	10
FLL	26	28	30	30	31	28	32	30	32	30

SE-0.56

Fig 4.35 Number of Panicle/plant of ten random selected plant of DHs line (S17 x RP-BIO(1246)



	1	2	3	4	5	6	7	8	9	10
PN	19	15	16	16	18	19	17	14	15	16

4.3. Molecular characterization of DHs rice.

SSR is very cost effective and codominant and powerful DNA marker to easly differentiate between double haploid and diploid. SSR marker was successfully used for evaluation of genetic diversity in rice.SSR analysis shows the uniformity as well confirms the origin of DH lines, which is consistent with an earlier study by Zhang.

In current experiment 6 different DH lines of 6 different crosses S-17 x RYT-3275(185) , S-17 x PB(717)), S-17 x IR-64(78), S-17 x IR-64(600), S-17 x IR-64(114), S17 x RP-BIO(1246) were selected for SSR marker analysis. SSR marker can be efficiently used to check the homozygosity or heterozygosity if there, with in lines. Allelic variation should be absent in doubled haploid , which can be revealed by using multiple SSRs for multiple locus of rice. Presence of multiple alleles (polymorphic) will confirm diploid plant out of unwanted anther walls (somatic diploids) during anther culture and presence of (monomorphic) band will confirm pollen generated haploid and DHs plants. In the 1st study we have use 130 no of SSRs marker of different locus of chromosome to check for homozygosity and uniformity of the 6 DHs line out 130 SSR marker 70 SSR marker has amplified and 6 has show monomorphic band conforms homozygous plant.ie DHs plant.(fig-4.34)

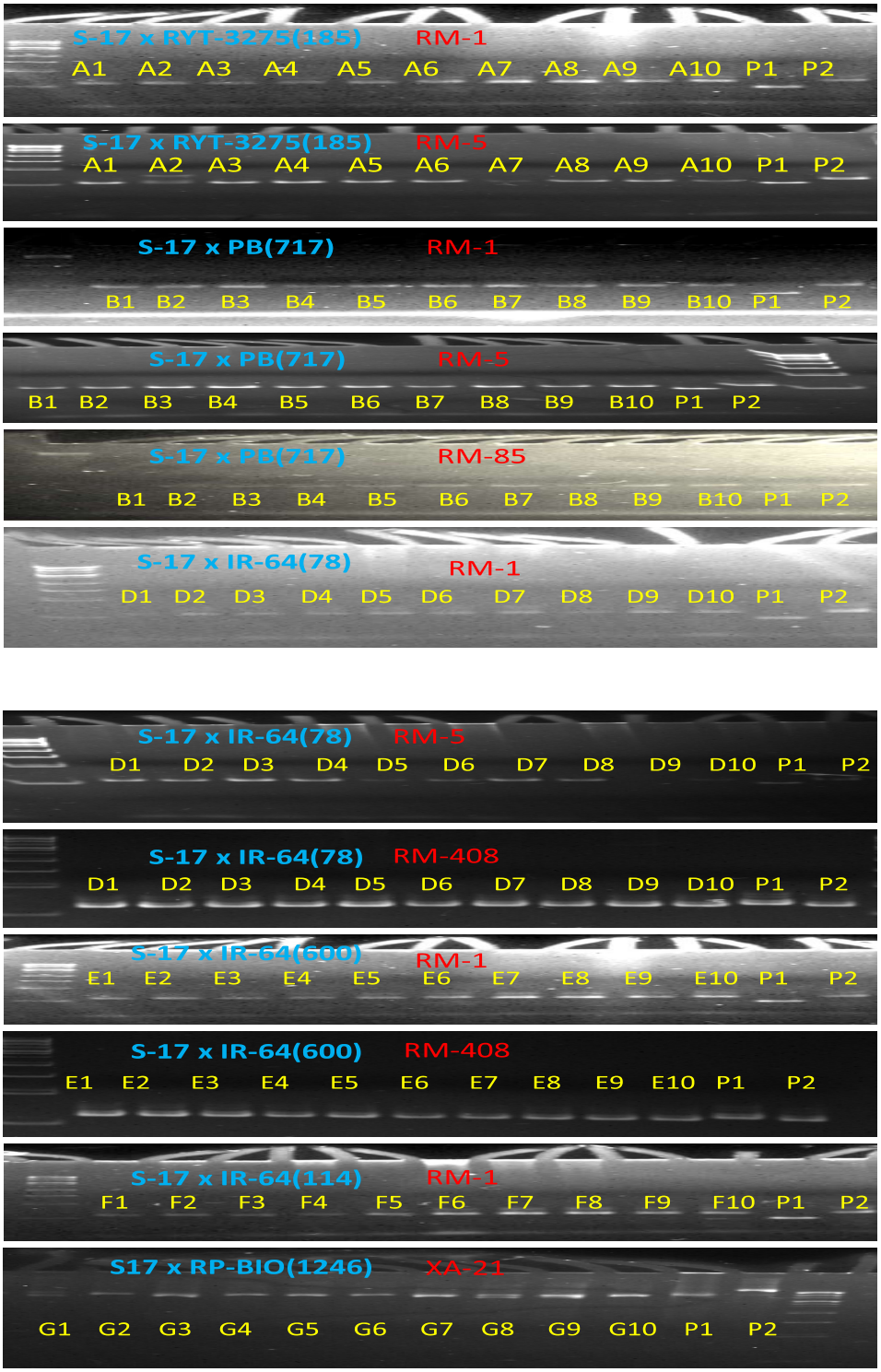


Fig no. 36 Assessment of homozygosity using SSR markers.

CHAPTER– V

SUMMARY AND CONCLUSION

In the present study, emphasis is given to haploid induction develop through anther culture. *In-vitro* androgensis *via* anther-microspore culture is most preferred techniques for obtaining haploids The production of haploids and DHs provides a particularly attractive biotechnological tool, to produce homozygous plants which has a significant impact on agricultural systems. Research efforts on the enhancement of response to anther culture have been confined mostly on manipulation of callus induction and plant regeneration protocols (Munesh *et al*., 2015).

5.1 Production of doubled haploid rice through anther culture.

The collected wrapped boots were sterilized with clean water then removed lower leaves and washed with 70 % ethanol and kept in 10 ˚C for 10 days in MTU1010 x Dagaddeshi (F3), and Swarna sub-1 x IR 90019-17-159-B (F5) to fix the meiotic division in pollen mid microspore and late uninuclear stage ensuring haploid large number of green plant generation. After cold pretreatment, the anthers were excised out and dusted to petriplates containing N6 media supplemented with 3 % maltose 0.8 % agar and 2 ml/l 2,4-D. In Swarna sub 1 x IR 90019-17-159-B (F5) T11,T15 media callus greening percent was 12.91% and 21.21% observed after 15 days of callus transfer. Highest callus greening was T15 (21.21%) followed by T11 (12.91%). There was no callus greening observed in control (without hormone).

In MTU1010 x Dagaddeshi on an average 461 callus was subjected to 2 different treatments T11, T15 along with control (without hormone) shown in (table 4.4). These 2 treatments along with control, T11 (01: 01: 02 (NAA:KIN:BAP) mg/l, (1/2 MS + 1.5S+0.8AGAR) and T15 (0.5 : 0.5 : 1.5 (NAA:KIN:BAP) mg/l ,1/2 MS + 1.5S + 0.8AGAR) has responded for greening of the callus. In T11 and T15 callus greening percent was 2.47% and 3.15% observed after 15 days of callus transfer. Highest callus greening was with T15 (3.15%) followed by T11 (2.47%) . There was no callus greening observed in treatment without hormone.

In Swarna sub 1 x IR 90019-17-159-B (F5), callus regeneration percentage was higher compare to MTU1010 x Dagaddeshi (F3). The callus regeneration % was moderate in both the crosses. Several works has report the callus regeneration % in anther culture to be very low. (Munesh *et al*., 2015) reported that T15 was found to be the best with 8.33 % of green callus regeneration and 32 green plant regeneration. However some work report high callus induction %.(Herath *et al* 2007) reported that callus regeneration percent was 41.0% in F1 hybrids. Reiffers, *et al.* (1990) obtained frequency 29% in plant regeneration. (Rukmini *et al* 2013) reported callus regeneration was 36.22 % in CRHR-5 and CRHR-7

After callus greening, the callus were transferred to green plant regeneration media (shooting media). All the callus were transfer to the same media for green plant regeneration. In cross Swarna sub-1 x IR 90019-17-159-B , total 1647 number of callus were inoculated ,out of which 320 callus has became green which was further transferred to shooting media.T15 has generated highest 289 green callus. Out of which 25 contaminated by the time of subculture or incubation at growth chamber. And 254 total number of green plant were produced.(Tab.4.5)

In cross MTU1010 x Dagaddeshi , total 461 number of callus was inoculated out of which 14 callus became green which was further transferred to shooting media.T15 has generated highest 12 green callus. Out of which 5 green callus became contaminated by the time of subculture or incubation at growth chamber. 7 total number of green plant were produced (Munesh *et al.,* 2015). T15 was found to be best as it has produce 261 numbers of green plants (tab.no.4.5 and 4.6). The green plant are planted in hydroponics (greenhouse) and field. This is the result recorded till the submission of the thesis, observation for DHs production is still continued.

5.2 Morphological characterization of doubled haploid rice progenesis to establish the homozygosity.

To differentiate between diploid and DH plants. Morphological characters and molecular marker can be used. Morphological DUS assay and SSR marker are used for differentiate DHs from were selected (DHs line of kharif 2015). 60 plants randomly selected from all 6 DH lines (S-17 x RYT-3275(185) , S-17 x PB(717), S-17 x IR-64(78), S-17 x IR-64(600), S-17 x IR-64(114), S17 x RP-BIO(1246)

individually were assessed for their genetic stability and homozygosity using SSR markers. DUS assay using different character like plant height , Panicle length, Number of tiller , Number of panicle, Flag leaf length , Flag leaf width, Grain length, Grain width, 1000 seed weight , Seed weight per/plant were showed less degree of standard error (0.83-0.018) of all character that can be confirm that DHs lines are homozygous and uniform in nature.

5.3. Molecular characterization of doubled haploid rice.

SSR is very cost effective and codominant and powerful DNA marker to easly differentiate between double haploid and diploid. SSR marker was successfully used for evaluation of genetic diversity in rice.SSR analysis shows the uniformity as well confirms the origin of DH lines, which is consistent with an earlier study by Zhang. In current experiment 6 different DH lines of 6 different crosses S-17 x RYT-3275(185) , S-17 x PB(717)), S-17 x IR-64(78), S-17 x IR-64(600), S-17 x IR-64(114), S17 x RP-BIO(1246) were selected for SSR marker analysis. SSR marker can be efficiently used to check the homozygosity or heterozygosity if there, with in lines. Allelic variation should be absent in doubled haploid , which can be revealed by using multiple SSRs for multiple locus of rice. Presence of multiple alleles (polymorphic) will confirm diploid plant out of unwanted anther walls (somatic diploids) during anther culture and presence of (monomorphic) band will confirm pollen generated haploid and DHs plants. In the 1st study we have use 130 number of SSRs marker of different locus of chromosome to check for homozygosity and uniformity of the 6 DHs line out 130 SSR marker 70 SSR marker has amplified and 6 has show monomorphic band conforms homozygous plant.ie DHs plant.(fig-4.34)

CONCLUSION:-

- In cross Swarna sub-1 x IR 90019-17-159-B, callus induction response was best 23.44 % as compared to MTU1010 x Dagaddeshi, callus induction response was 12.19% during Kharif season .
- In cross Swarna sub-1 x IR 90019-17-159-B, best callus greening was 21.21 % in T15 regeneration media as compared to MTU1010 x Dagaddeshi (F3), callus greening was 3.15% in T15 regeneration media.
- T15 was found to be best as it has produce 254 number of green plant were in Swarna sub 1 x IR 90019-17-159-B and 5 number of green plant MTU1010 x Dagaddeshi.
- DUS assay character like plant height , Panicle length, Number of tiller , Number of panicle, Flag leaf length , Flag leaf width, Grain length, Grain width, 1000 seed weight , Seed weight per/plant has showen less degree of standard error (0.83-0.018) in all characters that confirms t homozygosity in these lines.
- Molecular analysis ,we have use 130 number of SSRs marker of different locus of chromosome to check for homozygosity and uniformity of the 6 DHs line out 130 SSR marker 70 SSR marker has amplified and 6 has shown monomorphic bands. Which confirms that the generated lines were complete DHs lines.

REFERENCES

Araújo, L. G. De, Prabhu, A. S., Antonio, P., Pereira, A., & Barata, G. (2010). Marker-assisted selection for the rice blast resistance gene Pi-ar in a backcross population, 23–31.

Brar, D.S. and Khush, G.S. (2007). Utilization of wild species of genus *Oryza* in rice improvement. Monograph on genus *Oryza*.,283-309.

Hemaprabha K, Hemalatha T, UmaMaheswari T, Anbukkarasi K, Shanmugasundaram P (2013).Genetic diversity analysis of anther culture derived rice plants using molecular markers. International Journal of Advanced Research, ISSN NO 2320-5407,Volume 1, Issue 5, 103-112.

Herath, H.M.I. and Bandara, D.C. (2011).J.Natn.Sci.Foundation Sri Lanka, 39 (2): 149-154.

Hung Nguyen, Xiao-Yang Chen, Meng Jiang, Qing Wang, Li Deng , Wei-Zhang Zhang and Qing-Yao Shu (2016). Development and molecular characterization of a doubled haploid population derived from a hybrid between japonica rice and wide compatible indica rice. Journal Of Breeding Science 66: 552–559.

Ishra, R. M., Jwala, G., Ao, N. R., Ao, R. N. R., & Aushal, P. K. (2015). Development and characterization of elite doubled haploid lines from two *indica* rice hybrids, 22(6), 290–299.

J. Zhang · H.G. Zheng · A. Aarti G. Pantuwan · T.T. Nguyen · J.N. Tripathy A.K. Sarial · S. Robin · R.C. Babu · Bay D. Nguyen S. Sarkarung · A. Blum Henry T. Nguyen (2001). Locating genomic regions associated with components of drought resistance in rice: Comparative mapping within and across species. Theor Appl Genet 103:19–29.

Javed, Muhammad A.,Takashige Ishii , Osamu Kamijima , Shuji Misoo (2007). "The role of alternating culture temperatures and maltose in enhancing the anther culture efficiency of salt tolerant indica rice (Oryza sativa L.)

cultivars, Pokkali and Nona Bokra." Plant biotechnology24.3: 283-287.

Ma, Liangyong, Bao J, Guo L, Zeng D, Li X, Ji Z, Xia Y, Yang C, Qian Q (2009).. "Quantitative trait loci for panicle layer uniformity identified in doubled haploid lines of rice in two environments." Journal of integrative plant biology 51.9: 818-824

Maria, A.M., Adriana S, Ana MG (2010). Plant regeneration from rice anthers cryopreserved by an encapsulation/deshydradationTechnique. In vitro cell. Dev. Biol. Plant 42: 31-36.

Mohamed Najeb Barakat, Abdullah Abdlulaziz Al-Doss, Adel Ahmed Elshafei, Abdelhalim Ibrahim Ghazy, Khaled Ahmed Moustafa (2013). Asutrailion journal of crop science. 7(1):104-111

Nupur, N., Prachitara, R., Lakhan, V. R. A. M., Ngangkham, U., Lal, K. J., Nath, S. O., & Sanghamitra, S. (2016). Research article development of promising doubled haploids from indica rice hybrid : evaluation of uniformity and stability through morphological and molecular markers, 8(47), 1987–1992.

Premvaranon, P., Vearasilp, S., Thanapornpoonpong, S., Karladee, D., & Gorinstein, S. (2011). "In vitro studies to produce double haploid in Indica hybrid rice." Biologia 66.6 (2011): 1074-1081.

Purwoko, Bambang S., Iswari S. Dewi, and Nurul Khumaida (2010). "Rice anther culture to obtain doubled-haploids with multiple tolerances." Proceedings Asia Pacific Conference on Plant Tissue and Agribiotechnology (APaCPA). Vol. 17.

Rasoazanakolona, V., Rabealaina, B. B., Andrianjaka, A., Rakotonjanahary, X., Sangwan, R. S., & Rakotoarisoa, N. V. (2016).l. "Evaluation of Grain Quality and Nutritional Quality of Double Haploid DHP6, An Elite Rice Line in Madagascar." Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences.. Vol. 70. No.

Rukmini, M., G. J. N. Rao, and R. N. Rao (2013). "Effect of cold pretreatment and phytohormones on anther culture efficiency of two *Indica* rice (*Oryza Sativa* L.) Hybrids-Ajay and Rajalaxmi." J. Exp. Biol. Agr. Sci 2: 69-76.

Sahu, M., Minj, A., Chopkar, R., Jha, Z., & Verulkar, S. B. (2015). Production of double haploid population in two *indica* rice (*Oryza sativa* L.) CROSS SAFRI-17XIR-64 AND MTU1010 VARIETY. Journal of Cell and Tissue Research 15.3 (2015): 5347.

Siddique, Romana (2015). "Impact of different media and genotypes in improving anther culture response in rice (*Oryza sativa*) in Bangladesh." European Scientific Journal, ESJ 11.6.

Usenbekov, B. N., Kaykeev, D. T., Yhanbirbaev, E. A., Berkimbaj, H., Tynybekov, B. M., & Satybaldiyeva, G. K. (2014). "Doubled haploid production through culture of anthers in rice." Indian Journal of Genetics and Plant Breeding (The) 74.1 (2014): 90-92.

Resume

Name : Prem Narayan Patel

Date of birth : 1 may 1992

Present Address : Room No. 105, Sundaram Boys Hostel, COA, IGKV, Raipur, C.G.

Phones : 07772993301

E. mail : patelpremnarayan47@gmail.com

Permanent address : Vill- Bairagiya Pukhri, Post- Hinota, Dist.-Chhatarpur(M.P.), Pin co.-471525

Academic Qualification:

Degree	Year	University/Institute
B.Sc.(Agriculture) Science	2015	Dr. PDKV, AKOLA, Maharastra
M.Sc. (Ag.)	2017	College of Agricultural, I.G.K.V. Raipur (C.G.) Plant Molecular Biologyand Biotechnology

Professional Experience (If any) : No

Membership of Professional Societies (If any) : No

Awards / Recognitions (If any) : JNU-DBT Fellowship (2015-2017), NTS-ICAR (2011-2015)

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