IN VITRO INDUCTION OF CHROMOSOME DOUBLING FOR THE PRODUCTION OF DOUBLED HAPLOID RICE AND HOMOZYGOSITY TESTING OF MORPHOLOGICAL CHARACTERIZATION AND MOLECULAR APPROACH

M.Sc. (Ag) Thesis

by

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FACULTY AGRICULTURE

INDIRA GANDHI KRISHI VISHWAVIDYALAYA RAIPUR
(Chhattisgarh) 2017

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Thesis

Submitted to the

Indira Gandhi Krishi Vishwavidyalaya, Raipur

by

YOGENDRA TEKAM

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

in

Plant Molecular Biology And Biotechnology

Roll No.120115124 **ID No. 20151622538**

JULY, 2017

CERTIFICATE-I

This is to certify that the thesis entitled "***In vitro* induction of chromosome doubling for the production of doubled haploid rice and homozygosity testing of morphological characterization and molecular approach**" 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 **Yogendra Tekam** 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: 31-7-17

Chairman

THESIS APPROVED BY THE STUDENT'S ADVISORY COMMITTEE

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CERTIFICATE-II

This is to certify that the thesis entitled "***In vitro* induction of chromosome doubling for the production of doubled haploid rice and homozygosity testing of morphological characterization and molecular approach"** submitted by **Yogendra Tekam** 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: 9/9/17

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Dean/ Dean Faculty

Approved / Not approved

Director of Instructions

ACKNOWLEDGEMENTS

My first and foremost gratitude to my beloved parents and my family members for their encouragement, sincere prayers, expectations and blessings which have always been the most vital source of inspiration and motivation in my life.

It is a pleasure to thank those who helped me to complete this work. First and foremost, I would like to thank my *major advisor, Dr. (Smt.) Zenu Jha Assistant Professor, Department of Plant Molecular Biology and Biotechnology; IGKV, Raipur for* his thoughtful guidance, *supervision* and *consistent support to my studies and research.*

I wish to record my sincere thanks to Dr. S. K. Patil Hon'ble Vice Chancellor, Dr. S. S. Rao Dean, Dr. J. S. Urkurkar Director Research Services and Dr. S. S. Shaw, Director of Instructions, Dr. Madhav Pandey Librarian IGKV, Raipur for their help both administrative and technical which facilitated my research work.

I would like to express my cordial appreciation to *Dr. S. B. Verulkar, Professor and Head; Dr. Girish Chandel, Professor; Dr. (Smt.) Shubha Banerjee Assistant Professor; Dr. (Smt.) Archana S. Prasad Assistant Professor; Dr. (Smt.) Kanchan Bhan Assistant Professor, Department of Plant Molecular Biology and Biotechnology, IGKV, Raipur; Dr. (Smt.) Shubha Banerjee Assistant Professor , Department of Plant Molecular Biology and Biotechnology, , IGKV, Raipur for their valuable suggestions and providing necessary facilities in completing the research work.*

I express my deep sense of gratitude and sincere thanks to my advisory committee member Dr. *R, R. Saxsena Professor, Department of Agriculture Statistics; Dr. S.Bhandarkar Professor, Department of genetics and plant breeding, College of Agriculture, Raipur for their valuable suggestions critical comments and help rendered as and when needed*.

My respected seniors *Arpita mahobia madam, Pratik sir, Ashish sir, Sujata madam ,Deepak sir , Ajit Mannade sir, Vikrant sir, Sanjay sir, Ashish sir, Vinay sir,Arun sir were involved*

In this work directly or indirectly. I hereby extend my sincere gratitude and appreciation to all of them who made this thesis possible.

I extend my deepest thanks to my beloved seniors, colleagues Mr.Prem patel, Mr.Rishi raghuvansi, Mr.Indrapal patel, Mr. Satish navrang, and juniors, whose supports were valuable and very helpful to my research work.

My deepest sense of gratitude towards my lab buddies, Ms. Rita madam, Ms. Manju, Mr. Khubchand, Mr. Nitish, Mr. Manish, Ms. Anjali, Ms. Madhu, for their supportive hearts and made it a convivial place to work.

I extend my sincere thanks to all helpers in department specially Mr. Moti, Mr. Anil, Mr.Sujeet, Mr. Mohit, Mr. Durgesh, Ms. Annu, Mr.Sanat, Mr. Manoj, Mr. Dikesh, Suraj, Ram and Ms Meena ,others for helping me throughout the work.

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Date: 21-7-17

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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)
COL	Cholchicine
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
MS	Murashige and Skoog (1962) basal medium
N6	Chu (1978) callus induction media
NAA	a-naphthalene acetic acid
NaOCl	Sodium hypochloride
Mm	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
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
SN	Serial number
Psi	pound per square inch
QTLs	Quality trait loci
H	Hours
uv	Ultraviolet light
v/v	Volume by volume
w/v	Weight by volume

THESIS ABSTRACT

a) Title of the Thesis	:	*In vitro* induction of chromosome doubling for the production of doubled haploid rice and homozygosity testing of morphological characterization and molecular approach.
b) Full name of the student	:	Yogendra Tekam
c) Major Subject	:	Plant Molecular Biology and Biotechnology
d) Name and Address of the Major Advisor	:	Dr. Smt. Zenu Jha Associate Professor, Department of Plant Molecular Biology and Biotechnology, Collage of Agricuture, IGKV Raipur
e) Degree to be Awarded	:	M.sc. (Agriculture, Plant Molecular Biology and Biotechnology)

Signature of the Student

Signature of Major Advisor

Date - 21-7-17

Signature of Head of the Department

ABSTRACT

Anther culture generates haploid plants and if spontaneous chromosome doubling cannot bear seeds. so it is no use until being, diploidized by chromosome doubling agents. The current research is being focused on production of double haploid plants from haploid plants using *in vitro* colchicines treatment. Two crosses Swarna sub 1 x IR 159-B , MTU 1010 x Dagardesi and one variety RF-75 were studied for their responses in callus induction, green callus regeneration and

green plant regeneration by using N6 media and MS media with different doses of colchicine treatment under *in vitro* condition. Anthers and callus both were subjected to colchicines treatment anther treatment did not respond in all the 3 sample where as the callus treatment with colchicines repond well. In RF-75, 13.05 % of callus was inducing, 11.84 % in Swarna sub 1 x IR 90019-17-159-B and MTU 1010 x Dagardesi, callus induction was 8.61%. The induce callus was transferred to different media(T11 and T15) with different colchicine treatments (0.05% or 0.1%) for 2, 6, 24, 48 hours respectively along with control condition (without colchicine). In Swarna sub 1 x IR159-B highest callus greening percent (47.82%) was found in T15 media containing 0.1% Colchicine in 48 hours. In MTU 1010 x Dagardesi highest callus greening percent (12.5%) was found in T15 media containing 0.1% Colchicine in 48 hours.In RF-75 highest callus greening percent (26.92.%) was found in T15 media containing 0.1% Colchicine in 48 hours. Total of 25 plant has been generated in Swarna sub 1 x IR159-B . In MTU 1010 x dagardesi total of 6 plant generated and total of 23 plant has been generated in RF 75. These plants are present in field.

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). 70 plants randomly selected from all 7 DH lines S-17 x PR-122 (150), S-17 x RP BIO (112A), S-17 x RP BIO (235), S-17 x IR64 (185), S-17 x IR64 (203), S-17 x RP BIO (1353), S-17 x RP BIO (228) individually were assessed for their genetic stability and homozygosity using SSR markers. In morphological 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 of all character that can be confirm that DHs lines are homozygous and uniform in nature.

In molecular analysis we have use 130 no of SSRs marker of different locus of chromosome to check for homozygosity and uniformity of the 7 DHs line out of 66 SSR marker are amplified and 5 marker are shown parental polymorphism that confirm to DH lines. These lines were at trail under MLT for there evaluation.

शोध सारांश

अ)	शोध का शीर्षक	:	कृत्रिम परिवेषीय में गुणसूत्र द्विगुणन द्विगुणित धान के उत्पादन में और आकारिकीय व आण्विक समरुपता परिक्षण
ब)	छात्र का पूरा नाम	:	योगेन्द्र टेकाम
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

छात्र के हस्ताक्षर

दिनांकः - 21-7-17

विभागाध्यक्ष के हस्ताक्षर

शोध सरांश

परागकोष संवर्धन में अगुणित पौधों का उत्पादन होता है अगुणित पौधे स्वतः बीज का उत्पादन नही कर पाते इसलिए इनका उपयोग गुणसूत्र द्विगुणन कारक के उपयोग ना किये जाने तक नही किया जा सकता वर्तमान अनुंसधान अगुणित पौधों में कृत्रिम परिवेषीय कोलचीसिन उपचार के द्वारा द्विगुणित पौधों के उत्पादन पर केन्द्रित है। दो संकर स्वर्णा आई आर 159 बी , एम टी यू –1010 x दगडदेषी व एक किस्म आर एफ 75 के कैलस प्रेरण, हरे कैलस प्रेरण और हरे पौधे के प्रेरण के लिए एन6 व एम एस माध्यम का उपयोग करके उनकी प्रतिकियाओं के लिए अध्ययन किया गया। कृत्रिम परिवेषीय दषायें में कोलचीसीन की उपचार परागकोष व कैलस दोनों में थी लेकिन परागकोष के उपचार द्वारा तीनों नमूनों में कोई भी परिणाम नही मिला जबकि कैलस में कोलचीसीन उपचार

अच्छा रहा। आर एफ 75 में 13.05 प्रतिषत् और स्वर्णा आई आर 159 बी में 11. 84 प्रतिषत् और एम टी यू–1010 x दगडदेषी में 8.61 हरे कैलस प्राप्त हुए । प्रेरित कैलस को अलग–अलग कोलचीसीन उपचार 0.05 प्रतिषत् व 0.1 प्रतिषत् के साथ क्रमषः 2,6,24,48,घण्टों के साथ अलग–अलग माध्यम टी11, टी15, माध्यम में स्थानांतरित किया गया था। साथ ही साथ नियत्रंण की स्थिति (कोलचीसीन रहित) में भी स्थानांतरित किया गया उच्चतम कैलस हरियाली प्रतिषत् स्वर्णा आई आर 159 बी 47.82, एम टी यू–1010 x दगडदेषी 12.5 व किस्म आर एफ 75 में 26.92 प्रतिषत् उच्चतम हरियाली प्रतिषत् टी15 0.1 प्रतिषत् कोलचीसीन के माध्यम में 48 घण्टों में रहा । स्वर्णा में कुल 25, एम टी यू–1010 x दगडदेषी में 6 व आर एफ 75 में 23 हरे पौधे प्राप्त हुए जो क्षेत्र में मौजूद है।

द्विपादीय और द्विगुणित पौधे के बीच अन्तर करनें के लिए आकारिकी लक्षण और आण्विक 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 7 द्विगुणित लाईनों एस 17 x आई आर 64 (185), एस 17 x आई आर 64 (203), एस 17 x आर पी बायो (112 ए), एस 17 x पी आर 122 (150),एस 17 x आर पी बायो (1353), एस 17 x आर पी बायो (228),एस 17 x आर पी बायो (235) से अनियमित रुप से 70 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 सभी मानक में जों इन पंक्तियों में समरुपता की पुष्टि करता है आण्विक विष्लेषण में हमनें 130 एस एस आर मार्कर उपयोग किया जिसमें 66 एस एस आर मार्कर प्रदर्षित हुए पर इनमें पैतृक विविधता नही पाई गई पर 5 मार्कर मे पैतृक विविधता पाई गई और 10 फसल नमूने में समरुपता व समानता की पुष्टि हुए 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 is the most important staple food in Asia. More than 90% of the world's rice is grown and consumed in Asia, where 60% of the world's population lives. The production of haploid plants through anther culture, followed by chromosome doubling, provides plant breeders with a useful method for the development of doubled haploid (DH) lines. Production of DH through anther culture is a rapid approach to shortens the time required for the development of new rice cultivars as compared to conventional methods, which require at least 6-7 generations. Most of these applications have been limited to crosses involving at least one *japonica* parent due to the recalcitrant nature of *indica* rice varieties. *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.

The creation of high yielding rice varieties, good grain quality begins with knowledge of biotechnology combined with traditional experiences will produce results faster and more accurate. Many varieties of rice produced by tissue culture techniques and *in vitro* selection has improved some of the characteristics of shape, nutritional quality, pest resistance and the harsh conditions of the environment.

India is an important centre of rice cultivation. Rice is an important crop grown in nearly 44 million hectare of land in the country with the productivity of 2.2 tonn/hectare which is less than the productivity of many countries. Historians believe that while the *indica* variety of rice was first domesticated in the area covering the foothills of the Eastern Himalayas (i.e. north-eastern India), stretching through Burma, Thailand, Laos, Vietnam and Southern China, the japonica variety was domesticated from wild rice in southern China which was introduced to India. Perennial wild rice still grow in Assam and Nepal. It seems to have appeared around 1400 BC in southern India after its domestication in the northern plains. It then spread to all the fertiled alluvial plains watered by rivers.

The Chhattishgarh State has about 3.7 million hectare under rice cultivation which is mostly rainfed, covering both uplands and shallow low lands. The state average productivity is about 1.3 tonnes/hectare. The major constraints in production are low coverage under high yielding varieties, drought in uplands, infestation of gall midge, Blast and bacterial leaf blight. Cultivation in rainfed shallow lands with MTU 1010, Bambleswari, IR64, Swarna and with the hybrid, Indira sona.

Anther culture uses haploid cells for *in vitro* culture. Development of new varieties with rapid approach is required to increase the rice production in Chhattishgarh. Anther culture is one of the promising techniques by which new varieties can be developed in very less period of time. Haploid cells are produced from pollen or egg cells or from other cells of the gametophyte.

Anther culture in general produced haploid plants. Haploid plants can not bear seeds. So no use until being diploidized by choromosome doubling agent on haploid plants. So the current research is being focused on produced of double haploid plants from haploid plants using *in vitro* colchicines treatment.

Colchicine inhibits the mitotic spindle of dividing cells and thus induces chromosome doubling. Precisely, colchicine inhibits the formation of microtubules by binding to tubulin, the protein subunit of microtubules.

As the DH is being produced from anther culturing in which we use anthers. Anther contains haploid pollens (from which haploid plants will regenerate) and diploid anther walls. The plants can be regenerated from diploid anther walls which will be identified at very later stages. To avoid the diploid plants and to selected only haploid / DH screening test at DNA leval. Should be adopted markers can be used to differentiate haploid / DH plants and diploid plants by revealing the homozygosity DH / haploid and heterozygosity in diploid plants.

Keeping in mind the above facts the objective of the there is as under.

1. *In vitro* application of colchicine at various stages of anther culture.

2. Morphological characterization of doubled haploid rice progenies to establishing the homozygosity.
3. Assessment of homozygosity in individual doubled haploid population using molecular marker.

CHAPTRE-II
REVIEW OF LITRATURE

Work done in India and else where 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 *In vitro* colchicine application –

The relatively high frequency of doubled haploids needed by plant breeders, requires the application of efficient techniques to induce doubling which must involve high doubling rates, low frequency of damage and minimum plant mortality less time consuming and easy to handle on a practical scale.

Wong (1989) have studied that the Rice nodal segments from three flowering haploids were excised and treated for different lengths of time with 0.3% or 0.4% colchicine (dissolved in 2% DMSO) in an attempt to induce fertile seeds. A combination of higher colchicine concentration and longer hours of treatment reduced the survival rate of treated segments, but more fertile plants were transformed. Pooled data showed that of the 842 segments used, 42.2% survived the treatment and sprouted, but only 31.9% were successfully established and grown to maturity. Among the 269 mature plants, 29,4% produced fertile seeds (panicles) with an average of 146.2 seeds per diploidized plant. We have also noticed same results with application of colchicines in our media for chromosome doubling and regeneration.

Hassawi and Liang (1991) stated that colchicine is one of the most effective antimitotic agents for chromosome doubling.

Matzk and Mahn (1994) cultured the plantlets with one or two leaves and roots in vitro on medium containing 0.02% colchicine for 30 hours for doubling the chromosome number.

Alemanno *et al.,* (1994) have reported that plating rice anthers on a semisolid induction medium containing 250 or 500 mg/l colchicine for 24 or 48

hours incubations followed by transfer to colchicine-free medium and standard anther culture procedures resulted in overall 1.5 to 2.5 fold increases in doubled haploid green plant productions compared to control anther cultures. The addition of colchicine had no detrimental effects on the different anther culture efficiency parameters, but in some treatments led to significant enhancement of anther callusing frequency or callus green plant regenerating ability.

The most efficient treatment raised doubled haploid plant recovery from 31% to 65.5%. These results suggest that post-plating colchicine treatment of anthers, since it was found to improve both anther culture efficiency and doubled haploid plant recovery frequency, could be integrated into rice doubled haploid plant production programmes.

Navarro-Alvarez et al. (1994) found that addition of colchicine to wheat anther culture medium increased the doubled haploid plant production. Increasing the colchicine concentration reduced the number of embryoids produced from 77.4 to 29.9 embryoids/ 100 anthers but did not significantly affect the frequency of plant regeneration and increased the frequency of doubled haploid plants (19.0 to 72.3 DH plants/100 green plants)

Hensen and Andersen (1998) isolated microspore culture of two doubled haploid lines of wheat and treated with 0, 3, 10, 30, 1000 and 3000 µM of colchicine for 24 hours and 48 hours during microspore culture. Highest number of embryos regenerated from 24 hours colchicine treatments while highest frequencies of green plants and fertile plants were obtained from 48 hours colchicines treatment. The highest number of DH plants per spike resulted from treatment with colchicine concentrations of 300-1000 µM.

Zamani et al. (2000) studied the effect of colchicine added to induction medium for the production of doubled haploid plants after in vitro anther culture in one winter and two spring wheat genotypes. They reported that in case of winter wheat variety colchicine treatment resulted in 100% completely fertile plants

Chen et al. (2002) investigated the effect of colchicine treatment on doubled haploid production efficiency. They reported that MS media supplemented

with 100 ml/L colchicine gave maximum chromosome doubling frequency (100%) while in case of colchicine treatment by immersing leaves and roots of plants in colchicine solutions (500 mg/L) yielded 98.2% doubling frequency.

Klistov and Artemeva (2004) utilized two methods for doubled haploid production in spring and winter wheat ecotypes and their hybrids obtained with Aegilops speltoides and Agropyron erectus. In the first method they utilized wheat x maize system in which emasculated wheat and hybrid heads were hand pollinated with maize pollen. Embryos were excised 12-14 days after pollination and cultured on B5 medium. The resulting seedlings were immersed in 0.1% colchicine solution, rinsed and transplanted in pots which were kept in green house. In second method, they used anther and microspore culture technique for doubled haploid production. Wheat and hybrid anthers were isolated immersed in P11 nutrient agar medium for 20-30 days. Embryo like structures were transplanted into medium with 0.5 mg/L Kinetin. They obtained four doubled haploid lines from in vitro culture and 102 doubled haploid lines through wheat x maize system.

Burun *et al.,* (2008) have reported on tobacco (*Nicotiana tabacum* cv. Karabalar 6265) colchicine was applied at 3 different stages of anther culture. Before culture, anthers were treated with 0.4% aqueous solution of colchicine for 0, 2, 4, 6, 8, 10, and 12 hours Culture response of anthers decreased as the treatment duration increased (except 12 hour) and the highest diploidization of 29.7% was obtained with 6 hours. When plantlets with 4 to 8 leaves immersed in 0.2% colchicine for 0, 7, 24 and 48 hours on a shaker, besides 4.3%, 42.3%, 37.8% and 33.3% doubled haploids, respectively, haploids, tetraploids, aneuploids, and mixedploids were also found among the treated plants. When chromosome doubling rate and viability are taken into consideration, among the 3 methods tested, plantlet treatment with 0.2% colchicine for 7 hours appeared to be more efficient with 42.3% dihaploids.

Premvaranon *et al.,* (2011) have reported that the anthers of KDML 105×SPR 1 (*Indica* × *Indica*) were cultured in Linsmaier and Skoog (LS) medium, which contained nutrients, growth regulators for haploid generation. 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(Embryo like structure) 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.

Wurschum *et al.,* (2012) have investigated the applicability of an in vitro approach for chromosome doubling based on microspore culture. Our results show a pronounced increase in the proportion of doubled haploid triticale plants compared to the spontaneous doubling rate, but also compared to the doubling obtained by the standard *in vivo* approach. In addition, the frequency of plants surviving from culture medium to maturity is also much higher for the *in vitro* approach. Colchicine concentrations of 1 mM for 24 hours or 0.3 mM applied for 48 or 72 hours during the first hours of microspore culture performed best

Rukmini *et al.,* (2013) has 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.

2.2 Morphological characterization of doubled haploid rice progenies to establishing the homozygosity

Dewi et al., 2004. studied the Plants with high heterozygous (F1 or F2 is already selected) are used as a source for obtaining anther double haploid lines with genetic diversity and agronomic traits desired. Double haploid plants homozygous (pure strain) from heterozygous plants can be obtained in the first generation.

Bindeshwar Prasad Sah et al.(2007) reported that 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. Despite the sterility and preponderance of wild traits, some of the plants showed useful traits such as increased in panicle and spikelet length, reduced shattering and awning, and large size anther as in O. rufipogon. Two of the regenerants were superior for panicle related traits over either parent. The genotyping study using SSR markers also revealed the substantial variation among regenerants.

Liu et al., (2011) studied the Tillering in rice is one of the most important agronomic traits for grain production because tiller number per plant determines panicle number, a key component of grain yield. Zhu et al., (2011). Furthermore, tiller number usually serves as a suitable model trait for the study of developmental characteristics, since it changes over time. Hence, the genetic elucidation of tiller number has become a focus in rice genetic and breeding research.

Syafii *et al.*, (2011) 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.

Mohiuddin et al.(2014) studied that Data based on seed morphological characteristics of well developed DHLs were subjected to cluster analysis for grouping into types; designated as AC (anther culture) and numbered in ascending order of transferring to earthen pots. At least eight types of variants with a wide range of differences were conceivable including a type similar to the parent, BR802-78-2-1-1. Comparative analysis showed that all the variants had plant height lower than the parent. In respect of effective tillers, an important indicator of grain yield, AC156 and AC192, produced 100% effective tillers and only 4 and 3% grain sterility, respectively. The spikelet number per panicle was 306 and 294 with long-bold and long-slender grain in AC156 and AC192, respectively.

Rukmini Mishra et al.(2015) studied that In vitro culture of the rice anthers resulted in 243 and 186 fertile DH lines of CRHR5 and CRHR7, respectively. Flow cytometric and pollen fertility analyses confirmed the DH ploidy status of the regenerations. 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 5 097–6 965 kg/hm2 for CRHR5 and 5 141–7 235 kg/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, CR712 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 double haploid rice varieties.

2.3 Assessment of homozygosity in individual doubled haploid population using molcular marker.

C. Lu, et al (1996) studied that RFLP mapping of quantitative triat loci (QTLs) that affect some important agronomic traits in cultivated rice. An anther culture derived doubled haploid (DH) population was established from a cross between an indica and a japonica rice variety. On the basis of this population a molecular linkage map comprising 137 markers was constructed that covered the rice genome at intervals of 14.8cM on average. Interval mapping of the linkage map was used to locate QTLs for such important agronomic traits as heading date, plant height, number of spikelets per panicle, number of grains Per panicle, 1000-grain weight and percentage of seed set. Evidence of genotypebyenvironment interaction was found by comparing QTL maps of the same population grown in three diverse environments. A total of 22 QTLs for six agronomic traits were detected that were significant in at least one environment, but only 7 were significant in all three environments, 7 were significant in two environments and 8 could only be detected in a single environment. However, QTL-by-environment

interaction was traitdependent. QTLs for spikelets and grains per panicle were common across environments, while traits like heading date and plant height were more sensitive to environment.

P. He,et al.(2001) reported that comparison of molecular maps and mapped agronomic trait loci between DH and RIL populations derived from the same rice cross, ZYQ8 (indica) × JXI7 (japonica). We investigated six agronomic traits (days to heading, plant height, number of spikelets per panicle, number of grains per panicle, 1000-grain weight, and seed set percentage) and found that five of them did not show significant differences between the two populations. Restriction fragment length polymorphism (RFLP) and microsatellite markers were selected to construct two linkage maps of the DH and RIL populations. All the DNA markers except G39 showed the same linkage groups and orders between the two populations. The genetic distance per chromosome in the RIL population was shorter than that in the DH population, and the total genetic distance of genome in the RIL population (1465 cM) was 70.5% of that in the DH population (2079 cM). In the RIL population, 27.3% markers showed distorted segregation at $P < 0.01$ level, of which 90% markers favored indica alleles, while in the DH population, the skewed markers favoring indicia and japonica alleles were in accordance with 1:1 ratio. Eight commonly distorted regions on chromosomes 1, 3, 4, 7, 8, 10, 11, and 12 were detected in both RIL and DH populations, of which seven skewed toward indica alleles and one toward japonica allele. Five of them were located near gametophytic gene loci and/or sterility gene loci . We also compared the quantitative trait locus (QTL) mapping results between the DH and RIL populations and found a number of similarities in the QTL locations between these two populations. So both RIL and DH populations are equally effective in rice breeding and genetic analysis.

Deepinder Grewal et al.(2011) studied that 3000 doubled haploid (DH) lines through anther culture of 28 crosses involving indica and japonica rice (Oryza sativa L.) cultivars. Cultivars indica showed low anther culturability (1.2% callus induction) whereas japonica cultivars had 20-fold higher (28.1%) anther culturability. A set of 121 and 124 DH lines was used for phenotypic and

molecular analysis, respectively, generated from the japonica cultivar (IR69428) × indica variety (IR64). Significant variation was observed among DH lines for agronomic traits including Zn content. However, the phenotypic variance within each DH line was comparable with the mean phenotypic variance of the parents, suggesting no variation within DH line(s). A set of 209 simple sequence repeat (SSR) markers was selected to construct a linkage map with total genetic distance of 2148.8 cM. Simple sequence repeat analysis showed 1:1 ratio of indica and japonica alleles. Of the 209 markers, 21 showed distorted segregation and these markers are randomly located over 12 chromosomes. Homozygosity was detected for all the marker loci in 124 DH lines and 28 were heterozygote. Results show that indica cultivars are recalcitrant and genes for anther culturability are partially dominant. Molecular and phenotypic trait analysis of the DH lines showed that the origin of DHs is from pollen and these 121 DH lines are thus a valuable genetic resource in mapping quantitative trait loci (QTL) for grain Zn content and other agronomic traits.

CHAPTER-III
MATERIALS AND METHODS

This chapter deals with the investigation methodologies implemented to fulfill the objective of "*In vitro* induction of chromosome doubling for the production of doubled haploid rice and homozygosity testing of morphological characterization and molecular approach". All the experiments were conducted in Tissue Culture Lab, Green House of Department of Plant Molecular Biology and Biotechnology, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.).

3.1 Experiment Materials

Three Indica rice (Oryza sativa L.), Cross Swarna sub 1 x IR 90019-17-159-B (F5), Cross MTU 1010 x Dagardesi (F3) and Variety RF-75 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 was used as material. The material was three objective has been proposed for this work. Under objective 1st for *in vitro* chromosome doubling 2 Cross and 1 Variety has been then Cross Swarna sub 1 x IR 90019-17-159-B (F5), Cross MTU 1010 x Dagardesi (F3) and variety RF-75 for objective 2 & 3 for morphological characterization & molecular approach. Anther culture line of 7 differents crosses (2016), S-17xPB-122(150), S-17xRP BIO(112A), S-17xRP BIO(235), S-17xIR64 (185), S-17xIR64(203), S-17xRP BIO(1353), S-17xRP BIO(228) has been used. kindly provided by Dr.S.B.Verulkar HOD, Department of PMBB has been used for the current experiment.

3.2 Explants collection and Cold pre-treatment

Panicles were collected during 6.00 to 8.00 am and washed with water and sprayed with 70%ethanol. These Panicles were wrapped with whattmen paper (45 x 54 cm) and labeling the name of crosses and date. Cold pretreatment was given by placing them in refrigerator at 10^0C for 10-14 days.

3.3 Experimental Conditions

All the 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˚C ± 2˚C, 1000 lux and humidity was 60% maintained. Before starting the experiment, it is necessary to ensure all the material and tools used are sterile. complete sterilization of glassware, growth media, working area and surgical instruments was done. 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. The sterilization procedures adapted during the research were as under.

3.4 Methodology

Cross Swarna sub 1 x IR 90019-17-159-B , MTU 1010 x Dagardesi and Variety RF-75 was grown in field during Kharif Season in 2016. After 10-14 days of panicle emergence.The distance between flag leaf to second leaf was nearly 5-8 cm.The panicles along with the boot leaves in which panicles are still enclosed were pulled out of the tillers in the morning hours from a healthy crop of the Swarna sub 1 x IR 90019-17-159-B (F5), MTU 1010 x Dagardesi (F3),Var-RF-75 were collected. The extra leaves were removed with scissor. The panicle were washed with tap water, 2-3 times after that the panicle were surface sterilized with 70 % ethanol and kept at drying for 5-10 minutes.The panicles were wrapped with whattmen paper (45 x 54 cm) and labeling the name of crosses and date of pretreatment. The wrapped panicles were placed in a freeze that was maintained at 10^0C for 10-14 days for cold pretreatment. The panicle sheath was opened and the panicle portion (⅔) above the panicle emergence point was used for taking out anther for dusting.

3.4.1 Media preparation

Process of preparation of media (N6 Callus induction 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 were 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 make 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 121ºC, 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 no- 3.1- Composition callus induction 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

3.4.2-Stock solution of hormone

Stock solutions of 6-benzyl amino purine (BAP),2,4 dichlorophenoxyacetic acid(2,4-D) Naphthlenic acetic acid (NAA),were prepared by dissolving 50 mg of hormone first in 5ml of 1N NaOH or 70% ethanol and the final volume was made up to 50 ml with autoclaved double distilled water to prepare stock solution of 1mg/1ml (Table-3.2).Then hornmone stocks were filtered by using syring filter (0.25mm) under Laminar air flow cabinet and cap were covered by wrapping parafilm and kept in refrigerator at 4-8°C.2,4-D was used for callus induction and NAA,Kinetin and BAP was used for Regeneration.

Table no- 3.2- Stock solutions of hormones

Hormone	Required amount for stock solution (mg)	Amount of solvent required to dissolve	Amount of water added(ml)
BAP	50	5ml 1N NaOH	45
2,4-D	50	5ml 1N 70% ethanol	45
NAA	50	5ml 1N NaOH	45
Kinetin	50	5ml 1N NaOH	45

Table no- 3.3- Stock solutions of Colchicine

Colchicine	percentRequired amount (mg)	Amount of Solution water added(ml)
0.05%	**50**	**100**
0.1%	**100**	**100**

3.5 *In vitro* application of colchicine at various stages of anther culture.

3.5.1- Anther treatment

Treatment of anthers before culture, Anthers collect were dipped in 0.05% and 0.1% aqueous solution of colchicine for 1, 2 and 3 hours respectively along with control condition (without colchicine).

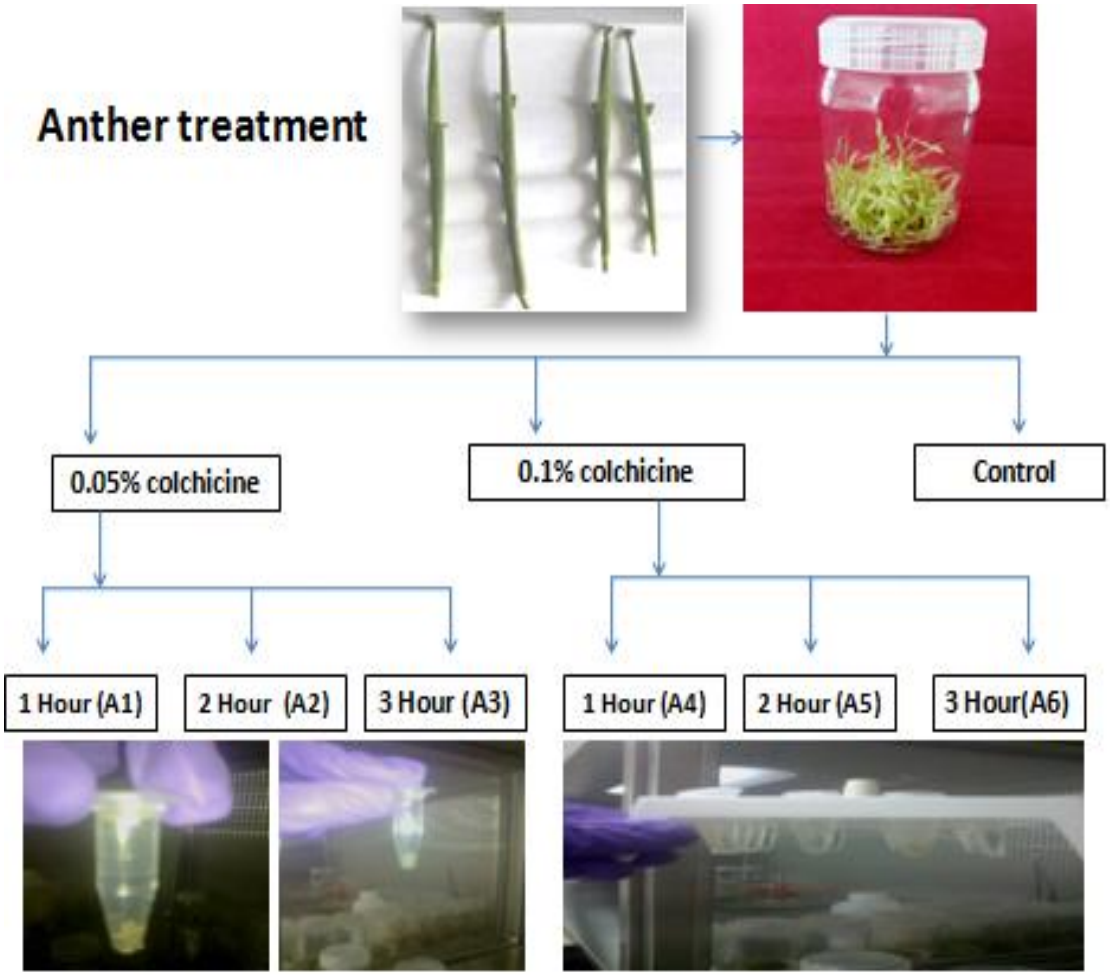


Fig no- 3.1 – Diagramic representation of anther treatment

3.5.2 Anther dusting for callus induction

After 10 days of panicle cold pretreatment in BOD at 25+_2˚C, Check the date of wrapped panicle. 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 sterilized in 0.1% HgCl2 for 5-10 minutes and washed with autoclaved distilled water, 4-5 times. Keep the panicle in tissue paper for drying. With the help forceps and scalpel the seeds were cut, The anther were squized out of the spiklet and dusted into N6 media with 2,4-D in petriplate with dusted anther were kept under 25+_2˚C in dark condition for callus induction. Dusted plate were checked every day and observation was taken for any

contamination (bacteria, fungus) and callus induction. Noted the callus induction date, days require for callus induction and contaminated plate. If contaminated plates found was subculture to fresh plates. Callus were allowed to grow up to 2mm size.

3. 5.3 Colchicine treatmentment on callus

The callus which was induced in control condition was subjected to colchicines treatment.

Process

1. The laminar air flow was cleaned by 70% ethanol and gives the UV light for 15 minute.
2. Size of 2 mm, callus was transferred in (T11+0.05% Col, T11+0.1% Col and T15+0.05% Col, T15+0.1% Col) for 2, 6, 24 , 48 hours (Fig 3.2) respectively along with control condition (without colchcine) with various combinations of hormones was used as given in (Table 3.4)
3. Using forceps and transferred 2mm size callus into Regenerative media.
4. Culture plate were labeled with treatment no, date of callus induction, date of callus transfer and variety name.
5. Kept in the growth chamber temp at 25± 2˚c for regeneration.
6. Then taken observation of plate every day and note the record.

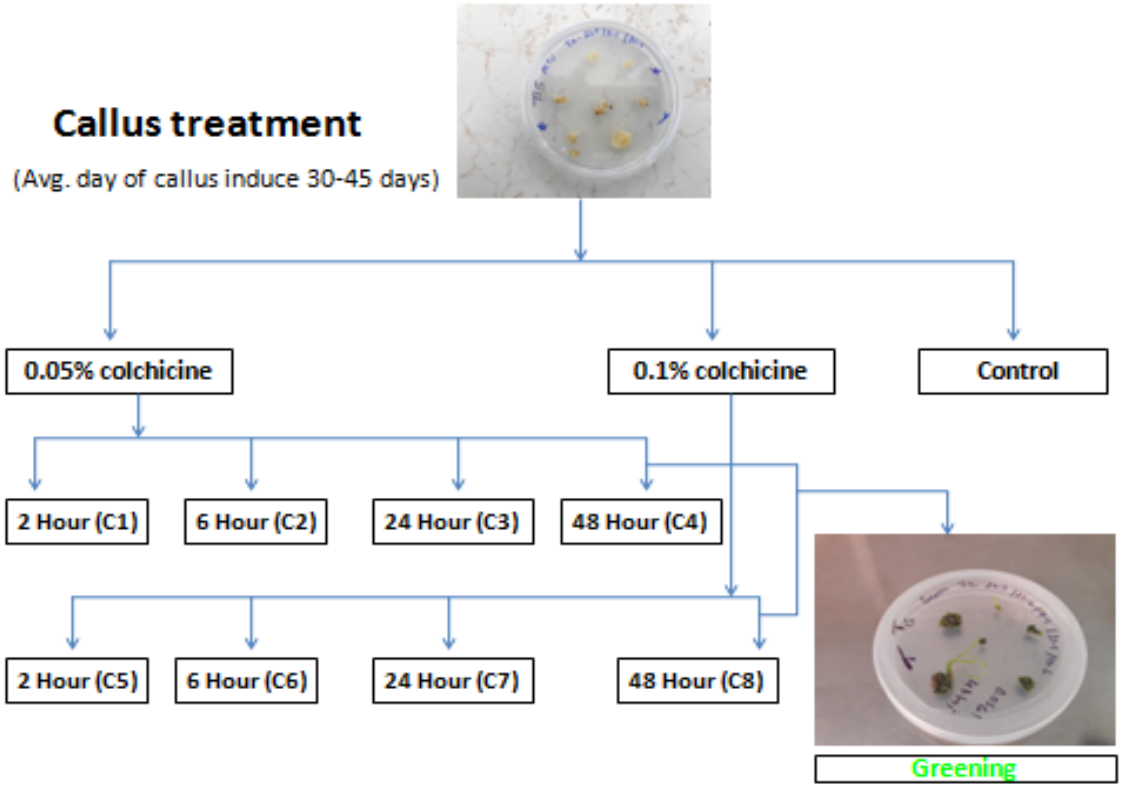


Fig no-3.2 – Diagramic representation of callus treatment

Table no- 3.4 - Constituents of regenerative media.

Treatment	Composition
T11	1/2MS +15gm.S + 8gm AGAR
	1:1:2 (NAA:KIN:BAP)mg/l + **0.05 or 0.1%Col**
T15	1/2MS +15gm.S + 8gm AGAR
	0.5 : 0.5: 1.5(NAA:KIN:BAP) mg/l+ **0.05 or 0.1%Col**
(T11)	1/2MS +15gm.S + 8gm AGAR
Control	1:1:2 (NAA:KIN:BAP)mg/l
(T15)	1/2MS +15gm.S + 8gm AGAR
Control	0.5:0.5:1.5 (NAA:KIN:BAP)mg/l

3.5.4-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.

Table no - 3.5 - Composition of shooting media

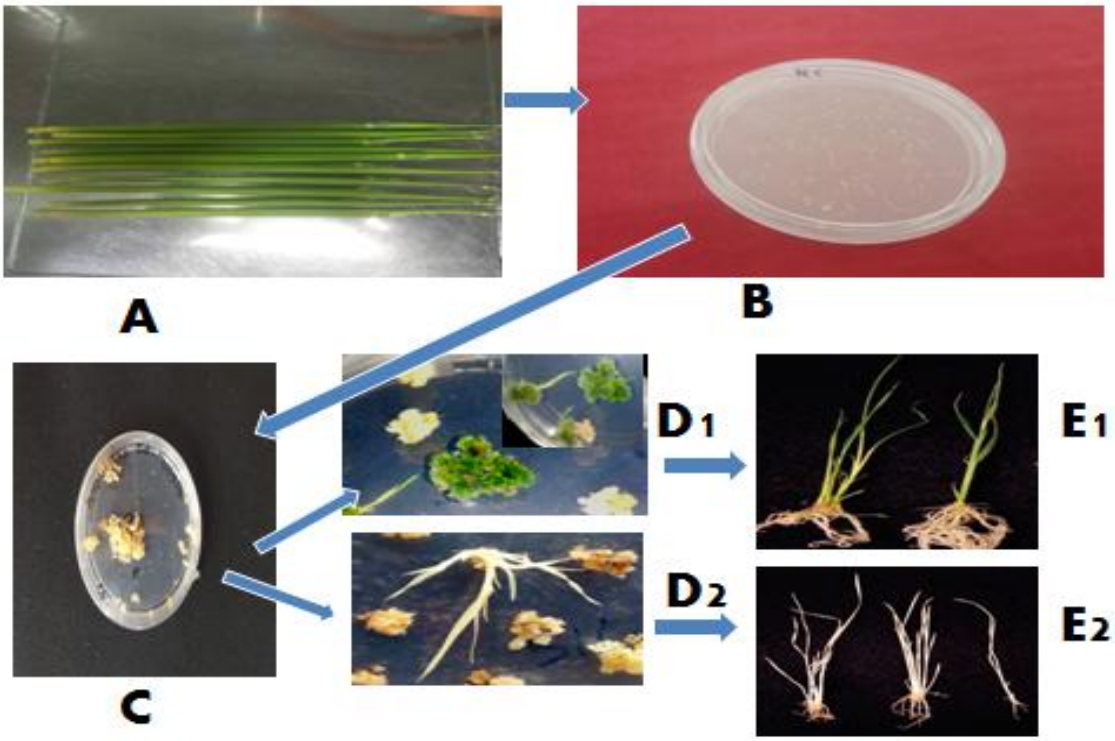
S. No.	Compositio for 1 lit.	For 1 lit.
1	MS media	4.41g
2	Sucrose	30 g
3	Inositol	100 mg
4	Adenine sulphate	0.18 g
5	BAP	4 mg
6	IAA	1 mg
7	pH	5.8
8	Agar- agar	8 gm

3.5.5- 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 no- 3.6 - Composition of rooting media.

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


A
B
C
D1
D2
E1
E2

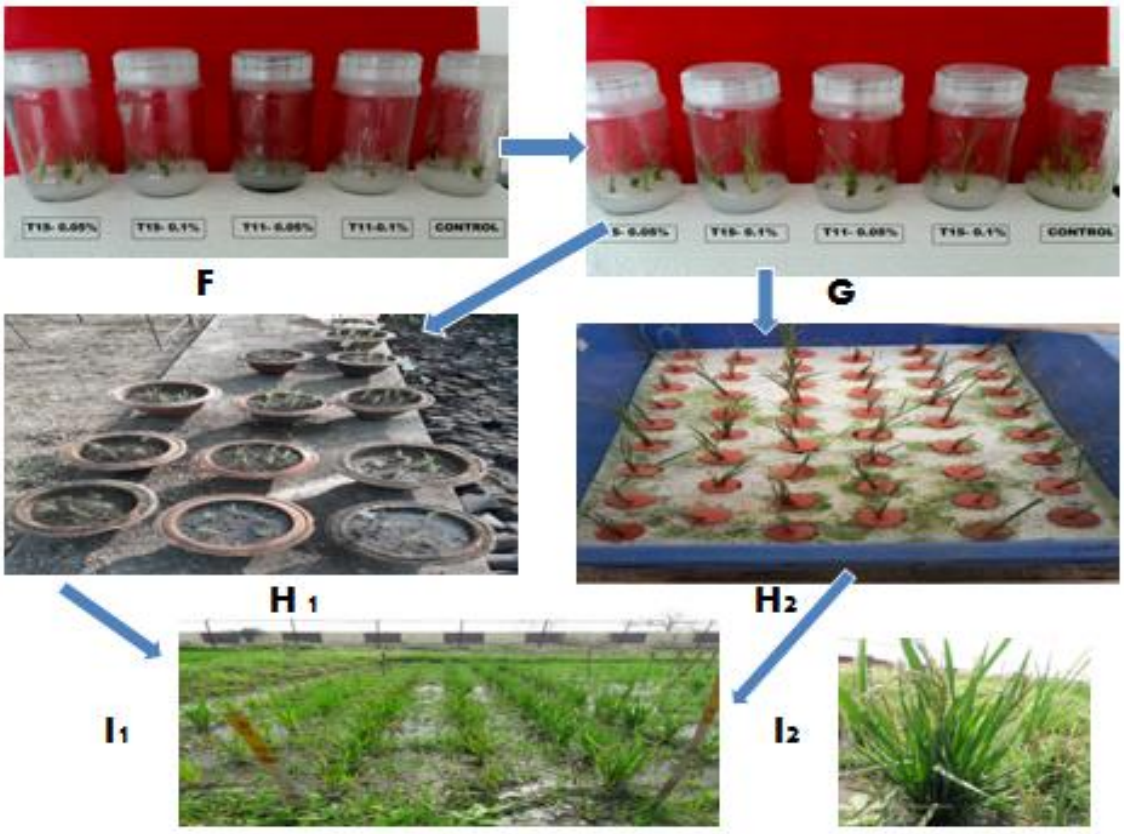

F
G
H1
H2
I1
I2

Fig no- 3. 3-Digrammatic representation of DH techniques

(A) Explant (B) Inoculated anthers (C) Callus (D1) Colchicine treated Green callus (E1) Green shoots (D2) White callus (E2) Albino shoots (F) Green plant in shooting media (G) Green plant in shooting media (H1,H2) Plants in green house (I1,I2) Plants in field.

3.6 Morphological characterization of doubled haploid rice progenies to establishing the homozygosity.

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 10x15 cm**.** Each line was sown in two replications. The NPK fertilizer was applied @ 100-60-40 kg per hectare.

Table 3.7 – List of Anther culture derived crosses

S.N	CROSS	CODE
1	S-17xPR-122 (150)	C
2	S-17xRP BIO (112A)	H
3	S-17xRP BIO (235)	I
4	S-17x IR64 (185)	J
5	S-17xIR64 (203)	K
6	S-17xRP BIO (1353)	L
7	S-17xRP BIO (228)	M

1. **Plant height** - Plant height was measured at the time of maturity from ground level to the tip of the panicle.
2. **No of tillers** - Number of total tiller per plant was counted form randomly selected plant.
3. **No of panicles** - 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.

4. **Panicle length** - 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).
5. **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.
6. **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.
7. **1000 seed weight**
8. **Flag leaf length**
9. **Flag leaf width**
10. **Seed weight per plant**

Fig no. -3.4 -Digramic representation of doubled haploid crosses in field

3.7 Assessment of homozygosity in individual doubled haploid population using molcular marker.

Table 3.8 - List of Marker

S/NO.	Marker
1	RM-1
2	RM-19
3	RM-154
4	RM-219
5	RM-215
6	RM-171
7	RM-586
8	RM-259
9	RM-527
10	RM-144
11	Xa- 13
12	RM-125
13	Xa- 21

3.7.1 - 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 tissue homogenizer.

1. Then 700 µl of solution transferred into 1.5 ml eppendorf tube.
2. Incubated at 65°C on water bath for 15-20 min further cooled briefly and add 700 µl of Chloroform: Isoamylalcohol (24:1).

3. The content were shaken by hands intermittently and kept at room temperature for 15 min. tubes were centrifuged at 13000 rpm for 3 min.
4. 600 µl of upper aqueous phase was transferred into a new 1.5 ml eppendorf tube. 900 µl of absolute ethanol was added and mixed gently and the tubes were kept for 2 hrs at -20°c.
5. The samples were centrifuged for 3 min at 10,000 rpm, the supernatant was decanted. The pellet was washed with 70% ethanol and air-dried.
6. DNA pellet was air dried and then dissolved in 50 µl of TE buffer.

3.7.2 - 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 8µl of cocktail was added to each sample.

3.7.3 - Dilution of DNA

The crude DNA after quantification was diluted to 50 ng/ul with TE buffer. The diluted DNA was subsequently used for PCR amplification.

Table no. 3.9 - PCR mix for one reaction

Reagent	Stock concentration	Volume (µl)
Nuclease Free Water		5.25
PCR buffer with 15 mM MgCl2	10X	1.0
dNTPs (Mix)	1mM	1.0
Primer (forward+ reverse)	5ρmol	0.5+0.5 (forward+ reverse)
Taq polymerase	3 U/µl	0.25
DNA template	50 ηg/µl	1.5
	Total	10

Table 3.10 - Temperature profile used for PCR amplification using microsatellite markers

Steps	Activity	Temperature(°C)	Cycle	Time (min)
1	Initial denaturation	95 °C	1	5
2	Denaturation	95 °C		1
3	Annealing	55 °C	35	1
4	Extension	72°C		1
5	Final extension	72 °C	1	7
6	Store	4°C	1	∞

3.7.4 - 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.

3.7.5 - 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.

3.7.6 - 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^{+}.

3.7.7 .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.

3.7.8 - Reagents and solutions

3.7.8.1- 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 (GeNei™) 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)

GeNei™make was used.

d. *Taq* DNA polymerase

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

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

f. Orange loading dye

3.7.8.2 - Stock solutions

A. DNA extraction buffer

Tris HCl (1M; pH-8) - 5 ml

EDTA (0.5M; pH-8) - 10 ml

NaCl (4M) - 7.5 ml

SDS (20% W/V) - 5 ml

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)

3.7.8.3 - 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. 50bp DNA Ladder (GeNei™ Merck Specialities Private Limited)

1.5µl ladder per well is used.
Step Up 50bp ladder 0.1ml
Gel loading buffer (6x) 0.2ml
Water 0.4ml

3.7.8.3 - 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-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. Anther culture in general produced haploid plants. Haploid plants can not bear seeds. So no use until being diploidized by choromosome doubling agent on haploid plants. So the current research is being focused on produced of double haploid plants from haploid plants using *in vitro* colchicines treatment. 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. Cold pretreatment of explants

The collected panicles of Cross Swarna sub 1 x IR 90019-17-159-B (F5), Cross MTU 1010 x Dagardesi (F3) and Variety RF-75 were washed with water and sprayed with 70% ethanol These Panicles were wrapped with whattmen paper (45 x 54 cm) and labeling the name of crosses and date. and kept in 10^0C for 10 days to fix the meiotic division in pollen Pretreated under cold condition is very important to ensure green haploid plant regeneration.

4.2 *In vitro* application of colchicine at various stages of anther culture.

4.2.1 Anther treatment

Treatment of anthers before culture, Anthers collect were dipped in 0.05% and 0.1% aqueous solution of colchicine for 1, 2 and 3 hours respectively along with control condition (without colchicine).

Table no-4.1 - Anther treatment in Swarna sub 1 x IR 90019-17-159-B (F5)

Treatment No.		Total no of Anther treated	Total no. of callus induce	Callus induction%
A1 (1h)	0.05% Col	318	0	0
A2 (2h)		445	0	0
A3 (3h)		321	0	0
A4 (1h)	0.1% Col	349	0	0
A5 (2h)		312	0	0
A6 (3h)		309	0	0
Control		3806	451	11.84

Table no- 4.2 -Anther treatment in MTU 1010 x Dagardesi (F3)

Treatment No.		Total no of Anther treated	Total no. of callus induce	Callus induction %
A1 (1h)	0.05% Col	380	0	0
A2 (2h)		327	0	0
A3 (3h)		420	0	0
A4 (1h)	0.1% Col	325	0	0
A5 (2h)		365	0	0
A6 (3h)		341	0	0
Control		3679	317	8.61

Table no- 4.3 - Anther treatment in RF-75

Treatment No.		Total no of Anther treated	Total no. of callus induce	Callus induction %
A1 (1h)	. 05% Col	467	0	0
A2 (2h)		454	0	0
A3 (3h)		341	0	0
A4 (1h)	0.1% Col	318	0	0
A5 (2h)		346	0	0
A6 (34)		371	0	0
Control		3700	483	13.05

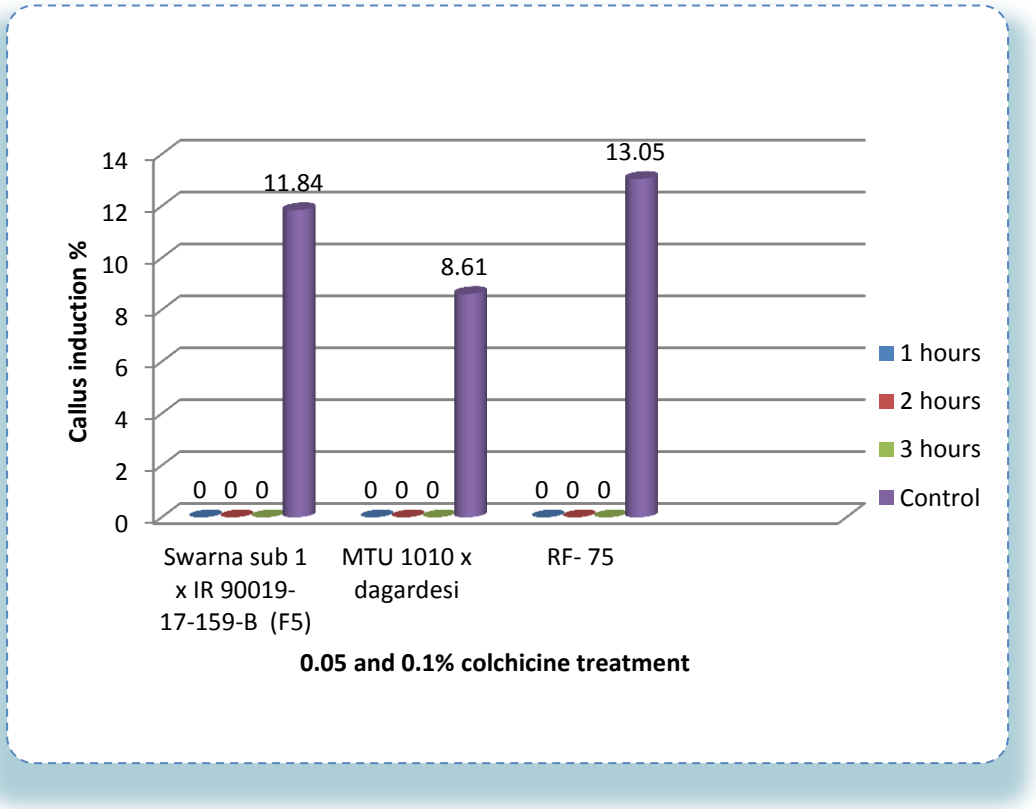


Fig -4.1 Graphical representation of callus induction

In Swarna sub 1 x IR 90019-17-159-B , MTU 1010 x Dagardesi and RF-75 Anthers were dipped in 0.05% and 0.1% aqueous solution of colchicine for 1, 2 and 3 hours respectively along with control condition (Table 4.1, 4.2 & 4.3)**.** After treatment these anthers were soaked in filter paper till dry (2-5 min) & transferred in separately into callus induction media. But anther treatment did not respond in all 3 sample while control condition callus induction percentage was respectively 11.84%, 8.61% and 13.05%.

4.2.2 Anther dusting for callus induction

After 10 days of panicle cold pretreatment in BOD at 25+_2˚C, Check the date of wrapped panicle. 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 sterilized in 0.1% HgCl2 for 5-10 minutes and washed with autoclaved distilled water, 4-5 times. Keep the panicle in tissue paper for drying. With the help forceps and scalpel the seeds were cut, The anther

were squized out of the spiklet and dusted into N6 media with 2,4-D in petriplate with dusted anther were kept under 25+_2˚C in dark condition for callus induction.

4.2.3 - Colchicine treatment on callus

The callus which was induced in control condition was transferred to different media (T11 and T15) with different colchicine treatments (0.05% or 0.1%) for 2, 6, 24, 48 hours respectively along with control condition (without colchicine).

Table no-4.4 Callus treatment in Swarna sub 1 x IR 90019-17-159-B (F5) in T11Media

Treatment	Composition	No. of callus inoculated	Callus greeing	Greening%	Total green plant
C1 (2h)	(T11 (1/2 MS + 1.5S +0.8AGAR) + (1:1:2 (NAA:KIN:BAP) mg/l + **0.05% Col}**	19	1	5.2	3
C2 (6h)		21	1	4.7	3
C3 (24h)		25	9	36	14
C4 **(**48h)		20	9	45	20
C5 (2h)	**(T11** (1/2 MS + 1.5S +0.8AGAR) **+** (1:1:2 (NAA:KIN:BAP) mg/l + **0.1% Col.**	16	1	6.25	2
C6 (6h)		15	1	6.66	2
C7 (24h)		15	5	33.33	13
C8 (48h)		16	7	43.75	19
Control	(Without colchicine)	28	6	21.42	11

Table no-4.5 Callus treatment in Swarna sub 1 x IR 90019-17-159-B (F5) in T15 Media

Treatment	Composition	No. of callus inoculated	Callus greeing	Greening%	Total green plant
C1 (2h)	((**T15** (1/2 MS + 1.5S +0.8AGAR) + (0.5 : 0.5: 1.5) (NAA:KIN:BAP) mg/l + **0.05% Col**}	20	1	5	5
C2 (6h)		20	1	5	3
C3 (24h)		23	5	21.73	18
C4 **(**48h)		24	10	41.66	28
C5 (2h)	**(T15** (1/2 MS + 1.5S +0.8AGAR) **+** (0.5 : 0.5: 1.5) (NAA:KIN:BAP) mg/l + **0.1% Col**}	18	1	5.55	2
C6 (6h)		5	1	6.66	5
C7 (24h)		17	6	35.29	17
C8 (48h)		24	11	47.82	25
Control	(Without colchicine)	30	8	26.66	15

Callus generated from the anther of cross Swarna sub 1 x IR 90019-17-159-B was transferred to different media(T11 and T15) with different colchicine treatments (0.05% or 0.1%) for 2, 6, 24, 48 hours respectively along with control condition (without colchicine). shown in (Table 4.4 &.4.5). Out of 2 colchicine treatments T11(1/2MS+15gm.S+8gm agar,1:1:2 (NAA:KIN:BAP) mg/l+0.05 or 0.1% Col) and T15(1/2MS +15gm.S + 8gm agar, 0.5 : 0.5: 1.5(NAA:KIN:BAP) mg/l+0.05 or 0.1% Col) given (Table 3.4) has responded for greening of the callus. Highest callus greening percentage was T11 (0.05% Col), C4 (45%) followed by C3 (36%), C1(5.2%),C2 (4.7%) and T11 (0.1% Col) Highest callus greening percentage was C8 (43.75%) followed by C7 (33.33%), C6 (6.66%), C5 (6.25%). And control (without colchicine) greening percent was (21.42%). and T15 (0.05% Col) Highest callus greening percentage was C4 (41.66%) followed by C3 (21.73), C2 (5%), C1 (5%) and T15 (0.1% Col) Highest callus greening percentage was C8 (47.82%) followed by C7 (35.29%), C6 (6.66%), C5 (5.55%) and control greening percentage was 26.66%.

Table no - 4.6 Callus treatment in MTU 1010 x Dagardesi (F3) in T11 Media

Treatment	Composition	No. of callus inoculated	Callus greeing	Greening%	Total green plant
C1 (2h)	(T11 (1/2 MS + 1.5S +0.8AGAR) + (1:1:2 (NAA:KIN:BAP) mg/l + **0.05% Col}**	14	0	0	0
C2 (6h)		16	0	0	0
C3 (24h)		21	1	4.2	3
C4 **(**48h)		21	5	5	3
C5 (2h)	**(T11** (1/2 MS + 1.5S +0.8AGAR) **+** (1:1:2 (NAA:KIN:BAP) mg/l + **0.1% Col.**	16	0	0	0
C6 (6h)		14	0	0	0
C7 (24h)		17	1	5.88	3
C8 (48h)		23	2	8.69	6
Control	(Without colchicine)	27	1	3.7	2

Table no - 4.7 Callus treatment in MTU1010 x Dagardesi (F3) in T15 Media

Treatment	Composition	No. of callus inoculated	Callus greeing	Greening%	Total green plant
C1 (2h)	((**T15** (1/2 MS + 1.5S +0.8AGAR) + (0.5 : 0.5: 1.5) (NAA:KIN:BAP) mg/l + **0.05% Col**}	18	1	5.55	0
C2 (6h)		14	0	0	0
C3 (24h)		16	1	0	0
C4 **(**48h)		17	1	5.88	4
C5 (2h)	**(T15** (1/2 MS + 1.5S +0.8AGAR) + (0.5 : 0.5: 1.5) (NAA:KIN:BAP) mg/l + **0.1% Col**}	14	0	0	0
C6 (6h)		15	0	0	0
C7 (24h)		15	0	0	0
C8 (48h)		16	2	12.5	6
Control	(Without colchicine)	29	1	3.44	3

Callus generated from the anther of cross MTU1010 x dagardesi transferred to different media (T11 and T15) with different colchicine treatments (0.05% or 0.1%) for 2, 6, 24, 48 hours respectively along with control condition (without colchicine). shown in (Table 4.6 & 4.7). Out of 2 colchicine treatments T11 (1/2MS +15gm.S + 8gm agar, 1:1:2 (NAA:KIN:BAP)mg/l + 0.05 or 0.1% Col) and T15 (1/2MS +15gm.S + 8gm agar, 0.5 : 0.5: 1.5(NAA:KIN:BAP) mg/l+ 0.05 or 0.1% Col) given (Table 3.4) has responded for greening of the callus. T11 (0.05% Col) Highest callus greening percentage was C4 (5%) followed by C3 (4.2%), C2 (0%), C1 (0%) and T11 (0.1% Col) Highest callus greening percentage was C8 (8.69%), followed by C7 (5.88%), C6 (0%), C5 (0%), and Control greening percent was (3.7%). In T15 (0.05% Col) Highest callus greening percentage was C4(5.88%) followed by C1 (5.5%), C2 (0 %), C3(0 %) and T15 (0.1% Col) Highest callus greening percentage was C8 (12.5%) followed by C7 (0%), C6 (0%), C5 (0%) and control condition greening percentage was (3.44%).

Table no - 4.8 Callus treatment in RF-75 in T11 Media

Treatment	Composition	No. of callus inoculated	Callus greeing	Greening%	Total green plant
C1 (2h)	(T11 (1/2 MS + 1.5S +0.8AGAR) + (1:1:2 (NAA:KIN:BAP) mg/l + **0.05% Col}**	25	5	20	16
C2 (6h)		16	1	6.25	3
C3 (24h)		17	3	17.64	14
C4 **(**48h)		21	4	19.04	18
C5 (2h)	**(T11** (1/2 MS + 1.5S +0.8AGAR) **+** (1:1:2 (NAA:KIN:BAP) mg/l + **0.1% Col.**	23	3	13.04	15
C6 (6h)		14	1	7.1	2
C7 (24h)		21	4	19.04	14
C8 (48h)		24	5	20.83	16
Control	(Without colchicine)	28	4	14.28	9

Table no – 4.9 Callus treatment in RF-75 in T15 Media

Treatment	Composition	No. of callus inoculated	Callus greeing	Greening%	Total green plant
C1 (2h)	((**T15** (1/2 MS + 1.5S +0.8AGAR) + (0.5 : 0.5: 1.5) (NAA:KIN:BAP) mg/l + **0.05% Col**}	27	6	22.22	21
C2 (6h)		18	1	5.55	4
C3 (24h)		21	5	23.80	9
C4 **(**48h)		23	6	26.08	22
C5 (2h)	**(T15** (1/2 MS + 1.5S +0.8AGAR) **+** (0.5 : 0.5: 1.5) (NAA:KIN:BAP) mg/l + **0.1% Col**}	24	5	20.83	21
C6 (6h)		17	1	5.88	4
C7 (24h)		26	5	19.23	17
C8 (48h)		26	7	26.92	23
Control	(Without colchicine)	28	5	17.85	7

Callus generated from the anther of Variety RF- 75 was transferred to different media (T11 and T15) with different colchicine treatments (0.05% or 0.1%) for 2, 6, 24, 48 hours respectively along with control condition (without colchicine) given (Table 4.8 & 4.9). Out of 2 colchicine treatments T11 (1/2MS +15gm.S + 8gm agar, 1:1:2 (NAA:KIN:BAP) mg/l + 0.05 or 0.1% Col) and T15 (1/2MS +15gm.S + 8gm agar, 0.5 : 0.5: 1.5(NAA:KIN:BAP) mg/l+ 0.05 or 0.1% Col). given (Table 3.4) has responded for greening of the callus. In T11 (0.05% Col) Highest callus greening percentage was C1(20%) followed by C4 (19.04%),

C3 (17.64%), C2 (6.25) and T11 (0.1% Col) Highest callus greening percentage was C8 (20.84%) followed by C7 (19.04%),C5 (13.04%),C6 (7.1%) and control (14.28%). In T15 (0.05% Col) Highest calluse greening percentage was C4 (26.08%) followed by C3 (23.80%), C1 (22.22%), C2 (5.55%) and T15 (0.1% Col) Highest callus greening percentage was C8 (26.92%) followed by C5 (20.83%),C7 (19.23%), C6 (5.88) and Control (17.85%).

In both 0.05% colchicine & 0.1% colchicine treatment in T11 and T15, C4 (48H) & C8 (48H) callus greening percentage was very high then the control which should a positive result by involve colchicine treatment.

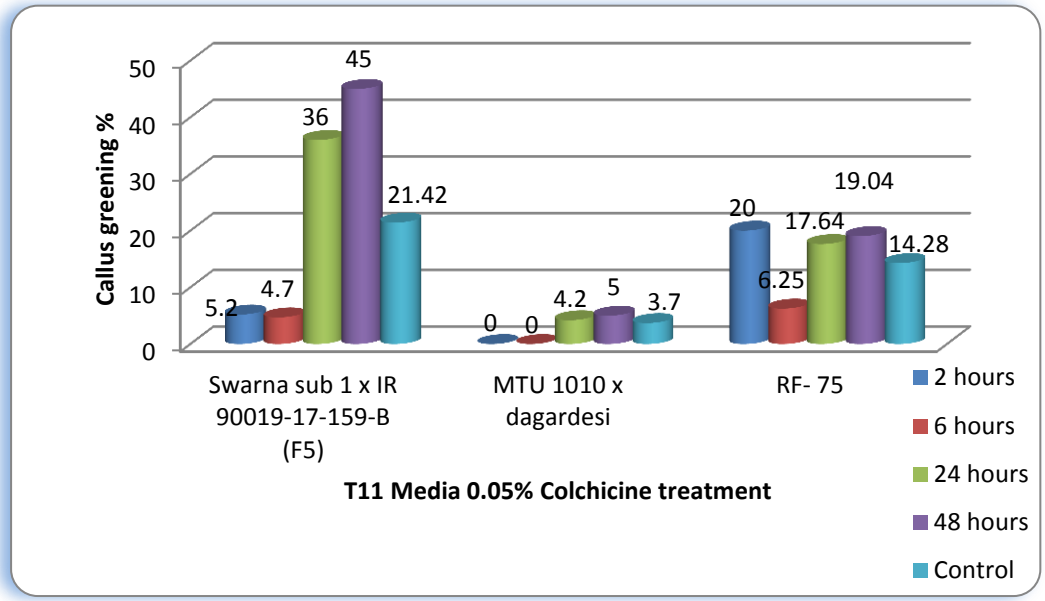


Fig no – 4.2 -Graphical representation of Relation between T11 media 0.05% colchicine treatment and greening %

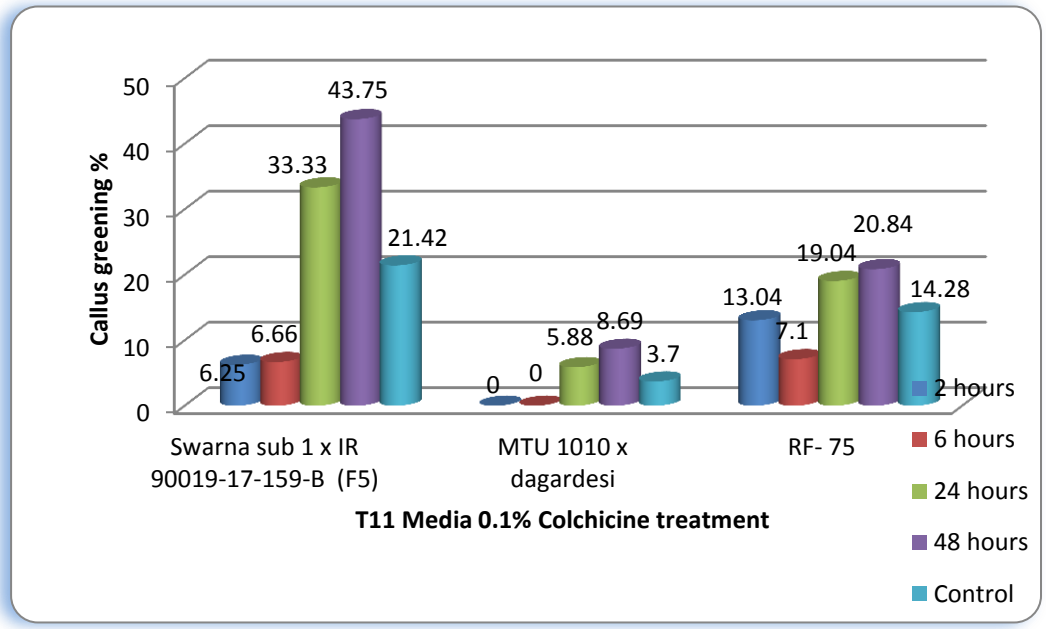


Fig no - 4.3 - Graphical representation of Relation between T11 media 0.1% colchicine treatment and greening %

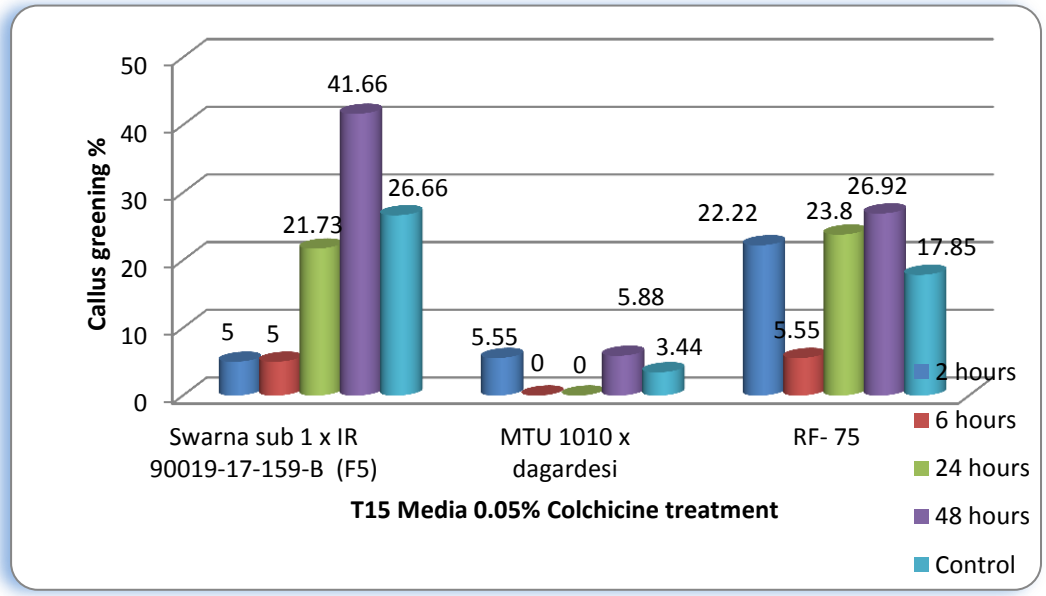


Fig no – 4.4 Graphical representation of Relation between T15 media 0.05% colchicine treatment and greening %

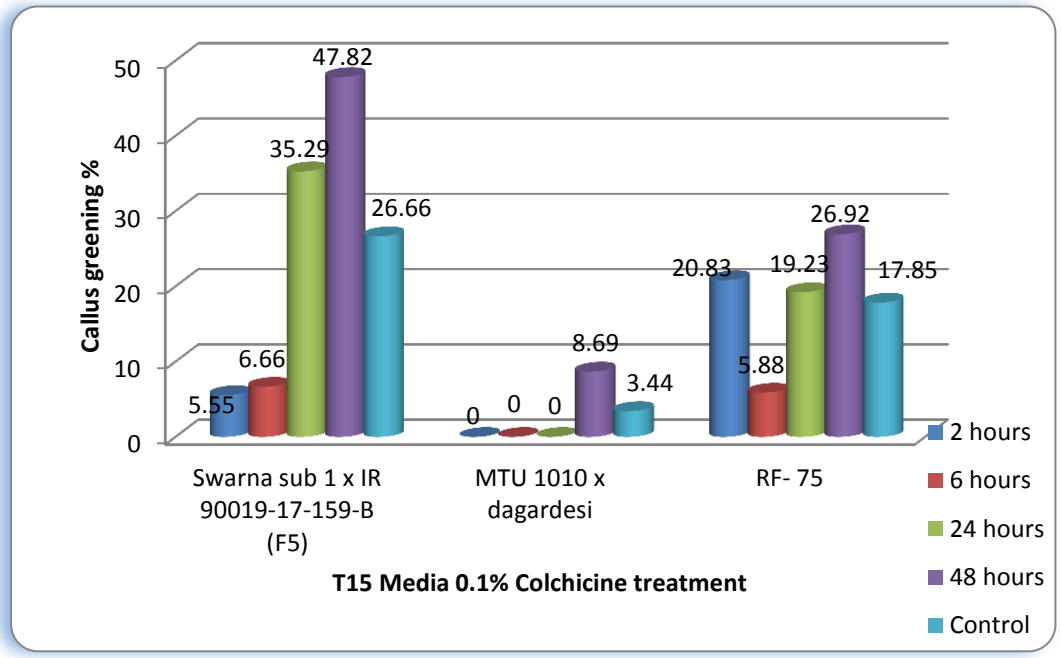


Fig no- 4.5- Graphical representation of Relation between T15 media 0.1% colchicine treatment and greening %

4.2.4 Green plants 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. out of which callus has became green which was further transferred to shooting media.

In cross Swarna sub-1 x IR 90019-17-159-B , total 451 callus was subjected to 2 different treatment T11(0.05% or 0.1% Colchicine supplemented media) and T15 (0.05% or 0.1% Colchicine supplemented media) for 2, 6, 24, 48 hours respectively along with control condition (without Colchicine) shown in(Table 4.4 &.4.5). Out of which callus has became green which was further transferred to shooting media.T11 0.05% col. C4 (48h) has generated highest 9 green callus. Out of which 1 contaminated by the time of subculture or incubation at growth chamber. and 20 total number of green plant were produced.(Table 4.5). and T11 0.1% col. C8 (48h) has generated highest 7 green callus.out of which 19 green plant were produced. In T15 0.05% col. C4

(48h) highest 28 green plants were produced while T15 0.1% col. C8 (48h) highest 29 green plants were produced.

In cross MTU 1010 x Dagardesi, total 317 callus was subjected to 2 different treatment T11(0.05% or 0.1% Colchicine supplemented media) and T15 (0.05% or 0.1% Colchicine supplemented media) for 2, 6, 24, 48 hours respectively along with control condition (without Colchicine) shown in (Table 4.6 &.4.7). Out of which callus has became green which was further transferred to shooting media. T11 0.05% col. both C3 (24h) & C4 (48h) highest 3 green plants were produced and T11 0.1% col.C8 (48h) highest 4 green plants were produced. T15 0.05% col. C4 (48h) highest 4 green plants were produced and T15 0.1% col. C8 (48h) highest 6 green plants were produced.

In variety RF-75, total 483 317 callus was subjected to 2 different treatment T11(0.05% or 0.1% Colchicine supplemented media) and T15 (0.05% or 0.1% Colchicine supplemented media) for 2, 6, 24, 48 hours respectively along with control condition (without Colchicine) shown in (Table 4.8 &.4.9). Out of which callus has became green which was further transferred to shooting media.T11 0.05% col. C4 (48h) highest 18 green plant were produced and T11 0.05% col. C8 (48h) highest 18 green plant were produced and T11 0.1% col. C8 (48h) highest 19 green plant were pro duced and T15 0.05% col. C4 (48h) highest 22 green plant were produced and T15 0.1% col. C8 (48h) highest 23 green plant were produced.

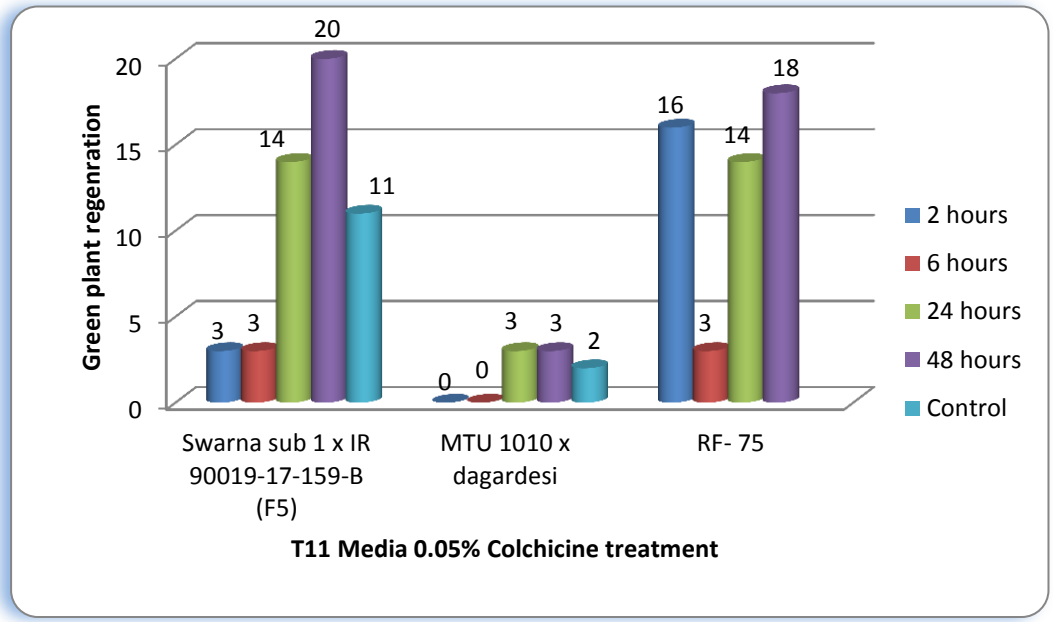


Fig no- 4.6- Graphical representation of Relation between T11 media 0.05% colchicine treatment and green plant regenration

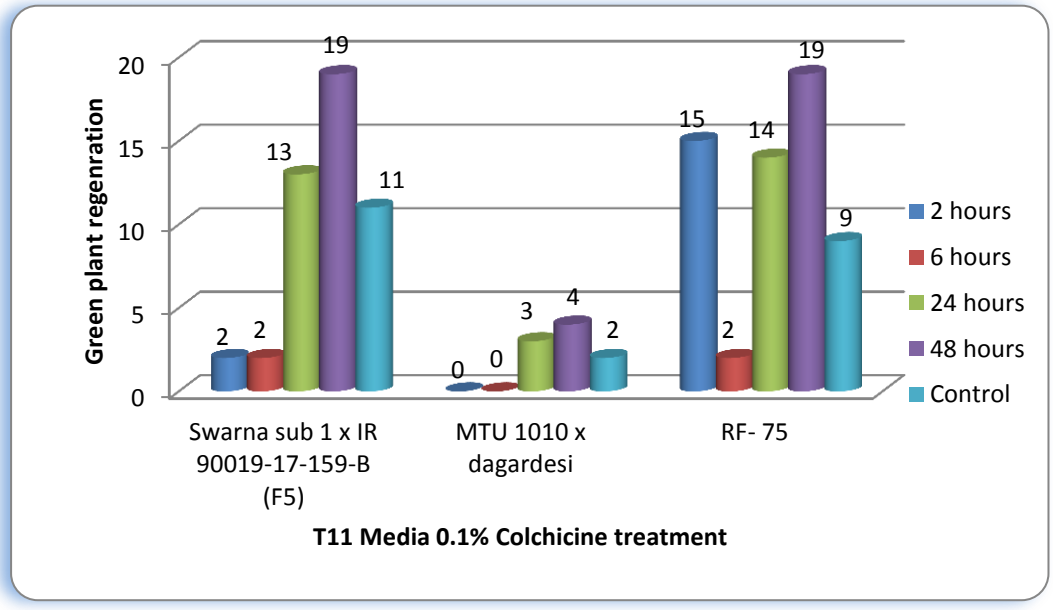


Fig no- 4.7- Graphical representation of Relation between T11 media 0.1% colchicine treatment and green plant regeneration

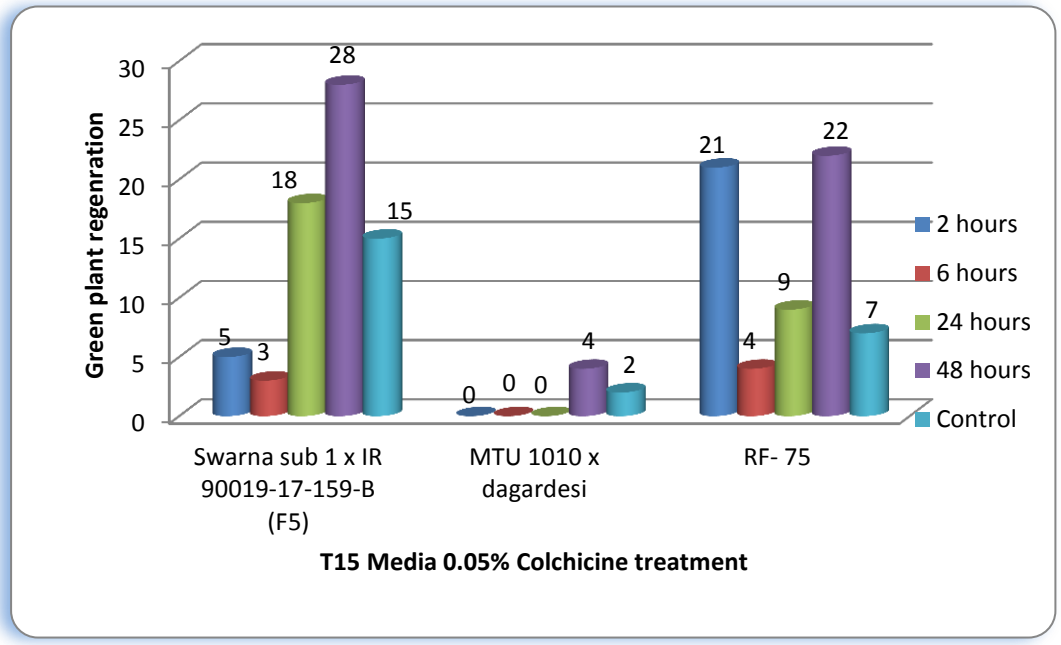


Fig no 4.8- Graphical representation of Relation between T15 media 0.05% colchicine treatment and green plant regeneration

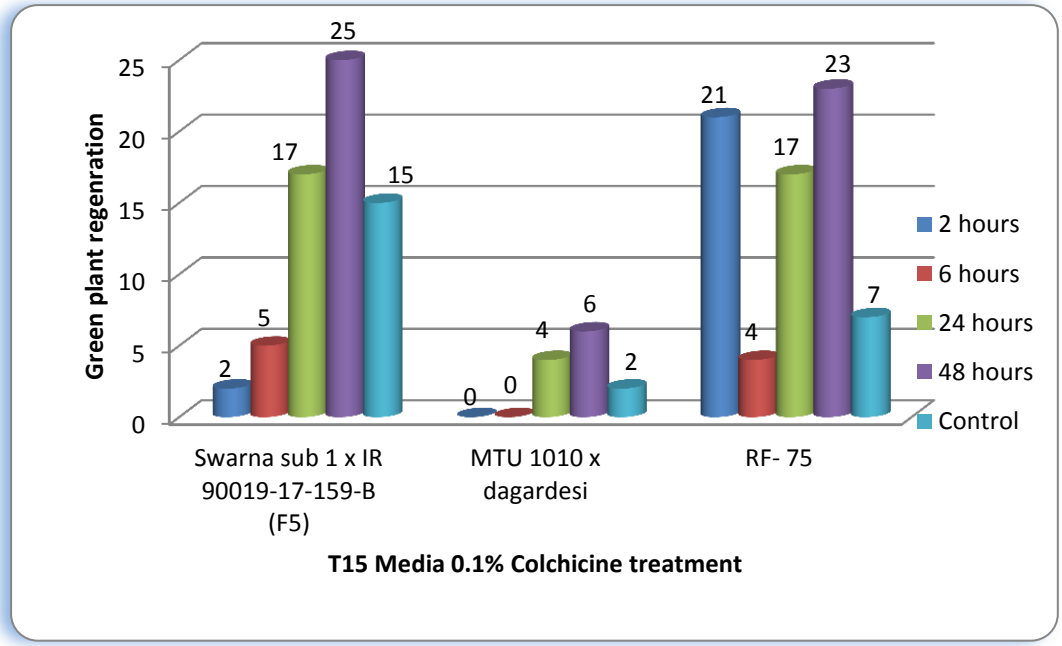


Fig no 4.9- Graphical representation of Relation between T15 media 0.01% colchicine treatment and green plant regenration

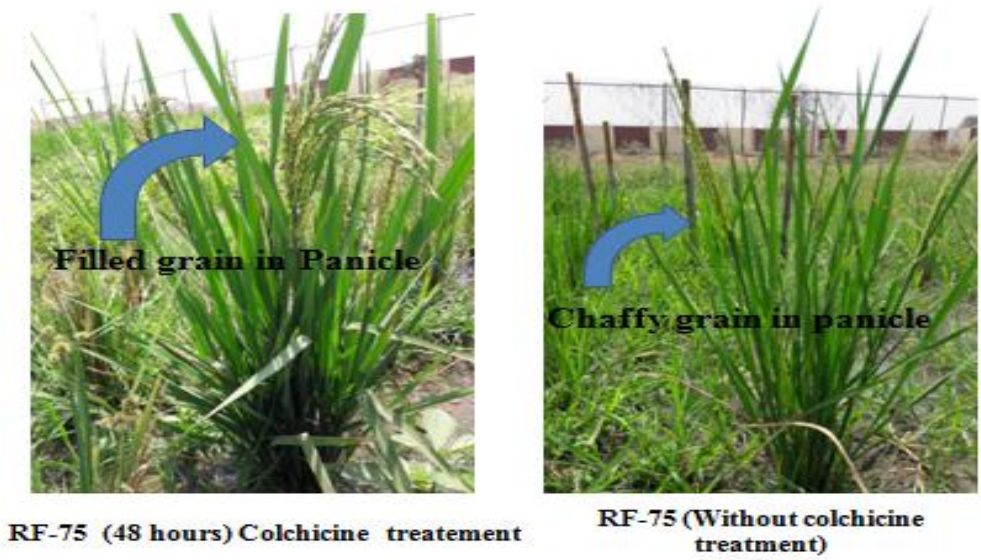


Fig-4.10 Anther culture derived plant in field

4.3 Morphological characterization of doubled haploid rice progenies to establishing 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 7 DHs line this are presented in table .

Table no 4.10 List of anther culure derived plant

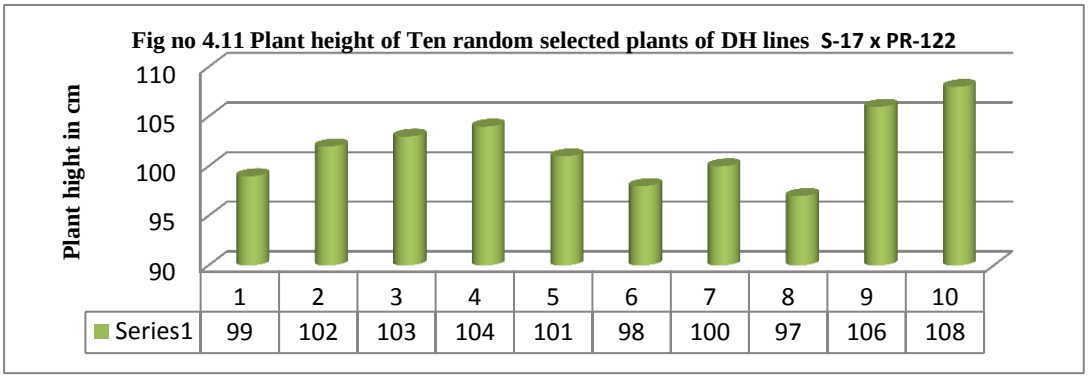
S.N	CROSS	CODE
1	S-17xPR-122 (150)	C
2	S-17xRP BIO (112A)	H
3	S-17xRP BIO (235)	I
4	S-17x IR64 (185)	J
5	S-17xIR64 (203)	K
6	S-17xRP BIO (1353)	L
7	S-17xRP BIO (228)	M

4.3.1 Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x PR-122(150).

The S-17 x PR-122(150) showed mean performance of different character like plant height 101.8 (cm), Number of tiller 19.3, Number of panicle 17.5, Seed weight per/plant 43.48(gm), Panicle length 27.1 (cm), Flag leaf length 44 (cm). Flag leaf width 1.63 (cm), 1000 seed weight 22.8(gm), Grain length 7.73 (mm), Grain width 3 (mm), 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 table-4.11.

Table no 4.11 - Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x PR-122 (150)

S.N	TRAIT	MEAN	SD	SE
1	Plant height	101.8	3.52	1.11
2	No. of tiller / plant	19.3	2.11	0.66
3	No. of panicle / plant	17.5	1.43	0.45
4	Seed weight / plant	43.48	4.33	1.37
5	Panicle length	27.1	4.74	1.49
6	Flag leaf length	44	4.66	1.47
7	Flag leaf width	1.63	0.23	0.07
8	1000 seed weight	22.8	0.52	0.16
9	Grain length	7.73	0.28	0.08
10	Grain width	3	0.04	0.01



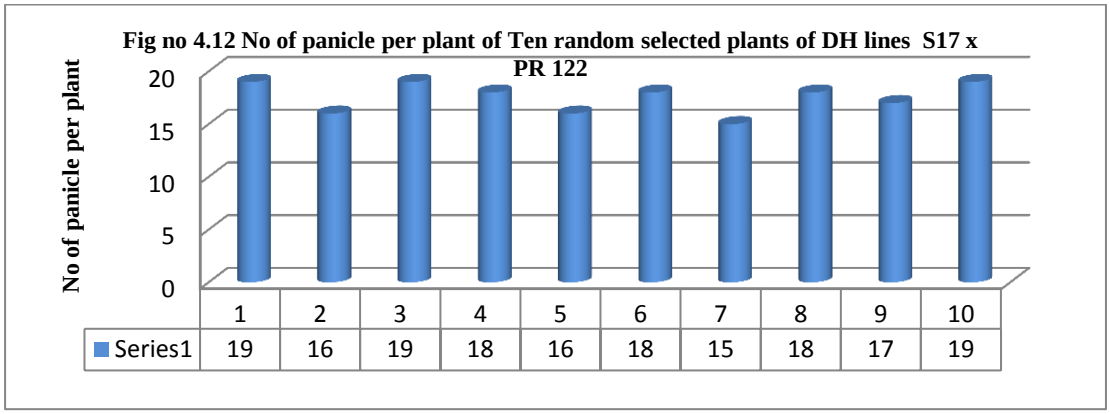


Fig no 4.12 No of panicle per plant of Ten random selected plants of DH lines S17 x PR 122
No of panicle per plant
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10
Series1 19 16 19 18 16 18 15 18 17 19

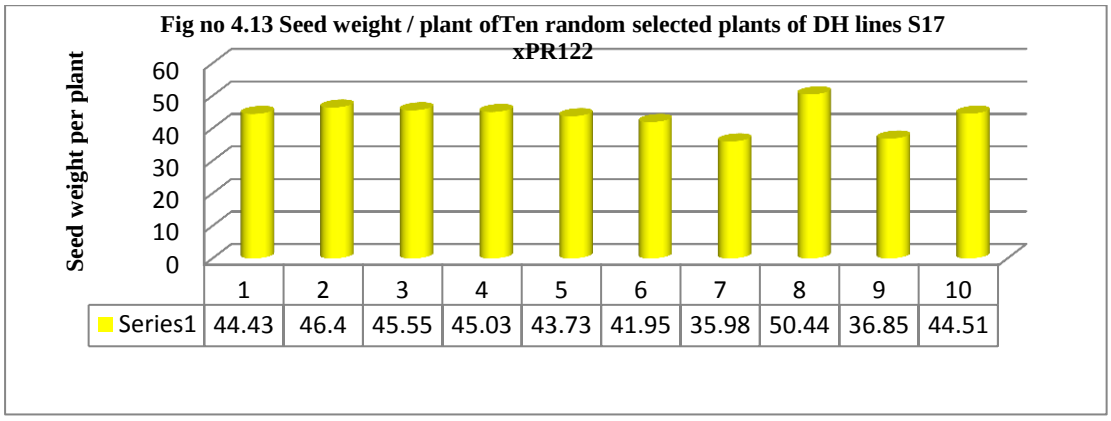


Fig no 4.13 Seed weight / plant ofTen random selected plants of DH lines S17 xPR122
Seed weight per plant
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10
Series1 44.43 46.4 45.55 45.03 43.73 41.95 35.98 50.44 36.85 44.51

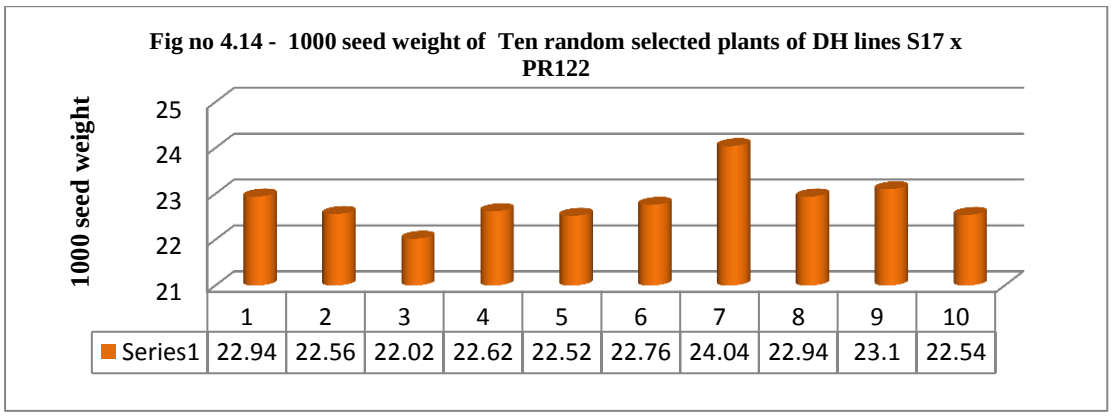


Fig no 4.14 - 1000 seed weight of Ten random selected plants of DH lines S17 x PR122
1000 seed weight
25
24
23
22
21
1 2 3 4 5 6 7 8 9 10
Series1 22.94 22.56 22.02 22.62 22.52 22.76 24.04 22.94 23.1 22.54

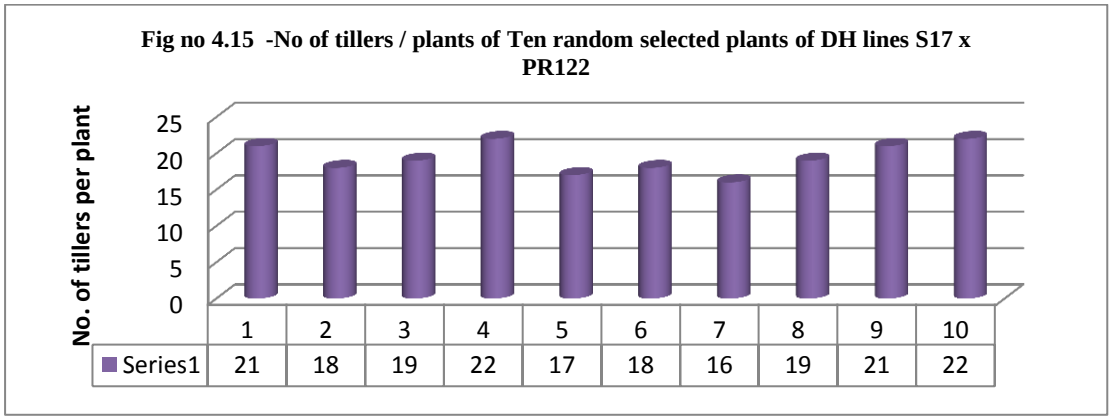


Fig no 4.15 -No of tillers / plants of Ten random selected plants of DH lines S17 x PR122
No. of tillers per plant
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10
Series1 21 18 19 22 17 18 16 19 21 22

4.3.2 Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x RPBIO-112(A).

The S-17 x RPBIO-112(A) showed mean performance of different character like plant height 119.7 (cm), Number of tiller 21.9, Number of panicle 20.6, Seed weight per/plant 47.2(gm), Panicle length 24.4 (cm), Flag leaf length 42.2 (cm). Flag leaf width 1.41 (cm), 1000 seed weight 25.96(gm), Grain length 9.19 (mm), Grain width 2.66 (mm), 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 (Table 4.12)

Table no 4.12 - Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x RPBIO-112(A)

S.N	TRAIT	MEAN	SD	SE
1	Plant height	119.7	4.92	1.55
2	No. of tiller / plant	21.9	4.79	1.51
3	No. of panicle / plant	20.6	3.89	1.23
4	Seed weight / plant	47.2	7.67	2.42
5	Panicle length	24.4	1.5	0.47
6	Flag leaf length	42.2	2.29	0.72
7	Flag leaf width	1.41	0.12	0.03
8	1000 seed weight	25.96	1.31	0.41
9	Grain length	9.19	0.11	0.03
10	Grain width	2.66	0.05	0.01

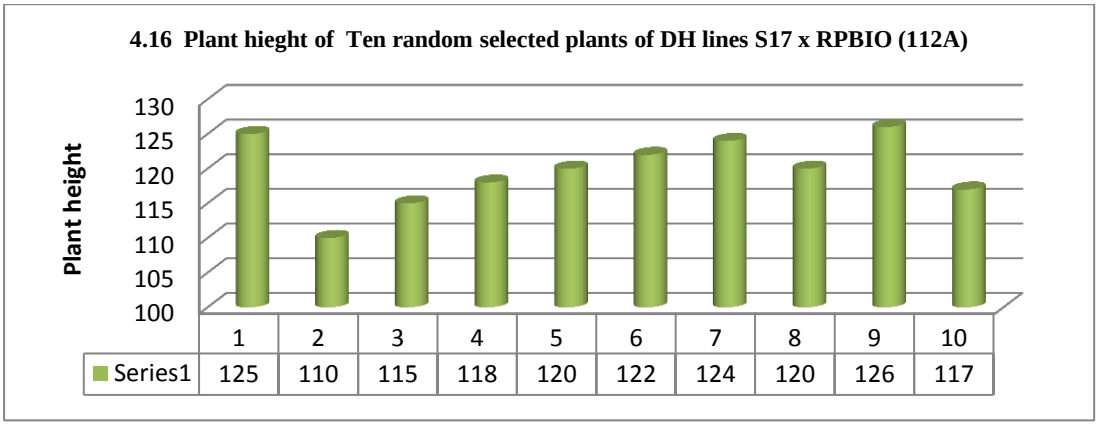
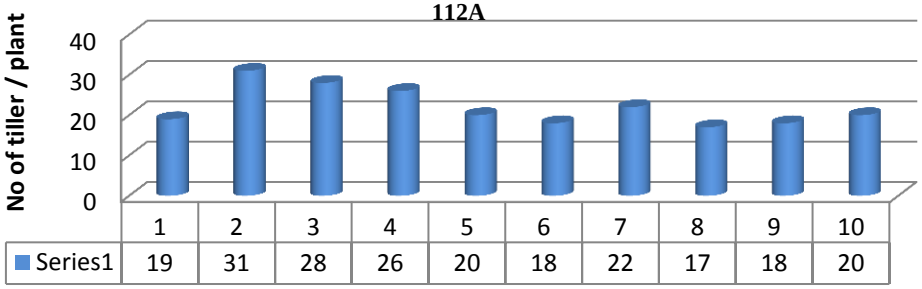
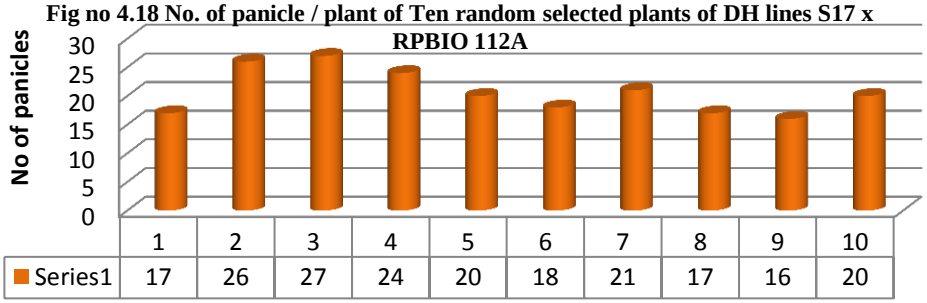


Fig no 4.17 No of tillers of Ten random selected plants of DH lines S17 x RPBIO 112A



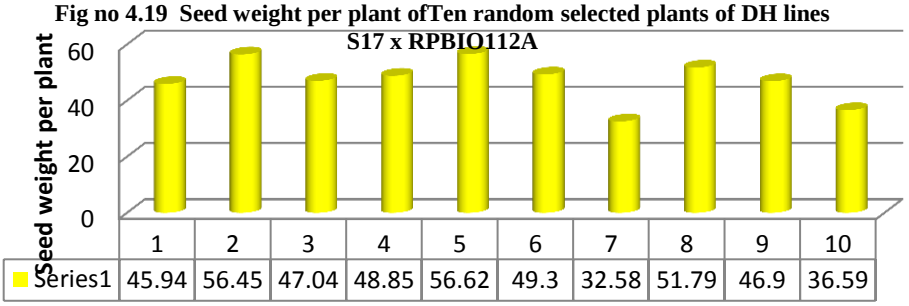
	1	2	3	4	5	6	7	8	9	10
Series1	19	31	28	26	20	18	22	17	18	20

Fig no 4.18 No. of panicle / plant of Ten random selected plants of DH lines S17 x RPBIO 112A



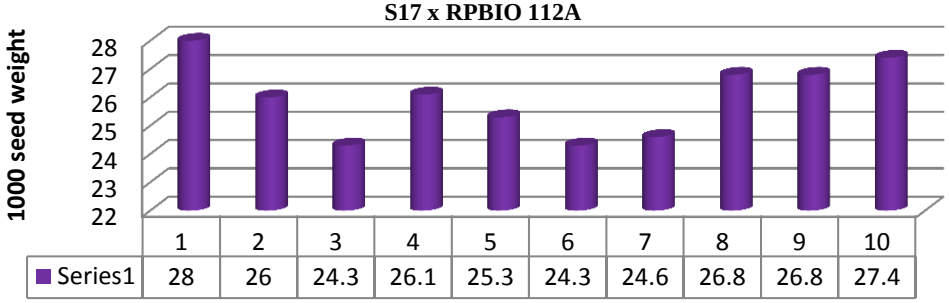
	1	2	3	4	5	6	7	8	9	10
Series1	17	26	27	24	20	18	21	17	16	20

Fig no 4.19 Seed weight per plant ofTen random selected plants of DH lines S17 x RPBIO112A



	1	2	3	4	5	6	7	8	9	10
Series1	45.94	56.45	47.04	48.85	56.62	49.3	32.58	51.79	46.9	36.59

Fig no of 4.20- 1000 Seed weight of Ten random selected plants of DH lines S17 x RPBIO 112A



	1	2	3	4	5	6	7	8	9	10
Series1	28	26	24.3	26.1	25.3	24.3	24.6	26.8	26.8	27.4

4.3.3 Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x RPBIO-235

The S-17 x RPBIO-235 showed mean performance of different character like plant height 110.4 (cm), Number of tiller 19, Number of panicle 17.7, Seed weight per/plant 47.2(gm), Panicle length 25.4 (cm), Flag leaf length 27.8 (cm). Flag leaf width 1.48 (cm), 1000 seed weight 25.06(gm), Grain length 8.94 (mm), Grain width 2.44 (mm), 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 (Table no 4.13)

Table no 4.13 - Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x RPBIO-235

S.N	TRAIT	MEAN	SD	SE
1	Plant height	110.4	2.75	0.87
2	No. of tiller / plant	19	1.82	0.57
3	No. of panicle / plant	17.7	1.63	0.51
4	Seed weight / plant	47.2	5.31	1.68
5	Panicle length	25.4	0.96	0.3
6	Flag leaf length	27.8	2.25	0.71
7	Flag leaf width	1.48	0.12	0.08
8	1000 seed weight	25.06	2.14	0.67
9	Grain length	8.94	0.05	0.01
10	Grain width	2.44	0.05	0.01

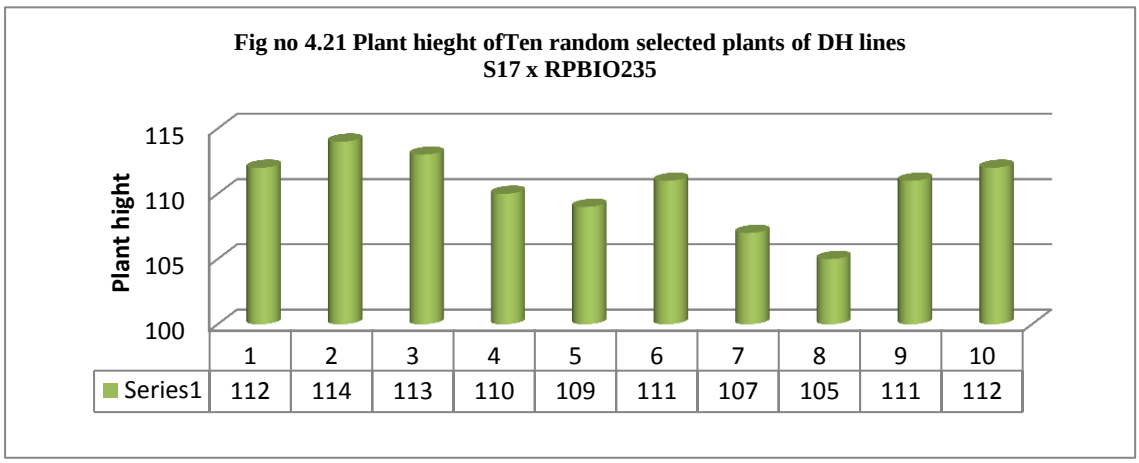


Fig no 4.22 No of tillers of Ten random selected plants of DH lines S17 x RPBIO235

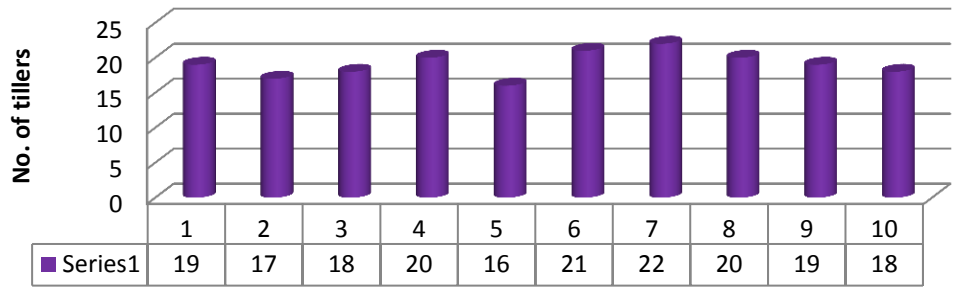


Fig no 4.23 seed weight of Ten random selected plants of DH lines S17 x RPBIO235

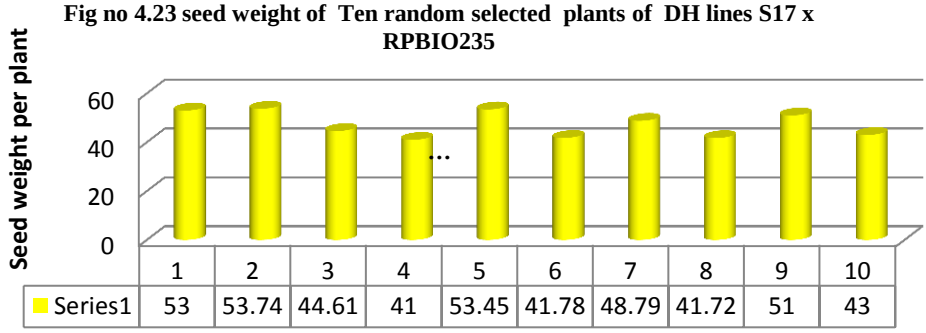


Fig no 4.24 No of panicles / plantts of DH lines S17 x RPBIO 235

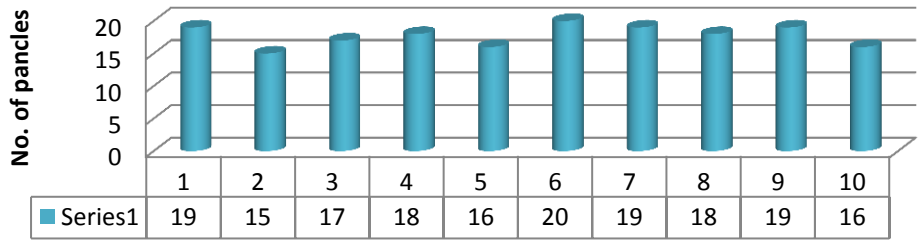
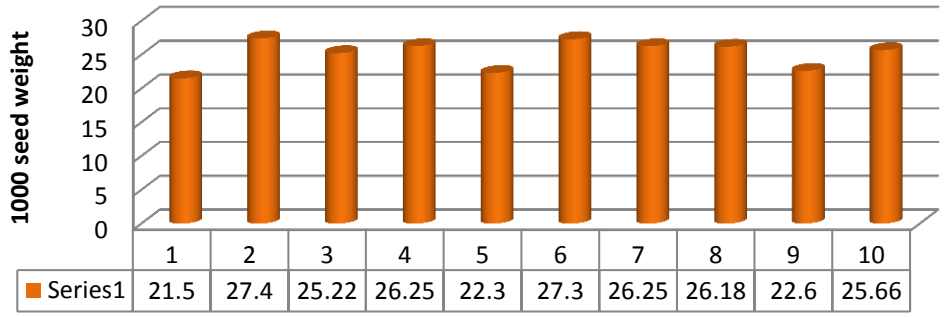


Fig no 4.25 1000 of Ten random selected plants of DH lines S17 x RPBIO 235



4.3.4 Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x IR64-185

The S-17 x IR64-185 showed mean performance of different character like plant height 127.2 (cm), Number of tiller 19.3, Number of panicle 18.4, Seed weight per/plant 49.31(gm), Panicle length 28.9 (cm), Flag leaf length 38.9 (cm). Flag leaf width 1.48 (cm), 1000 seed weight 25.27(gm), Grain length 8.81 (mm), Grain width 2.38 (mm), 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 4.14- Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x IR64-185

S.N	TRAIT	MEAN	SD	SE
1	Plant height	127.2	3.25	1.02
2	No. of tiller / plant	19.3	3.68	1.02
3	No. of panicle / plant	18.4	3.56	1.12
4	Seed weight / plant	49.31	9.08	2.87
5	Panicle length	28.9	2.46	0.77
6	Flag leaf length	38.9	3.69	1.16
7	Flag leaf width	1.48	0.27	0.08
8	1000 seed weight	25.27	0.92	0.29
9	Grain length	8.81	0.07	0.02
10	Grain width	2.38	0.06	0.01

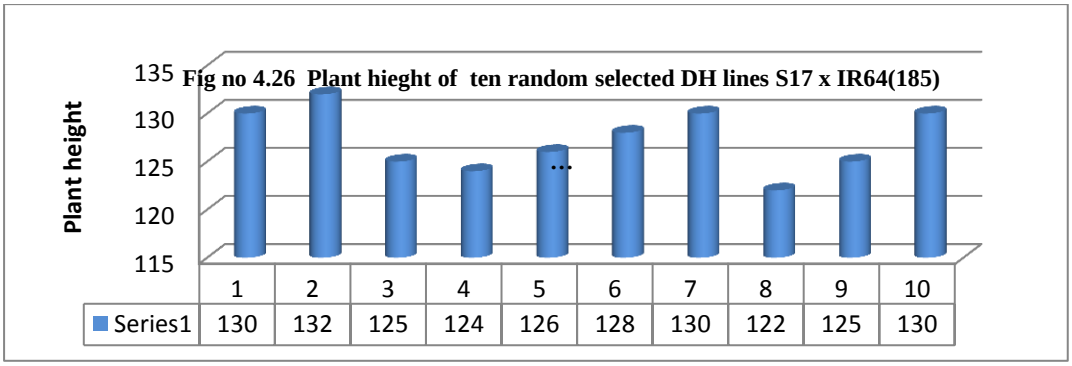


Fig no 4.27 No of tillers / plant od ten random selected DH lines S17 x IR64(185)

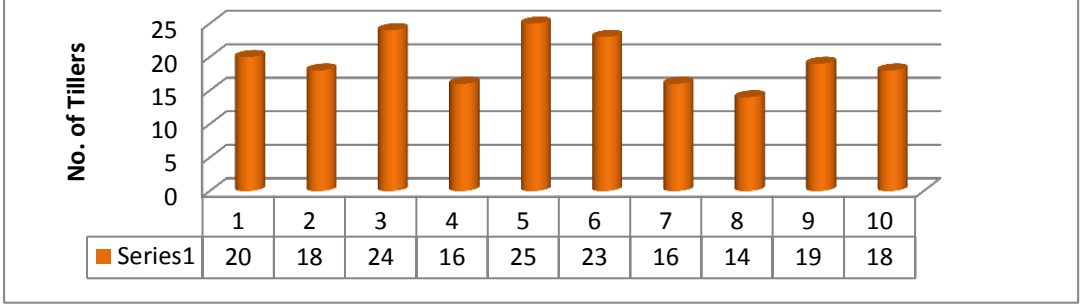


Fig no 4.28 No of panicles / plant of ten random selected DH lines S17 x IR64(185)

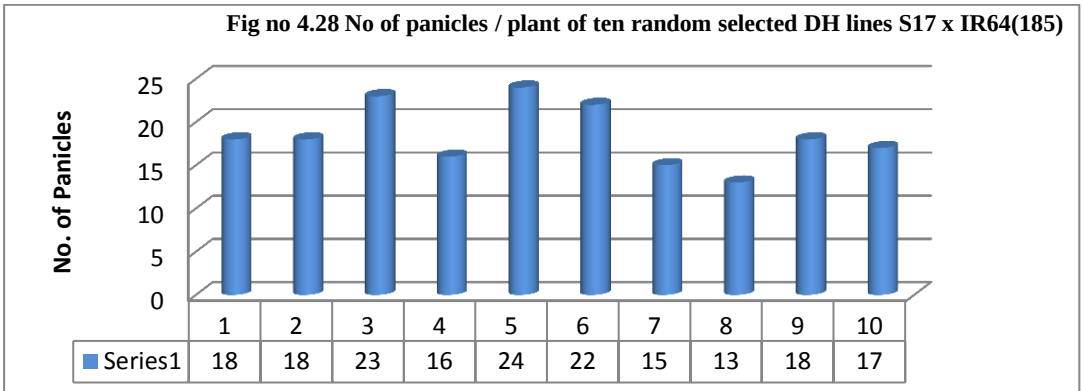


Fig no. 4.29 Seed weight of / plant of ten random selected DH lines S17 x IR 64(185)

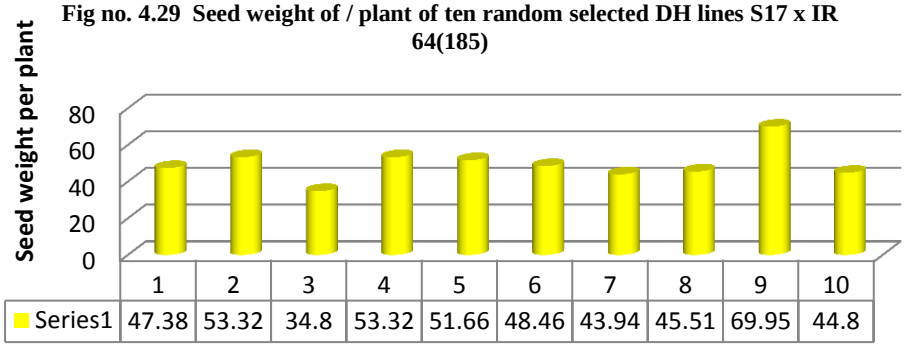
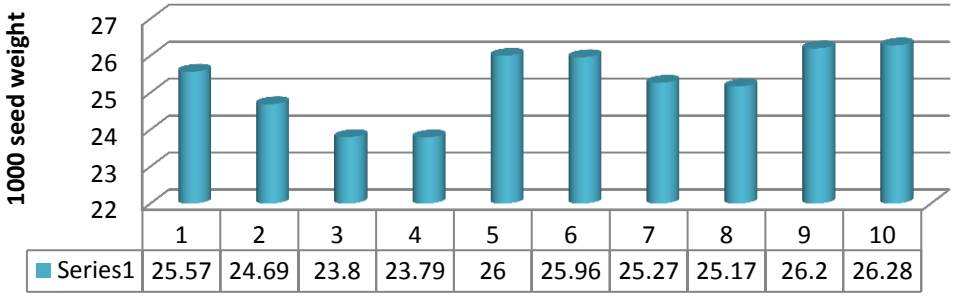


Fig no 4.30 - 1000 Seed weigtht / plantof Ten random selectected plants of DH linesS17 xIR64 (185)



4.3.5 Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x IR64-203

The S-17 x IR64-203 showed mean performance of different character like plant height 112.1 (cm), Number of tiller 21.4, Number of panicle 18.4, Seed weight per/plant 37.36(gm), Panicle length 26.7 (cm), Flag leaf length 37.8 (cm). Flag leaf width 1.58 (cm), 1000 seed weight 27.8(gm), Grain length 11.09 (mm), Grain width 2.11 (mm), 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 (Table 4.15).

Table no 4.15 - Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x IR64-203

S.N	TRAIT	MEAN	SD	SE
1	Plant height	112.1	3.51	1.11
2	No. of tiller / plant	21.4	3.16	1
3	No. of panicle / plant	18.4	2.83	0.89
4	Seed weight / plant	37.36	10.57	3.34
5	Panicle length	26.7	2.66	0.084
6	Flag leaf length	37.8	2.25	0.071
7	Flag leaf width	1.58	0.13	0.04
8	1000 seed weight	27.8	0.85	0.26
9	Grain length	11.09	0.18	0.05
10	Grain width	2.11	0.07	0.03

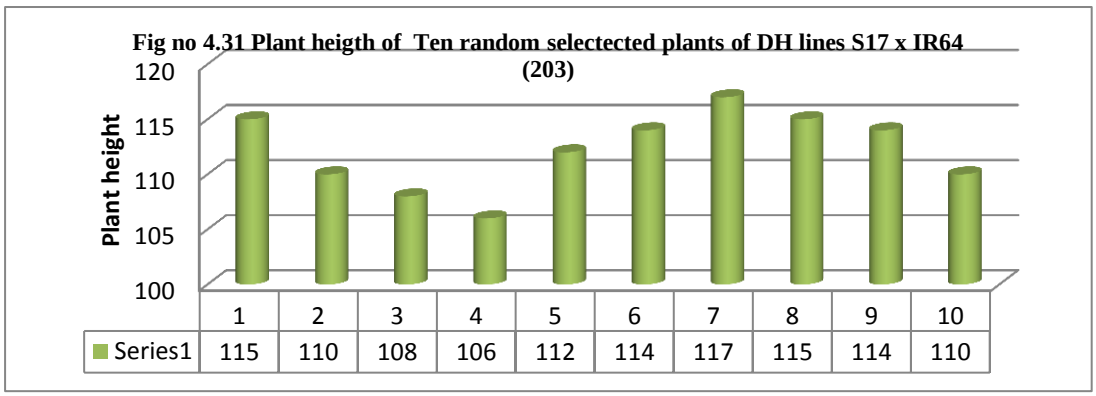


Fig no 4.32 No of tillers / plantof Ten random selectected plants of DH lines S17 x IR64 (203)

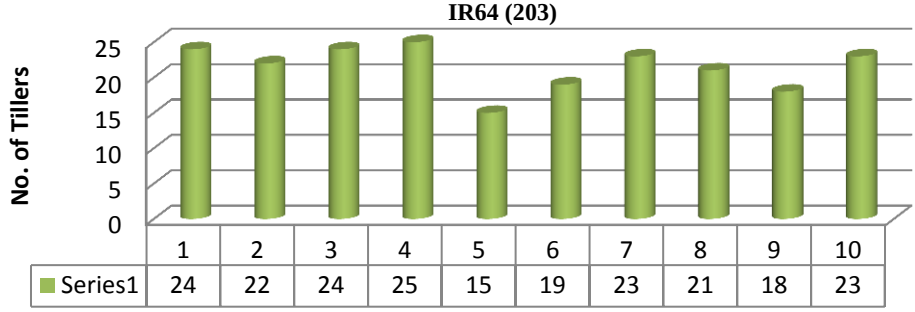


Fig no 4.33 No of panicles / plants of Ten random selectected plants of DH lines S17 x IR64 (203)

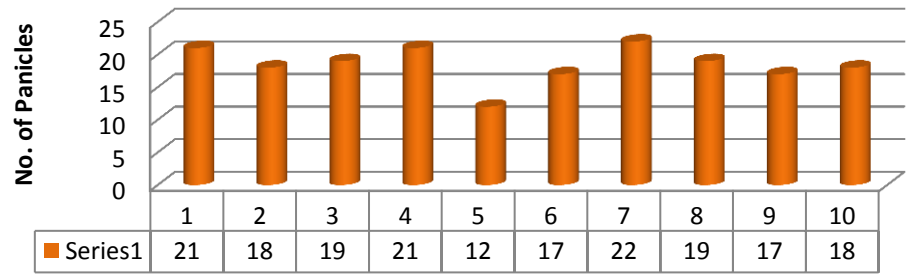


Fig no 4.34 Seed weight / per plant of Ten random selectected plants of DH lines S17 x IR64(203)

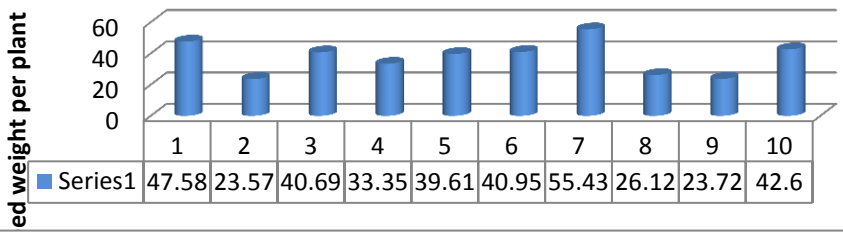
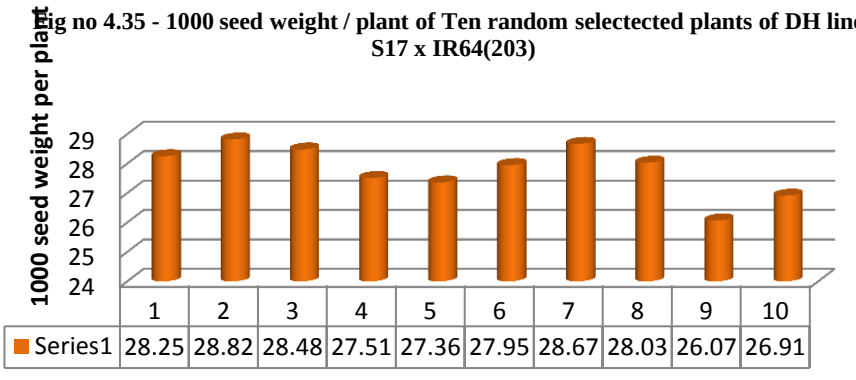


Fig no 4.35 - 1000 seed weight / plant of Ten random selectected plants of DH lines S17 x IR64(203)



4.3.6 Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x RPBIO 1353

The S-17 x RPBIO 1353 showed mean performance of different character like plant height 138 (cm), Number of tiller 11.2, Number of panicle 10.7, Seed weight per/plant 54.58 (gm), Panicle length 28.7 (cm), Flag leaf length 50 (cm). Flag leaf width 2.05 (cm), 1000 seed weight 26.82(gm), Grain length 7.92 (mm), Grain width 2.85 (mm), 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 (Table no 4.16)

Table no 4.16 - Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x RPBIO 1353

S.N	TRAIT	MEAN	SD	SE
1	Plant height	138	2.21	0.69
2	No. of tiller / plant	11.2	1.75	0.55
3	No. of panicle / plant	10.7	1.7	0.53
4	Seed weight / plant	54.58	6.28	1.98
5	Panicle length	28.7	0.67	0.21
6	Flag leaf length	50	2.16	0.68
7	Flag leaf width	2.05	0.15	0.04
8	1000 seed weight	26.82	0.39	0.12
9	Grain length	7.92	0.09	0.02
10	Grain width	2.85	0.05	0.01

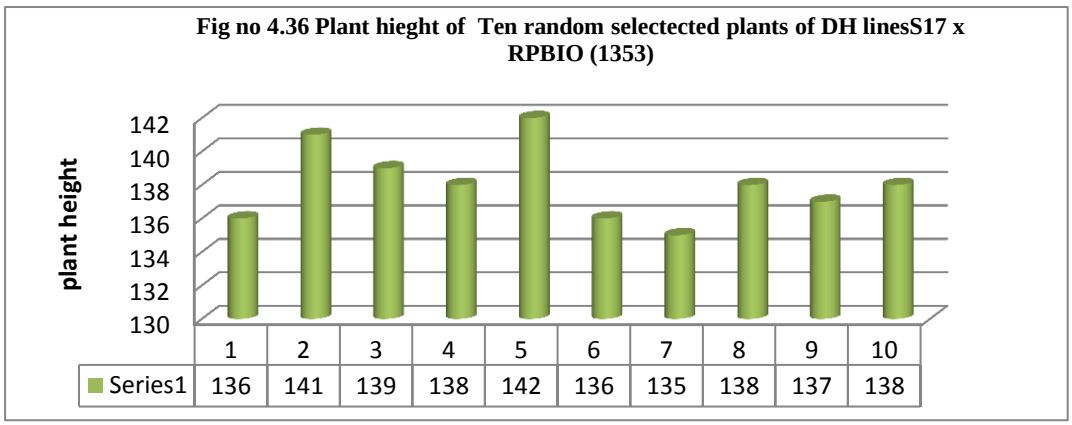


Fig no 4.37 No of Tillers/ plant ofTen random selectected plants of DH lines S17 RPBIO(1353)

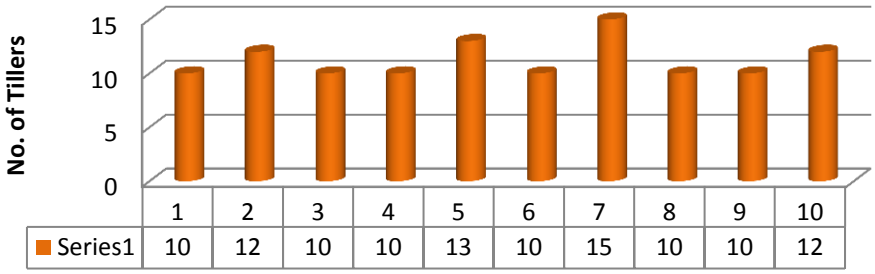


Fig no 4.38 No of panicle / plant of Ten random selectected plants of DH lines S17 x RPBIO (1353)

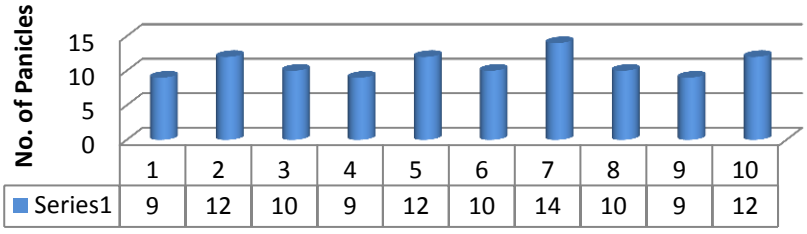


Fig no 4.39 Seed weight / plant of Ten random selectected plants of DH lines S17 x RPBIO (1353)

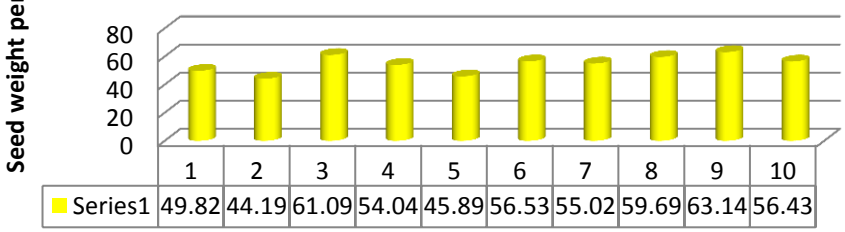
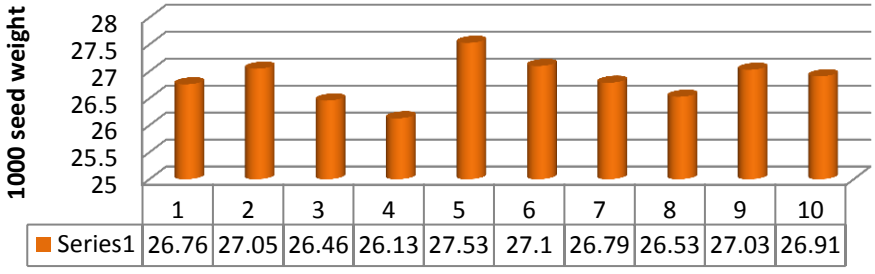


Fig no 4.40 - 1000 seed weight / plant of Ten random selectected plants of DH lines S17 xRPBIO (1353)



4.3.7 Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x RPBIO 228

The S-17 x RPBIO 228 showed mean performance of different character like plant height 140.1 (cm), Number of tiller 17, Number of panicle 16.2, Seed weight per/plant 54.51 (gm), Panicle length 28.6 (cm), Flag leaf length 54.5 (cm). Flag leaf width 2.26 (cm), 1000 seed weight 26.76(gm), Grain length 7.94 (mm), Grain width 2.97 (mm), 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 4.17 - Mean performance, standard deviation (SD) and standard error (SE) for quantitative traits of DHs line S-17 x RPBIO 228

S.N	TRAIT	MEAN	SD	SE
1	Plant hight	140.1	3.07	0.97
2	No. of tiller / plant	17	2.4	0.75
3	No. of panicle / plant	16.2	2.39	0.14
4	Seed weight / plant	54.51	3.44	1.08
5	Panicle length	28.6	1.95	0.61
6	Flag leaf length	54.5	1.58	0.5
7	Flag leaf width	2.26	0.1	0.03
8	1000 seed weight	26.76	0.64	0.2
9	Grain length	7.94	0.1	0.01
10	Grain width	2.97	0.09	0.03

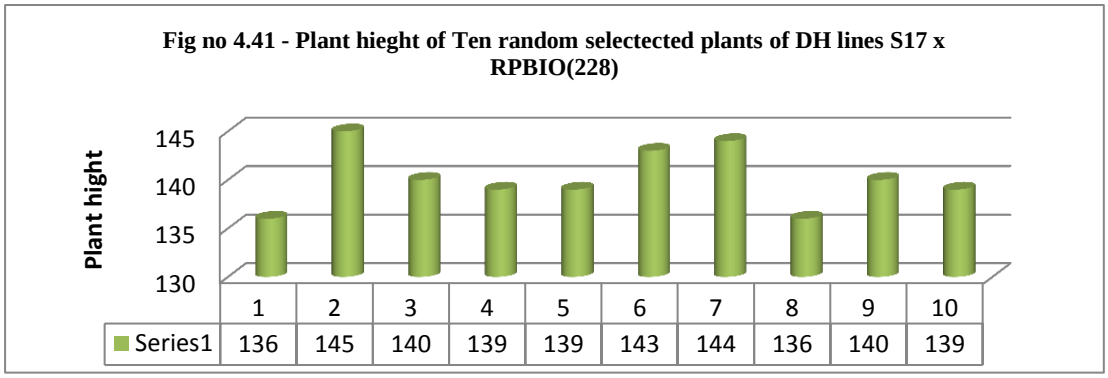


Fig no 4.41 - Plant hieght of Ten random selectected plants of DH lines S17 x RPBIO(228)

Fig no 4.42 No of tillers / plants of Ten random selectected plants of DH lines S17 x RPBIO (228)

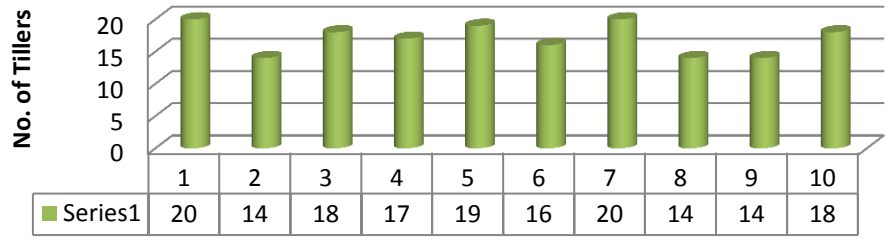


Fig no 4.43 No of Panicle / plant of Ten random selectected plants of DH lines S17 x RPBIO(228)

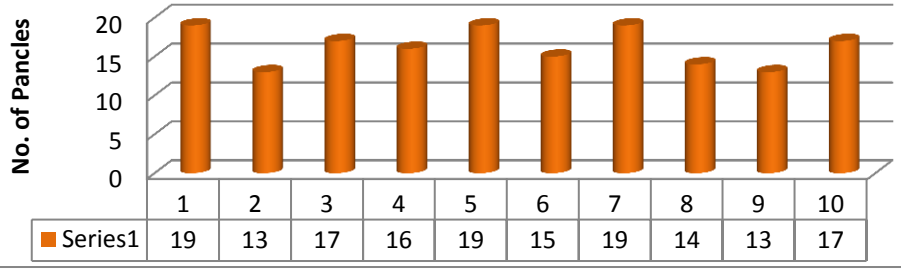


Fig no 4.44 Seed weight / plant of Ten random selectected plants of DH lines S17xRPBIO(228)

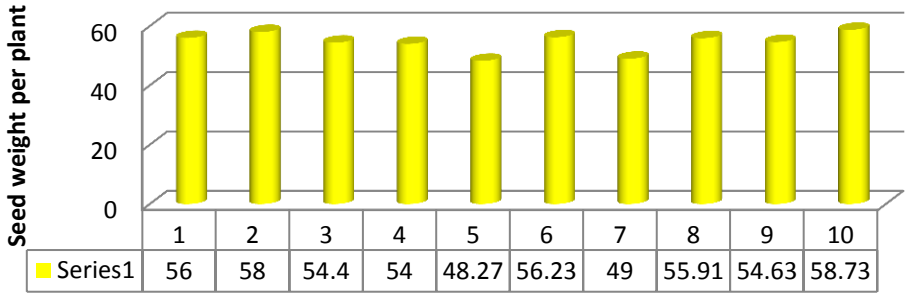
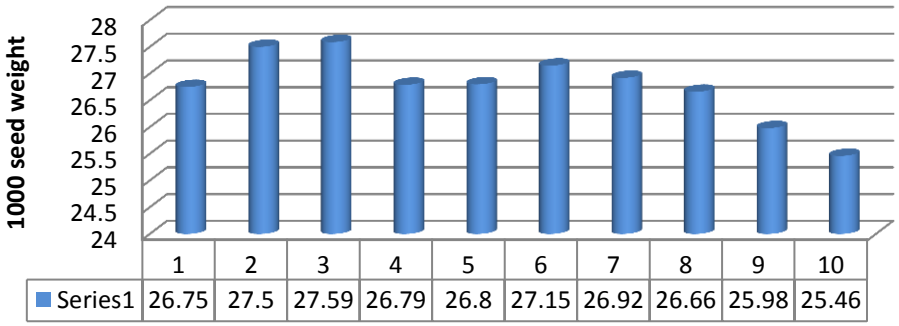


Fig no 4.45 - 1000 seed weight of Ten random selectected plants of DH lines S17 xRPBIO(228)



4.4 Assessment of homozygosity in individual doubled haploid population using molecular marker.

SSR is very cost effective and co dominant and powerful DNA marker to easily 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 7 different DH lines of 7 different crosses S-17 x PR-122(150),S-17 x RP BIO(112 A), S-17 x RP BIO(235), S-17x IR64 (185), S-17 x IR64(203), S-17 x RP BIO(228),S- 17 x RP BIO-(1353) 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. we have use 130 no of SSRs marker of different locus of chromosome to check for homozygosity and uniformity of the 7 DHs line out of 66 SSR marker are amplified and 5 marker are shown parental polymorphism that confirm to DH lines. (fig-4.46)

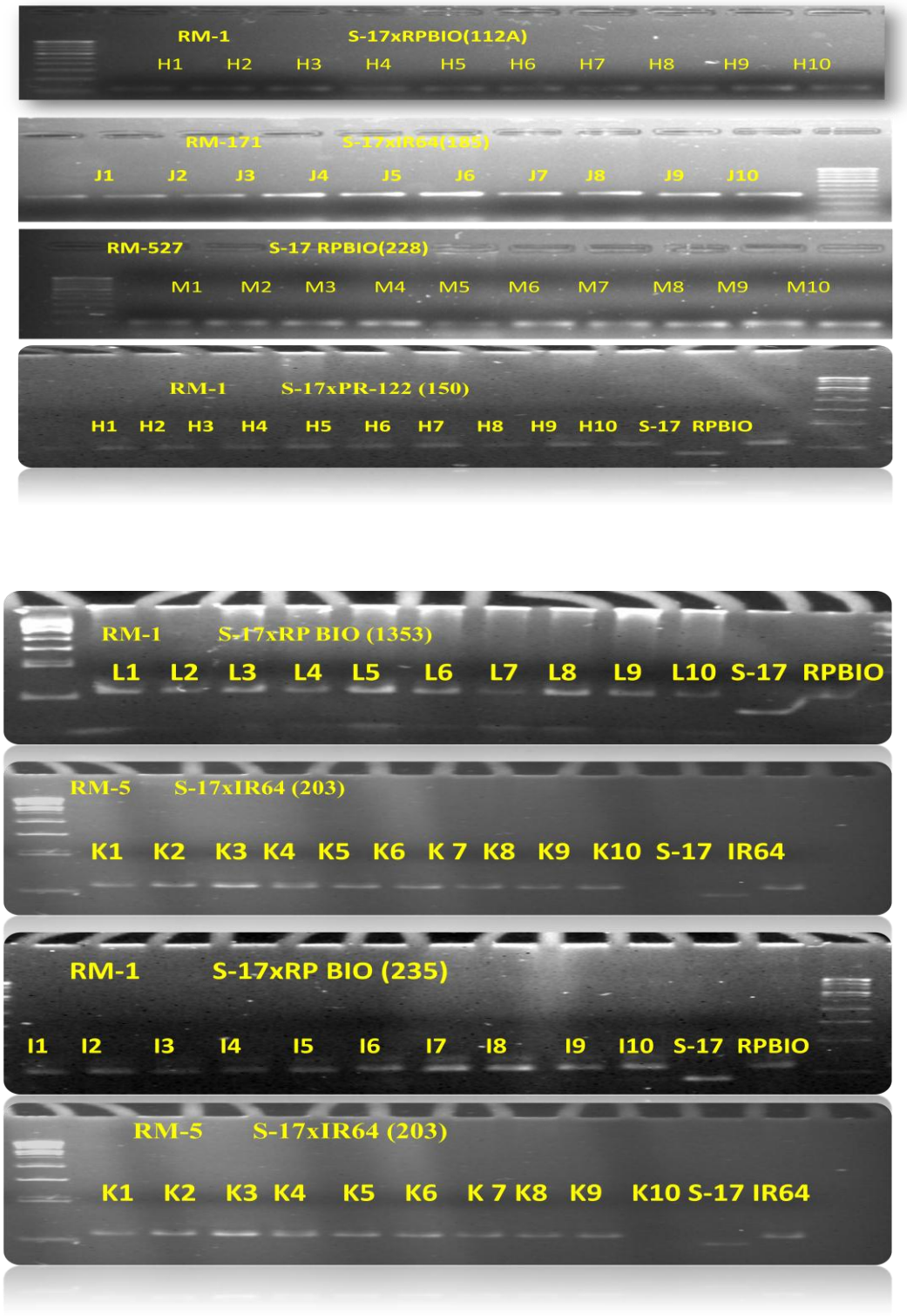


Fig 4.46 - Digrammatic representation of gel image

CHAPTER – V
SUMMARY AND CONCLUSIONS

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 The production of haploids and Double haploids (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).

A. *In vitro* application of colchicine at various stages of 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. After pretreatment of boots, 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 were dusted on each petri plate. Then the petri plates were incubated in 25±2˚C inside a BOD incubator for callus induction.

In Swarna sub 1 x IR 90019-17-159-B , MTU 1010 x Dagardesi and RF-75 Anthers were dipped in 0.05% and 0.1% aqueous solution of colchicine for 1, 2 and 3 hours respectively along with control condition. There was no callus induction observed in colchicine treatment. And control condition callus induction percent respectively 11.84%, 8.61% and 13.005%. In Variety RF- 75 callus induction percentage was higher (13.05%) as compare to Swarna sub 1 x IR 90019-17-159-B (11.84 %) and MTU1010 x Dagardeshi (F3) (8.61%).

In Swarna sub 1 x IR 90019-17-159-B was transferred to different media(T11 and T15) with different colchicine treatments (0.05% or 0.1%) for 2, 6, 24, 48 hours respectively along with control condition (without colchicine). In

Swarna sub 1 x IR159-B highest callus greening percent (47.82%) was found in T15 media containing 0.1% Colchicine in 48 hours. shown in (Table 4.5).

In MTU1010 x dagardesi (F3) was transferred to different media(T11 and T15) with different colchicine treatments (0.05% or 0.1%) for 2, 6, 24, 48 hours respectively along with control condition (without colchicine) for 2 ,6, 24, 48 hours respectively along with control condition (without Colchicine). In MTU 1010 x Dagardesi highest callus greening percent (12.5%) was found in T15 media containing 0.1% Colchicine in 48 hours. shown in (table 4.7).

In RF- 75 was transferred to different media (T11 and T15) with different colchicine treatments (0.05% or 0.1%) for 2, 6, 24, 48 hours respectively along with control condition (without colchicine) for 2 ,6, 24, 48 hours respectively along with control condition (without Colchicine). In RF-75 highest callus greening percent (8.69%) was found in T15 media containing 0.1% Colchicine in 48 hours. Shown in (Table 4.9)

Total of 25 plant has been generated in Swarna sub 1 x IR159-B . In MTU 1010 x dagardesi total of 6 plant generated and total of 23 plant has been generated in RF 75.

B . Morphological characterization of doubled haploid rice progenies to establishing the homozygosity.

In morphological 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 of all character that can be confirm that DHs lines are homozygous and uniform in nature.

C. Assessment of homozygosity in individual doubled haploid population using molecular marker.

SSR marker are used for differentiate DHs from were selected (DHs line of kharif 2015). 70 plants randomly selected from all 7 DH lines S-17xPR-122 (150), S-17xRP BIO (112A), S-17xRP BIO (235), S-17x IR64

(185), S-17xIR64 (203), S-17xRP BIO (1353), S-17xRP BIO (228) individually were assessed for their genetic stability and homozygosity using SSR markers. In molecular analysis we have use 13 of no of SSRs marker of different locus of chromosome to check for homozygosity and uniformity of the 7 DHs lines. we have use 130 no of SSRs marker of different locus of chromosome to check for homozygosity and uniformity of the 7 DHs line out of 66 SSR marker are amplified and 5 marker are shown parental polymorphism that confirm to DH lines trail under MLT for there evaluation.

CONCLUSION:-

- In Variety RF- 75 callus induction response was best (13.05%) compare to Swarna sub 1 x IR 90019-17-159-B (11.84%) and MTU1010 x Dagardeshi . (8.61%).
- In cross Swarna sub-1 x IR 90019-17-159-B, MTU1010 x Dagardeshi and variety RF-75 best callus greening was respectively 47.82 % , 12.5 %, 26.92 % was found in T15 media containing (0.01% colchicine) in 48 hours.
- 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 in all characters that confirms homozygosity in these lines.
- Molecular analysis , we have use 130 no of SSRs marker of different locus of chromosome to check for homozygosity and uniformity of the 7 DHs line out of 66 SSR marker are amplified and 5 marker are shown parental polymorphism that confirm to DH lines.
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SUGGESTIONS FOR FUTURE RESEARCH WORK

- The plants generated after *in vitro* treatment with colchicine is needed to be analyzed for its ploidy level to confirm whether the *in vitro* treatment is efficiently for doubling of chromosome or not.

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