PRODUCTION OF SEMI-DWARF AND BLAST RESISTANT DERIVATIVES OF RANBIR BASMATI USING ANTHER CULTURE AND MARKER-ASSISTED-SELECTION

THESIS

By

PRABHUDUTT SAMAL
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CHAUDHARY SARWAN KUMAR
HIMACHAL PRADESH KRISHI VISHVAVIDYALAYA
PALAMPUR – 176 062 (H.P.) INDIA

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Dr. Rajeev Rathour
Senior Scientist

Department of Agricultural Biotechnology
College of Agriculture, CSK HPKV,
Palampur (H.P.) India – 176062

CERTIFICATE – I

This is to certify that the thesis entitled **"Production of semi-dwarf and blast resistant derivatives of Ranbir Basmati using anther culture and marker-assisted-selection"** submitted in partial fulfillment of the requirements for the award of the degree of **Master of Science (Agriculture)** in the discipline of **Agricultural Biotechnology** of CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur is a bonafide research work carried out by **Prabhudutt Samal (Admn no. A-2016-30-007)** son of **Smt. Bidyut Lata Bhuyan** and **Sh. Bhagaban Samal** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

Place : Palampur
Dated: February 2019

(Dr. Rajeev Rathour)
Major Advisor

CERTIFICATE- II

This is to certify that the thesis entitled **"Production of semi-dwarf and blast resistant derivatives of Ranbir Basmati using anther culture and marker-assisted-selection''** submitted by **Prabhudutt Samal (Admn no. A-2016-30-007)** son of **Sh. Bhagaban Samal** to the CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur in partial fulfillment of the requirements for the degree of **Master of Science (Agriculture)** in the discipline of **Agricultural Biotechnology** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

(Dr. Rajeev Rathour) Chairperson Advisory Committee	( ) External Examiner
(Dr. K.D. Sharma) Member	(Dr. Nageswer Singh) Member
	(Dr. Virender Kumar) Dean's nominee

_____
Head of the Department

_____
Dean, Postgraduate Studies

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Place: Palampur
Dated February 2019 ***(Prabhudutt Samal)***

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LIST OF ABBREVIATIONS USED

Abbreviation	Meaning
%	Per cent
@	At the rate of
µl	Microlitre
µM	Micro molar
2,4-D	2,4-Dichlorophenoxyacetic Acid
BAP	Benzylaminopurine
cm	Centimetre
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
Fe	Ferrous
g	Grams
h	Hours
HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
i.e.	Id Est.
I_2	Iodine
IARI	Indian Agricultural Research Institute
IBA	Indolebutyric Acid
Indel	Insertion-Deletion
IRRI	International Rice Research Institute
Kb	Kilo base
KCl	Potassium chloride
KI	Potassium iodide
KOH	Potassium hydroxide
L	Litre
lbs	Libra (Latin)

M	Molar
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minutes
ml	Millilitre
mM	Millimolar
mm	Millimetre
N	Normal
NAA	Naphthaleneacetic Acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometre
^oC	Degree Celsius
PCR	Polymerase Chain Reaction
pH	Puissance de hydrogen (Ion Conc.)
PVP	Polyvinylpyrrolidone
R^2	Cofficent of Determination
SS	Stock Solution
SSR	Simple Sequence Repeats
STS	Sequence Tagged Site
Syn	Synonym
Taq	*Thermus aquaticus* DNA Polymerase
TBE	Tris Borate EDTA Buffer
TE	Tis EDTA Buffer
Tris	Tris (Hydroxy Methyl) amino methane
UV	Ultra violet
vis-à-vis	In relation to
viz.	Namely

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Department of Agricultural Biotechnology
CSK Himachal Pradesh Krishi Vishvavidyalaya
Palampur-176062

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ABSTRACT

Rice blast is one of the most important fungal diseases in rice, which is caused by the fungus *Magnaporthe oryzae* (syn: *Pyricularia oryzae Cav*.). The disease not only causes reduction in yield but also results in chalky and sterile grains thus ultimately deteriorating the grain quality in Basmati rice. The present investigation was aimed at development of the semi-dwarf and blast resistant fixed derivatives of a traditional Basmati rice variety Ranbir Basmati through introgression of blast resistance gene *Pi-9* and a recessive semi-dwarfing gene *sd-1* from a Basmati donor PB1(Pi9). Of the total 388 BC_2F_2 plants of cross Ranbir Basmati*^{3}/ PB1(Pi9) subjected to marker-assisted foreground selection, 23 were found to be homozygous for both the genes *Pi-9* and *sd-1*. Of the 12459 anthers of BC_2F_2 progenies of plant JKR-1-29-100 only 68 formed calli, whereas 28 calli were induced from the 6043 anthers obtained from BC_2F_2 progenies of JKR1-34-16. A total of 37 plantlets were regenerated from the 96 anther derived calli of the selected BC_2F_2 progenies with overall regeneration frequency of 38.54%. A significant proportion of regenerated plantlets were albinos. Of the total 37 regenerated plantlets 21 **(**56.75%) were green, while the remaining 16 (43.24%) were albinos. The overall anther culture response of the BC_2F_2 derivatives of cross Ranbir Basmati*^{3}/ PB1(Pi9) was very low (0.11%) as only 21 green plantlets could be regenerated from the 18502 cultured anthers. The analysis of yield and its component traits in anther culture derived doubled haploid (DH) plants revealed the presence of superior transgressive segregants for the traits *viz.,* effective tillers, grains per panicle and yield per plant; some of the DH plants out performed both the parental genotypes for these traits. Two DH lines, DH-6 and DH-11, besides being semi-dwarf and highly resistant to blast, exhibited yield, maturity and grain quality attributes (except grain length) comparable or better than Ranbir Basmati. These lines can be further evaluated for yield and blast resistance under multi-location yield trials to assess their potential as new varieties.

(Prabhudutt Samal)
Student
Date: February, 2019

(Dr. Rajeev Rathour)
Major Advisor
Date: February, 2019

Head of the Department

1. INTRODUCTION

Rice (*Oryza sativa* L.) is the world's most important cereal crop and a primary source of food for more than half of the world's human population. It is grown worldwide over an area of 167.24 million hectares with total production of 769.65 million tonnes (FAO 2017). In India, rice is grown on an area of 43.78 million hectares with a production of 168.50 million tonnes (FAO 2017). Rice is endowed with a great diversity with more than thousand landraces and improved cultivars maintained in the germplasm collections throughout the world. This amazing varietal diversity has arisen mainly due to diverse agro-climatic conditions coupled with conscious and continuous selection by the humans for their diverse quality preferences. A unique varietal group that has evolved as a result of natural and human selection, which found wider acceptance all over the world as a unique rice is called ''Basmati rice''.

Basmati rice has its origin in the Indian subcontinent (Singh et al. 2000) and belongs to group V of *O. sativa* L. (Glaszmann 1987). In India, it is mainly cultivated on an area of 15.54 lakh hectares with annual production of 56.46 lakh tonnes (Anonymous 2017) within a limited geographic region comprising parts of Punjab, Haryana, West Uttar Pradesh, Uttarakhand, Delhi, three districts of Jammu & Kashmir (Jammu, Samba and Kathua) and to a limited extent in Himachal Pradesh. The basmati rices grown in the Indo-Gangetic plains and foot-hills of Himalayas are highly priced in the international market for their unique grain and eating quality attributes like pleasing aroma, fluffy texture, and better kernel elongation with minimum swelling, good palatability and longer shelf life. These attributes have evolved in traditional basmati through centuries of selection and have been retained in their purest form in these varieties (Nagaraju et al. 2002). The traditional basmati varieties like Taroari Basmati, Basmati 370 and Type 3 (Dehraduni) still maintain their supremacy in domestic and international markets due to their exclusive quality features (Rani et al. 2006). Despite having excellent grain quality, these traditional varieties are tall statured, prone to lodging even under low fertilizer applications, susceptible to diseases and low yielding (Rani and Singh 2003).

All traditional basmati cultivars display a very high degree of susceptibility to blast disease caused by the fungus *Magnaporthe oryzae* (syn: *Pyricularia oryzae Cav*.) (Khanna et al. 2015). The disease not only causes reduction in yield but also results in chalky and sterile grains thus ultimately deteriorating the grain quality. Although effective fungicides are available to control the disease, their use often leads to presence of pesticide residues in grains thus raising the concerns of food safety. Therefore breeding for resistance through incorporation of broad spectrum resistance (R) genes is the most effective approach to combat the menace of blast disease. In rice, about 114 blast resistance genes have been identified through genetic analyses of different rice varieties and 25 of them have been cloned (Xu et al. 2014; Ma et al. 2015). These developments have provided an array of tightly linked as well as functional gene-derived markers that can be used for marker-assisted selection of blast resistance genes in breeding programmes. The blast resistance gene *Pi-9* sourced from *Oryza minuta* has shown broad spectrum and durable resistance to blast world-wide (Amante-Bordeos et al.1992; Imam et al. 2014; Thakur et al. 2013). The gene is also highly effective against blast in Northern parts of India including the states of Jammu Kashmir, Himachal Pradesh and Uttarakhand which have been earmarked as geographical indication (GI) areas for basmati (Khanna et al. 2015; Rathour et al. 2011). The *Pi-9* gene has been cloned and efficient gene-based markers have been developed for its indirect selection in rice breeding programmes (Tian et al. 2016; Rathour et al. 2016).

The semi-dwarfing gene (*sd-1*) identified in the Chinese variety Dee-geo-woo-gen (DGWG) is one of the most important genes deployed in modern rice breeding. The gene confers semi dwarf stature, reduces culm length, and improves lodging tolerance (Pinthus 1974), thus allowing increased use of nitrogenous-fertilizers for realizing high yields (Murai et al. 2002). Since the 1960s, the *sd-1* has remained the most prominent dwarfing gene in semi-dwarf rice cultivars developed worldwide (Tomita and Ishii 2018). The gene has been cloned and encodes loss of function mutations in the gibberellin (GA)-20-oxidase, which is the key enzyme in the gibberellin biosynthesis pathway in rice (Monna et al. 2002; Spielmeyer et al. 2002). The gene-derived markers developed from the sequence information of cloned *sd-1* have been used for marker assisted-selection of recessive *sd-1* allele in breeding populations (Ellis and Spielemeyer 2002; Rajpurohit et al. 2011).

The protracted nature of classical breeding approaches impedes speedy development of varieties intorgressed with new genes. Developing a new rice variety through conventional breeding requires more than 6-7 years of painstaking effort to get true breeding lines which are sufficiently homozygous for advanced testing. The constraint even applies to most efficient maker-assisted backcross breeding strategies that are widely hailed to hasten the return to the recurrent parent genome; the foreground and background selected progenies are required to be subjected to several generations of selfing to eliminate residual heterozygosity and stabilize the recombinants. The *in vitro* technique of anther culture, however, offers a mean to achieve homozygosity over the course of few months, rather than several generations required in conventional breeding approach (Snape 1989). The technique accelerates the process of obtaining pure lines with numerous advantages: shortening breeding cycle by immediate fixation of homozygosity, which allows an easy selection of phenotypes for quantitative characters; increased selection efficiency, widening of genetic variability through the production of gametoclonal variants and allowing early expression of recessive genes (Biswas and Zapata 1992). Since the first release of an anther culture raised rice variety by Guang-Chu et al. (1976), the technique has extensively been used to develop rice varieties possessing desirable characteristics like superior grain quality, resistance to diseases and pests like blast, bacterial blight, brown plant hopper and tolerance to abiotic stresses (Lee et al. 1989; Patil et al. 1997; Senadhira et al. 2002; Pauk et al. 2009).

Ranbir Basmati, an early maturing pure line selection of traditional Basmati 370, is widely cultivated basmati variety in the Jammu, Samba and Kathua districts of the Indian state of Jammu and Kashmir. The variety is very popular among the farmers due to its palatable taste and early-maturity due to which it fits well in the prevailing rice-wheat cropping system. It covers more than 90% of the area planted with basmati varieties in the region. Similar to parental genotype Basmati 370, Ranbir Basmati is also tall statured, prone to lodging and highly susceptible to rice blast. The development of blast resistant, and semi-dwarf versions of Ranbir Basmati is, therefore, urgently needed to improve the productivity of this variety and prevent the losses caused by blast disease and lodging.

Considering the above facts, the present study was planned to bring about genetic improvement of Ranbir Basmati for semi dwarf phenotype and resistance to blast by introgressing blast resistance gene *Pi-9* and semi-dwarfing gene s*d-1* from a basmati donor line PB1(Pi9). The non-conventional breeding tools like marker-assisted selection and anther-culture were used to ensure speedy conversion of Ranbir Basmati into semi-dwarf lines with in-built resistance to blast. The following were the broad objectives:

1. Marker assisted selection of *sd-1* and *Pi-9* homozygous recombinants among the BC_2F_2 derivatives of cross Ranbir Basmati/ PB1(Pi9).
2. Anther culture of selected recombinants to generate homozygous doubled haploid lines

2. REVIEW OF LITERATURE

Rice is the second most widely grown cereal crop and is a staple food for more than half of the world's population. Basmati rice, owing to its characteristic aroma and long slender grains, is a specialized group of rice (*Oryza sativa* L.) and is high in demand. The rice production has been continuously challenged by many biotic and abiotic stresses. Among the diseases, rice blast disease, which is caused by the fungal pathogen *Magnaporthe oryzae* (syn: *Pyricularia oryzae Cav*.), is one of the most devastating plant diseases worldwide (Ou 1985). Disease resistant varieties represent the most economical, environmentally-safe and sustainable means of controlling the disease. In rice, about 114 blast resistance genes have been identified through genetic analyses of rice varieties belonging to both *japonica* and *indica* subspecies and 25 of them have been cloned and characterized till date (Xu et al. 2014; Ma et al. 2015).

These developments have provided an array of tightly linked as well as functional gene-derived markers that can be used for marker-assisted selection of blast resistance genes in breeding programmes (Jia et al. 2002; Shang et al. 2009). Transfer of several genes with potential characteristics into a single genotype is possible through marker assisted selection (MAS), which can quicken the advancement of tolerant/resistant cultivars in less time as compared to traditional breeding methods. MAS has several advantages such as speeding up recovery of recurrent parent genome, consistency, minimize the linkage drag, biosafety and more accurate selection, which made this approach more popular over the conventional one (Hasan et al. 2015). MAS is an efficient technique for transferring genes from donor lines into new plants and improved varieties. Marker assisted selection has been extensively used by breeders to develop resistant "pyramid" lines by pyramiding several resistance genes/QTLs with the help of closely linked markers against diseases such as rice blast (RB), bacterial blight (BB), gall midge in rice, leaf rust resistance and powdery mildew resistance in wheat, and insect pest resistance in cotton, as well as for several abiotic stresses such as submergence, salinity, drought, and cold stress in rice, wheat, and cotton (Das and Rao 2015; Pradhan et al. 2015; Suh

et al. 2013; Shamsudin et al. 2016). MAS is gaining considerable importance as it can improve the efficiency of plant breeding through precise transfer of genomic regions of interest and acceleration of the recovery of the recurrent parent genome (Wijerathna 2015).

Anther culture is a tissue culture technique which can be applied in plant breeding to accelerate the process of obtaining pure lines with numerous advantages: shortening breeding cycle by immediate fixation of homozygosity, which allows an easy selection of phenotypes for quantitative characters, increased selection efficiency, widening of genetic variability through the production of gametoclonal variants and allowing early expression of recessive genes (Biswas and Zapata. 1992). Since the first release of an anther culture raised variety by Guang-Chu et al. (1976), anther culture has extensively been used to develop rice varieties possessing desirable characteristics like high yield and resistance to various biotic and abiotic stresses. Use of anther culture has contributed more than 100 new high yielding varieties in China (Li 1992). In the following paragraphs, the relevant literature has been reviewed under the following heads:

2.1 Marker assisted selection for disease resistance and semi-dwarfing genes.

2.2 Anther culture in rice.

2.1 Marker assisted selection for disease resistance and semi-dwarfing genes

2.1.1 Marker assisted selection for disease resistance genes

Hittalmani et al. (2000) used marker assisted selection to combine three blast resistance genes. *Pi-1* on chromosome 11 , *Pi-z5* on chromosome 6 and *Pi-ta* on chromosome 12, into a single cultivar Co39. The study demonstrated the effectiveness of MAS in developing gene pyramids and showed that the lines containing all three resistance genes had a broader resistance spectrum compared to those harboring single genes. At Punjab Agriculture University (PAU), Ludhiana, three bacterial blight (BB) resistance genes *xa-5*, *xa-13* and *Xa-21* have been pyramided in two widely adapted cultivars PR106 and Pusa 44 using marker-assited breeding (Singh et al. 2001). The pyramid lines having gene combinations *xa-l3+Xa-21* and *xa-5+xa-13+xa-21* have shown

resistance to all the prevalent pathotypes of *Xanthomonas oryzae pv. oryzae*. Jia et al. (2002) developed three STS markers *viz* YL153/YL154, YL155/YL87 and YL100/102 that perfectly co-segregate with the dominant *Pi-ta* allele in a range of genetic backgrounds.

Plaha et al. (2005) used marker-assisted selection to pyramid three blast resistance genes *Pi-1, Pi-54* and *Pi-ta* in an elite genotype HPU741. The PCR-based allele-specific and InDel markers sets have been developed for nine blast resistance genes, *Pi-z Pi-z*^{t}, *Pi-k*^{m}, *Pi-*^{k}, *Pi-k*^{p} *Pi-t, Pi-b, Pi-ta, Pi-ta*^{2}, providing an efficient system for MAS of blast resistance genes (Hayashi et al. 2006).

By combining marker assisted foreground selection with background analysis in a backcross breeding program, two bacterial blight resistance genes *xa-l3* and *Xa-21* have been transferred to the parental lines Pusa6B and PRR78 of a superfine grain aromatic rice hybrid Pusa RH 10 (Basvaraj et al. 2010). Yuan et al. (2011) combined anther culture and marker-assisted selection to introgress blast resistance gene into Y58S, an elite two-line photo-thermo sensitive genic male sterile line that is widely used for hybrid production in China.

Jiang et al. (2012) introgressed three blast resistance genes *Pi-t, Pi-t* and *D12* into Jin23B, a maintainer line for number of hybrid rice varieties in China using marker assisted backcross breeding. The improved versions of Jin23B were highly resistant to blast under field conditions. The improved Jin 23B and its derived hybrid rice were taller than or similar to control under disease free conditions. Singh et al. (2012) used marker-assisted backcross pedigree strategy to transfer blast resistance genes *Pi-2* and *Pi-54* into PRR78, the restorer line of a popular high yielding superfine grain aromatic rice hybrid Pusa RH10.

A major dominant gene conferring resistance against BB i.e. *Xa-21* and *Pi-54*, a blast resistance gene, have been introgressed into DRR17B, an elite, fine-grain type maintainer line of a stable wild-abortive cytoplasmic male sterile line DRR17A through marker-assisted backcross breeding to improve resistance against BB and blast (Balachiranjeevi et al. 2015). Hasan et al. (2015) demonstrated successful incorporation of blast resistance gene *Pi-kh* into a susceptible rice variety MR264 from local resistant genotypes, Pongsu Seribu 2 through marker-assisted

backcrossing (MABC) approach. Lee et al. (2015) developed rice variety 'Saeilmi' which showed resistance to panicle blast by introducing *Pb-1* resistance gene from 'Hwayeong' into susceptible variety 'Ilmi', by marker assisted backcross approach using DNA marker RM206.

Suh et al. (2015) successfully developed a multiple gene pyramided breeding lines (GPLs) for resistance to bacterial blight, blast, and brown plant hopper by introducing five resistance genes *viz.*, *Pi-40, Xa-4, xa-5, Xa-21* and *Bph-18* into a Korean *japonica* rice variety Jinbubyeo using marker-assisted selection (MAS).

Arunakumari et al. (2016) introgressed *Xa21* and *xa13,* two major bacterial blight resistance genes and a major gene for blast resistance i.e. *Pi-54* into an Indian rice variety MTU1010 by marker assisted backcross breeding. Rathour et al. (2016) developed co-dominant gene-derived STS and CAPS markers for marker assisted selection of blast resistance gene *Pi-9.* These markers have shown polymorphism between *Pi-9* donors and diverse varieties that include some of the most popular IRRI bred and many prominent mega varieties of India and other parts of Asian subcontinent. Tian et al. (2016) developed allele-specific marker for *Pi-2* and *Pi-9* genes that confer broad-spectrum resistance against diverse blast isolates. Two new markers named *Pi9-Pro* and *Pi2-LRR* were developed by targeting the unique polymorphism of the resistant susceptible alleles of *Pi-2* and of *Pi-9*, respectively. The InDel marker *Pi9-Pro* differentiated three genotypes corresponding to the *Pi-2/Piz-t*, *Pi-9* and non *Pi-2/Piz-t/Pi-9* alleles and the CAPS marker *Pi2-LRR* differentiated the *Pi-2* allele from the non Pi-*2* alleles.

Krishnamurthy et al. (2017) introgressed two broad-spectrum blast resistance genes *Pi-2* and *Pi-5* independently into a popular variety, Samba Mahsuri (BPT5204) through marker-assisted backcross breeding approach using tightly linked markers, AP5930 and 40N23r, respectively. Luo et al. (2017) developed a gene-targeted and co-dominant marker for the *Pi-2* and *Pi-9* genes. This robust and specific marker was derived from polymorphisms within the target gene, and could potentially distinguish *Pi-2* and *Pi-9* from other alleles through high-resolution melting of a small amplicon. The marker has been successfully used to transfer *Pi-2* gene into an elite restorer line R179 through marker assisted backcrossing. Luo et al. (2017) reported

pyramiding of rice blast R gene *Pi-9*, bacterial blight R genes *Xa-21* and *Xa-27*, and submergence tolerance gene *Sub-1A* in the genetic background of 93-11through backcrossing and marker-assisted selection. The newly improved rice line 49311 and its hybrid rice, GZ63S/49311, have shown resistance to rice blast and bacterial blight and tolerance to submergence for over 18 days without significant loss of viability. Miah et al. (2017) developed 13 improved blast resistant lines by introducing *Pi-z, Pi-2* and *Pi-9* broad-spectrum blast resistance genes into the cultivated rice variety MR219 by using SSR markers RM6836 and RM8225 for foreground selection.

Suwannual et al. (2017) pyramided four QTLs for blast resistance located on chromosomes 1, 2, 11 and 12, into *Thai* rice cultivar RD6 through marker-assisted selection using 8 flanking microsatellite markers to enable the selection in $BC_2F_{2:3}$ lines. Xiao et al. (2017) developed monogenic near-isogenic lines (NILs) NIL*Pi*9, NIL*Pizt* and NIL*Pi*54 by introgressing *Pi-9*, *Pi-zt*, and *Pi-54*, respectively into an agronomically superior *japonica* genetic background by marker assisted backcross breeding. They also developed polygene pyramid lines (PPLs) combining *Pi-9* with *Pi-54*, and four combining *Pi-zt* with *Pi-54* using corresponding NILs.

Khan et al. (2018) introduced major blast resistance genes *Pi-54, Pi-1* and *Pi-ta* into a popular susceptible aromatic landrace *Mushk Budji* known for its aroma and exquisite quality, from non-aromatic three-gene donor DHMAS70Q 164-1b through marker-assisted foreground and background selection. Mi et al. (2018) used marker assisted selection to introduce R genes *Pi-2* and *Xa-7* conferring resistance to rice blast and bacterial blight respectively, into Guangzhan63-4S, an elite photoperiod- and thermo-sensitive male sterile (P/TGMS) line which is widely used in two-line hybrid rice in China.

Rekha et al. (2018) introgressed two major blast resistance genes, *Pi-2* and *Pi-54* into Improved Samba Mahsuri (ISM), the bacterial blight resistant version of the popular variety Samba Mahsuri, through marker-assisted backcross breeding. Shankar et al. (2018) developed blast resistance variety of rice by inrogressing blast resistance gene *Pi-54* from Tetep into blast susceptible scented rice variety Kalanamak by marker assisted foreground selection and visual phenotyping for targeted traits.

2.1.2 Marker assisted selection for semi-dwarfing gene in rice.

Semi dwarfism is one of the most important traits in cereal crops, including rice. The rice Semidwarf-1 (*sd-1*) gene well known as the "green revolution gene", has contributed to the significant increase in crop production seen in the 1960s and 1970s, especially in Asia. This gene originally derived from the Chinese cultivar Dee-Geo-Woo-Gen (DGWG), has been exploited in rice breeding to develop cultivars with short, thick culms, raises the harvest index, improves lodging resistance and responsiveness to nitrogen fertilizer, resulting in high yields without affecting panicle and grain quality. The *sd-1* gene has been isolated by positional cloning and revealed to encode gibberellin 20-oxidase, the key enzyme in the gibberellin biosynthesis pathway (Monna et al. 2002). Using the sequence information of cloned *sd-1*, gene-derived markers have been developed for marker assisted-selection of recessive *sd-1* allele in breeding populations (Ellis and Spielemeyer 2002). Kim et al. (2009) reported that the majority of modern US cultivars contained *sd-1* alleles were traced back to either Dee-geo-woo-gen (DGWG) or Calrose 76 (CR76) according to pedigree information. In addition to DGWG and CR76 allele, a novel *sd-1* allele was also identified from the California cultivar M-401.

Bhatia et al. (2011) developed dwarf and bacterial blight resistant versions of the two traditional basmati rice varieties Basmati 370 and Basmati 386 by integrating marker assisted selection for *sd-1* and BB resistance genes with stringent phenotypic selection during back-crossing. Rajpurohit et al. (2011) pyramided blight resistance genes i.e. *Xa-21* and *xa-13* and a semi dwarfing gene *sd-1* into Type 3 Basmati, a traditional basmati rice cultivar from a rice cultivar PR106-P2 using marker-assisted selection (MAS). Foreground selection for BB resistance genes, *Xa-21* and *xa-13* and reduced plant height gene, *sd-1* was carried out using linked markers pTA248, RG136 and 'h', respectively. Foreground selection for the target genes was continued till F_5 generation to ensure recovery for Type 3 Basmati and BC_2F_5 pyramid lines T3-4, T3-5, T3-6 and T3-7 homozygous for the three target genes i.e. *Xa-21, Xa-13* and *sd-1* were identified. Luo and Yin (2013) developed semi-dwarf and disease resistant version of traditional Thai aromatic rice Khao Dawk Mali 105 (KDML 105) by pyramiding of semi-dwarf gene *sd-l,*

submergence tolerance gene *Sub-1A,* blast resistance gene *Pi-9* and bacterial blight resistance genes *Xa-21* and *xa-27* by marker-assisted selection. The improved line T5105 exhibited semi-dwarf phenotype with improved lodging resistance and a greater harvest index.

Luo et al. (2014) developed an improved version of Indonesian traditional tall rice variety 'Siputeh" by introgressing *sd-1, Wx^b, xa-4* and *Xa-21* genes from the donor line WH421. The gene linked markers were used to select the gene positive plants in different backcross generations eventually resulting in the selection of a semi-dwarf line TS4 with *sd-1, Wx^b, Xa-4* and *Xa-21* genes. TS4 had semi-dwarf phenotype with reduced growth duration, produced high yield with good grain quality and exhibited broad-spectrum resistance to *Xoo* strains.

Wu et al. (2017) reported an intragenic recombination between two parental non functional *sd-1* alleles resulting in the generation of a functional SD1 allele in RI92, a tall line (160 cm height) obtained from the cross between two semi-dwarf *indica* cultivars Zhenshan 97 and Minghui 63. Liu et al. (2018) reported a new rice lodging-resistance gene, *Shortened Basal Internodes (SBI)*, which encoded gibberellin 20-oxidase which was also encoded by *sd-1* gene in semi dwarf rice varieties and controlling the elongation of culm basal internodes through deactivating GA activity. *SBI* was predominantly expressed in culm basal internodes and found to show partial dominance in affecting plant height and lodging resistance.

Tomita and Ishii (2018) developed isogenic semi dwarf lines by continuously backcrossing of *sd-1* of Jukkoku (Jukkoku *sd-1*), *sd-1* of Kinuhikari (IR8 *sd-1*) and *d60* as well as both genes into the Koshihikari background to detect differences between *d60* and *sd-1* carrying NILs in relation to yield and to assess the utility of *sd-1* and *d60* semi-dwarf genes in rice breeding. The *d60* allele had stronger effect on culm length than *sd-1* and exerted similar effects on other phenotypic traits as *sd-1*. The *d60* did not have any negative effect on phenotypic traits of rice plants and was inferred to be useful for adding genetic diversity to semi-dwarf varieties.

2.2 Anther culture in rice

In rice the first successful report of anther culture was made by Niizeki and Oono (1968) in *japonica* rice. Since then a lot of research has been undertaken on this technique and anther culture has been well integrated into *japonica* breeding. Lee et al. (1989) developed a rice variety "Hwacheongbyeo" by anther culture of F_1 plants of Suweon 298/Milyang 64 cross. This variety was resistant to blast besides having resistance to bacterial blight and rice stripe tenuivirus.

Martinez et al. (1996) compared anther culture and pedigree method in terms of their efficiency in producing rice lines resistant to blast. Anther culture produced significantly higher proportion of lines with blast resistance than pedigree method across all the 11 crosses made between blast susceptible and resistant cultivars. Anther culture was also found to be superior to pedigree method in generating stable resistance (assessed based on field performance over 3 years) in some of the crosses. Additionally, transgressive doubled haploid lines showing resistance to blast were also obtained by the anther culture of F_2 plants of the crosses between blast susceptible cultivars

The potential of the technique has not yet been fully exploited in *indica* rice due to their poor anther culturability. Several studies on *indica* and *japonica* cultivars and their hybrids aimed towards enhancing the anther culture response have been conducted by different researchers.

Senadhira et al. (2002) have reported the development of first anther culture derived line from *indica/indica* cross to be released as a cultivar for cultivation in salinity prone areas. Seventy nine di-haploid lines were produced through anther culture of two *indica* breeding lines (IR5657-33-2 x IR4630-22-2-5-1-3) with an aim of combining high yield and salt tolerance ability of the parents. The anther cultured derived lines were evaluated in the green house, field and salinity affected areas resulting in the release of line IR51500-AC11-1 named as PSBRc50 "Bicol" for commercial cultivation in salt affected rice lands of the Philippines.

Sharma et al. (2003) developed doubled haploids through anther culture of F_1s of crosses between blast susceptible (LCH-7 and Ram Jawain) and resistant (Fukunishiki and Tetep) genotypes. Of the 30 anther culture derived doubled haploids from the cross

Fukunishiki/LCH-7, sixteen (53.33%) were resistant to blast, whereas two of the four doubled haploids of cross Ram Jawain/Tetep were resistant.

Purwoko et al. (2010) attempted anther culture of sixteen different crosses involving cultivars and accessions tolerant to Aluminium (Al) and shade with an aim of obtaining doubled haploid plants with tolerances to multiple stresses. Of the 16 crosses subjected to anther culture only 13 regenerated green plants. Selection to Al stress in a hydroponics test, a quick test of a full darkness, and screening against 4 races of blast fungi resulted in the selection of 5 lines with tolerance to Al and shade and resistance to 4 races of blast fungi. The study demonstrated that anther culture can be used to speed upland rice breeding programs.

Syafii et al. (2011) investigated agronomic characters of double haploid lines developed from anther culture vis-à-vis popular rice varieties of Indonesia. Analysis of 18 genotypes showed that there was diversity of agronomic characters among the rice double haploid genotypes. Yield per hectare in double haploid line KP44223 was the highest (4.5 tons/ha) among the lines, even higher than that of control varieties, Ciherang and Celebes.

Several rice varieties have been developed in Korea,China India, Japan, Hungary and USA using this technique (Siddique 2015). Several anther-derived doubled haploid lines with superior grain quality characteristics, resistant to diseases like blast, bacterial blight, brown planthopper and tolerance to abiotic stress have been released in different countries (Mishra and Rao 2016).

Naik et al. (2017) conducted anther culture of a popular rice hybrid BS 6444G known for its high yield potential and BLB resistance with an aim to fix a part of heterosis due to dominance for developing high yielding pure line varieties. A total of 200 doubled haploids were developed from this hybrid employing the anther culture technique. The lines were screened for 3 BLB resistance genes (*viz*. *Xa4*, *xa5*, *Xa21*) using identified molecular markers to select the lines with different combinations of resistance genes. When the selected lines were evaluated in the field under heavy disease pressure all showed substantial resistance to BLB. Two lines containing all the three resistance genes were shortlisted for possible release as new varieties.

2.2.1 Factors affecting rice androgenesis

Anther culture is influenced by many external as well as internal factors which can be considered limiting for the success of anther culture. Genotype of plant, physiological status of donor plant, stage of microspore development, pretreatments, culture medium, growth regulators and application of chemicals and type of carbon source are the foremost factors upon which the response of anther culture is mainly dependent.

The available literature on these factors is briefly reviewed below.

2.2.1.1 Genotype of plant

Genotypic variation had been found predominantly in anther culture response of rice in many instances (Raina 1997; Shahnewaz et al. 2003) after the very first report of success by Niizeki and Oono (1968). *Japonica* rice varieties have proven to be more responsive than *indica* cultivars (Chen and Lin 1976; Chu 1982; Woo and Chen 1982; Bishnoi et al, 2000). In general, the subsequent regeneration of green plants is also evidently better in *japonica* cultivars. More than 10 % callus induction frequency has been reported in the cultured anthers of *japonica* cultivars where as it was only 1% in case of *indica* cultivars (Hu 1985). The best anther culture response has been recorded from *japonica* × *japonica* hybrids, followed by *indica* × *japonica* hybrids. While, the poorest anther culture response has been recorded in *indica* ×*indica* hybrids (Miah et al. 1985; Tao et al. 2002).

While attempting anther culture of recalcitrant basmati rice variety Basmati 370, Raina et al. (1987) have recorded a very low level of callusing frequency (0.46 to 1.25%) from the cultured anthers of this variety. Likewise, a very low level of plant regeneration was achieved from the anther derived calli and a great majority of the regenerated plants were albinos.

Bishnoi et al. (2000) compared androgenic plant production efficiencies of six *indica*, two basmati rice (Basmati 370, Taraori Basmati) varieties and 14 heterotic *indica* ×Basmati F_1/F_2 on modified agarose-solidified N6M, Heh5M and RZM media. The cold treated anthers of F_2 plants were more responsive to anther culture compared to F_1 hybrids and parental rice varieties. Androgenesis frequencies of 31–78% were

obtained for *indica* × Basmati F_2 plants in RZM medium in just 30 d which were comparable to or higher than that reported for japonica rice varieties. The regeneration frequency of anther derived calli ranged from in 0–51% depending upon the genotype and 8–78% of the regenerants were diploid.

Lee et al. (2004) studied callus induction and plant regeneration frequency in anther culture of backcrossed hybrids between a responsive *japonica* variety (Mankeumbyeo, recurrent parent) and a recalcitrant *indica* variety (Ranta Emas, donor parent). The mean callus induction and plant regeneration of BC_4F_1 hybrids was almost same to that of *japonica* recurrent parent and there were no statistical differences between BC_4F_1 and BC_5F_1 hybrids.

He et al. (2006) investigated the inheritance of callus induction and green-plant regeneration in a 3×4 incomplete diallel crosses between three genotypes believed to possess relatively high callus induction capacity and the other four with relatively high green-plant regeneration capacity. Analysis of combining abilities and heritabilities revealed that both additive and non-additive effects were important in the genetic control of callus induction. In case of green-plant regeneration, non-additive effects were predominant. Broad sense heritability values suggested that more than 90% of the observed variation was due to genetic effects. It was therefore concluded that the frequency of anther culture response can be improved by suitable hybridization and selection strategies.

Dewi et al. (2009) evaluated the regeneration abilities in six *indica* genotypes tolerant to aluminum (Al) toxicity through anther culture. The callus growth and plant regeneration varied within the tested genotypes. The genotypes Grogol, Krowal, and Sigundil had good anther culture abilities, and therefore were recommended for inclusion as parents in breeding programs for development of new rice varieties tolerant to Al toxicity through anther culture.

Grewal et al. (2011) had reported the production of 3000 doubled haploid (DH) lines through anther culture of 28 crosses involving *indica* and *japonica* rice cultivars. *Indica* cultivars were found to show low anther culturability (1.2% callus induction), whereas *japonica* cultivars had 20-fold higher (28.1%) anther culturability.

Singh (2014) compared the anther culture efficiency of rice varieties belonging to *japonica, indica* and basmati groups. The results indicated that the *japonica* type lines had a good callus response compared to *indicas* and basmati types. The mean callus induction frequencies of *japonica*, *indica* and basmati genotypes observed were 38, 5 and 4.6%, respectively. Low regeneration frequencies were observed among the *indica (*0.6-0.8 per cent) and basmati genotypes (0.7-1.2 per cent) and a great majority of the plantlets obtained were albinos or light green with poorly developed chlorophyll.

2.2.1.2 Physiological status of donor plant

During anther excision, the physiological state of the plant plays a crucial role in anther culture response in a number of plant species. Many physiological factors *viz*., plant age, photoperiod, light intensity, temperature, growing conditions and nutritional status, are reported to considerably affect the anther culture response. It is reported that anthers collected from the plants at the beginning of flowering period were more responsive than those cultured at the end of flowering period (Chen and Lin 1976). When plants were provided bright sun light at 18-20 °C, anther responded comparatively well (Hu et al. 1981). Exposure of donor plants to high temperature has also been reported to be harmful for green plant regeneration (Huang et al.1983). The anthers collected from tertiary tillers exhibited significantly lower callus formation frequency than those from primary and secondary tillers (Tsay and Chen 1984).

Ling et al. (1990) observed that there is no difference in callus forming ability of photoperiod sensitive and insensitive rice genotypes. Sun et al. (1992) reported that photoperiodism and thermoperiodism played an influential role in the anther culture response in *indica* rice cultivars. Callus induction rate was the highest when donor plants were grown under mid or short photoperiod and at mid or low temperature. The ratio of green to albino plantlets was also higher under a short photoperiod. In the rice crop, plants grown during the dry season have provided the best microspore response (because of dehiscence and pollen viability). The anther culturability trait is heritable and has independent inheritance, have no maternal influence and have dominant gene action. In general, *japonica* rice genotypes responded better than *indica*. Rice cultivar IR43 that reached the panicle emergence stage under long days (> 12 h) and sunshine

(> 8 h) and day/night temperature (34 °C/24 °C) showed highest anther culture response (Raina and Zapata 1997).

Apart from temperature, adequate nitrogen supply is also required to maintain the photosynthetic activity of the plants which ultimately improve the pollen development. Optimum level of nitrogen in donor plants results in anthers having superior microspores with high embryogenic ability (Lapitan and Villegas 1999). Guzman et al. (2000) reported higher frequency of microspore-calli/embryo-like structures (ELS) in anther cultures initiated from ratooned plants.

In rice, anthers with the early or mid- to late-uninucleate pollen stages of microspore development are reported to be most responsive and suitable for culture (Chen 1977). Usually the distance between the collar of the flag leaf and ligule of the penultimate leaf of the tiller serves as a reliable guide to anther maturity (Bishnoi et al. 2000). The most appropriate distance between flag leaf and subtending leaf that correlates with late-uninucleate pollen stages of microspore is known to vary slightly in different genotypes; 5-10 cm (Gueye and Ndir 2010), 4 to 8 cm (Abbasi et al. 2011) and 5-10 cm (Xa and Lang 2011).

2.2.1.3 Effects of pretreatments

Application of a variety of stresses, such as temperature pre-treatment, osmotic shock and sugar starvation, during the labile development period of pollen grains are known to be useful for the induction of androgenesis as these will change normal gametophytic pathway to sporophytic pathway of pollen (Bhojwani and Razdan 1996; Bhojwani et al. 1999). Trejo-Tapia et al. (2002) studied the effects of a cold pretreatment, the concentration of different auxins (2,4-D, NAA and IAA) and the type of carbon source (maltose and sucrose) on the induction of callus from anthers of three parental lines and four F_1 hybrids (*japonica* × *indica*, *indica* × *japonica*). The results indicated that cold pretreatment was essential for the induction of callus from anthers of the parental lines and the F_1 hybrids. These effects were found to be genotype dependent.

Another pre-treatment which can substitute cold treatment for the induction of androgenesis is osmotic shock. Raina and Irfan (1998) reported that treatment of anthers in 0.4 *M* mannitol solution for 4 days at 25 ^{o}C was essential to induce androgenesis in microspore cultures of *indica* and *japonica* cultivars. For *indica*

cultivars, pre-treatment at 33 ^{o}C was better than at 25 ^{o}C with respect to induction of number of embryo-like structures or calli.

Sandaruvini and Ratnasekera (2013) studied the effect of cold pretreatment duration on anther culture response of selected *indica* rice varieties. The effect of variety and cold pre treatment was significant ($p<0.05$) for the callus induction frequency. Cold pre treatment for 7 days at 10°C had the highest callus induction frequency in genotypes of Kahata wee, BG379-2 and BW267-3 where as AT362 had highest callus development in 12 days thereby suggesting that the optimum duration of cold pre-treatment was genotype dependent.

Tian et al. (2015) reported a positive relationship between pollen fertility and callus induction in anthers of the F_1 progeny of the cross 'Nanjing 46' × 'Zaxima subjected to low temperature treatment and exogenous plant growth hormones like NAA and Kinetin for several days. They concluded that low temperature treatment for up to 5 days before anther inoculation in the callus induction medium delayed degradation of the innermost layer of the anther wall, the tapetum, by altering the insoluble polysaccharide and protein contents in the anther wall. Nurhasanah et al. (2016) studied the effect of panicle cold pretreatment and the addition of putrescine, a plant growth regulator of polyamines group, into culture medium on anther culturability of local upland rice varieties. The effect of duration of cold-pretreatment in influencing the ability of rice microspores to develop into callus and green plantlet regeneration was genotype-dependent. They also observed that the effect of putrescine addition in the culture medium was also genotyped-dependent which either increased the number of calli, plantlets and green plantlets or decreased them in specific genotypes.

2.2.1.4 Culture medium

Culture medium composition is one of the predominant factors affecting the production of embryos/callus from cultured anthers/microspores. Nutrient profile of both the media i.e. induction medium for androgenesis and regeneration medium for growth of developing embryos differs significantly (Wang et al 1974). Chu et al. (1975) developed N_6 medium which is considered to be the most suitable for anther culture of *japonica* type rice genotypes. The medium was reported to be the best for

callus induction (Chen 1986), while the best differentiation of anther derived calli was recorded on MS medium. However, the N_6 medium initially developed for *japonica* cultures has not proven suitable for anther culture of *indica* varieties. Therefore, several modifications have been made in the nutrient media to improve anther culture response of *indica* rice. A modified N_6 medium had been developed by replacing $(NH_4)_2SO_4$ and KH_2PO_4 with NH_4HPO_4 to meet the requirements of *indica* cultivars (Yang et al. 1980). Zapata et al. (1991) compared genotypic response differences by changing basal medium and reported that N_6 medium gave best results in isolated pollen culture of "Taipei 309", a *japonica* cultivar, on the other hand *indica* cultivar IR-43 responded better to E10 medium, a modification of B5 medium.

Laxmi and Reddy (1997) developed an efficient and improved medium which resulted in high frequency callusing and green plant regeneration from anther culture of *indica* rice cultivars 'Rasi Tellahamsa', 'Ptb 33' and 'IR 24'. They reported that N_6 medium supplemented with 6% maltose, 10 mg/l $AgNO_3$, 2 mg/l 2,4-D and 0.5 mg/l Kinetin for promoting high frequency of embryogenic calli within 20 days of culture. A new medium named by MO-19 was developed, which differs from N_6 by having reduced level of $(NH_4)_2SO_4$ and higher level of KH_2PO_4 and KNO_3 to suit the requirements of *indica* cultivars (Raina and Zapata 1997). Raina (1997) has also observed increment in anther culture efficiency in highly recalcitrant *indica* cultivars by using 9% maltose as carbon source instead of sucrose in MO-19 basal medium.

Trejo-Tapia et al. (2002) also recorded the highest callus induction frequency with maltose at the concentration of 30 g/1. The use of liquid RZM culture medium was found to enhance the frequency of androgenesis in *indica* × Basmati hybrids. Javed et al. (2007) studied the effects of different culture temperatures, culture media and saccharides on the anther culture efficiency in two salt tolerant rice *indica* cultivars, Pokkali and Nona Bokra. Comparison of two culture temperatures (constant temperature at 25°C and alternating temperatures at 30°C/20°C) indicated that alternating temperatures showed remarkable effects not only on the callus induction but also on the plant regeneration. Among the basal media examined, higher callus productivity was observed in SK-I medium and higher shoot productivity was obtained in SK-II medium than other basal media. When alternating temperatures and revised N_6 medium including maltose were used throughout anther culture, callus

productivity and green shoot productivity showed the highest score in both the salt tolerant cultivars.

Silva and Ratnayake (2009) studied the anther culture ability of two local *indica* rice varieties on N_6 and SK-I callus induction media. The traditional rice variety, Kurulu Thuda displayed good anther culture potential with a callus induction frequency of 17.2%. Anthers bearing bi-nucleate pollen were found to be most suitable for culture. Callus induction was nearly three times higher on N_6 medium than on SK-I medium in var. Kurulu Thuda. Shoot regeneration in var. Kurulu Thuda on SK-II medium was achieved only from the callus induced on N_6 medium. The studies conducted by Mishra et al. (2011) on the nutritional requirements of two elite *indica* hybrid rice cultivars, Rajalaxmi and Ajay, using three artificial media indicated that the responses of genotypes to media were highly significant for all the three parameters of anther culture examined i.e. callus induction frequency (%), total regeneration frequency (%) and anther culture response (%). Among the media, the callus induction frequencies were higher on N_6 medium (34.56%) followed by MO-19 (28.29%) and SK-1 (25.58%) and the callus regeneration and green plant regeneration efficiency was also high in N_6 media as compared to MO-19 and SK-1. The results suggested that both genotype and culture media played a critical role in the anther culture response of *indica* rice.

While comparing the efficacy of different media, Kaushal et al. (2014) reported that He2 medium produced highest callus induction, green plant regeneration and least albino plant development compared to the other 3 other induction media *viz*., N_6, B5 and SK1. They also concluded that out of 13 genotypes evaluated, IR58025B with *eui* (25eB) was highly responsive for both callus induction as well as green plant regeneration.

2.2.1.5 Growth regulators and application of chemicals

The effect of several auxins like α naphthalene acetic acid (NAA), phenyl acetic acid (PAA), picloram and dicamba have also been tested either alone or in combination with 2,4-D (Lentini et al. 1995). Considering the auxin/cytokinin balance, the growth regulator combination that was favorable for enhanced anther response in the large number of genotypes proved to be 2,4-D (2.00 mg/l), picloram

(0.07mg/l) and Kinetin (0.5mg/l). A lower level of 2,4-D (0.5mg/l) in combination with the milder auxin NAA (2.5mg/l) and Kinetin (0.5mg/l) have been used effectively to induce callus from several *indica* varieties (Shahnewaz et al 2003; Shahnewaz and Bari 2004). Roy and Mandal (2005) studied the effect of organic adjuvants, synthetic plant hormones and diverse carbon sources in influencing anther culture response in five *indica* rice genotypes (IR72, Mansarovar, Taraori Basmati, Pusa Basmati and Karnal local 95). Androgenic callus induction as well as green plantlet regeneration was more when callus was induced on N6 fortified with 100 mg/l yeast extract. Green plantlet regeneration was observed when callus induction medium (CIM) was supplemented with 2, 4-D and IBA in Taraori Basmati and Karnal local 95.

Grewal et al. (2006) studied the effects of amino acid cysteine on microspore-derived callus induction as well as plantlet regeneration. Isolated pollens of basmati cultivars, Pusa Basmati 1, Basmati 370 and Basmati 386 were cultured in a medium based on N_6 salts supplemented with or without cysteine following pollen embedment in agarose. The induction and regeneration medium with cysteine gave twice as effective androgenesis and plantlet regeneration in recalcitrant Basmati rice cultivars compared to medium lacking cysteine.

Dewi and Purwoko (2008) reported that addition of 10^{-3}M putrescine into the culture media inhibited ethylene biosynthesis, increased the polyamines contents, and decreased the ACC content as well as ACC oxidase activity in anthers and anther-derived calli of *indica* rices. This study suggested that the application of 10^{-3} M putrescine in anther culture of rice subspecies *indica* improves androgenesis by inhibiting early senescence of cultured anthers and enhancing embryo or callus formation from microspores.

Hooghvorst et al. (2018) reported that application of 2 mg/l 2,4-D and 1.0 mg/l Kinetin on colchicine-free induction medium gave the best anther culture response in two *japonica* rice genotypes, NRVC980385 and H28, while colchicine at 300 mg/l for 48 h enhanced callus induction 100-fold in H28. They showed that the post-anther

culture treatment of haploid plantlets at 500 mg/l of colchicine permitted the regeneration of fertile doubled haploid plantlets.

Mayakaduwa and Silva (2018) reported that stressful environmental conditions and inorganic nitrogen stress applied on-field and *in vitro* could be used as effective approaches for substantial enhancement of *indica* rice anther response. They showed a better response in standard N_6 medium with complete withdrawal of $(NH_4)_2SO_4$ compared to the control and relatively improved anther response when the donor plants were grown under the stressed conditions of the year.

3. MATERIALS AND METHODS

The present investigation entitled, "Production of semi dwarf and blast resistant derivatives of Ranbir Basmati using anther culture and marker assisted selection" was carried out in the Department of Agricultural Biotechnology, CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur during 2016-2018. The materials used and methods followed during the course of investigation are described below:

3.1 Plant material

The popular rice variety Ranbir Basmati was used as a recipient parent. The variety has tall stature and highly susceptible to rice blast. A semi-dwarf line PB1(*Pi9*), an isogenic line of popular basmati variety Pusa Basmati-1, was used as donor of blast resistance gene *Pi-9* and semi-dwarfing allele *sd-1*. The BC_2F_1 progenies of the cross Ranbir Basmati/ PB1(Pi9) already developed in the department were used as starting material for the present study. The BC_2F_2 seeds obtained from two superior BC_2F_1 recombinants JKR1-34-16 and JKR1-29-100 of cross Ranbir Bamsati*^{3}/ PB1(Pi9) were used as starting material in the present study (Table 3.1). BC_2F_2 progenies of plants JKR1-29-100 and JKR1-34-16 were raised in plastic pots in the cage house of the Department of Agricultural Biotechnology, CSKHPKV Palampur during July, 2017 using standard agronomic practices to obtain healthy BC_2F_2 plants. The BC_2F_2 plants were initially subjected to marker-assisted selection to select *Pi-9* and *sd-1* double homozygous plants that were subsequently used as the source of anthers for developing double haploid plants.

Table 3.1. List of rice genotypes used in the study

Sr. No.	Genotype	Genes	Remarks	Source
1	Ranbir Basmati	-	Recurrent parent, tall statured and susceptible to rice blast	School of Biotechnology SKUAST, Jammu
2	PB1(Pi9)	*Pi-9* + *sd-1*	Donor of blast resistance and semi-dwarfing gene	Division of Genetics, IARI, New Delhi
3	JKR1-29-100	*Pi-9* + *sd-1*	BC_2F_1 plant of cross Ranbir Bamsati*^{3}/ PB1(Pi9) with 96.15% recovery of recurrent parent genome as determined through SSR based background analysis	Department of Agricultural Biotechnology, CSKHPKV, Palampur
4	JKR1-34-16	*Pi-9* + *sd-1*	BC_2F_1 plant of cross Ranbir Bamsati*^{3}/ PB1(Pi9) showing phenotypic similarity to recurrent parent	Department of Agricultural Biotechnology, CSKHPKV, Palampur

3.2 Marker assisted selection for blast resistance and semi-dwarfing gene

3.2.1 Isolation of genomic DNA

DNA of parents and 388 BC_2F_2 plants, comprising 296 and 92 plants of JKR1-29-100 and JKR1-34-16, respectively was isolated using the CTAB method of Murray and Thompson (1980). Young and soft leaves were harvested from plants, washed with deionized water and dried on tissue paper. About 250 mg of fresh leaf tissue was taken from each plant and ground into fine powder using autoclaved mortar and pestle in the presence of liquid nitrogen. The powder was then transferred into the eppendorf tube containing 800 µl of extraction buffer (2% CTAB, 100 mM Tris, 20 mM EDTA, 1.4 M NaCl and 1% PVP, pH 8.0), maintained at 60 ^oC and mixed vigorously. The tubes were then incubated at 60 ^oC in water bath for 1 h with gentle shaking. Equal volume of chloroform:isoamyl alcohol (24:1) was added to each tube and mixed gently before centrifugation. After centrifugation at 10,000 g for 10 min at 4 ^oC, the upper layer (aqueous phase) was transferred to eppendorf tubes and 2 µl of *RNase* (10

mg/ml) was added to each tube. After adding *RNase,* the eppendorf tubes were incubated at 37 ^{o}C for 1 h. After *RNase* treatment, 600 µl of pre-chilled isopropanol was added, gently mixed with the aqueous phase and kept at -20 ^{o}C for 30 min. DNA was precipitated by centrifugation at 10,000 g for 10 min. The supernatant was drained and pellet was retained. The pellet was washed twice with 300 µl of chilled 70 per cent ethanol. The pellet was dried in a laminar air flow cabinet for 3 to 4 h. Dried DNA pellet was dissolved in 100 µl TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.0). The yield and quality of DNA was checked by agarose gel electrophoresis using 1.0 % gel. The final concentration of DNA in working solution was adjusted to10 ng/µl.

3.2.2 Markers used for foreground selection of *Pi-9* and *sd-1*

The gene-derived markers linked to target resistance genes were used to identify the plants carrying the respective resistance genes in parents and BC_2F_2 progenies. The information on the chromosomal location, marker type, linkage phase, annealing temperature and source of the markers used for foreground analysis of the target resistance genes in presented in Table 3.2. These markers were initially validated in the donor and the recurrent parent 'Ranbir Basmati' with respect to polymorphism and subsequently used for marker assisted selection of target genes in BC_2F_2 plants.

3.2.3 PCR Amplification and visualization of PCR products

DNA amplification was carried out in a 12.5 µl reaction volume containing 20 ng template DNA, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.5 mM $MgCl_2$, 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3) and 1 unit *Taq* polymerase (Gotaq® DNA Polymerase, Promega). PCR amplification was carried out in a thermocycler (Proflex PCR System; Applied Biosystems, Life Technologies, USA) using specific temperature profiles. To conform the presence of *Pi-9* gene PCR amplification was done with the marker *Pi9-Pro* having temperature profile as follows: initial denaturation at 94 ^{o}C for 5 min followed by 35 cycles at 94 ^{o}C for 30 seconds, 55 ^{o}C for 30 seconds, 72 ^{o}C for 30 seconds and a final extension at 72 ^{o}C for 5 min followed by rapid cooling to 4 ^{o}C. PCR amplification for *Nbs2-Pi9* was carried out as follows: initial denaturation at 94 ^{o}C for 5 min followed by 35 cycles at 94 ^{o}C

Table 3.2 Markers used for foreground election of *Pi-9* and *sd-1* genes

Gene (trait)	Linkage group	Marker	Sequence	Type of marker	Linkage phase	Source
Pi-9 (Blast resistance)	6	*Pi9-Pro*	F: 5'TGATTATGTTTTTTATGTGGGG 3' R: 5'ATTAGTGAGATCCATTGTTCC 3'	Gene-derived Indel	Co-dominant	Tian et al. (2016)
Pi-9 (Blast resistance)	6	*Nbs2-Pi9*	F: 5'ATGGTCCTTTATCTTTATTG 3' R: 5'TTGCTCCATCTCCTCTGTT 3'	Gene-derived STS	Dominant	Qu et al. (2006)
sd-1 (Semi dwarfing)	1	*SD-1*	F: 5'CACGCACGGGTTCTTCCAGGTG 3' R: 5'AGGAGAATAGGAGATGGTTTACC 3'	Gene-derived Indel	Co-dominant	Eliss and Spielmeyer (2002)
sd-1 (Semi dwarfing)	1	*GA20-1*	F: 5' CGTGATTTCTCTGTTCCAATCA 3' R: 5' TCCAAAATACACATCCAACGAG 3'	Gene-derived Indel	Co-dominant	In this study

for 1 min, 55 ^{o}C for 1 min, 72 ^{o}C for 2 min and a final extension at 72 ^{o}C for 5 min followed by rapid cooling to 4 ^{o}C. Similarly to verify the presence of *sd-1* gene, amplification was carried out with the markers *SD-1* and *GA20-1*. The conditions for PCR amplification of *SD-1* and *GA20-1* were essentially same as described in the preceding section for *Pi9-Pro* marker except that 35 reiterative PCR cycles were carried out at 94 ^{o}C for 45 seconds, 55 ^{o}C for 45 seconds, 72 ^{o}C for 1 min with a final extension at 72 ^{o}C for 5 min.

Ten µl of each PCR product was analyzed on 2 to 4% agarose gel prepared in 1X Tris borate-EDTA (TBE) buffer (0.05 M Tris, 0.05 *M* boric acid, 1 mM EDTA, pH 8.0). The PCR amplicons for marker *Pi9-Pro* were analyzed on 4% agarose and those obtained with markers *Nbs2-Pi9*, *SD-1* and *GA20-1* were resolved on 2% agarose gel. The gel was run at a constant voltage of 100 volts for 1 h. The gel was stained with ethidium bromide (0.5µg/ml) for 5 min after the completion of electrophoresis. The gel was destained for 10 min by keeping under running tap water. The resolved PCR products were visualized over an ultraviolet transilluminator and a photograph of the gel was stored in Gel-Doc system (BIO-RAD).

3.3 Anther culture of *Pi-9* and *sd-1* homozygous BC_2F_2 plants

The foreground selected BC_2F_2 plants that were homozygous for both the genes *Pi-9* and *sd-1* were used as source of anthers for producing homozygous double haploid lines. For anther culture the protocol previously standardized in our laboratory was used (Chopra 2006).

3.3.1 Media Preparation.

i. Preparation of Stock solutions

Three different basal nutrient media *viz*., MO-19, N_6 and MS were used during the different stages of anther culture. Compositions of these basal nutrient media are given in Appendix I. To avoid errors and inconvenience in weighing of small quantities of multiple constituents, four concentrated stock solutions (SS-I to SS-IV) corresponding to macro nutrients, micro nutrients, Fe-EDTA and vitamins, respectively were prepared for each tissue culture media. For macro nutrients 20X stock was prepared, whereas for micro nutrients, Fe-EDTA and vitamins 200X stocks were prepared by dissolving the analytical grade chemicals in the required quantities of double distilled water.

ii. Preparation of medium

The compositions of media used for different stages of anther culture are given in the Table 3.3 To prepare each of these media, the required quantities of the stock solutions (SS-I @50ml/l, SS-II @5ml/l, SS-III @5ml/l, SS-IV @5ml/l) along with the required quantity of carbon source and growth regulators were added to glass beaker and final volume was made up with distilled water. The pH of the medium was adjusted to 5.6-5.8 using 0.1 N NaOH and 0.1 N HCl. The required quantity of agar (8 g L^{-1}) was added and the mixture was then heated for dissolving the agar. The medium was transferred to 150 ml conical flasks, ensuring that each flask was filled with not more than half of its capacity to ensure proper autoclaving. The flasks were covered with aluminum foil and autoclaved at 121ºC temperature and 15 lbs pressure for 15 min. After autoclaving, the media along with other materials (culture containers, parafilm, etc.) were shifted to a laminar hood, which was previously sterilized by UV for 15 min. The medium was poured into the pre-sterilized Petri plates (9*0* x 15mm) and allowed to solidify.

Table 3.3. Composition of media used for different stages of anther culture

Composition	Callus induction medium	Regeneration medium	Rooting medium
Basal medium	MO19	N_6	MS (50%)
2,4-D (mg/l)	0.5	-	-
NAA (mg/l)	2.5	0.5	-
IBA (mg/l)	-	-	1.0
BAP (mg/l)	-	1.0	-
Kinetin (mg/l)	0.5	1.0	-
Carbon source	9% maltose	3% maltose	3% sucrose
Agar (g/l)	8.0	8.0	8.0

MO19 (Raina and Zapata 1997); N_6 medium (Chu et al. 1975); MS (Murashige and Skoog 1962).

3.3.2 Excision of panicles and pretreatment

In rice, the anthers having microspores of uninucleate stage are reported to be the best for anther culture (Raina 1997). The panicles from the plants having 4-5 cm distance between the collar of flag leaf and auricle of penultimate leaf are reported to contain uninucleated microspores and were, therefore, used for anther culture. The selected panicles at appropriate stage were excised and kept in polythene bags containing sterilized distilled water to avoid physiological wilting. Thereafter, the panicles were brought to laboratory and given the following pretreatments:

a) Mannitol pretreatment: Anthers were removed aseptically from the panicles still enclosed in the boot leaves. From the pool of florets, about 400-500 anthers were plated for pretreatment in 60 mm petri plates containing 10 ml of preautoclaved 0.4 M mannitol solution and kept for 72 hours in the dark at 25 °C. The pretreated anthers were washed with sterilized distilled water, followed by blot drying on a sterilized filter paper. About 80-100 dried anthers were plated in each petri plate containing 25 ml of the callus induction medium.

b) Cold pretreatment: The panicles of appropriate stage having anthers in the uninucleate stage were collected in polythene bags containing sterile distilled water and kept at 4°C for 7 to 10 days prior to anther excision.

3.3.3 Plating of anthers in the callus induction medium and plantlet regeneration.

The panicles were uncovered by gently removing the flag leaf with a sterilized forceps inside a laminar air flow cabinet. The panicles were surface sterilized with 0.1% $HgCl_2$ for 2-3 minutes in a pre-autoclaved beaker followed by three washings with sterilized distilled water. The spikelets from the top and bottom $1/3^{rd}$ of the panicle were chopped with a pair of sterilized scissors and the spikelets from the middle portion were selected for the excision of anthers. Six anthers from each floret were excised carefully without causing any injury to the anthers.

Approximately, 80-100 anthers were inoculated in each Petri plate containing the callus induction medium (Table3.3). The inoculated plates were sealed with parafilm strips and kept in dark at 25 ± 1 °C. Petriplates were observed regularly for callus formation starting 4 weeks after the inoculation of anthers. Recording of data

on callus induction was continued up till 12 weeks after anther plating. When the calli originating from the anthers grew 5-6 mm in diameter, they were transferred to the flasks containing 90 ml of the regeneration media (Table 3.3) and the flasks were incubated at 25±1 °C under 18/6 hours light/dark cycle for plantlet regeneration. Regenerated green plantlets with well developed shoots were transferred to the rooting medium for induction of roots (Table 3.3).

Data on different components of anther culture response *viz.*, callusing frequency (%), regenerating calli (%), green plantlet regeneration (%), albino plantlet regeneration (%), and anther culture efficiency were recorded as follows:

a) Callusing (%) $= \frac{\text{No. of calli formed}}{\text{Total anthers plated}} \times 100$

b) Regenerating calli (%) $= \frac{\text{No of regenerating calli}}{\text{Total calli transferred to regeneration medium}} \times 100$

d) Green plantlet (%) $= \frac{\text{No. of green plantlets formed}}{\text{Total regenerated plantlets}} \times 100$

d) Albino plantlet (%) $= \frac{\text{No. of albino plantlets formed}}{\text{Total regenerated plantlets}} \times 100$

e) Anther culture efficiency $= \frac{\text{No of green plantlet formed}}{\text{Total anthers plated}} \times 100$

3.3.4 Hardening and raising of anther culture derived plants

Regenerated green plantlets with well developed roots and shoots were washed with luke warm water to remove the adhering medium and subsequently transferred to plastic pots (4'' diameter) containing potting mixture (soil: sand :: 3: 1). After about 10-12 days of hardening, the plantlets were transferred to large sized pots (8'' diameter) and kept in a glasshouse for their development and further growth until maturity. Data were collected on total number of plantlets transferred and number of plantlets that survived to calculate the frequency of plantlet survival. The 21 days-old seed derived plantlets of parental genotypes Ranbir Basmati and PB1(Pi9) were also raised alongside the anther culture derived plants for the comparison of agro-morphological and seed physico-chemical traits of regenerants vis-à-vis parental genotypes.

3.3.5 Foreground selection in anther cultured derived plants to confirm the presence of *Pi-9* and *sd-1*

The anther culture derived lines were subjected to foreground selection to ensure fixation of *Pi-9* and *sd-1* in these lines using gene-derived markers. The methodology for genomic DNA isolation, PCR amplification and analysis of PCR products was essentially same as described previously in Section 3.2.

3.4 Evaluation of agro-morphological and seed quality attributes of doubled haploid derivatives

The doubled haploid derivatives obtained through anther culture along with the parental lines Ranbir Basmati and PB1(Pi9) were evaluated for agronomic performance and seed quality attributes using standard methods. The data on agro-morphological attributes was recorded as per the standard evaluation system for rice (IRRI 2002) as explained below:

i) **Days to flowering:** Number of days was recorded from the date of sowing to flowering on each plant separately.

ii) **Plant height:** Height of each doubled haploid plant was recorded in cm from ground to tip of each plant.

iii) **Number of effective tillers per plant:** Number of effective tillers i.e. tillers having panicle per plant were counted at maturity and recorded.

iv) **Panicle length:** Length of panicle was measured in cm from base to tip of the panicle for each selected panicle.

v) **Number of grains per panicle:** The total number of filled grains per panicle was counted at maturity and the data were recorded for each of the doubled haploid plants.

vi) **1000-grain weight:** Total of 1000 filled grains were counted from each doubled haploid plant and weighed on the electronic balance. The weight was expressed in grams per plant.

vii) **Grain yield:** Grain harvested from each plant was dried to optimum moisture levels (13-14%) and weighed on electronic balance. This was expressed as grams per plant.

3.4.1 Physico-chemical characteristics of harvested seeds

A part of the harvested seed of each of the doubled haploid lines was used for the analysis of physico-chemical characteristics. The seed quality analysis was conducted in the Quality Analysis Laboratory of the Division of Genetics, IARI, New Delhi following standard methods as explained below:

i) **Grain length [L]:** Length of five dehusked grains of each doubled haploid plants was recorded in millimeters (mm) using digital Vernier caliper (Aerospace Digimatic, Sudershan Measuring & Engineering Pvt Ltd, New Delhi). Based on grain length the grains were characterized as Extra long: > 7.5 mm, long: 6.61 to 7.5 mm, medium: 5.51 to 6.60 mm and short: < 5.50 mm.

ii) **Grain breadth [B]:** Five dehusked grains of each doubled haploid plant, whose length was earlier recorded, were used for recording grain breadth in millimeters (mm) using digital Vernier caliper.

iii) **L/B ratio of seed:** L: B ratio was calculated by dividing the grain length by its breadth as recorded above.

iv) **Grain type:** Based on the L/B ratio, the harvested seeds were categorized into different types as slender (L/B ratio > 3.0), medium (L/B ratio 2.1 to 3.0), bold (L/B ratio 1.1 to 2.0) and round (L/B ratio less than 1.1).

v) **Alkali spreading value (ASV):** The alkali spreading value was measured by the alkali digestion technique (Little et al. 1958). Briefly, six milled rice gains were soaked in 1.7% KOH at 39^{o}C for 24 hours. The degree of spreading was measured following seven-point numerical spreading scale: (1) grain not affected; (2) grain swollen; (3) grain swollen and collar incomplete and narrow; (4) grain swollen and collar complete and wide; (5) grain split or segmented and collar complete and wide; (6) grain dispersed and merging with collar and (7) grain completely dispersed and intermingled.

Alkali spreading values were used to infer the gelatinization temperature (GT) of the samples as per the scale of Lanceras et al. (2000) as given below:

Table 3.4. Scale for scoring of gelatinization temperature based ASV values

Classification	Alkali Spreading Value	Gelatinization temperature (GT)
1-2	Low	High (74.5 -80.0°C)
3	Low, intermediate	High, intermediate
4-5	Intermediate	Intermediate (70-74°C)
6-7	High	Low (55-69°C)

v) Amylose Content: Amylose content was estimated as per the following procedure described by Juliano (1971).

i. Preparation of amylose standard curve

1. Forty mg standard potato amylose was dissolved in 9 ml of 1N sodium hydroxide and 1 ml of ethyl alcohol and heated for 5 to 10 min in boiling water bath, cooled and final volume made up to 100 ml with distilled water. The aliquots of 1 ml, 2ml, 3 ml, 4 ml and 5 ml of the standard amylose solution were pipetted out into 100 ml volumetric flasks in three replicates.
2. The 1 ml, 2 ml, 3 ml, 4 ml and 5 ml of standard amylose solutions were acidified with 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1.0 ml of acetic acid, respectively.
3. Two ml of freshly prepared I_2-KI solution (0.2 g iodine and 2.0 g potassium iodide in 100 ml of aqueous solution) was added to each sample and final volume was made to 100 ml. The flasks were shaken, covered with black cloths and allowed to stand for 20 min.
4. After 20 minutes the absorbance was recorded in spectrophotometer at 620 nm.

ii. Procedure for analysis amylose content in rice samples:

1. 100 mg of fine powdered rice flour was taken in long test tubes (2 x 19.5 cm) from each sample and 1 ml of ethyl alcohol and 9 ml of 1.0 N sodium hydroxide was added to it.
2. After shaking thoroughly, the sample was heated in boiling water bath for 10 min.
3. The sample was cooled and poured in to 100 ml volumetric flasks and volume brought up to 100 ml with distilled water.
4. From each sample, 5 ml of starch solution was pipette into 100 ml volumetric flasks followed by the addition of 1 ml of acetic acid and 2 ml of I_2 -KI solution. The final volume was made up to 100ml with distilled water.
5. The flasks were shaken, covered with black cloths and allowed to stand for 20 min.
6. After 20 minutes the absorbance was recorded in spectrophotometer at 620 nm.

iii. Calculation of amylose content

1. Standard graph was drawn by plotting amylose concentration on x-axis and net optical density on y-axis.
2. The established equation from the graph was used to get the amylose content in the samples.
3. The obtained value was converted into percentage by multiplying it with the dilution factor.

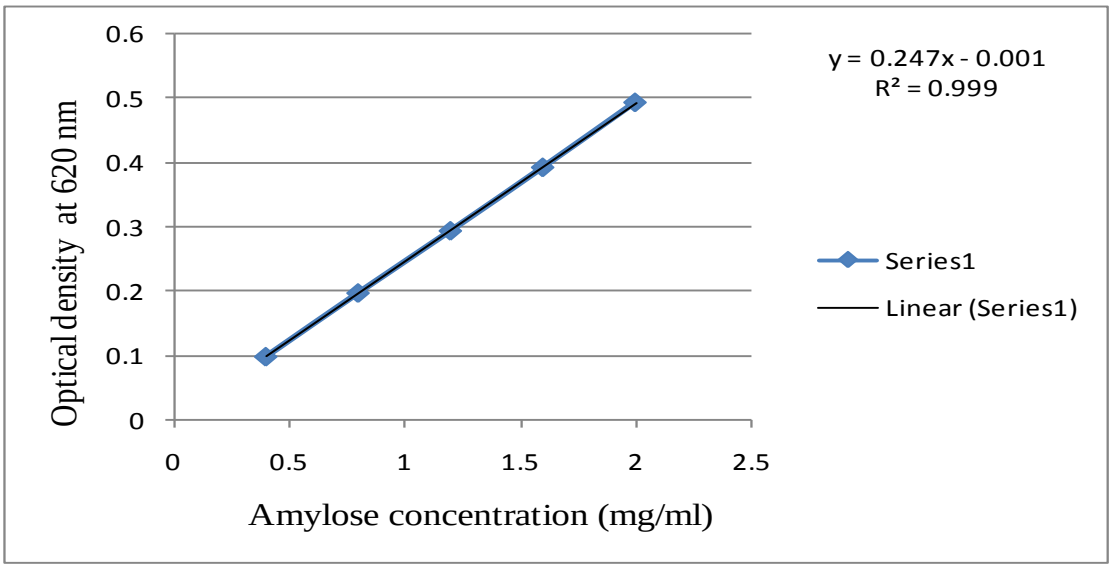

Fig 3.1 Standard curve for amylose

Based on amylose content the grains were categorized into five categories: waxy (1-2%); very low (2-9%); low (10-20%); intermediate (20-25%) and high (25-30%).

vi) **Aroma:** Grain aroma was determined using the sensory test given by Sood and Siddiq (1978). Thirty grains of each doubled haploid plants were soaked in 7.5 ml of 1.7% KOH solution at room temperature in a closed 15ml conical polypropylene tube for 2-3 hours. Based on sensory evaluation the samples were rated for aroma using as 0-3 scale: 0: absence of aroma; 1: mildly aromatic; 2: moderately aromatic and 3: strongly aromatic.

3.5 Evaluation of doubled haploid plants for blast resistance

The doubled haploid derivatives were screened for their reaction to rice blast isolate *PO-RB1* using the procedure described by Rathour et al. (2004). The blast isolate *PO-RB1* is virulent on Ranbir Basmati and avirulent on PB1(Pi9) and its culture is maintained in the Pathological Laboratory of the Department. About 20-25 seeds of each doubled haploid and parental genotypes, Ranbir Basmati and PB1(Pi9), were raised in a plastic tray (40 x 30 x 10 cm) filled with potting mixture (soil : sand :: 3 : 1) till the seedlings grew 21-days old. The 21-days old seedlings were inoculated with spore suspension of blast isolate *PO-RB1* containing 40-50 conidia/microscopic field (40x). The inoculated seedlings were kept in a growth chamber maintained at 25 ± 1 ^{o}C and sprayed three to four times a day with distilled water to maintain high humidity. Disease reaction was recorded after 7 days of inoculation using 0-5 scale given by Mackill and Bonman (1992) (Table 3.5).

Table 3.5. Disease assessment scale for determining the reaction of rice lines to rice blast.

Reaction type	Description
0	No evidence of infection
1	Brown specks smaller than 0.5 mm in diameter
2	Brown specks about 0.5-1.0 mm in diameter
3	Roundish to elliptical lesions about 1-3 mm in diameter with gray centre and brown margins
4	Typical spindle-shaped blast lesions
5	Same as 4 but half of one or more leaves killed by coalescence of lesions.

The genotypes with 0-3 score were considered resistant and those with 4-5 score as susceptible.

4. RESULTS AND DISCUSSION

The present investigation was aimed at fast-track development of the semi-dwarf and blast resistant fixed derivatives of a traditional basmati rice variety Ranbir Basmati through introgression of blast resistance gene *Pi-9* and a recessive semi-dwarfing gene *sd-1* from a basmati donor PB1(Pi9). To achieve these objectives, the biotechnological tools of marker-assisted selection and anther culture were used to ensure precise selection of gene positive homozygous progenies and instant fixation of the background genotype of the derivatives, respectively. The results pertaining different aspects of the present study are presented and discussed under the following sub-heads

4.1 Marker-assisted foreground selection for *Pi-9+ sd-1* double homozygotes

Three hundred and eighty-eight BC_2F_2 plants of cross Ranbir Basmati*^{3}/ PB1(Pi9) obtained from two background selected BC_2F_1 plants, JKR1-29-100 and JKR1-34-16, were subjected to marker-assisted foreground selection to select *Pi-9* and *sd-1* double homozygous plants (Fig 4.1 & 4.2). Of the 296 BC_2F_2 progenies derived from plant JKR1-29-100, 70 were found to be homozygous for the *Pi-9* gene when analyzed with *Pi-9* gene-derived marker *Pi9-Pro*, whereas of the 92 BC_2F_2 derived from JKR1-34-16, 34 were homozygous for the gene (Table 4.1). The *Pi-9* homozygous plants were analyzed with a *sd-1* gene-derived marker *GA20-1* developed in our laboratory to select *sd-1* homozygous plants. Out of 104 *Pi-9* homozygous plants (representing 70 and 30 progenies of plants JKR1-29-100 and JKR1-34-16, respectively) 23 were found to be homozygous for recessive gene *sd-1*. Of the total 388 BC_2F_2 plants of cross Ranbir Basmati*^{3}/ PB1(Pi9), 23 were found to be homozygous for both the genes *Pi-9* and *sd-1* (Table 4.1). The segregation of non-double homozygotes and *Pi-9+ sd-1* double homozygotes plants showed a good fit to segregation ratio of 15: 1 as expected for the F_2 progeny of a dihybrid cross segregating for two genes. The selected *Pi-9+ sd-1* double homozygotes were used as a source of anthers for producing doubled haploid plants via anther culture.

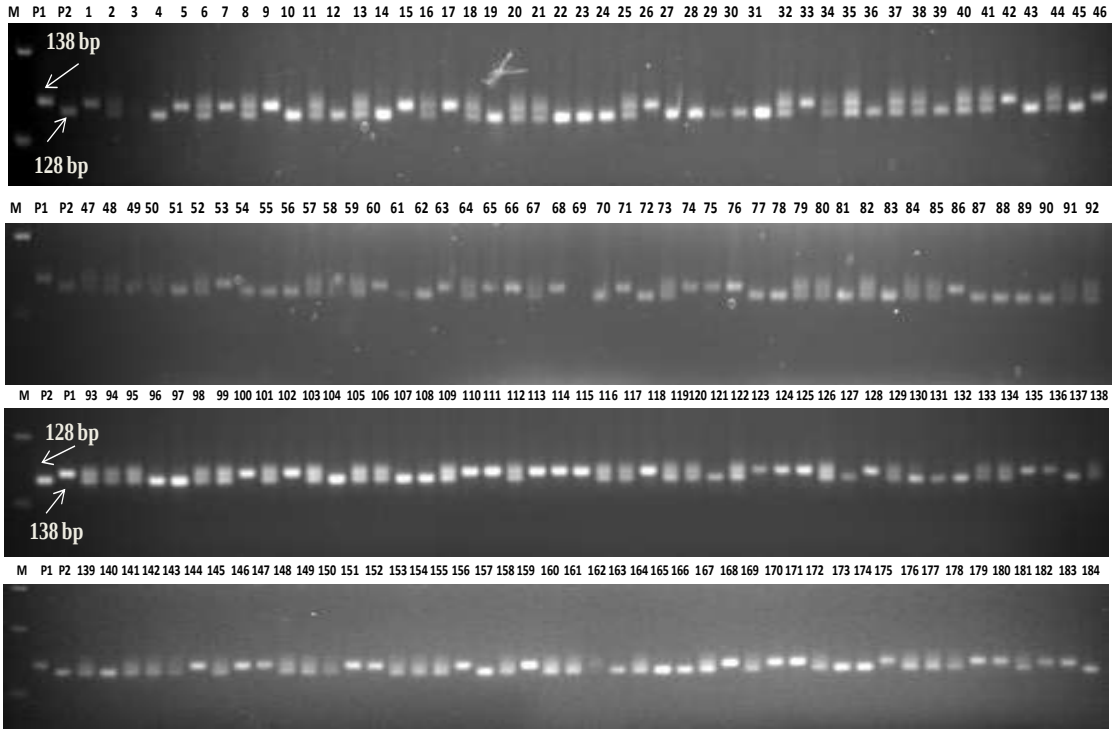

Fig 4.1. A representative gel depicting the genotyping of BC_2F_2 plants of cross Ranbir Basmati*[3]/ PB1(Pi9) with *Pi-9* gene-derived Indel marker *Pi9Pro*. P1= Ranbir Basmati; P2 = PB1(Pi9). PCR products were resolved in 4.0 % agarose gel and stained with ethidium bromide..

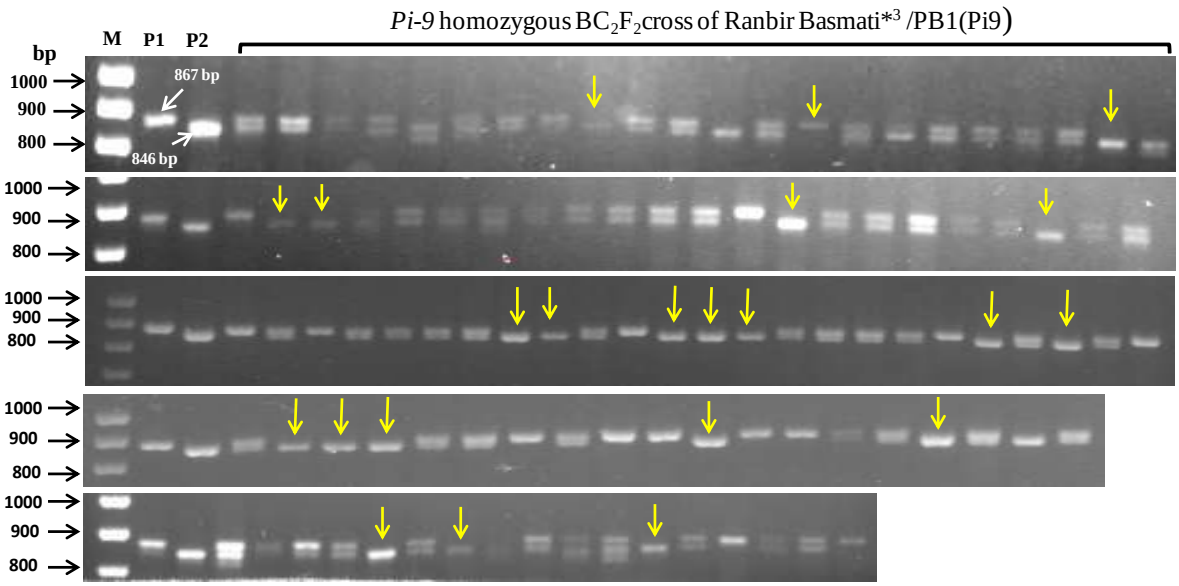

Fig 4.2. Genotyping of 104 *Pi-9* homozygous BC_2F_2 plants of cross Ranbir Basmati*[3]/PB1(Pi9) with semi-dwarfing gene s*d-1* specific marker *GA20-1*. PCR products were resolved in 2.0 % agarose gel and stained with ethidium bromide. The *sd-1* homogzyous recessive plants have been marked with arrows. P1= Ranbir Basmati; P2 = PB1(Pi9).

Table 4.1. Summary of foreground analysis for target resistance genes among BC_2F_2 progenies of cross Ranbir Basmati*[3]/ PB1(Pi9)

Sr. No.	Plant no.	# of BC_2F_2 plants genotyped	# of *Pi-9* homozygous plants	# of *Pi-9+ sd-1* double homozygous plants	Expected ratio [a]	χ^2	P value
1	JKR1-29-100	296	70	18	15 : 1	0.014	0.90 < P< 0.95
2	JKR1-34-16	92	34	5	15 : 1	0.103	0.50 < P< 0.75
3	Overall	388	104	23	15 : 1	0.068	0.75 < P< 0.90

a: ratio of non-double homozygotes to *Pi-9+ sd-1* double homozygotes

4.2 Anther culture of *Pi-9* and *sd-1* homozygous BC_2F_2 plants

4.2.1 Callus induction and Plantlet regeneration

The anthers obtained from the foreground selected *Pi-9+ sd-1* double homozygous BC_2F_2 plants were given two different pretreatments *viz.*, cold and mannitol and subsequently cultured on callus induction medium (MO-19 + 0.5 mg/l 2,4-D + 2.5 mg/l NAA + 0.5 mg/l Kinetin + 9% maltose). Calli started emerging asynchronously from the anthers 4-6 weeks after culture initiation and continued till 12^{th} week. Out of 12123 cold pretreated anthers plated on callus induction medium 92 formed calli, whereas of the 6379 anthers which were pretreated with mannitol only 4 formed calli (Table 4.2). Although a very low percentage of anthers responded to callusing, the callus induction frequency was higher in cold pretreated anthers (0.75%) compared to mannitol pretreatment (0.06%).

Table 4.2. Callusing percentage in anthers subjected to different pretreatments

Pretreatment	No of anthers plated	No of calli formed	Callusing Percentage (%)
Cold	12123	92	0.75
Mannitol	6379	4	0.06
Overall	18502	96	0.51

The data pertaining to the anther culture response of *Pi-9+sd-1* double homozygous plants selected amongst the progenies of JKR-1-29-100 and JKR1-34-16 is presented in Table 4.3. Of the 12459 anthers of BC_2F_2 progenies of plant JKR-1-29-100 only 68 formed calli, whereas 28 calli were induced from the 6043 anthers obtained from BC_2F_2 progenies of JKR1-34-16. The callusing induction frequency in the BC_2F_2 progenies of JKR-1-29-100 and JKR1-34-16 was 0.54 and 0.46 %, respectively with an overall callus induction frequency of 0.51%. When the anther derived calli grew to 4-6 mm in size, they were transferred to regeneration medium and data were recorded on different parameters, namely, regenerating calli (%), calli regenerating green plantlets (%), calli regenerating albino plantlets (%) and the overall anther culture efficiency. Different stages in the development of doubled haploids through anther culture are shown in Fig 4.3

Only 33.82% of the calli derived from the progenies of JKR-1-29-100 regenerated into plantlets while the calli originating from JKR1-34-16 anthers exhibited relatively higher regeneration potential (50.00%). A total of 37 plantlets were regenerated from the 96 anther derived calli of the selected BC_2F_2 progenies with overall regeneration frequency of 38.54%. A significant proportion of regenerated plantlets were albinos. Of the total 37 regenerated plantlets 21 **(**56.75%) were green, while the remaining 16 (43.24%) were albinos. The overall anther culture efficiency (number of green plantlets regenerated/ 100 anthers) for the BC_2F_2 progenies of JKR-1-29-100 and JKR1-34-16 was 0.10 and 0.15, respectively. The overall anther culture response of the BC_2F_2 progenies was very low (0.11%) as only 21 green plantlets could be regenerated from the 18502 cultured anthers.

Table 4.3. Anther culture response of *Pi-9+sd-1* double homozygous plants selected amongst the progenies of JKR-1-29-100 and JKR1-34-16.

Plant no.	No. of anthers plated	No. of calli formed	Callusing (%)	No. of plantlets regenerated	Regeneration (%)	No. of green plants regenerated	Green plantlets (%)	No. of albino plants regenerated	Albino plantlets (%)	Anther culture efficiency (%)
JKR-1-29-100	12459	68	0.54	23	33.82	12	52.17	11	47.82	0.10
JKR1-34-16	6043	28	0.46	14	50.00	9	64.29	5	35.71	0.15
Overall	**18502**	**96**	**0.51**	**37**	**38.54**	**21**	**56.75**	**16**	**43.24**	**0.11**

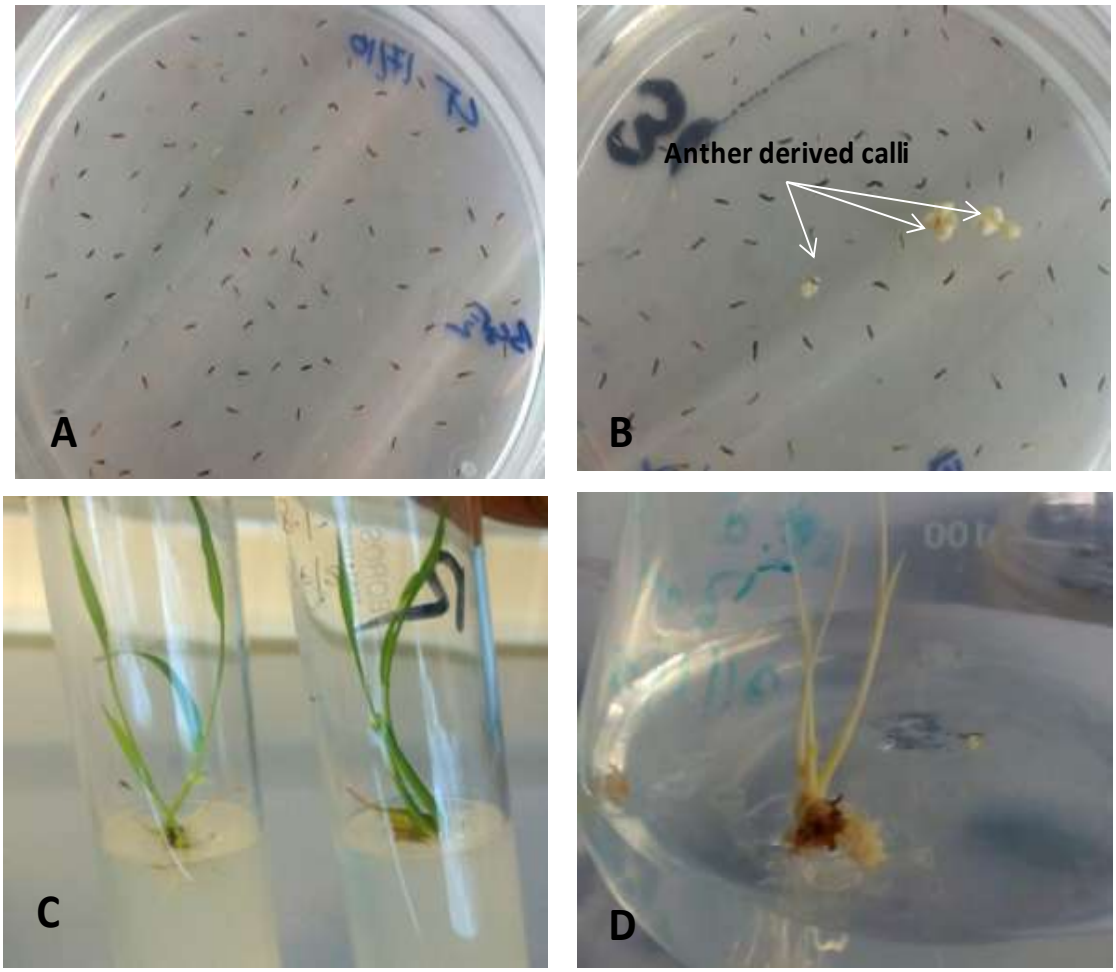

Fig 4.3. Different stages in the development of androgenic plants from cultured anthers of *Pi-9* and *sd-1* homozygous plants. A): Anthers plated on callusing medium; B): Callus induction from plated anthers; C): Green plantlets regenerated from anther derived calli; D): Albino plantlets obtained from anther derived calli.

4.2.2 Hardening of anther culture derived plantlets and plant survival.

A total of 21 anther culture regenerants were transferred to pots for hardening. Of the total transplanted regenerants 19 survived till maturity with an overall survival rate of 90.47% (Table 4.4). Based on morphological features and fertility data, the surviving plants could be classified into haploids, doubled haploids. Haploids were characterized by thin light green leaves, reduced height and excessive tillering resulting in a bushy appearance. They had small panicles with miniature spikelets and were completely sterile. Doubled haploids were morphologically similar to the seed derived diploids and had well proportioned leaves, stems and panicles and high spikelet fertility. Among anther culture derived plantlets a great majority was represented by doubled haploids as 15 of them were fertile. The frequency of spontaneous doubled haploids was 78.95%. The overall percentage of haploids was 21.05% (Table 4.4).

Table 4.4. Frequency of plant survival and spontaneous recovery of haploids and doubled haploids among anther culture derived plants of cross Ranbir Basmati*3/PB1(Pi9)

Cross	No. of plants transferred to pots	No. of Plants survived	Survival (%)	Doubled haploids[a]		Haploids	
				(no.)	(%)	(no.)	(%)
Ranbir Basmati*3/PB1(Pi9)	21	19	90.47	15	78.95	4	21.05

[a]Calculated as percentage of total surviving plants

4.3 Confirmation of zygosity of doubled haploids at *Pi-9* and *sd-1* loci

To confirm the presence and zygosity of the 15 doubled haploids plants at *Pi-9* and *sd-1* gene loci these plants were analyzed with suitable gene derived markers. To confirm the presence of *Pi-9* gene, the doubled haploids were analyzed with a co-dominant marker *Pi9-Pro* as well as a dominant marker *Nbs2-Pi9*. All the doubled haploids were found to be positive for gene *Pi-9* gene showing the presence of a ~ 2kb gene-specific amplicon inherited from PB1(Pi9) (Fig 4.4). When these doubled

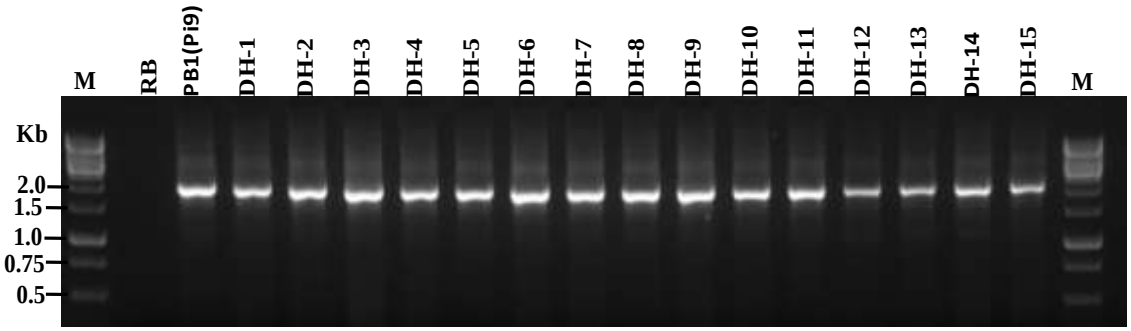

Fig 4.4. Genotyping of parental genotypes and doubled haploids regenerated from BC_2F_2 plants of cross Ranbir Basmati x PB1(Pi9) with *Pi-9* gene-derived marker *Nbs2-Pi9*. RB= Ranbir Basmati; PCR products were resolved in 2.0 % agarose gel and stained with ethidium bromide.

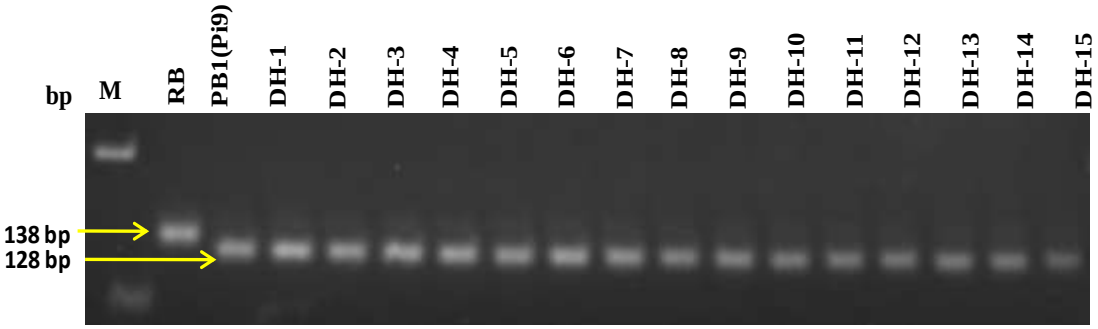

Fig 4.5. Genotyping of parental genotypes and doubled haploids regenerated from BC_2F_2 plants of cross Ranbir Basmati^{*3}/PB1(Pi9) with *Pi-9* gene-derived Indel marker *Pi9-Pro*. RB= Ranbir Basmati; PCR products were resolved in 4.0 % agarose gel and stained with ethidium bromide.

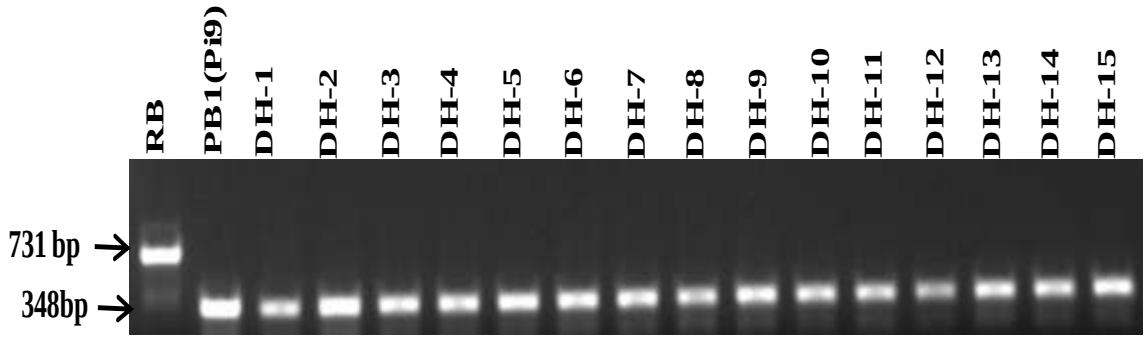

Fig 4.6. Genotyping of parental genotypes and doubled haploids regenerated from BC_2F_2 plants of cross Ranbir Basmati^{*3}/PB1(Pi9) with semi-dwarfing gene s*d-1* specific marker *SD-1*. RB= Ranbir Basmati; PCR products were resolved in 2.0 % agarose gel and stained with ethidium bromide.

haploids were analyzed with co-dominant marker *Pi9-Pro*, all were found to have inherited a single 128 bp amplicon from the *Pi-9* donor thereby confirming their homozygosity for *Pi-9* locus (Fig 4.5). Similarly, to confirm the presence of the semi-dwrafing gene *sd-1*, the doubled haploids were genotyped with gene-derived co-dominant marker *SD-1*. All the doubled haploids were found to have inherited a *sd-1* specific 348 bp amplicon from PB1(Pi9) thereby confirming the homozygosity of recessive *sd-1* gene in these plants (Fig 4.6).

4.4 Agronomic performance of anther culture derived doubled haploids obtained from BC_2F_2 plants of cross Ranbir Basmati*3/PB1(Pi9)

The data on yield and yield related traits of the anther culture derived doubled haploid plants of cross Ranbir Basmati*3/PB1(Pi9) and seed derived parental genotypes Ranbir Basmati and PB1(Pi9) are presented in Table 4.5.

4.4.1 Days to flowering

The parental genotypes Ranbir Basmati and PB1(Pi9) flowered after 90 and 108 days of sowing, respectively. Days to flowering varied from 86 to 115 days in case of doubled haploid (DH) plants. Most of DH plants flowered a couple of days later than the parental genotype Ranbir Basmati. DH-9 took most number of days for flowering (115 days) while DH-8 and DH-10 took least number of days (86 days) for flowering.

4.4.2 Height of the plants (cm)

All the anther culture derived DH plants were short-statured compared to Ranbir Basmati with plant height ranging from 88 to 122 cm (Table 4.5; Fig 4.7). There was 9.6 to 43.6 cm reduction in the height of different DH plants introgressed with *sd-1* compared to Ranbir Basmati. Majority of the DH plants exhibited intermediate height compared to the parental genotypes and only two plants DH-1 and DH-9 had height similar to the dwarf parent PB1(Pi9).

4.4.3 Number of effective tillers per plant

The number of effective tillers in DH plants ranged from 7 to 30. Maximum number of effective tillers (30) was found in DH-12 and least effective tillers (7) were recorded in DH-4. Seven DH plants *viz.*, DH-3, DH-6, DH-7, DH-12, DH-13, DH-14 and DH-15 showed improvement in effective tillers/plant compared to both the parents.

4.4.4 Panicle length (cm)

Most of the anther culture derived DH plants possessed longer panicle length than the both parental genotypes. The length of the panicle varied from 23.5 cm to 31.6 cm. among the anther culture derived DH plants. The longest recorded panicle was found in DH-6 (31.6cm), while shortest panicle length was observed in DH-1 (23.5cm). The seed derived parental genotypes Ranbir Basmati and PB1(Pi9) were having panicle length 26.8cm and 24.2cm, respectively.

4.4.5 Number of grains per panicle

In DH plants the number of seeds per panicle ranged from 87.4 to 162.6. Maximum seeds per panicle were recorded in DH-6 (162.6) and minimum in DH-1 (87.4). Three anther culture derived DH plants *viz*., DH-6, DH-14 and DH-15 exhibited significantly superior grain number/panicle compared to both the parental genotypes with grain number ranging from 152 to 162.6.

4.4.6 1000 seed weight (g)

All the DH plants were inferior to both the parental genotypes for the trait 1000 seed weight. In case of DH plants 1000 seed weight varied from 16.31 to 19.83 g. DH-9 showed maximum seed weight (19.83 g) and DH-1 possessed the minimum seed weight (16.31 g). The parental genotypes Ranbir Basmati and PB1(Pi9) depicted 1000 seed weight of 22.80 g and 23.79 g, respectively.

4.4.7 Yield per plant (g)

The grain yield in DH plants varied from 5.04 to 30.10 g. Highest grain yield per plant was recorded in DH-14 (30.10 g), while the minimum was observed in DH-1 (5.04 g). Seven DH plants *viz.*, DH-3, DH-6, DH-11, DH-12, DH-13, DH-14 and DH-15 exhibited superiority over the parental genotypes for grain yield with yield/plant ranging from 21.40 to 30.10 g.

4.5 Physico-chemical characteristics of grains of anther culture derived doubled haploid plants

The anther culture derived DH plants and parental genotypes Ranbir Basmati and PB1(Pi9) were evaluated for grain dimension and quality parameters *viz*., grain length, grain breadth, length/breadth ratio, grain shape, amylose content, alkali

spreading value and aroma. The data pertaining to grain dimensions and quality parameters is presented in Table 4.5

4.5.1 Grain length [L] and breadth [B]

The grain length of DH plants varied from 6.33 mm to 7.22 mm. All the DH plants except, DH-10, had inferior grain length compared to parental genotypes. Only two DHs, DH-10 and DH-11 possessed grain length > 7.0 mm, while grain length in the remaining plants ranged from 6.33 6.99 mm. The grain breadth of DH plants varied from 1.54 mm to 1.74 mm. All the DH plants were superior to Ranbir Basmati for the trait grain breadth.

4.5.2 Grain L/B ratio

The grain L/B ratio for the anther culture derived doubled haploid plants ranged from 3.65 to 4.45. Six DH plants *viz*., DH-3, DH-5, DH-7, DH-10, DH-11 and DH-12 exhibited superiority over Ranbir Basmati for grain L/B ratio (4.11 to 4.45).

4.5.3 Grain Type

The grains of DH plants and parental genotypes were categorized into different classes based on their length and L/B ratio. Ten DH plants *viz*., DH-3, DH-5, DH-6, DH-7, DH-8, DH-9, DH-10, DH-11, DH-12 and DH14 had long-slender grains similar to Ranbir Basmati albeit grain length in a great majority of these plants was comparatively shorter than Ranbir Basmati.

4.5.4 Alkali spreading value

A great majority of doubled haploids possessed intermediate to high alkali spreading value, hence intermediate to low gelatinization temperature, similar to parental genotypes (Table 4.5; Fig 4.8). However, the grains from three DH plants *viz*., DH-8, DH-9 and DH-10 possessed low alkali spreading value with ASV score of 2.

4.5.5 Amylose content (%)

Amlyose content is one of the important quality determinants in rice. The basmati rice varieties are characterized by intermediate amylose content (20-25%), which imparts soft and fluffy texture to cooked rice. Majority of the doubled haploid derivatives possessed intermediate amylose similar to parental genotypes with amylose content ranging from 20.0 to 22.6%. However, four DH plants *viz.*, DH-7,

DH-12, DH-14 and DH-15 exhibited low values (16.33 to 18.92 %) for amylose and most likely were transgressive segregants.

4.5.6 Aroma

The grains of parental genotypes Ranbir Basmati and PB1(Pi9) possessed strong and moderate aroma, respectively. A great majority of doubled haploid regenerants exhibited only mild aroma. However, three doubled haploids, DH-4, DH-6 and DH-9 exhibited strong aroma similar to Ranbir Basmati.

Table 4.5. Agromorphological and grain quality characteristics of the doubled haploids and parental genotypes

Doubled haploid plant	Days to flowering	Plant height (cm)	Number of effective tillers	Panicle length (cm)	No of seeds per panicle	1000 seed weight (g)	Yield/plant (g)	Fertility (%)	Grain length (mm)	Grain breadth (mm)	L/B ratio	Grain type	Alkali spreading value	Amylose content (%)	Aroma
DH-1	92	88	11	23.5	87.4	16.31	5.04	76.91	6.41	1.65	3.88	MS	4	22.62	Mild
DH-2	99	109	16	27.1	125.6	17.57	12.53	66.24	6.46	1.74	3.70	MS	5	20.89	Mild
DH-3	108	116	20	29.1	119.4	17.96	21.40	79.56	6.99	1.69	4.14	LS	4	21.73	Mild
DH-4	92	96	7	26.7	93	19.03	8.05	71.83	6.43	1.68	3.83	MS	5	20.00	Strong
DH-5	94	101	11	26.6	120	17.25	16.54	80.44	6.67	1.62	4.11	LS	5	21.70	Mild
DH-6	96	112	19	31.6	162.6	18.00	23.94	89.79	6.76	1.70	3.99	LS	5	21.48	Strong
DH-7	110	108	20	28.6	122.6	16.88	10.42	69.66	6.61	1.54	4.30	LS	6	16.33	Mild
DH-8	86	101	9	28.5	127.2	19.62	14.34	93.71	6.90	1.71	4.04	LS	2	22.00	Mild
DH-9	115	88	14	27.6	101.6	19.83	12.75	77.17	6.83	1.69	4.05	LS	2	20.54	Strong
DH-10	86	122	15	28.1	130	19.54	19.75	94.77	7.22	1.73	4.17	LS	2	22.02	Mild
DH-11	91	100	17	27.1	134.2	18.42	21.73	79.88	6.93	1.69	4.11	LS	5	20.59	Mild
DH-12	103	95	30	27.8	117	18.98	29.44	78.29	7.08	1.59	4.45	LS	4	18.87	Mild
DH-13	102	107	21	30.2	131.6	18.79	26.65	83.89	6.46	1.72	3.76	MS	5	21.08	Mild
DH-14	100	105	22	29.2	152	19.31	30.10	81.84	6.61	1.69	3.91	LS	5	18.22	Mild
DH-15	107	112	23	29.4	152.8	18.82	27.26	82.59	6.33	1.73	3.65	MS	6	18.92	Mild
Ranbir Basmati	90	131.6	17	26.8	133	22.8	19.75	89.25	7.16	1.76	4.06	LS	4	22.75	Strong
PB1(Pi9)	108	88.6	12	24.2	125	23.79	18.29	84.57	8.35	1.60	5.21	ELS	7	22.67	Moderate

Grain shape: *extra long* >7.50 mm, *long* 6.61–7.50 mm, *medium* 5.51–6.60, *L/B ratio* = length/breadth ratio, Amylose content:9–20 %–*low*, 20–25 %—*intermediate, grain type: MS = medium-slender; LS = Long-slender; ELS= extra long-slender*

Fig 4.7. Comparison of plant height of individual doubled haploids with parental genotypes Ranbir Basmati and PB1(Pi9).

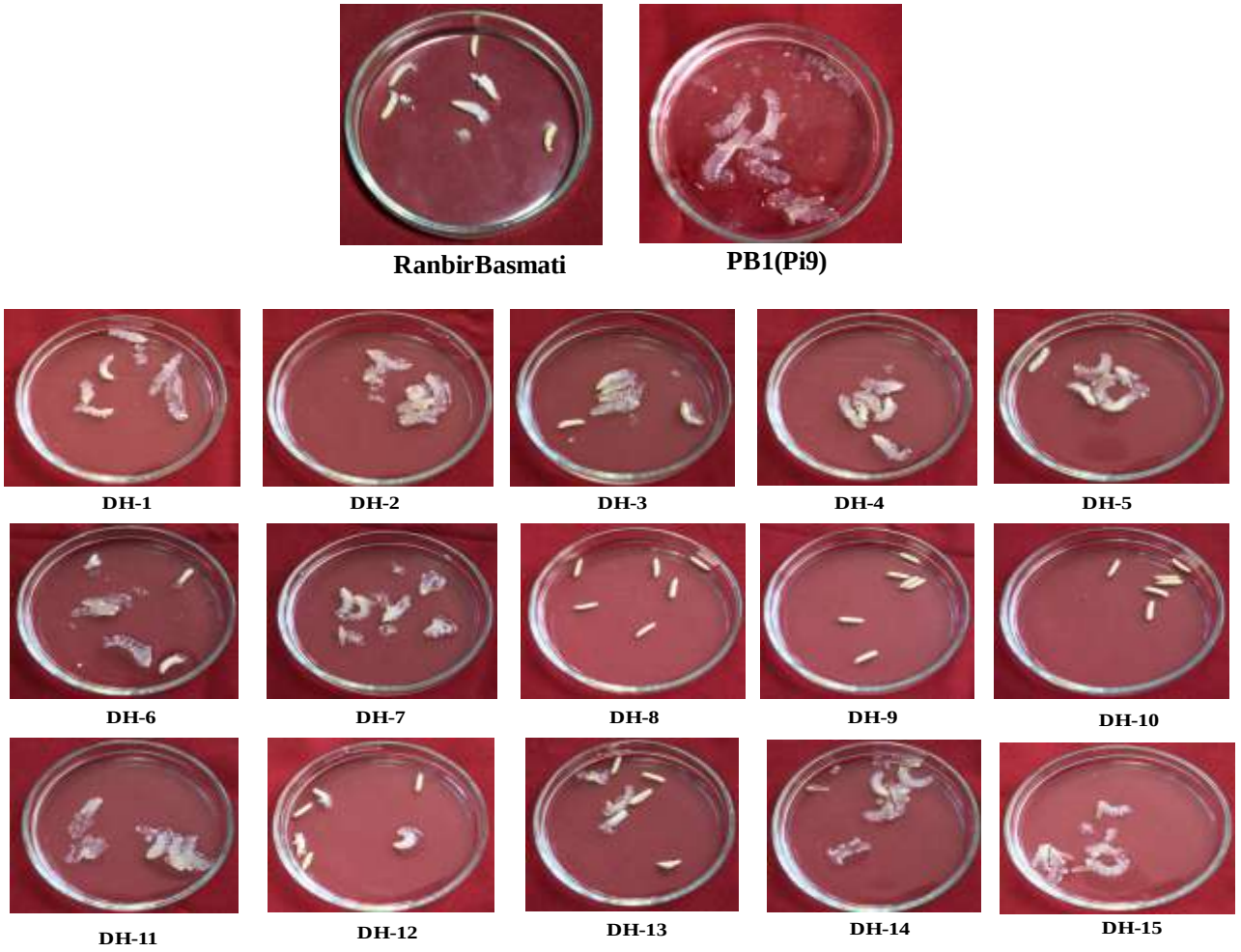

Fig 4.8. Alkali spreading of the anther culture derived doubled haploids plants regenerated from BC_2F_2 plants of cross Ranbir Basmati x PB1(Pi9) and parental genotypes Ranbir Basmati and PB1(Pi9).

4.6 Evaluation of doubled haploid plants for blast resistance

The anther culture derived DH plants and parental genotypes were tested under artificial epiphytotic conditions for their reaction to *Pi-9* diagnostic blast isolate *PO*-RB1. The parental genotype Ranbir Basmati exhibited highly susceptible reaction (4-5 reaction type), while the *Pi-9* donor PB1(Pi9) was highly resistant (0-1 reaction type) (Table 4.6). All the DH plants were found to be highly resistant to blast exhibiting disease reaction similar to *Pi-9* donor PB1(Pi9). These results confirmed the introgression of functional alleles of *Pi-9* in the anther culture regenerants.

Table 4.6. Disease reaction of doubled haploids and parental genotypes to rice blast

Genotype	Reaction type	Disease reaction
DH-1	0-1	R
DH-2	0-1	R
DH-3	0-1	R
DH-4	0-1	R
DH-5	0-1	R
DH-6	0-1	R
DH-7	0-1	R
DH-8	0-1	R
DH-9	0-1	R
DH-10	0-1	R
DH-11	0-1	R
DH-12	0-1	R
DH-13	0-1	R
DH-14	0-1	R
DH-15	0-1	R
Ranbir Basmati	4-5	S
PB1(Pi9)	0-1	R

R=Resistant reaction (reaction type 0-2); S= Susceptible (reaction type 3-5).

In present study, we attempted to bring about genetic improvement of Ranbir Basmati for semi dwarf phenotype and resistance to blast by introgressing blast resistance gene *Pi-9* and semi-dwarfing gene s*d-1* from a basmati donor line PB1(Pi9). Combined use of marker-assisted selection and anther culture ensured speedy conversion of Ranbir Basmati into semi-dwarf lines with in-built resistance to rice blast. The use of *Pi-9* and s*d-1* gene-derived markers in BC_2F_2 progenies facilitated speedy and precise selection of gene homozygous progenies without resorting to time consuming and imprecise disease screening procedures and also curtailing the need for progeny testing for selecting homozygous recombinants. In recent years considerable progress has been achieved in improving the disease resistance in traditional as well as evolved basmati rice varieties through marker assisted breeding approaches. Marker-assisted selection has been successfully used to incorporate single or a combination of various blast resistance genes *Pi-2*, *Pi-9, Pi-1, Pi-54, Pi-ta, Pi-b* and *Pi-5* into basmati varieties like Pusa Basmati-1, Pusa 1121, Pusa Basmati 6 and parental lines of basmati hybrids (Khanna et al. 2015; Ellur et al. 2016; Singh et al. 2012). The *Pi-9* gene sourced from the wild tetraploid species, *Oryza minuta* has been reported to be highly effective against blast in northern parts of India including the states of Jammu Kashmir, Himachal Pradesh and Uttrakhand which have been earmarked as geographical indication areas for basmati (Khanna et al. 2015; Rathour et al. 2011). The *Pi-9* introgressed double haploid lines developed in present study have also shown high level of resistance to blast thereby making them potential candidates for release in the basmati growing areas of the country.

The semi dwarfing gene *sd-1* sourced from Dee-geo-woo-gen (DGWG) has been extensively exploited since 1960s for developing semi-dwarf varieties of rice (Spielmeyer et al. 2002). The development of semi-dwarf varieties through the introduction of *sd-1* has been major reason why green revolution could double the yields in rice (Khush 1995). The *sd-1* gene confers semi-dwarf stature, improves lodging resistance and harvest index (Hedden 2003; Luo and Yin 2013). In present study, the introgression of *sd-1* gene has resulted in 9.6 to 43.6 cm reduction in plant height as compared to Ranbir Basmati. Although all the *sd-1* introgressed recombinants were shorter than Ranbir Basmati, only two (DH-1, DH-6) attained the dwarfness of PB1(Pi9). These results are in accordance with reported incomplete or

partial recessive nature of *sd-1* gene which can reduce the plant height by 20-25 % only (Spielmeyer et al. 2002; Tomita and Ishii 2018). Similar to our results, a partial reduction in height has been earlier reported in *sd-1* introgressed derivatives of tall basmati varieties Basmati370 and Type 3 Basmati (Rajpurohit et al. 2011; Bhatia et al. 2011).

In present study, we processed the foreground selected *Pi-9* + *sd-1* homozygous plants of cross Ranbir Basmati*^{3}/PB1(Pi9) via anther-culture to produce doubled haploids. The BC_2F_2 derivatives of cross Ranbir Basmati*^{3}/ PB1(Pi9) have shown very low level of response to anther culture as only 21 green plantlets could be regenerated from 18502 cultured anthers with overall anther culture efficiency of 0.11%. A great majority of (78.95%) anther cultured derived plants of cross Ranbir Basmati*^{3}/PB1(Pi9) were spontaneous diploids as we didn't use colchicine for inducing chromosome doubling in the regenerants. Both endo-reduplication and nuclear fusion have been reported to occur during rice anther culture (Chen and Wu 1983) and are the most likely causes of spontaneous chromosome doubling in anther culture regenerants. A comparable frequency of doubled haploids also been recorded earlier in rice anther culture derived plants by several workers (Guiderdoni et al 1992; Bishnoi et al. 2000; Germana 2011; Kaushal et al. 2014).

Anther culture response is a genetically controlled trait and fine grained rice varieties including Basmatis are known to exhibit poor anther culture response (Raina et al. 1987; Bishnoi et al. 2000; Roy and Mandal 2005; Singh 2014). Similar to present study, very low anther culture efficiency has been recorded in basmati variety Basmati 370, the parental genotype of Ranbir Basmati and in crosses involving Basmati 370 as one of the parents (Guiderdoni et al. 1992; Raina et al. 1987). Raina et al. (1987) reported very low anther culture response (0.04%) in Basmati-370 as they could regenerate only 9 green plants from 21990 cultured anthers. Although the low anther culture response of basmati varieties might be a hurdle in exploitation of doubled haploidy as a routine tool in conventional basmati rice breeding, its inclusion as an adjunct to marker-assisted backcross breeding scheme for eliminating the residual background heterozygosity of the marker-selected recombinants could be advantageous. The elimination of residual background heterozygosity in a regular

marker-assisted backcross breeding programme would otherwise require several rounds of self-pollination of the foreground selected individuals to get the lines that are stable enough for advanced evaluation trials.

Transgressive segregation is the phenomenon where by progenies are observed with the phenotypes that can fall outside the phenotypic range of the parental genotypes. In present study, the analysis of yield and its component traits in DH plants revealed the presence of transgressive segregants for the traits *viz.,* effective tillers, grains per panicle and yield per plant; some of the DH plants out performed both the parental genotypes for these traits. These results clearly suggest that these traits are controlled by multiple non-allelic genes and favorable alleles were contributed by both the basmati parents. Similar to our results, transgressive segregants with improved yield, effective tillers and grain number per panicle have earlier been reported among the progenies of crosses involving basmati parents (Ellur et al. 2016; Basavaraj et al. 2010; Rajpurohit et al. 2011).

Transgressive segregants showing inferior grain quality characteristics *viz*. alkali spreading value, aroma and amylose content were also obtained among double haploids of the cross Ranbir Basmati*^{3}/ PB1(Pi9). The situation was more so for grain aroma for which most of the DH plants were inferior having weak aroma compared to both the parents. Although a single recessive gene *fgr* located on chromosome 8 aroma is known to control aroma in rice, several other QTLs that affect the strength of aroma in rice have been identified in different rice varieties (Lorieux et al. 1996; Amarawathi et al. 2008). Therefore the obtention of great majority of double haploids with weak/mild aroma can be attributed to the failure to combine all the favorable aroma alleles in a single genotype. The amylose content in rice is controlled by a single dominant gene *Waxy gene* (*Wx*) located on chromosome 6 and number of other modifiers or QTLs with minor effects on different rice chromosomes (Kumar and Khush 1986; Takemoto-Kuno et al. 2015). These polygenic modifier genes can influence the amylose content in unexpected ways leading to transgressive segregation and can account for the recovery few doubled haploids with inferior amlylose content in our study. Similar to our study transgressive segregants with significantly low amylose content compared to parents have been recorded in the

progenies of Type 3 Basmati and Pusa 1121 (Amarawathi et al. 2008; Rajpurohit et al. 2011).

The introgression of semi-dwarfing gene *sd-1* not only conferred semi-dwarf phenotype but also improved grain yield in some of the recombinants. Seven DH plants *viz.*, DH-3, DH-6, DH-11, DH-12, DH-13, DH-14 and DH-15 exhibited higher grain yield and short-stature compared to Ranbir Basmati. These results are consistent with previous reports wherein introgression of *sd-1* gene has been reported to improve yield and harvest index in various rice varieties (Luo and Yin 2013; Bhatia et al. 2011). However, whether the improved yield of *sd-1* introgressed lines is due to pleiotropic action of *sd-1* or multiple linked genes is not clear. Co-localization of QTLs for grain yield and the *sd-1* gene in rice has been previously suggested due to linkage rather than pleiotropy (Trijatmiko et al. 2014). Two DH lines, DH-6 and DH-11 besides being semi-dwarf and highly resistant to blast exhibited yield, maturity and grain quality attributes comparable or better than Ranbir Basmati. These lines should be further evaluated for yield and blast resistance under multi-location yield trials to assess their potential as new varieties. These two lines and other DH lines with superior quality attributes can also be used as immediate donors in basmati improvement programmes.

5. SUMMARY AND CONCLUSIONS

The present investigation was aimed at development of the semi-dwarf and blast resistant fixed derivatives of a traditional basmati rice variety Ranbir Basmati through introgression of blast resistance gene *Pi-9* and a recessive semi-dwarfing gene *sd-1* from a basmati donor PB1(Pi9). Marker-assisted foreground selection was conducted to select BC_2F_2 plants homozygous for blast resistance gene *Pi-9* and semi-dwarfing gene *sd-1* by using suitable gene- derived markers. The foreground selected BC_2F_2 plants were processed via anther culture to produce homozygous doubled haploid lines. The doubled haploid derivatives obtained through anther culture along with the parental genotypes Ranbir Basmati and PB1(Pi9) were evaluated for agronomic performance, seed quality attributes and blast resistance using standard methods. The results of the study are summarized below:

1. Of the total 388 BC_2F_2 plants of cross Ranbir Basmati*^{3}/ PB1(Pi9) subjected to marker-assisted foreground, 23 were found to be homozygous for both the genes *Pi-9* and *sd-1*.The segregation of non-double homozygotes and *Pi-9+ sd-1* double homozygotes plants showed a good fit to segregation ratio of 15: 1 expected for the F_2 progeny of a dihybrid cross segregating for two genes.

2. Of the 12459 anthers of BC_2F_2 progenies of plant JKR-1-29-100 only 68 formed calli, whereas 28 calli were induced from the 6043 anthers obtained from BC_2F_2 progenies of JKR1-34-16. The callusing induction frequency in the BC_2F_2 progenies of JKR-1-29-100 and JKR1-34-16 was 0.54 and 0.46 %, respectively with an overall callus induction frequency of 0.51%.

3. A total of 37 plantlets were regenerated from the 96 anther derived calli of the selected BC_2F_2 progenies with overall regeneration frequency of 38.54%. A significant proportion of regenerated plantlets were albinos. Of the total 37 regenerated plantlets 21 (56.75%) were green, while the remaining 16 (43.24%) were albinos.

4. The overall anther culture efficiency (number of green plantlets regenerated/ 100 anthers) for BC_2F_2 progenies of JKR-1-29-100 and JKR1-34-16 was 0.10 and 0.15, respectively. The overall anther culture response of the BC_2F_2 derivatives of cross Ranbir Basmati*^{3}/ PB1(Pi9) was very low (0.11) as only 21 green plantlets could be regenerated from the 18502 cultured anthers.

5. Among 19 anther derived plantlets that survived after transplanting, 15 were fertile spontaneous doubled haploids while the remaining 4 were sterile haploids. All the 15 doubled haploids were found to be homozygous for *Pi-9* and *sd-1* genes when genotyped with gene-based markers *Pi9-Pro* and *SD-1*, respectively.

6. The analysis of yield and its component traits in anther culture derived doubled haploid (DH) plants revealed the presence of superior transgressive segregants for the traits *viz.,* effective tillers, grains per panicle and yield per plant; some of the DH plants out performed both the parental genotypes for these traits.

7. Transgressive segregants showing inferior grain quality characteristics *viz*. alkali spreading value, aroma and amylose content were also obtained among double haploids of the cross Ranbir Basmati*^{3}/ PB1(Pi9). The situation was more so for grain aroma for which most of the DH plants were inferior having weak aroma compared to both the parents.

8. All the anther culture derived DH plants were short-statured compared to Ranbir Basmati with plant height ranging from 88 to 122 cm. There was 9.6 to 43.6 cm reduction in the height of different DH plants introgressed with *sd-1* compared to Ranbir Basmati.

9. All the DH plants were found to be highly resistant to blast exhibiting disease reaction similar to *Pi-9* donor PB1(Pi9). These results confirmed the introgression of functional alleles of *Pi-9* in the anther culture regenerants.

10. Based on overall performance, several DH plants were found to possess superior grain yield and quality attributes. These included 2 for days to 50% flowering (DH-8 and DH-10), 7 for effective tillers/plant (DH-3, DH-6, DH-7, DH-12, DH-13, DH-14 and DH-15), 3 for grain number (DH-6, DH-14 and DH-15), 7 for grain yield/plant (DH-3, DH-6, DH-11, DH-12, DH-13, DH-14 and DH-15), 6 for

L/B ratio (DH-3, DH-5, DH-7, DH-10, DH-11 and DH-12), 8 for ASV (DH-2, DH-4, DH-5, DH-6, DH-7, DH-11, DH-14 and DH-15) and 3 for aroma (DH-4, DH-6 and DH-9).

11. Two DH lines, DH-6 and DH-11, besides being semi-dwarf and highly resistant to blast, exhibited yield, maturity and grain quality attributes (except grain length) comparable or better than Ranbir Basmati. These lines can be further evaluated for yield and blast resistance under multi-location yield trials to assess their potential as new varieties.

Practical Implications:

- The generated DH lines can be further evaluated for yield and blast resistance under multi-location yield trials to assess their potential as new varieties.
- The DH lines with superior quality attributes can also be used as immediate donors in basmati improvement programmes.
- Our study has demonstrated the utility of anther-culture as an adjunct to marker-assisted backcross breeding. The technique can be integrated with marker-assisted backcross breeding programmes for eliminating residual heterozygosity among the background selected recombinants to obtain the fixed lines for advanced evaluation trials.

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APPENDICES

Appendix-I

Composition of tissue culture media used in the present study.

Constituent	MO19 (mg/l)	N_6 (mg/l)	MS (mg/l)
NH_4NO_3	-	-	1650.0
KNO_3	3101.0	2830.0	1900.0
KH_2PO_4	540.0	400.0	170.0
$(NH_4)_2SO_4$	264.0	463.0	-
$MgSO_4.7H_2O$	370.0	185.0	370.0
$CaCl_2.2H_2O$	440.0	166.0	440.0
KI	0.8	0.8	0.8
H_3BO_3	6.2	1.6	6.2
$MnSO_4.4H_2O$	22.3	4.4	22.3
$ZnSO_4.7H_2O$	8.6	1.5	8.6
$Na_2MoO_4.2H_2O$	0.25	-	0.25
$CuSO_4.5H_2O$	0.025	-	0.025
$CoCl_2.6H_2O$	0.025	-	0.025
$FeSO_4.7H_2O$	27.8	27.8	27.8
$Na_2EDTA.2H_2O$	37.3	37.3	37.3
Inositol	100.0	-	100.0
Nicotinic acid	2.5	0.5	0.5
Pyridoxine HCl	2.5	0.5	0.5
Thiamine HCl	1.0	1.0	0.5
Glycine	2.0	2.0	2.0

MO19 (Raina and Zapata 1997); N_6 medium (Chu et al. 1975); MS (Murashige and Skoog 1962).

Brief Biodata of student

Name	:	Prabhudutt Samal
Father's Name	:	Mr. Bhagaban Samal
Mother's Name	:	Mrs. Bidyut Lata Bhuyan
Date of Birth	:	01 November 1993
Permanent Address	:	At- Charchika Sahi, Po- Banki, District-Cuttack, State -Odisha, Pin-754008

Academic Qualification

Qualification	Year	School/ Board/ University	Marks (%)/ OGPA	Division	Subjects
10th	June 2009	S.A.C.I.E Banki,Cuttack Odisha (BSE-Odisha)	86.83	First	Odia,English, Mathematics, Science, and Social Science
12th	June 2011	KBR COLLEGE Pira Bazzar, Cuttack Odisha (CHSE-Odisha)	75	First	English, Physics, Math, Chemistry and Biology
B.Sc. (Agriculture)	June 2016	College of Agriculture Bhawanipatna, Odisha Odisha University of Agriculture and Technology, Odisha, India	7.56	First	Agriculture
M.Sc. (Agricultural Biotechnology)	May 2019	CSK Himachal Pradesh Krishi Vishwavidyalaya, Palampur-176062 (H.P.), India	7.69	First	Agricultural Biotechnology

Fellowships/Scholarships/Gold Medals/Awards/Any Other Distinction

Recipient of JNU Fellowship during M.Sc. (Agri. Biotechnology) program from the Department of Biotechnology, Govt. of India, New Delhi