Marker assisted backcross breeding for pyramiding genes conferring resistance to bacterial blight in rice variety Uma

By

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **'Marker assisted backcross breeding for pyramiding genes conferring resistance to bacterial blight in rice variety Uma,' is** a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **'Marker assisted backcross breeding for pyramiding genes conferring resistance to bacterial blight in rice variety Uma,'** is a record of research work done independently by **Ms. Megha L. M.,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Dedicated to my parents, grandparents and to my dearest sister

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

%	Per cent
BB	Bacterial Blight
BC_1	1st backcross generation
BC_2	2nd backcross generation
BC_3	3rd backcross generation
bp	Base pairs
cm	Centimetre
cM	Centimorgan
CTAB	Cetyl Trimethyl Ammonium Bromide
COH	College of Horticulture
DAS	Days after sowing
DBT	Department of Biotechnology
DNA	Deoxy Ribo Nucleic acid
EDV	Essentially Derived Variety
F_1	1st filial generation
F_2	2nd filial generation
F_3	3rd filial generation
g	Gram
GGT	Graphical Geno Types
IRRI	International Rice Research Institute
ISM	Improved Samba Mahsuri
MABB	Marker Assisted Backcrossing Breeding
MAS	Marker Assisted Selection
Max.	Maximum
Min.	Minimum
MSL	Mean Sea Level

µg	Microgram
µl	Microliter
ml	Millilitre
mm	Millimetre
mM	Millimolar
No.	Number
OD	Optical Density
PCR	Polymerase Chain Reaction
POP	Package of Practices
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RP	Recurrent Parent
RLK	Receptor Like Kinase
RM	Rice Microsatellite
SAP	Specific Amplicon Polymorphism
spp.	Species
STS	Sequence Tagged Site
SES	Standard Evaluation System
TAE	Tris Acetic acid EDTA

Introduction

I. INTRODUCTION

Rice (*Oryza sativa* L.) is one of the important staple food crops grown worldwide. Rice yields have continued to increase across the globe since Green revolution. However, the annual growth rates are slowing. In 2017-2018, rice production in the world reached 484 million tons from an area of 161.1 million ha notching a productivity of 3.00 tons/hectare (www. statista.com). China followed by India has remained the world's leading producer of rice for past several years. During 2016-2017 rice production in India was 110.15 million tonnes from an area of 43.39 million hectors with the productivity of 2.40tons/ha (DES, 2018).

According to Khush (2005), for India to remain self-sufficient and meet the future demands, it need to produce about 135-240 million tonnes of rice by the year 2030. Ensuring food security demands an increase in the production and productivity of rice from the available resources. This increase is to be achieved in the face of challenges posed by abiotic factors including climate, land, water, labour or a constant battle against biotic forces such as pathogens and pests. Combating stress whether biotic or abiotic, limits rice crop productivity, forcing farmers to use higher amounts of inputs to obtain the same yield as before. Nevertheless, to meet the needs of increasing population, it is imperative that the rice productivity in the country needs to be improved on a sustainable basis.

Rice plays a significant role in moulding the culture and life in Kerala. There has been considerable decrease in area and production of rice in Kerala over the years. Rice area in the state has declined to 1.71 lakh ha in 2017 registering a total production of 4.36 lakh tons and productivity of 2.55 tons/ha (DES, 2017). Despite this decline, rice consumption in Kerala remains strong, driven by both population and economic growth.

Kerala too faces the daunting challenge of balancing the rice production and demand scenario. Unlike rice consumers in other regions of the country, people of Kerala prefer red-kernelled rice. This unique preference makes it crucial that

the state become self-sufficient in rice production. High soil acidity and iron toxicity are major detrimental factors affecting the productivity of rice in Kerala. Apart from these abiotic stresses, the humid environment prevailing in Kerala favours the occurrence of both insect pests and pathogens throughout the cropping period.

Like in other rice growing regions in the country, bacterial blight caused by *Xanthomonas oryzae* p.v. *oryzae (Xoo)* is the one of the most obliterating disease that affects rice crop in Kerala. According to Sonti (1998), this disease causes a yield losses ranging from 20 to 40 per cent though in severely infected fields the losses may reach up to 80-100 per cent (Dokku *et al.,* 2013a). In Kerala, the occurrence of disease in epidemic form was first reported in Palakkad district in the year 1998 (Priyadarisini and Gnanamanickam, 1999). Since then the disease has been observed in severe proportions almost every year in the state. Among the various disease management tactics like forecasting, intercultural practices, chemical and biological control, host plant resistance based on multiple genes, have been reported to impart durable resistance to the dreaded disease (Chen *et al.*, 2011).

Forty-two resistance (R) genes designated from *Xa1* to *Xa42* conferring host plant resistance against various strains of *Xoo* have been reported (Zhang *et al.*, 2014, Kim *et al.*, 2015, Hutin *et al.,* 2015 and Busungu *et al.,* 2016). These include 30 dominant and 12 recessive genes. Pyramiding resistance gene through marker assisted backcross (MAB) breeding approach is found to be an effective method to ensure durable resistance. According to Priyadarisini and Gnanamanickam (1999), rice line NH56 carrying four *R* genes (*Xa4* + *xa5* + *xa13* + *Xa21*) was found to be resistant to Kerala isolate of the *Xoo* pathogen. However, breakdown of resistance of cultivars with *Xa4* has been reported earlier by Mew *et al.* (1992). Successful introgression of bacterial blight resistance genes into rice crop have been reported (Shanti *et al.*, 2010; Salgotra *et al.,* 2012; Baliyan *et al.*, 2018; Das *et al.*, 2018; Sagar *et al.*, 2018). Unlike the conventional breeding approaches were phenotypic selection of plants is a

tedious procedure and can also be under epistatic influences, Marker assisted backcross breeding (MABB) has been found to be a reliable strategy for resistance breeding (Bharani *et al.*, 2010).

Among the elite rice cultivars grown in Kerala, Uma (Mo 16) is a high yielding variety with a yield potential of 6-6.5t/ha. Uma along with variety Jyothy (Ptb 39) occupies nearly 80 per cent of the rice growing tracts of the state. Although Uma exhibits resistance to Brown plant hopper (KAU, 2016), it is highly susceptible to Bacterial Blight (BB). Hence, occurrence of bacterial blight has become a recurring phenomenon in the state leading to heavy decline in rice production and productivity.

Considering the above, efforts were taken to pyramid three BB resistance genes (*xa5, xa13* and *Xa21*) from donor Improved Samba Mahsuri (ISM) into variety Uma (recurrent parent) through Marker Assisted Selection (MAS) at the College of Horticulture, Vellanikkara, Thrissur resulting in production of backcross generations BC_2F_1s and BC_1F_2s. Identification of genotypes pyramided with all the three resistance genes among the BC_2F_1s need to done before resorting to further backcrossing to recurrent parent Uma. Further, the selfed progenies (BC_1F_2) need to be evaluated for bacterial blight to identify BB resistant genotypes.

In line with the above, the present study was formulated to identify R-gene pyramided plants in BC_2F_1 population and to develop stable pyramided BC_3F_1 lines of variety Uma with low linkage drag from the donor parent. The study also perceived evaluation of BC_1F_2 population for resistance to bacterial blight pathogen and production of advanced breeding generations (BC_2F_2s and BC_1F_3s).

Review of literature

II. REVIEW OF LITERATURE

Rice is the major source of carbohydrate for over three billion people across the globe. Recurrence of bacterial blight (BB), which is one of the most devastating disease identified in rice till date, seriously affects the production and productivity of this staple (Gnanamanickam *et al.,* 1999). Though prophylactic measures and chemical control of the disease are available, durable resistance of varieties is a much better option to combat this biotic stress.

In Kerala, severe yield reductions are common in the widely grown elite cultivars Uma (Mo16) and Jyothi (PTB39) owing to occurrence of bacterial blight. The heavy monsoon showers received by the state during the rice growing seasons impair chemical and mechanical control of the disease. Ensuring durable resistance thus becomes essential and economical to sustain the yield rather than relying on the use of chemical control measures, at the time of disease incidence. Presently, exploiting host plant resistance through marker assisted selection is being emphasised.

Hence, imparting host plant resistance to variety Uma by incorporating BB resistance genes through MABB has been attempted. The literature related to the study is detailed below in brief under the following headings.

2.1. Etiology of bacterial blight (BB) disease in rice

2.1.1. Ecology of BB pathogen

2.1.2. Disease cycle and epidemiology

2.1.3. Disease symptoms

2.1.4. Disease management

2.1.4.1. Host plant resistance

2.1.4.2. Exploiting host plant resistance for management of BB pathogen

2.2. Phenotypic screening for BB resistance

2.3. Marker Assisted Selection (MAS) to incorporate BB resistance in rice

2.1. Etiology of bacterial blight (BB) disease in rice

Bacterial blight, caused by bacterium *Xanthomonas oryzae* pv. *oryzae (Xoo)* is a devastating disease of widespread occurrence in rice growing tracts of the world. The pathogen belongs to the family Xanthomonadaceae. Majority of the members of the genus *Xanthomonas* are found to cause diseases in a wide range of crops. According to Sonti (1998), rice and most of its wild relatives are found to be infected with *Xanthomonas oryzae* pv. *oryzae.*

Xanthomonas oryzae pv.*oryzae* is a rod shaped, yellow slime producing non-sporulating bacteria. It is an obligate aerobic bacterium with size varying from 0.7 to 0.2 µm in length and 0.4 to 0.7µm in width. The cells are motile possessing a single polar flagellum. Each cell is covered by a capsule of galactose, glucose, xylose and uronic acid. This extra cellular polysaccharide that the bacterium produces results in the formation of droplets or strands of bacterial exudates from infected leaves which not only prevents desiccation but also aid in dispersal of pathogen through wind and rain water (Swings *et al.,* 1990). The *Xoo* cells cannot reduce nitrate as they are catalase positive but can produce acids from carbohydrates (Bradbury, 1984).

This pathogen was initially discovered as a bacterial mass from the dew drops on rice leaves by Takaishi in 1908. It was first isolated by Bokura in 1911, who eventually studied its morphology and physiology and named it as *Bacillus oryzae* Hori and Bokura. The pathogen was further redesignated as *Pseudomonas oryzae* Iyeda and Ishiyama. Swings *et al.* (1990) elevated the pathogen to the status of species and named it as *Xanthomonas oryzae* pv. *oryzae.* Upon infecting the rice plants *Xoo* pathogen produces certain race specific effectors which target host cell nucleus, which in turn binds to host cell susceptibility or resistance genes resulting in infection or resistance respectively (Horgan and Henderson, 2015).

Occurrence of the disease in south India was first reported during 1959 (Parthasarathy *et al.*, 2014). There after the disease attained the status of epidemics in

the year 1962 in Bihar and other regions of North India. Now the disease has become prevalent in all rice growing tracts of the country.

2.1.1. Ecology of BB pathogen

Xanthomonas oryzae pv. *oryzae* has a wide host range including other graminaceous species apart from rice. For example, *Leersia oryzoides* var. *japonica*, *L. oryzoides* var. *zizanialatifolia*, and *Pharlaris arundinacea* are severely infected by the pathogen. However, members like *L. japonica*, *Phragmites communis* and *lsachne globose* are less likely to be infected (Goto *et al.*, 1953).

Xie *et al.* (1999) screened two hundred and eighty pathogenic bacterial isolates associated with rice seeds collected from tropical and subtropical regions of the world. It was found that six per cent of pathogenic bacteria were of subtropical and nine of tropical origin.

Mizukami (1961) reported that the roots of the plants affected by BB were found to harbour the pathogen. Besides roots, the pathogen in its inactive form is also found in seeds, straw and stubbles. Under favourable conditions they get activated and infect the rice plants.

Apart from the presence of inoculum in seeds, tillers, roots or stubbles and in alternative weed hosts, the pathogen is also detected to be airborne and water borne (Tagami *et al.*, 1964).

2.1.2. Disease cycle and epidemiology

The pathogen enters through openings like hydathodes present along the edge of the leaf or through wounds (Ou, 1985). The pathogen multiplies within xylem and spread to other parts of the plant. The bacterial ooze forms droplets that get collected on the surface of the leaf. It gets dispersed through rain and irrigation water to uninfected parts of the plant as well as uninfected plants. Bacterial blight outbreak is most common during monsoon season (June to September) in India and other regions of South-east Asia (Liu *et al.*, 2004). The high relative humidity (90% for 12-14 hours/day) and rainfall that prevails during the season results in the rapid building up

of disease inoculum. For disease development a temperature of 23-31°C is favoured during wet season. Seedling blight (*kresek*) is observed when the temperature ranges between 28-35°C.

According to Tagami *et al.* (1964), a progression of the bacterial population is observed from maximum tillering to panicle initiation stage. This may also be influenced by climatic factors like temperature, humidity etc. The bacterial population is found to be maximum during May to July. The invasion of bacteria occurs from older to younger leaves. Various cultural operations contribute to the intensity of disease development like nursery preparation, fertilizer application and selection of rice varieties etc. (Mizukami and Wakimoto, 1969). Disease development in young transplanted seedlings are also favoured by clipping of leaves during transplanting. The deeply irrigated or flooded nurseries have a greater chance of being affected by the disease than semi-irrigated or upland nurseries, where the severity of damage is much lower.

Increased vegetative growth provides micro-climate which favour pathogen multiplication, which is further enhanced by nitrogen application leading to increased lesion length. Reddy *et al.* (1979) reported increased BB incidences owing to increased N application. A direct relationship between the N application and BB severity and reduction in yield was elucidated.

Disease development was also found to depend on the type of soil. Acidic soils with poor drainage facilities enhances disease development. BB incidences are also reported to be severe in soils like clay or clay loam alluvium, sandy loam soils, while, in sandy tracts of dunes it was found to be negligible (Tagami *et al.,* 1966).

Pathogen that harbour in dead plants and seeds gets transported through irrigation water and gain entry into the host plants through wounds or natural openings. Pathogens was also found to survive in the base of tillers, rice stubbles and roots (Mizukami and Wakimoto, 1969). During winter season of temperate regions, pathogens are found to survive in rhizospheres of weedy plants of *Leersia* and *Zizania species. Xoo* can survives for one to three months in soil depending on soil moisture

and acidity. However, this does not serve as a major inoculum. The pathogen is also found to overwinter in piled straw, which may act as an inoculum where the occurrence of weedy host is limited (Ou, 1985).

2.1.3. Disease symptoms

According to the stage of the crop infected the symptoms may be classified as kresek (seedling blight) and leaf blight (mature plants). *Kresek* symptom occurs in seedlings soon after they are transplanted to field from nurseries (Nino-Liu *et al.*, 2006). Initially, small water soaked spots appear along the sides of older leaves, which gradually enlarges and turns yellow. Symptoms start from leaf blades and proceeds downwards reaching the basal part, finally reaching the sheath. Severely affected leaf sheath becomes discoloured and get decayed. In case of early infection, symptoms appear at third and fourth week of transplanting and eventually spreads upwards from older to younger leaves. The lesions are usually found on the upper part of leaf edges, where water pores which facilitates the bacterial invasion are most often distributed. As a result, the veins develop yellow coloured lesions after two or three days of infection, which then turns whitish or greyish and later on the infected leaves roll and wither. The transplanted seedlings showing *kresek* symptoms exhibits stunted growth and dies within one to six weeks after transplanting (Mew, 1987). Generally, the whole leaf or upper half of the leaf dries up turning pale white before withering. Infection in flowered plants results in the development of water soaked lesions on the glumes of young spikelets and grains. During ripening the lesions turn yellowish to greyish white in the middle with an indistinct margin (Yoshimura, 1960).

Gnanamanickam *et al.* (1999) reported that even though the disease is found to occur in all stages of the crop, disease incidence in majority of cases occurs between maximum tillering to flowering stage. Infection during pre-flowering and panicle initiation stages may highly impair grain development and also increases the chances of sterility. In case of post flowering infection, the grain yield of the crop is less affected.

2.1.4. Disease management

Management practices like disease forecasting, biological or chemical control, host plant resistance etc. need to be adopted either alone or in combination for combating the yield loss due to BB infection. Use of antibiotic is an effective chemical control approach but its frequent usage has resulted in the evolution and existence of drug resistant strains which imposes serious problem in identifying an appropriate management strategy for controlling bacterial blight in rice (Gnanamanickam *et al.*, 1999). Moreover, the climatic conditions prevailing in Kerala during rice growing seasons (*Virippu* and *Mundakan*) results in washing-off of the chemicals applied for controlling BB. Hence, a durable mechanism providing defence against pathogen within the host system need to be relied upon.

Peng *et al.* (2015) reported that apart from the environment and food safety issues raised by application of plant protection chemicals, the protection conferred by chemical pesticides and biocontrol agents (antagonistic organisms and plant extracts) are not satisfactory and their effectiveness decreases over time. Hence, till date host plant resistance is the most effective strategy to compact BB pathogen known.

2.1.4.1. Host plant resistance

Resistance to *Xanthomonas* is reported to be conferred by a multigene R-gene family found distributed throughout the rice genome. Receptor like kinase (RLK) class and nucleotide-binding site leucine-rich repeat (NBS)-LRR are two important R-gene classes related to bacterial blight in rice. These genes are named with a '*Xa*' prefix followed by a specific number assigned upon discovery. Six R-genes have been cloned (*Xa1, xa5, xa13, Xa21, Xa3/ Xa26* and *Xa27*) and six have been physically mapped (*Xa2, Xa4, Xa7, Xa30, Xa33* and *Xa38*) (Song *et al.*, 1997; Yang *et al.*, 1998; Sun *et al.*, 2003; Gu *et al.*, 2005; Liu *et al.*, 2006; Cheema *et al.*, 2008; Bhasin *et al.*, 2012; Natrajkumar *et al.*, 2012). About 42 resistant genes (Table 1) have been identified till date. Specific resistance against multiple races of the newly emerged *Xoo* pathogen has developed due to evolution of genes in these multigene families. These genes can be dominant or recessive in nature while some others are dependent on dose.

According to the development stage of the crop, the degree of resistance reaction varies, indicating that the expression of these genes are developmentally controlled. There exists a dynamic interaction between the R-genes and host genome and the genetic function of these genes are highly diverse, indicative of the evolution of R-genes in rice for combating new races of the pathogen (Horgan and Henderson, 2015).

The resistance in host against BB pathogen upon infection is activated by means of producing certain race specific effectors (transcription activator like effectors) which targets host cell nucleus and binds to genes that activate resistance to the pathogen. These factors which are used for binding are called a virulence factors (Horgan and Henderson, 2015) which results in the activation of a series of events finally leading to host resistance. This activated signalling pathways also results in hypersensitivity reactions that check pathogen spread to the rest of the plant by causing localized cell death or other changes in plants, leading to a lower degree of disease infection.

Table 1. R-genes conferring resistance against BB pathogen in rice

Gene identified	Resistance source	Origin	Reference
Xa1	Temperate *Japonica*	Japan	Sakaguchi (1967); Yoshimura *et al.* (1998)
Xa2	*Indica*	Vietnam	Kurata and Yamazaki (2006)
Xa3/Xa26	*Japonica*	Japan	Sun *et al.* (2006); Xiang *et al.* (2006)
Xa4	*Indica*	India	Wang *et al.* (2001)
xa5	Aus	Bangladesh	Petpisit *et al.* (1977)
Xa6/Xa3	–	USA	Sidhu *et al.* (1978)
Xa7	Aus	Bangladesh	Sidhu *et al.* (1978); Lee and Khush (2000)
xa8		USA	Sidhu *et al.* (1978); Singh *et al.* (2002)

(Contd...)

...Contd.

Table 1. R-genes conferring resistance against BB pathogen in rice

Gene identified	Resistance source	Origin	Reference
xa9	–	Laos	Singh *et al.* (1983); Ogawa *et al.* (1988)
Xa10	–	Senegal	Yoshimura *et al.* (1983); Kurata and Yamazaki (2006)
Xa11	*Indica*	Philippines	Kurata and Yamazaki (2006)
Xa12	*Japonica*	Japan	Ogawa (1987)
xa13	–	India	Ogawa *et al.* (1988); Kurata and Yamazaki (2006)
Xa14	*Japonica*	Taiwan	Sidhu *et al.* (1978); Kurata and Yamazaki (2006)
xa15	–	–	Nakai *et al.* (1988); Ogawa (1996)
Xa16	*Indica*	Vietnam	Kurata and Yamazaki (2006)
Xa17	*Japonica*	South Korea	Kurata and Yamazaki (2006)
Xa18	*Indica, Japonica*	Philippines, Japan	Liu *et al.* (2004); Kurata and Yamazaki (2006)
xa19	–	Japan	Ogawa *et al.* (1978)
xa20	–	–	Taura *et al.* (1992); Kurata and Yamazaki (2006)
Xa21	Wild spp. of *Oryza*	Mali	Song *et al.* (1995)

(Contd...)

...Contd.

Table 1. R-genes conferring resistance against BB pathogen in rice

Gene identified	Resistance source	Origin	Reference
Xa22 (t)	–	China	Sun *et al.* (2004); Kurata andYamazaki (2006)
Xa23	Wild spp. of *Oryza*	China/Cambodia	Zhang *et al.* (1998); (2001)
xa24	–	Bangladesh	Khush and Angeles (1999)
xa25(t)	*Indica*	China	Liu *et al.* (2011)
xa26(t)	*Indica*	China	Lee *et al.* (2003)
Xa27(t)	Wild spp. of *Oryza*	Philippines	Lee *et al.* (2003); Gu *et al.* (2004)
xa28(t)	*Indica*	Bangladesh	Lee *et al.* (2003)
Xa29(t)	Wild spp. of *Oryza*	–	Tan *et al.* (2004)
Xa30(t)	Wild spp. of *Oryza*	India	Cheema *et al.* (2008)
Xa31(t)	*Japonica*	China	Wang *et al.* (2009)
Xa32(t)	Wild spp. of *Oryza*	–	Ruan *et al.* (2008); Zheng *et al.* (2009)
Xa33	Wild spp. of *Oryza*	–	Natrajkumar *et al.* (2012)
xa33(t)	–	Thailand	Korinsak *et al.* (2009)
xa34 (t)	*Indica*	Sri Lanka	Chen *et al.* (2011)
Xa35 (t)	Wild spp. of *Oryza*	Philippines	Guo *et al.* (2010)
Xa36(t)	–	China	Miao *et al.* (2010)

(Contd...)

...Contd.

Table 1. R-genes conferring resistance against BB pathogen in rice

Gene identified	Resistance source	Origin	Reference
Xa38(t)	*Oryza nivara*	–	Bhasin *et al.* (2012)
Xa39	*Oryza rufipogan*	–	Zhang *et al.* (2014)
Xa40(t)	–	Korea	Kim *et al.* (2015)
Xa41(t)	–	–	Hutin *et al.* (2015)
Xa42(t)	–	Japanese	Busungu *et al.*(2016)

Majority of the genes used for conferring resistance to BB pathogen are dominant in nature. Some widely studied dominant genes are:

***Xa1* gene**

Sakaguchi (1967) first reported the presence of *Xa1* gene conferring resistance to Japanese race I of *Xoo*. The gene locus was tagged by using RFLP marker XNpb235 and was mapped on to chromosome 4 of rice (Yoshimura *et al.*, 1996). As part of rice genome project positional cloning of gene *Xa1* was carried out in Japan using 340-kb YAC clone (Y5212). Studies on *Xa1* gene by Yoshimura *et al.* (1998) reported that this gene encodes a nucleotide-binding site leucine-rich repeat (NBS-LRR) type of protein.

***Xa21* gene**

Xa21 was identified in *O. longistaminata.* It is a broad spectrum bacterial blight resistant gene. R-gene *Xa21* belonging to class RLK which induce broad spectrum resistance against BB, was the first gene to be transferred. It was transferred into the background of *O.sativa* (Khush *et al.*, 1989). RAPD 248 is a RAPD marker which was used to tag the gene (Ronald *et al.*, 1992). Another marker RG103 is also tightly linked at a distance of 1.2cM from *Xa21* gene. Based on these markers, a new STS marker pTA 248 at a distance 0.2cM from *Xa21*was designed. This can be efficiently used for marker assisted selection. The sequence of *Xa21* gene revealed that it codes for a receptor kinase domain with serine– threonine specificity. This gene is also present in

plasmid pC822. Ronald (1997) used map based cloning strategy for the first time to clone the disease resistance gene *Xa21* in rice.

Ponciano *et al.* (2006) reported that the resistance against bacterial blight offered by dominant gene *Xa21* to *Xoo* pathogen is developmentally controlled in rice. Expression of pathogenesis related defense genes (*OsPR1a, OsPR1b*, and *OsPR1c*) were analysed for their BB resistance at juvenile and adult stages. It was observed that the leaves in adult stages exhibit complete expression of genes and *Xa21* locus favours higher degree of disease resistance and the juvenile stage lacks complete activation of these genes resulting in moderate reaction. Peng *et al.* (2015) revealed that BB resistance conferred by *Xa21* is responsible for the activation of various signalling pathways associated with resistance.

***Xa7* gene**

Xa7 is a dominant gene which provides durable resistance against bacterial blight pathogen with avirulence (*Avr*) gene *AvrXa7*. It is located on chromosome 6, between two markers M1 (2.2 cM) and M3 (0.5 cM) (Porter *et al.*, 2003). However, Chen *et al.* (2008) integrated *Xa7* further at an interval of approximate 118.5 kb between two proximal markers GDSSR02 and RM20593. Recently, Mallikarjun and Kotasthane (2018) mapped *Xa7* gene to a 0.21cM interval between the markers GDSSR02 and RM20593 based on which a contig map corresponding to the *Xa7* gene was constructed. Similar to other cloned *Xa* genes, candidate gene analysis of *Xa7* revealed that the genes encode novel domains that have no amino acid sequence.

A small number of genes conferring resistance to BB pathogen are recessive in nature. A few of the frequently integrated recessive genes are detailed below.

***xa5* gene**

RFLP markers RG556 and RZ390 and rice microsatellites markers RM122 and RM390 was used to tag R-gene *xa5*. The gene was mapped onto chromosome 5 of rice genome (Blair and McCouch, 1997). RFLP marker RG556 was used to design an STS marker for incorporation of *xa5* gene in rice based on MAB approaches (Huang *et al.*, 1997). The PCR amplification product of STS marker does not exhibit polymorphism

in banding pattern among the resistant and susceptible plants. Therefore, it was suggested that the PCR product need to be digested with Dra I (restriction enzyme) to generate Specific Amplicon Polymorphism (SAP). Functional markers were also developed for the gene (Iyer and McCouch, 2006). R-gene *xa5* was found to be encoded with gamma subunit of transcription factor IIA (TFIIAγ) of the eukaryotes, giving it a novel form of disease resistance (Iyer and McCouch, 2004). Two nucleotide substitutions responsible for change in an amino acid were revealed on sequencing the factor in resistant and susceptible isolines.

***xa13* gene**

It is an important resistant gene which is used to confer resistance to Philippines race 6 of bacterial blight pathogen. Zhang *et al.* (1996) tagged the gene with RAPD marker OPAC05 900 and RFLP marker RG136. It was mapped on to chromosome 8 of rice genome. A PCR-based STS marker linked to *xa13* gene was developed from RFLP marker RG136. PCR product of RG136 also produced monomorphic banding pattern and hence, it was subjected to restriction digestion with HinfI to generate specific amplicon polymorphism.

2.1.4.2. Exploiting host plant resistance for management of BB pathogen

For a long time, conventional breeding was used as the only method for breeding rice cultivars resistant to BB. Several rice varieties with *Xa 4* gene resistant to BB were developed through conventional backcross breeding approach (Khush *et al.*, 1989). Initially, variety TKM6 was used as the BB resistant gene donor line in India. Several varieties like IR36, Karjat, Radha, Ramakrishna *etc.* were developed using TKM6. In a few cases genotype Sigadis was also used to develop resistant varieties like Ratnagiri and 68-1. Similarly, varieties like PR 4141 and IET 8585 (Ajaya) was developed from BJI through conventional breeding approach.

Thus R-gene *Xa 4* was introgressed to several high yielding varieties in India through conventional breeding approach but predominance of *Xoo* races that could overcome resistance conferred by *Xa 4* gene was reported (Khush *et al.*, 1989). In

another study, Joseph *et al.* (2004) screened 13 near-isogenic lines of rice against four isolates of bacterial blight pathogen from Basmati growing regions. R-genes *Xa4, xa8, xa13* and *Xa21* was reported to be effective against all the isolates tested. In comparison with the individual genes, enhanced resistance was imparted by two or more genes in combination as expressed by reduced average lesion length. These results pointed out that introgression of a single resistant gene was not satisfactory and might cause a shift in pathogen race frequency leading to subsequent breakdown of resistance (Khush *et al.*, 1989; Mew *et al.*, 1992; Joseph *et al.*, 2004; Joshi and Nayak, 2010).

When grown in Kerala the rice line IRBB21 was found to be susceptible to *Xoo* pathogen races isolated from the region (Gnanamanickam *et al.*, 1999). It was reported that the rice line NH56 carrying four R-genes, (*Xa4* + *xa5* + *xa13* + *Xa21*) was resistant to the *Xoo* pathogen isolates from Kerala (Priyadarisini and Gnanamanickam, 1999).

The presence of genes *xa5, xa13* and *Xa21* was found to give broad spectrum resistance to all predominant races of bacterial blight pathogen found in Philippines and Punjab, when introgressed into a susceptible *Indica* rice cultivar PR106 (Singh *et al.*, 2001). The advanced breeding lines were found to give higher yield advantage. Effectiveness of resistant genes towards bacterial blight is given by the order *Xa21*> *xa5*>*xa13*.

Hence, an urgent need to emphasise on the durability of resistance in cultivars was felt. Gene pyramiding refers to the simultaneous introgression of several 'undefeated' R genes into a single cultivar to ensure durable resistance that could out-weigh the simultaneous pathogen mutation to break the resistance (McDowell and Woffenden, 2003; Pink, 2002). This approach works towards accumulating the desirable R-genes identified in multiple parents into a single genotype (Joshi and Nayak, 2010).

Gene introgression through conventional breeding is a time consuming approach. It may take 8-12 years for the development of a resistant pyramided cultivar.

However, the resistance developed for a variety cannot be guaranteed owing to the evolution of new pathogen races (Ragimekula *et al.*, 2013).

In order to improve the efficiency of the conventional breeding, approaches like marker assisted selection (MAS) has been resorted to world over in the recent past. DNA markers are identifiable genetic sequence having specific locations in the genome of the organism and exhibit Mendelian inheritance. These markers, mostly codominant in nature are less influenced by epistatic and environmental factors. Compared to other marker systems, molecular markers exhibit a high degree of polymorphism. Marker and target gene linkage and co-segregation are important factors that determine the effectiveness of MAS (Dekkers, 2004).

Among the DNA based markers, microsatellites are widely used for efficient and effective selection of resistance genes. Blair and McCouch (1997) reported the existence of microsatellite markers linked to R-gene *xa5*. Salgotra *et al.* (2012) opined that the reliability of MAS in BB resistance breeding approach can be enhanced by the use of functional markers. These help in the direct selection of R-genes conferring BB resistance. By cloning some of the identified BB genes *Xa1, xa5, xa13, Xa21, Xa26* and *Xa27,* several functional markers have been designed and efficiently used for identifying BB resistance (Song *et al.*, 1995; Yoshimura *et al.*, 1998; Iyer and McCouch, 2004; Chu *et al.*, 2007).

2.2. Phenotypic screening for evaluation of BB resistance

Phenotyping provides information about the diversity in germplasm and identifies the resistant and susceptible lines which can be used for further breeding purposes (Cheema *et al.*, 1998). Artificial inoculation for screening of resistance in plants is conclusive because of the presence of adequate inoculation resulting in disease initiation (Mew, 1984).

Divya *et al.* (2015) used a virulent isolate of the BB pathogen, DX-020 to screen rice genotypes against BB pathogen following the methodology advocated by Kauffman *et al.* (1973). The leaves (3 to 6 leaves) of each plant to be evaluated were

clip-inoculated with a bacterial suspension (109 cfu/ml) at maximum tillering stage (45-55 days after establishment) and the disease reaction recorded after 14 days. Lesion length was measured and the disease severity was scored as per IRRI standard evaluation system (IRRI, 2002). Out of 35 intercross F_2's screened, only 13 plants were found to possess BB resistance.

Fred *et al.* (2016) screened 32 Korean rice cultivars, to identify their reaction to BB race K1. Pathogenicity was tested at maximum tillering stage at both green house and open field conditions and the lesion length was measured after 14 days of inoculation. It was found that the screening for BB resistance was more accurate in open field conditions than in green houses. Five cultivars Hanareum, Namcheon, Samgdeok, Samgang, and Yangjo were identified to be resistant in both open field and greenhouse conditions.

Mubassir *et al.* (2016) inoculated active strain of *Xanthomonas oryzae* pv. *oryaze* (*Xoo*) BXO-09 in ten International Rice Research Institute (IRRI) advanced lines and seventeen varieties of rice, collected from BINA, BRRI, and Gazipur district of Bangladesh. Morphological rating done following artificial inoculation by clipping method indicated that rice line RC251 was susceptible while the remaining lines exhibited moderately resistance or moderate susceptibility to the BB pathogen. Among the rice varieties screened, BR-11, Binadhan-8, and Binadhan-10 were found highly susceptible whereas, varieties BR-26, BRRI Dhan31, IRBB5, IRBB21, IRBB60, IRBB65, and Kumragur were identified to be resistant. The resistant reactions of the plants were further confirmed using molecular markers. Results indicated that morpho-molecular characterization could be used for further planning and development of bacterial blight resistant cultivars.

2.3. Marker Assisted Selection (MAS) to incorporate BB resistance in rice

Unlike conventional breeding programmes, marker assisted selection enhances the precision of selection and reduces the reliance on laborious and time consuming phenotypic screening procedures. Due to masking effect of genes, identification of

plants carrying genes would be cumbersome in absence of these markers (Khan *et al.*, 2014). In addition, Marker Assisted Backcross Breeding (MABB) can be used for the early fixing of the desirable genes in homozygous condition. It also allows for selection of desirable genotypes in seedling stage. This method also helps us to identify recessive alleles, which is difficult to be identified during conventional methods.

In a backcrossed individual there are chances of introgression of some undesirable segments from donor parent. Consequently, the progeny developed would fail to perform like that of recipient parent. Such linkage drags can be greatly reduced by integrating the use of molecular markers in breeding programmes (Frisch *et al.*, 1999; Joshi and Nayak, 2010). In addition, plants with multiple resistant genes can also be selected by Marker Assisted Selection (MAS). Action of gene conferring resistance to many races of pathogen may mask the action of another resistance gene, indicating that the selection solely based on the phenotype can be misleading due to epistatic gene actions (Tanksley *et al.*, 1989; Davierwala *et al.*, 2001; Rao *et al.*, 2002; Akhtar *et al*, 2010).

Three levels of selection are involved in marker assisted backcross breeding programmes. The first and foremost is the foreground selection which involves selection for target trait. The next being background selection, *i.e.,* selection of the plants that possess maximum recurrent parent genome recovery and the last one is the recombinant selection done to minimize the linkage drag. Tightly linked flanking markers are used for selecting the backcross progeny possessing target gene (Akhtar *et al.*, 2010). Background selection allows maximum recurrent parent genome recovery while recombinant selection reduces the size of donor chromosome segment containing the target locus.

BB resistant gene *Xa21* was the first one to be introgressed in rice variety IRBB21 using pTA 248 marker from the germplasm of *O. longistaminata* (Ronald *et al.*, 1992). Abenes *et al.* (1993) employed MAS for gene pyramiding to incorporate BB resistance, while, Yoshimura *et al.* (1995) transferred gene *xa5* from variety DZ192 into IRBB5 using marker RG556. Zhang *et al.*, (1996) incorporated resistant gene *xa13* into IRBB13 from Nang Som (long grain variety) using RG 136 marker.

Sanchez *et al.* (2000) demonstrated the usefulness of MAS in gene pyramiding. Marker aided backcrossing produce were used to transfer three resistant genes *xa5*, *xa13*, and *Xa21* to three new plant type (NPT) rice lines (IR65598-112, IR65600-42 and IR65600-96) using markers RG207 and RG556 for *xa5* gene, RM136 for *xa13* and pTA248 for *Xa21*. Polymorphism was observed for markers RG556 and RG207 when digested with restriction enzymes MaeII. The PCR product of marker RM136, was digested using HinfI to obtain polymorphic banding pattern. MAS helped in the identification of recessive genes like *xa5* and *xa13* which get masked due to dominance action of gene *Xa21* during conventional breeding approaches.

IR50, an elite *indica* rice was improved by Narayanan *et al.* (2002) through MAS approach. They succeeded in the genetic transformation for resistance against blight and blast diseases. C101A51 was used as the donor parent for blast resistance. Initially, blast resistance was confirmed, followed by resistance against blight by introgression of *Xa21* gene. Bioassay data revealed that the improved IR50 is resistant to both blight and blast pathogens. *Xa4* gene was also found to be endogenously present along with transformed *Xa21* gene in improved IR 50.

Nguyen and Nguyen (2004) introgressed three resistant genes *xa5, xa13* and *Xa21* into IR 24 (recurrent parent) from Nang Som (donor parent). The presence of gene was identified by STS markers RG556, RG136 and pTA248 for *xa5, xa13* and *Xa21* respectively. Out of 160 plants screened for resistance, 11 plants were found to have *Xa21* gene. Only *Xa21* gene was transferred from the donor parent. Attempts to introgress the above three genes into variety Samba Mashuri (BPT5204), a medium slender grained variety, with good cooking quality and yield was successful and resulted in production of essentially derived variety (EPV) Improved Samba Mahsuri (ISM) (Sundaram *et al.,* 2008). PCR based markers were used to introgress all the genes into Samba Mashuri from donor line (SS1113) which had all the three genes in homozygous condition. Microsatellite markers were used for background selection, for identification of maximum recurrent parent genome recovery. Two and three gene pyramided lines were reported and found to have high degree of disease resistance and yield advantage.

High yielding short duration variety ADT43 and ADT47 were also introgressed with the *xa5, xa13* and *Xa21* genes from rice variety IRBB60 (Bharani *et al.*, 2010). IRBB60 were found to provide resistance to *Xoo* isolates *Xoo12* and *Xoo17* prevalent in Tamil Nadu. A single plant that was identified in F_3 generation was found to have all the three R-genes in homozygous condition. Among different R-genes identified, *Xa21* was mostly preferred for development of resistant genotypes against widely prevalent bacterial blight isolates in the state.

Shanti *et al.* (2010) attempted to introgress four resistance genes (*Xa4, xa5, xa13* and *Xa21*) into hybrid rice parental lines KMR3, IR58025B, PRR78, Mahsuri and Pusa 6B. IRBB60 was used as the donor parent for all the four genes. DNA based markers were used for foreground selection. However, background selection was conducted by means of conventional breeding approaches. It was observed that the four R-gene introgressed lines showed high degree of resistance to ten virulent *Xoo* races isolated. The grain quality of the introgressed lines resembled the recurrent parent. This combination of four genes was found to combat the evolution of new pathogen races. These lines could either be used directly as a variety or as donors in resistance breeding against BB.

Salgotra *et al.* (2012) aimed to introgress BB resistant genes *xa5*, *xa13* and *Xa21* into basmati breeding line IRS 5441-2 from a non-basmati donor IRBB59 using both MAS and phenotypic selection. Recombinant lines derived from BC_1F_3 population were found to be effective against the most virulent bacterial blight isolates. The higher level of resistance was observed on pyramiding more than two resistant genes. This was attributed to be the result of quantitative complementation or gene interaction (Yoshimura *et al.*, 1995; Huang *et al.*, 1997; Sanchez *et al.*, 2000, Sundaram *et al.*, 2008). In this study, superior recombinants were identified with all the three genes along with basmati traits in homozygous condition. Hence, it became evident that durable resistance to bacterial blight in basmati genotypes could be developed by means of advanced basmati breeding lines through MAS and phenotypic selection. Further on, various basmati rice genotypes from Jammu and northern Himalayan

regions were also introgressed with BB genes through marker assisted approach (Bhat *et al.*, 2015).

Popular fragrant glutinous rice variety RD 6 of north and north-east Thailand was introgressed with bacterial blight resistant genes from IR62266 (donor parent). Twelve lines were identified to be successfully enhanced with bacterial blight resistance, which can be used for benefiting the farming community (Pinta *et al.*, 2013).

Suh *et al.* (2013) used the strategy of introgression of multiple R-genes into rice though MAS. IRBB57 was used as the donor parent for introgression of *Xa4, xa5* and *Xa21* into Mangeumbyeo, a susceptible elite *japonica* rice cultivar. Three advanced backcross breeding lines were identified to possess the three resistant genes with only negligible amount of linkage drag. The recovery of the recurrent parent genome was 92.1 per cent. The pyramided lines did not show any negative effect on yield. Moreover, they were extremely resistant to *Xoo* races compared to the lines with individual genes.

Two bacterial blight resistant genes (*xa13* and *Xa21*) were introgressed into MTU1010 (Cottondora Sannalu) from B95-1 (Magar *et al.*, 2014). MTU1010 (Cottondora Sannalu) is a fine grained, short duration, high yielding variety from Andhra Pradesh Rice Research Institute (APRRI), released in 1999. Foreground selection for the genes were done using markers *xa13* promotor and pTA *248*. The F_1 plants, that were confirmed to be true hybrids for both the genes were selfed to give F_2's. Genetic analysis of F_2 population confirmed mendelian pattern of inheritance for genes (*xa13* and *Xa21*) governing BB resistance.

Khoshkdaman *et al.* (2014) developed improved pyramided lines with gene *Xa7, Xa14* and *Xa21* that were moderately resistant to BB as compared with the susceptible local varieties in the Iranian provinces. Pyramided lines with three resistant genes were reported to provide higher level of disease resistance as compared with two or three gene pyramided lines.

Swarna was used as the background for the introgression of three gene *xa5, xa13* and *Xa21* from IR64. These genes were identified to give broad spectrum resistance against bacterial blight (Pradhan *et al.*, 2015a). The pyramided line (CRMAS2232-85) was found to be superior in agronomic performances along with resistance as compared to parents. It was concluded that the pyramided line with bacterial blight resistant genes can provide better resistance and also have agro-morphological and quality traits, similar to the recipient parent.

Sabar *et al.* (2016) evaluated 80 rice genotypes and identified 41 entries with *Xa4* gene, 14 lines with *xa5* gene and only one line with *Xa21* gene along with *Xa4*. Three isogenic lines, IRBB4 (carrying *Xa4*), IRBB5 (carrying *xa5*) and IRBB21 (carrying *Xa21*) was used as positive checks and IR24 (carrying none) was used as a negative check. This study was helpful in identifying donors to pyramid disease resistant genes in popular rice varieties of Pakistan.

Jalmagna is a popular deep water rice variety with prominent good yield under water logged conditions, but seriously affected by bacterial blight. Molecular marker assisted backcross breeding programme was employed to transfer *Xa21, xa13* and *xa5* genes into Jalmagna from a BB pyramided line Swarna (Pradhan *et al.*, 2015b). Homozygous BC_3F_2 plants was generated on selfing a selected BC_3F_1 plants. It was found that the BB gene pyramided lines exhibited higher yield advantage and had all agro-morphological traits of Jalmagna along with resistance genes against BB.

Das *et al.* (2015) tried stacking BB resistance genes along with other biotic and abiotic stress resistance genes in to rice variety CRMAS2621-7-1 (Improved Lalat) through marker assisted selection technique. They succeeded in introgressing genes conferring resistance/tolerance to blast (*Pi2, Pi9*), gall Midge (*Gm1, Gm4*), submergence (*Sub1*), and salinity (*Saltol*) along with bacterial blight genes *xa5, xa13* and *Xa21* into Improved Lalat. They identified two lines having all the 10 resistance genes against five target stress demonstrating the successful staking of genes into a single line with high recurrent parent genome recovery.

Arunakumari *et al.* (2016), introgressed the major BB resistance genes *Xa21* and *xa13* into Indian rice variety MTU1010 through MABB approach using ISM as the donor parent. They also tried to introgress blast resistance gene (*Pi54*) into the same variety using NLR145 as the donor parent. They evaluated BC_2 generations using functional markers for foreground selection and a set of parental polymorphic microsatellite markers for background selection. Selected BC_2F_1s from both the crosses having highest recurrent parent recovery were intercrossed to obtain intercross (IC) F_1s, which was then selfed to give ICF_2. They identified seven triple homozygous plants (*xa13+Xa21+Pi54*) with high recurrent parent recovery. These were selfed to give ICF_3. Several ICF_3 lines having high resistance against BB and blast, coupled with yield and grain quality and plant type which is almost on par with MTU1010 was recovered and advanced for further evaluation and selection.

Mianhui 725 (MH725) is an elite cultivar widely used as a restorer line in three-line hybrid rice production in China. Luo *et al.* (2016), introduced bacterial blight genes *Xa4* and *Xa21* into MH725 and obtained an introgressed rice line Wanhui 421 (WH421) with 96.9 per cent genetic background of line MH725. Later, efforts were taken to improve WH421 by introduction of resistance genes *Xa 27* and *Pi9* along with genes for submergence tolerance (*Sub1A*) and aromatic fragrance (*badh2.1*) through MABB resulting in the development of a new line designated as Wanhui 6725 (WH6725). The important agronomic traits of this newly developed lines was found to be almost similar to MH725. Hence, it was further used for the production of three line rice hybrid in China.

Research on host plant resistant gene introgression in cytoplasmic male sterile lines having Kalinga 1 (other than WA cytoplasm) as male sterile cytoplasm (Dash *et al.*, 2016) resulted in the development of improved bacterial blight resistant line CRMS 32A and its maintainer line CRMS 32B. These were used for the production of Rajalaxmi, an elite popular hybrid in India. Resistance genes (*Xa4, xa5, xa13*, and *Xa21*) were transferred to the A and B lines from a BB pyramid of IR64 through MABB. Foreground selection was applied to transfer BB resistant genes into CRMS 32B using markers associated with the genes, and plants having resistance alleles of

the donor were selected. Through background selection using genome-wide based SSR markers, lines having more than 95 per cent of the recurrent parent genome was identified. From the newly introgressed CRMS 32B, all the four resistance genes were then transferred to CRMS 32A through repeated backcrosses. These improved CMS line and maintainer line can be directly used for hybrid rice breeding in India.

Multiplex PCR method was used for simultaneous identification of genotypes with five BB resistance genes *Xa4, xa5, Xa7, xa13* and *Xa21* in backcross population with donor parent IR BB66 and recipient parent 'Tainung 82'. Two plants homozygous for all the four genes were identified from among 1100 BC_2F_2 plants. The study pointed out that multiplex PCR can assist in pyramiding genes against BB in elite rice cultivars (Yap *et al.,* 2016).

Verma (2016), resorted to MAS approach to introgress three resistant genes *Xa4, xa5* and *Xa21* from the *Indica* BB resistant donor IRBB57 into a susceptible cultivar Karma Mashuri, Among the progenies of crosses between Karma Mashuri and IRBB57, 17 lines carrying two R-genes (*Xa21+ xa5*), 13 lines with *Xa21+ xa5* and four lines carrying all the three gene combinations were identified on using known R-gene linked markers (*Xa4:* RM224; *xa5:* RM13pro, xa5R, xa5S and *Xa21*: *Xa21* and RM21).

Marker-assisted backcross breeding in RPHR-1005, a stable restorer line of hybrid DRRH-3 was initiated for the introgression of two major BB resistance genes, *Xa21* and *Xa33* and a major blast resistance gene, *Pi2* (Kumar *et al.,* 2016). Functional markers were used for foreground selection and 59 simple sequence repeat (SSR) markers showing polymorphism between the donors and recipient parents were used for background selection. Promising homozygous lines in BC_2F_2 possessing *Xa33* and another possessing *Xa21* and *Pi2* were identified. These lines were intercrossed to stack the target resistance genes into the genetic background of RPHR-1005. Ten promising lines with better panicle exertion and complete fertility restoration that possessed all the resistance genes in homozygous condition was identified at ICF_4.

Initiative was taken by Ramalingam *et al.* (2017) to pyramid *xa5, xa13*, and *Xa21* in maintainer lines CO 2B, CO 23B, and CO 24B of three WA male sterile lines CO 2A, CO 23A, and CO 24A through MABB strategy. IRBB60 was used as the donor parent for the transfer of three BB resistance genes into the maintainer lines. Further, backcrossing of the male sterile lines with the maintainer lines resulted in production of *xa5, xa13* and *Xa21* introgressed male sterile lines that were used for hybrid seed production. Hybrids obtained by crossing BB introgressed CMS lines *viz.*, CO 23A × AD08009R and CO 24A × IET20898R were found to give higher yield and were stable at different locations.

Efforts were taken to introgress bacterial blight resistant gene *Xa21* into BRRIdhan 52, a submergence tolerance rice variety of Bangladesh with high yield even during incidence of flash flood conditions (Kabir *et al.*, 2017). IRBB60 (near isogeneic line of IR24) with R-genes *Xa4, xa5, xa13* and *Xa21* was used as a donor. STS and SSR markers which are closely linked to *Xa21* and *SUB1* QTL were used to select desirable plants. Microsatellite markers were used to identify the maximum recurrent parent contribution. They identified three plants in BC_1F_1 population which were double heterozygous for the genes.

Efforts to incorporate bacterial blight resistance genes (*xa5, xa13* and *Xa21*) through MABB into popular high yielding red rice variety Jyothi were taken in Kerala Agricultural University. Improved Samba Mashuri (ISM) served as the donor parent Sequence Tagged Sites (STS) markers (RG556, RG136 and pTA 248) and functional markers (xa5SR and xa13 promoter) were used for foreground selection of R-genes *xa5, xa13* and *Xa21* in BC_2F_4s. Rice microsatellite (RM) markers were used for evaluation of the recurrent parent genome recovery in the desirable plants selected in foreground selection. Kabade *et al.* (2017) found that all the BC_2F_4s plants evaluated were introgressed with *xa5* gene and only two plants were found to have both *xa5* and *Xa21* genes. The advanced breeding lines of these R-gene introgressed plants were forwarded for further evaluation.

Das *et al.* (2018) attempted to pyramid R-genes *Xa4, xa5, xa13* and *Xa21* along with abiotic stress tolerant genes into improved Tapaswini through MABB strategy.

Bioassays showed that the pyramids exhibited higher levels of resistance against target stresses.

Attempts to introgress *Xa* genes into popular high-yielding, salt-tolerant Basmati variety CSR-30 widely cultivated in Haryana was taken by using IRBB- 60 as the donor variety (Baliyan *et al.*, 2018). The intercrossed breeding lines obtained, (IC-R28, IC-R68, IC-R32, and IC-R42) were found to be promising and are advanced to BC_3F_2 generation for further evaluation. In addition, MABB was also used for the simultaneous transfer of both bight and blast resistant genes *xa13, Xa21, Pi2* and *Pi54* into an early maturing Basmati rice variety Pusa Basmati 1509 (recurrent parent) from donor Pusa 1790 (Sagar *et al.*, 2018).

Materials & Methods

III. MATERIALS AND METHODS

Incidence of bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* is a common occurrence in the elite cultivar Uma (Mo 16) widely grown in Kerala. Decline in production and productivity of rice crop is thus an annual phenomenon in most rice growing belts of the state. Hence, in order to confer resistance to rice variety Uma against bacterial blight, efforts to pyramid three R-genes (*xa5, xa13* and *Xa21*) into the variety from donor Improved Samba Mashuri (ISM) through Marker Assisted Backcross Breeding (MABB) were taken at the College of Horticulture, Vellanikara, Thrissur. This resulted in the production of backcross generations BC_2F_1s and BC_1F_2s.

The backcross progenies (BC_2F_1s and BC_1F_2s) thus generated formed the basis of the present investigation 'Marker assisted backcross breeding for pyramiding genes conferring resistance to bacterial blight in rice variety Uma (Mo16).' The study was conducted in the Department of Plant Breeding and Genetics, College of Horticulture, Kerala Agricultural University Vellanikkara, Thrissur, between 2016 and 2018. It comprised of four experiments *viz.,* I) Genotyping of BC_2F_1 population, II) Morphological characterisation of pyramided lines of BC_2F_1 population, III) Production of BC_3F_1s and BC_2F_2s and IV) Morphological characterisation and pathotyping of BC_1F_2 population, and production of BC_1F_3 population. The materials and methods employed in this investigation are detailed below.

3.1. Experimental location

The experimental site was located at the College of Horticulture (COH), Kerala Agricultural University, Vellanikkara P.O., Thrissur 680656, located 40m above MSL between 10°31'N latitude and 76°13'E longitude and experiences humid tropic climate.

The laboratory and field facilities at the Department of Seed Science and Technology, College of Horticulture, Vellanikara, Thrissur 680656 were utilised for the study.

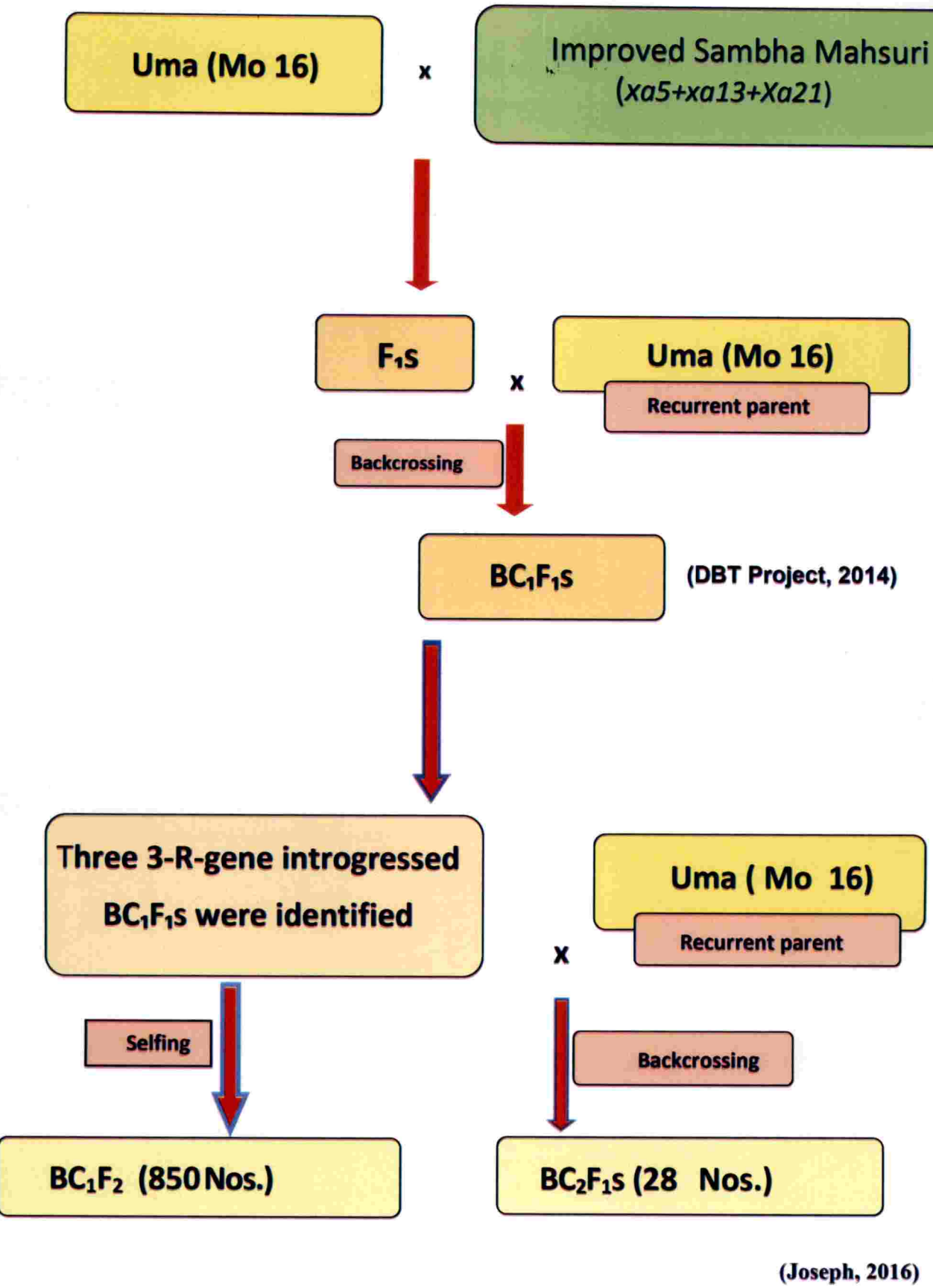


Figure 1. Pedigree of the experimental material

3.2. Experimental material

Improved Samba Mashuri (ISM), also developed through MABB served as the donor of the three bacterial blight resistance genes *xa5*, *xa13* and *Xa21* to be introgressed into the high yielding variety Uma (Mo16) that is used as the recipient parent. ISM is an EDV developed from variety Samba Mashuri, at the Indian Institute of Rice Research (Former Directorate of Rice Research), Hyderabad.

Pyramiding three bacterial blight resistant genes *viz., xa5 + xa13 + Xa21* from Improved Samba Mahsuri into elite cultivar Uma was attempted under the DBT project: 'Rice-Gene pyramiding to develop cultivars with durable resistance to Bacterial Leaf Blight through Marker Assisted Selection,' implemented at the College of Horticulture, Vellanikkara, Thrissur. It had resulted in production of backcross lines (BC_1F_1s). Marker assisted evaluation of the BC_1F_1s was done to discern the R-gene pyramids. As a result, three R-gene pyramids were identified in the study (Joseph, 2016). The BC_2F_1s (28 no's) and BC_1F_2s respectively produced through further backcrossing (Recurrent Parent (RP) Uma) and simultaneous selfing of the R-gene pyramids formed the base material for the present study. The details and schematic representation of the origin of the experimental material are given in Table 2 and Figure 1 respectively. The BC_2F_1 and BC_1F_2 plants along with their parents formed the basis of the study.

Table 2. Genotypes used to generate the experimental material

Genotype	Parentage	Year of release/ production	Salient features
Recurrent parent: Uma (Mo16)	Mo 6 x Pokkali	1998	Medium duration (115 - 120) Red kernelled rice variety
Donor parent: Improved Samba Mahsuri (ISM)	Samba Mahsuri x 4/SS1113	2008	Long duration (135 - 140) White kernelled rice variety
BC_2F_1 generation			
Progenies of BC_1F_1 Plant No. 8.3.2	BC_1F_1 Plant No. 8.3.2 x Uma (Recurrent parent)	2016	BC_1F_1 Plant No. 8.3.2: Long duration (234 days) Red kernelled genotype
Progenies of BC_1F_1 Plant No. 8.3.3	BC_1F_1 Plant No.8.3.3 x Uma (Recurrent parent)	2016	BC_1F_1 Plant No. 8.3.3: Long duration (228 days) Red kernelled genotype
Progenies of BC_1F_1 Plant No. 8.3.9	BC_1F_1 Plant No. 8.3.9 x Uma (Recurrent parent)	2016	BC_1F_1 Plant No. 8.3.9: Long duration (158 days) Red kernelled genotype
BC_1F_2 generation			
BC_1F_2 progenies of Plant No. 8.3.2	Selfing of BC_1F_1 Plant No. 8.3.2	2016	–
BC_1F_2 progenies of Plant No. 8.3.3	Selfing of BC_1F_1 Plant No. 8.3.2	2016	–
BC_1F_2 progenies of Plant No. 8.3.9	Selfing of BC_1F_1 Plant No. 8.3.9	2016	–

3.3. Methods

3.3.1. Experiment 1: Genotyping of BC_2F_1 population

A non-replicated experiment block of BC_2F_1s (Table 3) was raised on 12th December, 2016. Seeds of BC_2F_1 Plants No. 8.3.2, Plant No. 8.3.3 and Plant No. 8.3.9, numbering 28 (Table 3) along with the recurrent parent Uma and donor parent ISM, were sown in separate trays containing sterilized soil [Plate 1(a)]. Twenty-one day old seedlings of parents as well as backcross progenies were then transferred to pots (30 cm diameter). Care was taken to transplant only a maximum of three seedlings per pot [Plates 1(b) and 1(c)]. Standard agronomic practices as per package of practices (KAU, 2016) were followed during the entire crop period to obtain good crop stand.

Table 3. Details of BC_2F_1s used in the study

Source	No. of BC_2F_1 seeds sown	No. of BC_2F_1 seeds germinated	Designation of BC_2F_1 plants
BC_1F_1 Plant No. 8.3.2/ Uma	6	4	Plant No. 8.3.2.1 to Plant No. 8.3.2.4
BC_1F_1 Plant No. 8.3.3/ Uma	12	7	Plant No. 8.3.3.1 to Plant No. 8.3.3.7
BC_1F_1 Plant No. 8.3.9/ Uma	10	10	Plant No. 8.3.9.1 to Plant No. 8.3.9.10
Total	**28**	**21**	

3.3.1.1. DNA isolation, quality and quantity assessment

Cellular DNA (Deoxy Ribonucleic Acid) of the BC_2F_1's along with their parents were isolated and their quality and quantity determined.

Plate 1(a). BC_2F_1s in nursery

Plate 1(b). Transplanted seedlings of BC_2F_1s

Plate 1(c). BC_2F_1s (1 MAS)

3.3.1.1.1. Extraction of genomic DNA

Fresh leaf samples collected from plants in the early morning hours were labelled, covered in aluminium foil and transported immediately to the laboratory in ice box to prevent degradation. The samples were then stored in -20° C until used for extraction. Modified CTAB method (Dellaporta *et al.*, 1983) was used for extraction of DNA from the collected samples.

Reagents used

1.CTAB buffer

Table 4. Composition of CTAB buffer

Contents	Concentration	Quantity for 250 ml
CTAB (W/V)	2%	5g
NaCl	1.4M	20.455g
EDTA (pH 8)	20mM	1.86g
Tris HCL (pH 8)	100mM	3.025g
Polyvinylpyrrolidin (PVP)	1%	2.5g
β mercaptoethanol	10mM	0.25 ml
Distilled water	-	250ml

2. Chloroform - Isoamyl alcohol (100ml)

Chloroform - 96ml
Isoamyl alcohol - 4ml

3. 3M Sodium acetate (100ml)

Dissolved 24.6g sodium acetate in 50ml distilled water using magnetic stirrer. Before making up to 100ml a pH of 5.2 was ensured.

4. 70% ethanol

70ml - ethanol
30ml – distilled water

5. Chilled isopropanol (100%)

6. RNAses (10mg/ml)

10mg/ml RNase was prepared by adding powdered RNase A in 10Mm sodium acetate solution with pH adjusted to 5.2 by adding 1N HCL or 1N NaOH. This was then boiled in a waterbath to 100^0C for 5 minutes and then cooled at room temperature finally adjusting the pH to 7.4.

Procedure

1. To extract the DNA, 0.2g of leaf sample was first weighed out. The weighed sample was then washed with sterile distilled water and then wiped with 70% ethanol.

2. The leaf was then cut into small pieces and ground into fine paste in a pre-chilled mortar and pestle using 1ml CTAB (Cetyl Trimethyl Ammonium Bromide) buffer along with 50µl of β mercaptoethanol and a pinch of Polyvinyl Pyrrolidin (PVP).

3. The fine ground sample was then transferred into a centrifuge tube and incubated at 65°C in water bath for 15 minutes with intermittent shaking of tubes at every 5 minutes' interval.

4. After incubation, equal volume of chloroform: isomyl alcohol (24:1) was added to the sample and the contents were gently mixed. The samples were then centrifuged at 10,000rpm for 10 minutes at 4°C. The contents in the tube separated out into three phases:

Aqueous top layer – DNA with small quantity of RNA
Middle layer – Protein and other cell debris
Bottom layer – Chloroform, pigments etc.

5. The upper aqueous phase obtained was transferred into a new tube and the process was repeated until a clear solution was obtained.

6. To the fresh tube containing the transferred solution, 0.2 µl of RNase (10mg/ml) was added and incubated it for 30 minutes.

7. After incubation, equal volume of chloroform: isoamyl alcohol (24:1) was added and the samples were centrifuged at 10,000rpm for 10 minutes.

8. The top aqueous phase obtained was then transferred to a 1.5ml fresh tube and an equal volume of ice cold isopropanol and 3M sodium acetate (1:20) was added. The samples were then incubated at -20^{0}C for 24 hours for DNA precipitation.

9. After 24 hours the samples were centrifuged at 10,000 rpm for 10 minutes at 4^{0}C and the supernatant was decanted carefully, retaining only the pellet.

10. The pellet obtained was washed using 100-200µl of 70 per cent ethanol and was again centrifuged at 10,000rpm at 4^{0}C for 10 minutes.

11. The supernatant was then carefully discarded and the tubes were air dried. The DNA pelleted was resuspended in 50-100µl distilled water and stored in vials at -20^{0}C.

3.3.1.1.2. Determination of quality and quantity of isolated DNA

The quality and quantity of the DNA isolated was analysed using Nanometer (JH Bioinovations, India). For nucleic acid and proteins, the maximum absorbance occurs at 260 nm and 280nm respectively. 1µl volume of the samples were used for the measurement of absorbance at wavelengths 260nm and 280nm. The quantity of DNA in the samples were calculated based on Beer-Lambert's law.

3.3.1.2. Foreground selection

Three STS markers, RG556, RG136 and pTA248 (Table 5), closely linked to the BB resistance genes, *xa5, xa13 and Xa21*, respectively were used to confirm the presence of the resistance allele of each gene in the backcross generation as per the procedure advocated by Sundaram *et al.* (2008). The RG556 marker is located ~0.1 cM from *xa5*, RG136 marker is ~1.5 cM from *xa13* and pTA248 marker is ~0.2 cM from *Xa21*. The functional marker *xa13* promoter, *xa5S/R* were also used for further confirmation of presence of resistance genes.

Table 5. Markers used for foreground selection

Gene	Primer name	Primer sequence	Marker distance (cM)	Product size (bp)	Reference
xa5	xa5SR F	AGC TCG CCA TTC AAG TTC TTG AG	0.0	410, 310, 180	Petpisit *et al.* (1977)
	xa5SR R	TGA CTT GGT TCT CCA AGG CTT			
	RG 556 F	ATA CTG TCA CAC ACT TCA CGG	0.1	440, 410	
	RG 556 R	GAA TAT TTC AGT GTG TGC ATC			
xa13	RG 136 F	TCC CAG AAA GCT ACT ACA GC	3.8	530, 490	Sundaram *et al.* (2008)
	RG 136 R	GCA GAC TCC AGT TTG ACT TC			
	xa13 pro F	GGC CAT GGC TCA GTG TTT AT	0.7	500	
	xa13 pro R	GAG CTC CAG CTC TCC AAA TG			
Xa21	pTA 248 F	AGA CGC GGA AGG GTG GTT CCC GGA	0.2	1000	Sundaram *et al.* (2008)
	pTA 248 R	AGA CGC GGT AAT CGA AAG ATG AAA			

3.3.1.2.1 Polymerase chain reaction (PCR)

The good quality DNA isolated from each backcross progeny was amplified for both foreground and background selection using PCR which was performed using thermal cycler Eppendorf Master Cycler (Eppendorf, Germany Model: Hamburg 22331).

3.3.1.2.1.1 DNA dilution for PCR

For PCR reactions to be carried out, a concentration of 50µg/ml DNA was required. The required concentration of DNA was obtained by dilution of the samples based on the optical density (OD) values and DNA quantity (µg/ml) recorded. DNA samples were diluted so as to obtain a concentration of 50µg/ml of 100µl solution using distilled water as per the formula $N_1V_1=N_2V_2$.

3.3.1.2.1.2 Dilution of the primers and PCR

Primer stocks were diluted with distilled water to give 1M stock solutions of both forward and reverse primers. Primer dilutions for PCR was prepared by dissolving primer stock and distilled water in the ratio 9:1. The amplification of diluted genomic DNA was carried out in 0.2ml PCR tubes using 15µl reaction mixture. The PCR reaction mixture for each tube was prepared as detailed in Table 6. After preparing the reaction mixture the tubes were centrifuged for 1 minute at 4°C and then placed in the thermal cycler for amplification. The PCR was carried out as per the profile (Table 7) given by Sundaram *et al.* (2008).

Table 6. Composition of PCR reaction mixture

Aliquot	Quantity
10x Taq buffer	2µl
dNTP mix	1 µl
$MgCl_2$(25mM)	1.5 µl
Taq DNA polymerase (1U)	0.3 µl
Primers (Forward and Reverse)	2µl each
DNA sample	3µl
Distilled water	3.2 µl
Total	**15 µl**

Table 7. Reaction profile for PCR

Stage	Temperature (°C)	Time	Number of cycles
Initial denaturation (hot start)	94	5 min	
Denaturation	94	30 sec	35 cycles
Primer annealing	55	30 sec	
Primer elongation	72	1 min	
Final extension	72	7 min	
Cold storage	4	Till required	

3.3.1.2.1.3 Restriction digestion of PCR product of STS markers

Initially five µl of the PCR amplified product of STS markers RG556 and RG 136 were used to detect the amplification of DNA. The remaining 10µl of the amplified DNA using the STS markers RG556 and RG 136 were subjected to restriction digestion with enzymes Dra1 and Hind1 respectively. The reaction mixture (30µl) detailed in the Table 8 was then centrifuged for 1 minute and incubated at 37°C for 4-5 hours.

Table 8. Reaction mixture for restriction digestion

Aliquot	Quantity
Distilled water	17µl
10X- Fast digest green buffer	2 µl
DNA samples after PCR	10 µl
Fast digest enzyme (Dra1/Hinf1)	1 µl
Total	**30 µl**

3.3.1.2.2 Gel electrophoresis of PCR products

Amplified PCR products of markers used for foreground and background selection were separated by running them (Table 9) in 1.5% agarose gel stained with ethidium bromide. The bands obtained were then visualised using gel documentation software.

Reagents used:

1) Agarose 1.5%
2) 50X TAE buffer

Table 9. Composition for TAE buffer

Contents	50X TAE (1 Litre stock solution)
Tris base	242g
Glacial acetic acid	57.1ml
EDTA	100ml of 500Mm (pH 8)
Distilled water	842.9ml

Working stock (1X TAE) 1 litre was prepared by dissolving 20ml of 50X TAE in 980ml distilled water

3) Ethidium Bromide (0.5µg/ml)

Procedure:

Agarose gel 1.5% was prepared by dissolving 1.5g agarose in 100ml of 1X TAE buffer in a microwave until the solution became clear. The solution was then cooled to about 42-45°C, *i.e.*, the point at which ethidium bromide can be added (0.5µg/ml). This warm solution was then poured into the gel casting tray to a depth of 5cm and the combs were placed. The gel was then allowed to solidify at room temperature. After 30-45 minutes the combs were removed and the casting tray was placed in the electrophoresis unit filled with electrophoretic buffer (same buffer used for gel preparation) until the wells were submerged. The PCR digested samples were then loaded into the wells, with one lane loaded with suitable molecular weight marker. The gel was then electrophoresed at 100 volts for 45

minutes until the dye has migrated to two third the length of the gel. The electrophoresed gel was then carefully transferred to gel documentation unit (GeNei^{TM} – UVITEC, Merck, UK + Dell computer system) and the banding pattern was observed under UV exposure.

3.3.1.2.3. Visualization of PCR product and screening

UVITEC Fire Reader software (Merck, UK) was used for the proper visualization of bands in the electrophoresed gel and to score the well resolved and distinct amplicons for polymorphism /monomorphism. In comparison to the known molecular weight marker that were run along with amplified samples, the location of amplicon position and its molecular weight were assessed. Amplicons having different size were scored as polymorphic and that of same size were scored as monomorphic

3.3.1.3. Background selection

For background selection of BC_2F_1s, Rice microsatellite (RM) markers were used. A set of 22 RM markers (Table 10) that were reported to be polymorphic between the parents in the DBT project (mentioned under Section 3.2.1) were selected for background selection. Care was taken to ensure that the markers selected for background selection covered the 12 linkage group in rice. The list of markers used for foreground and background selection is detailed below.

Table 10. Markers used for background selection

Primer	Sequence		Annealing temperature (°C)	Product size (bp)
	Forward	Reverse		
RM 1	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC	55	113
RM 16	CGCTAGGGCAGCATCTAAA	AACACAGCAGGTACGCGC	55	181
RM 205	CTGGTTCTGTATGGGAGCAG	CTGGCCCTTCACGTTTCAGTG	55	122
RM 214	CTGATGATAGAAACCTCTTCTC	AAGAACAGCTGACTTCACAA	55	112

(Contd...)

...Contd.

Table 10. Markers used for background selection

Primer	Sequence		Annealing temperature (°C)	Product size (bp)
	Forward	Reverse		
RM 252	TTCGCTGACGTGATAGG TTG	ATGACTTGATCCCGA GAACG	55	216
RM 254	AGCCCCGAATAAATCC ACCT	CTGGAGGAGCATTT GGTAGC	55	165
RM 307	GTACTACCGACCTACCG TTCAC	CTGCTATGCATGAAC TGCTC	55	174
RM 5586	CTCCATAATCAAGGAA GCTA	ATGAGTTCTTTCGTC AGTGT	55	134
RM 11554	AGGACT TAG GGT ACG TTT GAA TCT CC	GAC GAT GAT TGT CTC CTA AGT CTG C	55	318
RM 10871	TGA GGC TGT AAC GTA GAC GAT GAA CC	AAG CCT GCT AGA GAG GCC CAA CC	55	234
RM 13910	GAG CGA GCT ATA CCA CCG TGA CC	ATC GCG TCC AAG AAA GGT GTC G	55	188
RM 14725	CCA CAT AAG TAT TGG AGT GCA TCG	AGA TGT TAA CCC ACG AGG AAT GG	55	469
RM 15026	GCA TGC TCT TCC ATG ACT GC	CAT ATC AGA GGG TAC GAA ATG ACC	55	378
RM 15303	GAA TCG GGT CTA CGG TTT AGG	AAA GGA AGA GAA GAG GCA ACG	55	199
RM 15561	ATT AGC TTG GGC GTC TTC CTC TGG	TGC AAA CAA TGG CTT CAC ATC G	55	266
RM 15583	CCC AAA TAG TCA CCA GCA TTA TCG	TTG CCT GTG CAA CCT TAT GAA CC	55	174
RM 17182	TGCAGCGTCTCATCATA AAGTCG	GCTTAGTGCTGTGAA CTGTGAAGACC	55	199
RM 19199	GCTCTACCAGGTATTAT AGCCGATCC	AACTCCTCCAAGGTT CCATAGCC	55	158
RM 24616	CACCTTGGCCAACTAAC TAATCG	GGGCAAGAGGAATT CACAACC	55	287
RM 26213	GCCACAGGAGACAGCA AGAACC	CGATCCAATTCCAGC CTAGATAGC	55	345
RM 26868	CAACTGTACTGTGCTGA CCATCG	AGTAGGGACGAGGA TTTCATGG	55	168
RM 28267	GCATAGCCCTGTTTGTT GCATGG	CGGTCCTTCCTCTTC TGTCATAACG	55	382

Amplification of DNA samples with markers, the separation of PCR product and documentation of gel picture was done following the procedures enumerated under 3.3.1.2.1. However, as only SSR markers were used for background selection, restriction digestion of PCR product followed in case of STS markers used for Foreground selection (enumerated in Item 3.3.1.2.1.3) was not done.

3.3.2. Experiment II: Morphological characterisation of pyramided lines of BC_2F_1 population

Morphological characterization of each BC_2F_1 and the parents (5 plants each) raised were recorded as per the descriptor for rice (IRRI, 1996).

3.3.3. Experiment III: Production of BC_2F_2s and BC_3F_1s

3.3.3.1. Production of BC_2F_2s

One half of the panicles of the BC_2F_1 plant found to be introgressed with all the three R-genes were selfed to obtain BC_2F_2s. At maturity the seeds were harvested and dried to 13 per cent moisture to aid prolonged storage.

3.3.3.2. Production of BC_3F_1s

Staggered sowing of the recurrent parent (Uma) was done at weekly intervals staring from 25th November, 2016 up to 20th February, 2017 [Plate 2(a) and 2(b)]. This was done to ensure sufficient pollen load for the production of BC_3F_1s. The BC_2F_1s identified to be introgressed with all the three BB resistance genes *xa5*, *xa13* and *Xa21* were backcrossed to the recurrent parent Uma to obtain BC_3F_1 seeds.

3.3.3.2.1. Emasculation

BC_2F_1 (female parent) panicles that exhibited fifty to sixty per cent emergence from the flag leaf were selected for emasculation. For the ease of emasculation, the spikelets were further exposed by slightly detaching the leaf sheath from the panicle. Late afternoon (after 3 p.m.) was the most preferred time for emasculation. Very young florets with height of anthers less than half the florets,

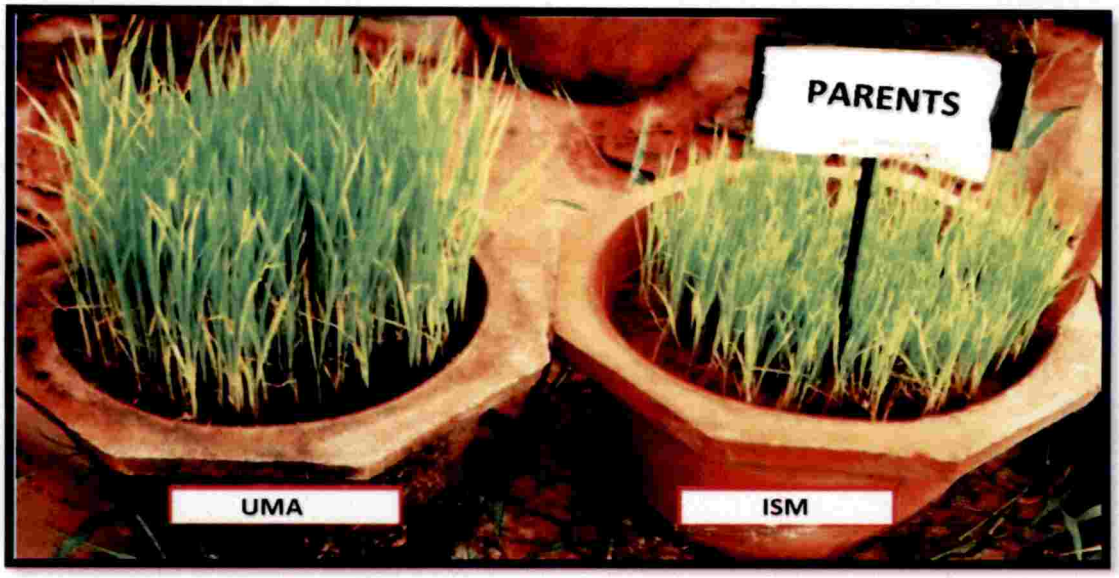


Plate 2(a). Raising parents (nursery)

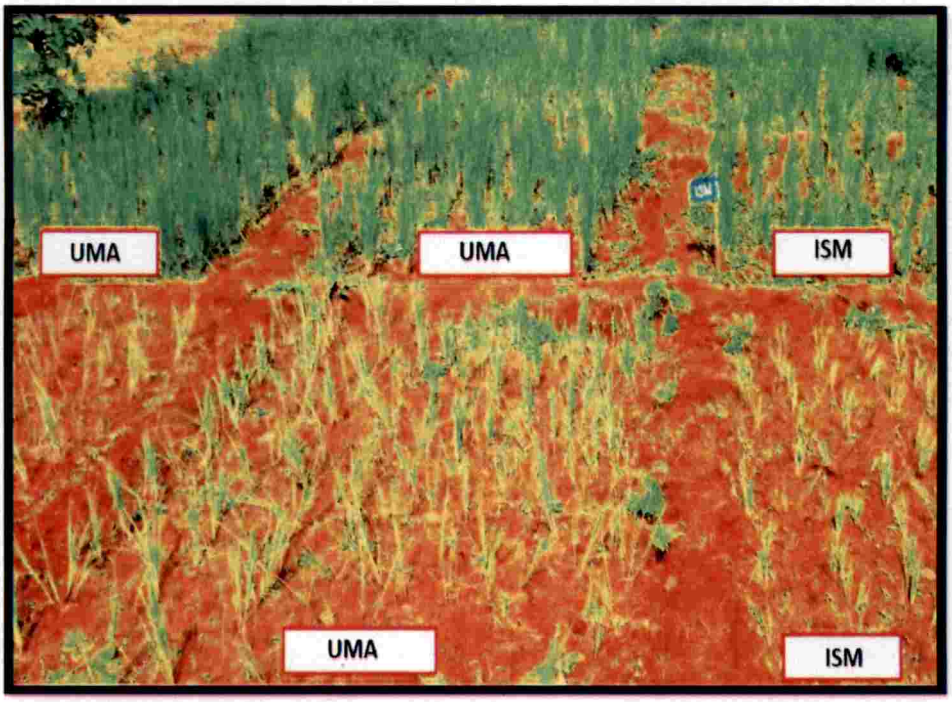


Plate 2(b). Staggered sowing of parents

located at the base of the panicle were cut away. Florets with height of anthers equal to or more than half the florets, which are likely to open on the following day were selected for emasculation. With the help of scissors, top one-third of each selected floret for emasculation was clipped off to expose anthers. The anthers were then removed by pressing it against the side of the floret and lifting them out with the tip of the forceps prong. Butter paper bags were used to cover the emasculated panicles, which was then tagged and labelled. Before tagging, bottom edge of the butter paper bags was folded against peduncle for holding it securely in place with a clip.

3.3.3.2.2. Hybridisation

In the morning (8.00 am) on the subsequent day of emasculation, panicles of male parent (Uma), which were ready to dehisce were selected. Pollen grains were collected by gently tapping the full bloomed panicle in a petridish containing distilled water. With the help of a thin brush, the collected pollen grains were then transferred to the stigma of each emasculated spikelets of the female parent. Re-bagging of pollinated panicles was done immediately to avoid contamination by foreign pollen. Seed set was checked on the fifth day after hybridization. Although the stigma remained receptive for three to seven days, maximum seed set was reported when it was pollinated on the day subsequent to emasculation. At maturity, seeds were harvested, dried and stored to 13 per cent moisture.

3.3.4. Experiment IV: Morphological characterisation and pathotyping of BC_1F_2 population, and production of BC_1F_3 population.

3.3.4.1 Pathotyping of BC_1F_2 population

BC_1F_2 plants (Table 11) were phenotypically screened for bacterial blight resistance, for identification of resistant and susceptible genotypes following the leaf clip method (IRRI, 2002).

Table 11. Details of BC_1F_2s used in the study

Source	No. of BC_1F_2 seeds obtained
Selfing of BC_1F_1 Plant No. 8.3.2	273
Selfing of BC_1F_1 Plant No. 8.3.3	284
Selfing of BC_1F_1 Plant No. 8.3.9	293
Total	**850**

3.3.4.1.1 Preparation of bacterial culture (*Xanthomonas oryzae* pv. *oryzae* isolates)

The isolate of *Xanthomonas oryzae* pv. *oryzae (Xoo)* collected from Regional Agricultural Research Station, Pattambi, Kerala was used for pathotyping the BC_1F_2 population and parents to evaluate their reaction to pathogen infection.

3.3.4.1.2. Bacterial inoculation

Bacterial suspension used for inoculating the plants was prepared by mixing two loops of pure culture of bacteria in 500ml sterilized distilled (Kauffman *et al.*, 1973). This suspension was immediately used for inoculation.

Clip inoculation technique was followed as the crop reached maximum tillering stage (Kauffman *et al.*, 1973). In each plant, a minimum three leaves were inoculated early in the morning by cutting away 1-2 cm of the leaf tip with scissors that was previously dipped in bacterial suspension on the cutting edge.

3.3.4.1.3. Phenotypic evaluation of BC_1F_2 population

Individual plants of BC_1F_2 population and parents were evaluated for field infection to bacterial blight. Disease incidence was scored after 15 days of inoculation. Physical measurement of lesion was recorded and per cent leaf area was worked out. The resistant and susceptible plants were categorised based on scores 1-9 corresponding to lesion length.

3.3.4.2. Production of BC_1F_3s

All the panicles of the BC_1F_2s exhibiting resistance or moderately resistance to BB pathogen infection were selfed to obtain BC_1F_3s and the seeds harvested at maturity, dried to 13 per cent moisture content and stored.

3.4. Observations recorded

3.4.1. Genotyping of BC_1F_1 population

3.4.1.1. Quality and quantity of DNA isolated

Quality and quantity of DNA isolated was assessed using Nanodrop. The purity of the DNA samples was assed based on A_{260}/A_{280} ratio. The ratio of 1.8 to 2 indicate pure DNA. Protein contamination was evident if the ratio was less than 1.8 and for values greater than 2, RNA contamination was inferred. The quantity of DNA obtained was calculated based on the relation that optical density (OD) for a DNA sample with a concentration of 50µg/ml (double stranded) at 260nm equals one.

1 OD 260 = 50 µg/ml (ds)

Quantity of DNA (µg/ml) = Absorbance at OD 260 x 50

The quantity of DNA in the samples were calculated based on Beer-Lambert's law. OD 260 = 1 is equivalent to 50 µg of double stranded DNA. The quantity of DNA (µg/ml) in the sample is therefore calculated by formula OD 260x 50.

3.4.1.2. Nature of amplification

UVITEC Fire Reader software, gel documentation system was used to capture the image for analysing the banding pattern resolved by gel electrophoresis. Observations on nature of banding pattern was recorded as monomorphic or polymorphic.

3.4.1.3. Number of amplicons

UVITEC Fire Reader software used for gel documentation was used to count the number of amplicons resolved on the electrophoresed gel.

3.4.1.4. Size of amplicons

Using UVITEC Fire Reader software the size of amplicons for each marker resolved on the gel were estimated in base pairs (bp) by comparison with a known molecular weight marker that were run along with PCR product.

3.4.1.5. Uniqueness of amplicons

UVITEC gel documentation system was also used for identifying the uniqueness of amplicons in terms of size in base pairs (bp), by comparing the banding pattern observed for the BC_2F_1s with that of parents (ISM and Uma).

3.4.2. Pathotyping of BC_1F_2 population

Lesion length was measured in centimetre and per cent diseased area was worked out. The resistant and susceptible plants were categorised based on scores 1-9 corresponding to lesion length (IRRI, 2002) as in Table 12.

Table 12. Scale for rating BB resistant lines and varieties

Scale	Diseased leaf area (%)	Description
1	1-5	Resistant(R)
3	6 – 12	Moderately resistant (MR)
5	13 – 25	Moderately susceptible (MS)
7	26 – 50	Susceptible(S)
9	>50 %	Highly susceptible (HS)

3.4.3. Morphological characterisation of BC_2F_1 population

1. Plant height (cm)

Distance measured from plant base (ground level) to the tip of flag leaf at maturity and expressed in centimetre.

2. Days to flowering

The number of days taken for the first panicle emergence from the date of sowing was recorded.

3. Leaf blade width (cm)

Measurement across the broadest point of the leaf lamina of three randomly selected leaves were taken and the average expressed in centimetre.

4. Leaf blade length (cm)

Measurement was taken from the base to tip of three representative leaves and average computed and expressed in centimetre.

5. Productive tillers

The total number of grain bearing tillers of each plant was counted at maturity.

6. Panicle length (cm)

Panicle length was measured from the panicle base to the tip and expressed in centimetre.

7. Spikelets/ panicle

Number of spikelets in each of three randomly selected panicles was counted and the average was computed.

8. Grain length (mm)

Length of grains was measured from ten seeds selected at random and the average computed and expressed in millimetre.

9. Grain width (mm)

Width of grains was measured from ten seeds selected at random and the average expressed in millimetre.

10. Decorticated grain length (mm)

The seeds were decorticated and the length measured from ten seeds selected at random and the average expressed in millimetre.

11. Decorticated grain width (mm)

The seeds were decorticated and the width measured from ten seeds selected at random and the average expressed in millimetre.

3.6. Statistical analysis

3.6.1. Genotyping of BC_1F_1 population

Analysis of the genomic contribution of the parent in the BC_2F_1 based on SSR data was carried out by using Graphical Geno Types (GGT) Version 2.0 (Van Berloo, 1999) software. Similarity matrix was generated as per Sneath and Sokal, 1973 and clusters based on default similarity coefficient and dendrogram were generated.

3.6.2. Parameters of variability

The variability in morphology of backcross progenies were estimated.

1. Mean

It is calculated from the ration of sum of individual observations to the corresponding number of individuals on which the observation was made.

$$\bar{X} = \sum X_i / N$$

Where,

X_i - any observation in i^{th} treatment

N - Total number of observations

2. Range

It is measured as the lowest and highest values of the sample observations made.

Results & Discussion

IV. RESULTS AND DISCUSSION

Although rice is the main staple food in Kerala, it is in short supply. A huge gap exists in the production-demand scenario of rice in the state. Kerala constantly faces the daunting challenge of sustaining food security. Enhancing food security warrants, increasing the production and productivity of rice. However, bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae (Xoo)* is a serious threat to rice crop in the recent past. The yield losses due to bacterial blight in severely infected fields may reach up to 81 per cent (Srinivasan and Gnanamanickam, 2005).

In order to mitigate this stress, efforts were taken at College of Horticulture, Vellanikkara, Thrissur, to introgress three BB resistance genes *xa5*, *xa13* and *Xa21* into popular high yielding variety Uma (Mo 16) from donor Improved Samba Mahsuri (ISM) through MABB approach under the DBT project 'Rice-Gene pyramiding to develop cultivars with durable resistance to Bacterial Leaf Blight through Marker Assisted Selection'. Backcrossing the F_1s obtained to the recurrent parent Uma, resulted in the production of BC_1F_1s. Genotypic and phenotypic evaluation of the BC_1F_1s lead to the identification of three plants (Plant No. 8.3.2, Plant No. 8.3.3 and Plant No. 8.3.9) introgressed with all the three resistant genes. The three BC_1F_1s were backcrossed and selfed further to produce the advanced breeding generations BC_2F_1 and BC_1F_2 (Joseph, 2016).

The present study aimed to identify R-gene pyramided plants in BC_2F_1 population and to develop stable pyramided BC_3F_1 lines of variety Uma with low linkage drag from the donor parent. It also perceived the evaluation of BC_1F_2 population for resistance to bacterial blight pathogen (pathotyping). Production of advanced breeding generations (BC_2F_2s and BC_1F_3s) was also envisaged. The results obtained from the study has been enumerated and discussed below.

4.1. Genotyping of BC_2F_1s

Owing to the climatic conditions prevailing and the fragmented/ terraced rice ecosystems in Kerala, the occurrence and spread of bacterial blight disease is very rapid. Forecasting and management are the recommended approaches to reduce crop devastation due to the disease. However, according to Murty and

Devadath (1982) the use of disease forecasting is limited and it is also highly impractical to undertake chemical control measures for the management of the disease. This is all the more true in the Kerala rice growing scenario as both rice growing seasons (*kharif* and *rabi*) coincides with monsoons. Resorting to only chemical and biological control measures like spraying of antibiotics (streptocyclin) or cow dung extract (KAU, 2016) is not much effective, as it can be washed-off with monsoon showers. Apart from the ecological and food safety concerns, the effectiveness and efficiency of chemical pesticides and biocontrol agents considerably decreases on continuous application (Peng *et al.,* 2015). Moreover, the evolution and occurrence of drug resistant strains also impairs the effectiveness of chemical management (Gnanamanickam,1999).

According to Liu *et al.* (2004), the disease spread is fundamentally influenced by the amount and pattern of rainfall. High relative humidity (90 per cent for 12-14h/day) and rainfall favours quick disease development. In addition, a temperature between 23^0C and 31^0C that prevails in the state is also said to favour disease development during wet season. Chen *et al.* (2011) opined that durable resistance against BB for varieties to combat this stress is a better option over chemical and biological control measures of BB pathogen.

Considering the above, resorting to host plant resistance along with good management practices is the most effective, environment safe method to combat BB pathogen (Sing *et al.*, 2015). About 42 resistant genes imparting resistance against BB have been reported till date (Khan *et al.*, 2014; Zhang *et al.*, 2014; Busungu *et al.*, 2016). The R-gene combinations *Xa4* + *xa5* + *xa13* + *Xa21* has been reported to confer broad spectrum resistance to *Xoo* isolates identified in Kerala (Priyadarisini and Gnanamanickam, 1999; DRR, 2015). Through conventional breeding approaches, the R-gene *Xa4* has been widely incorporated in many high yielding cultivars (Khush, 1989). The widespread cultivation of these varieties had led to the predominance of *Xoo* races that could overcome the resistance conferred by the R-gene (Mew *et al.*, 1992). The results thus pointed out the usefulness of deployment of multiple BB resistance genes into a single cultivar to confer durable resistance.

Gene introgression through conventional breeding is a time consuming approach. It may take 8 to 12 years for the development of a resistant pyramided cultivar. In a backcross individual there are chances of introgression of some undesirable segments from donor parent. Such linkage drags can be greatly reduced by integrating the use of molecular markers in breeding programmes (Frisch *et al.*, 1999; Joshi and Nayak, 2010).

Of the three genes (*xa5*, *xa13* and *Xa21*) under consideration, only *Xa21* is dominant. Conventional breeding tools are generally inefficient for introgression of recessive genes (Ullah *et al.,* 2012; Singh *et al.,* 2015). Moreover, as action of a gene conferring resistance to many races of pathogen may mask the action of another resistance gene, the selection of desirable plant solely based on the phenotype can be misleading due to epistatic gene actions (Tanksley *et al.*, 1989; Davierwala *et al.*, 2001; Rao *et al.*, 2002; Akhtar *et al*, 2010).

MAS therefore has been reported to be an effective alternative to conventional breeding approach for pyramiding R-genes (Tanksley *et al.*, 1989; Frisch *et al.*, 1999; Davierwala *et al.*, 2001; Rao *et al.*, 2002; Akhtar *et al*, 2010; Joshi and Nayak 2010; Salgotra *et al.*, 2012; Pinta *et al.,* 2013; Khoshkdaman *et al.*, 2014; Pradhan *et al.*, 2015b; Sabar *et al.*, 2016; Arunakumari *et al.*, 2016; Luo *et al.*, 2016; Ramalingam *et al.*, 2017; Das *et al.*, 2018; Sagar *et al.*, 2018; Baliyan *et al.,* 2018).

4.1.1. Quality and quantity analysis of genomic DNA of BC_2F_1s and parents.

Quality and quantity for the genomic DNA of BC_2F_1s and parents was analysed (Table 13, Appendix I and II).

The A260/A280 ratio of genomic DNA isolated from BC_2F_1s ranged from 1.73 to 2.01, while, the average value of the parents Uma (recurrent parent) and ISM (donor parent) was 1.89 and 1.90 respectively. The results indicated that the genomic DNA extracted from the parents as well as BC_2F_1s were of good quality as the A260/A280 ratio of DNA extracts was between 1.9 and 2.01. A value less

than 1.8 or greater than 2.0 would have indicated a high degree of protein contamination and RNAse respectively (Manchester, 1996).

The quantity of the isolated DNA in the BC_2F_1 individual varied from 7.7µg/ml to 1341.6µg/ml. In case of parents, Uma and ISM, the concentration of DNA extracted was 79.6µg/ml and 257.7µg/ml respectively. The concentration of DNA (50µg/ml DNA) required for genotyping was obtained by dilution of the samples based on the optical density (OD) values and DNA quantity (µg/ml) as per the methodology elaborated under 3.4.1.1.

Table 13. Quality and quantity of genomic DNA of BC_2F_1s and parents

Individuals	Quantity of DNA (µg/ ml)			Quality of DNA		
	Mean	Range		Mean	Range	
		Max.	Min.		Max.	Min.
Uma (Recurrent parent)	79.60	145.30	15.80	1.89	1.94	1.81
ISM (Donor parent)	257.70	281.90	233.50	1.90	1.92	1.87
BC2F1s	243.94	1341.60	7.70	1.90	2.01	1.73

4.1.1. Foreground selection

The good quality total genomic DNA thus extracted from backcross individuals (BC_2F_1: 21 Nos.) and parents (Uma and ISM) were then subjected to foreground selection.

4.1.2.1. Foreground selection for R-gene *xa5*

The R-gene *xa5* is reported to be tightly linked with STS marker, RG556 located at a distance of 0.1 cM (Sundaram *et al.*, 2008). The presence of the R-gene can also be confirmed by using a functional marker xa5SR (Pradhan *et al.*, 2015b; Verma, 2016).

Amplification of genomic DNA of backcross individuals and parents (Uma and ISM) using STS marker RG556 produced monomorphic bands. Restriction digestion of the amplified products using restriction enzyme Dra 1 produced fragments of size 460bp, 318 bp, 268bp and 216bp (Plate No. 3, Table 14 and Table 15) in both the parents as well the BC_2F_1s indicating absence of polymorphism between the parents (Uma and ISM) and the BC_2F_1s examined. The donor parent ISM is an EDV introgressed with resistant allele of R-gene *xa5* in homozygous state. According to Huang *et al.* (1997) digestion of the amplified products using restriction enzyme Dra 1 would generate Specific amplicon polymorphism (SAP) that would help differentiate the susceptible and resistant genotypes. Therefore, monomorphic banding pattern observed in both parents as well as the BC_2F_1s evaluated indicated that the recurrent Uma and all the BC_2F_1s possessed the resistant allele of R-gene *xa5*.

Further confirmation of presence of resistant allele of R-gene *xa5* was done using functional marker xa5SR (Plate 4, Table 14 and Table 15). When the PCR products were resolved on 1.5 per cent agarose gel, monomorphic banding pattern was observed as amplicons of size 200bp was detected in all BC_2F_1 as well as the parents (ISM and Uma). The result thus pointed out the endogenous presence of resistant allele of R-gene *xa5* gene in both the parents as well as in the BC_2F_1s. This result is in confirmation with the findings of Joseph (2016).

Such endogenous presence of *xa5* gene in genotypes have been reported by earlier workers (Narayanan *et al.*, 2002; Kabade *et al.*, 2017). Singh *et al.* (2015) reported the presence of *xa5* gene in 20 cultivars out of 34 rice cultivars evaluated with an amplicon size of 219bp. Monomorphic banding pattern were also observed by Tuyen and Lang (2004) among the several local rice cultivars evaluated for *xa5* gene. Ullah *et al.* (2012) had also reported absence of polymorphism for RG556.

It is to be noted that in spite of the presence of resistant allele of R-gene *xa5*, the recurrent parent is susceptible to BB pathogen. This indicated that presence of *xa5* alone is insufficient to combat the attack by isolate of the BB pathogen prevalent in Kerala. Li *et al.* (2001) *and* Sundaram *et al.* (2008) had reported that

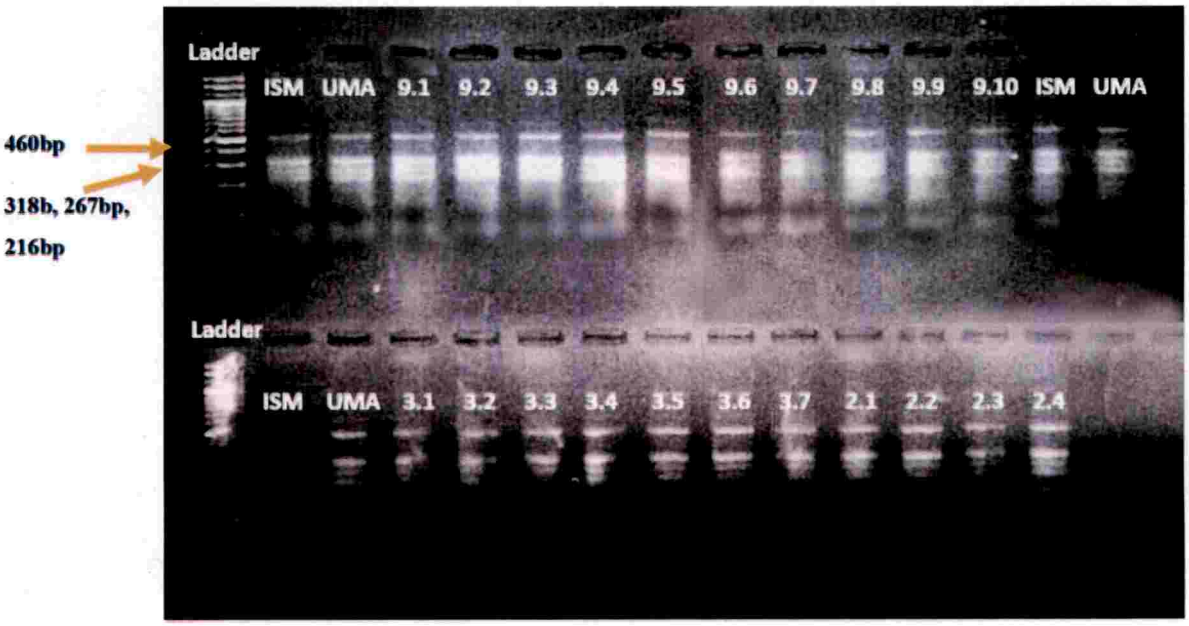
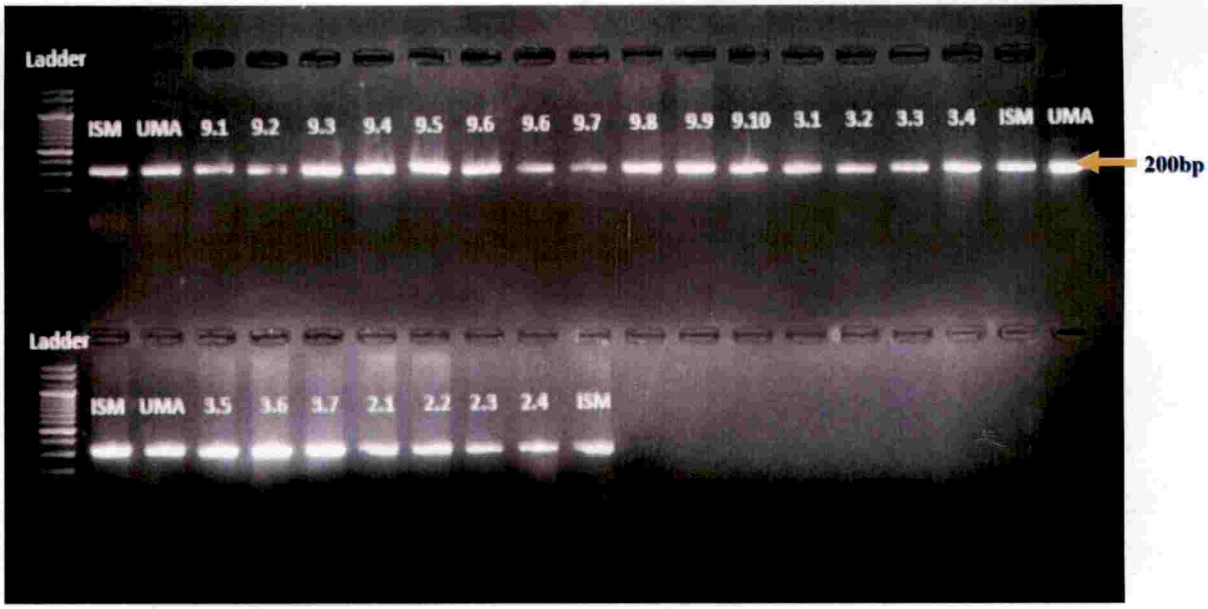


Plate 3. Specific amplicon polymorphism in BC_2F_1s on restriction digestion of PCR product of *xa5* linked STS marker RG556



ISM: Donor parent
Uma: Recurrent parent

9.1 – 9.10: BC_2F_1 Plant No. 8.3.9.1 to 8.3.9.10
3.1 – 3.7: BC_2F_1 Plant No. 8.3.3.1 to 8.3.3.7
2.1 – 2.4: BC_2F_1 Plant No. 8.3.2.1 to 8.3.2.4

Plate 4. Foreground selection of BC_2F_1s using *xa5* linked functional marker xa5 SR

xa5 provides only moderate resistance to BB pathogen. According to *Li et al.* (2001), *xa5* displays partial dominance and additivity to avirulent races of BB pathogen and possesses only relatively small but significant residual effect. Bharathkumar *et al.* (2008) had confirmed that resistance conferred by single gene was prone to breakdown in the field and therefore suggested pyramiding R-genes as a strategy to gain durable resistance against BB pathogen. Multiple BB resistance gene pyramiding into lines have been confirmed to confer higher level of resistance to the *Xoo* pathogen compared to those with single resistance gene (Yoshimura *et al.*, 1996; Huang *et al.*, 1997; Sundaram *et al.*, 2008; Pradhan *et al.*, 2015a, Baliyan *et al.*, 2018).

4.1.2.2. Foreground selection for R-gene *xa13*

Introgression of R-gene *xa13* was confirmed by using STS marker RG136 and functional marker xa13 promoter.

Monomorphic banding pattern was observed in the BC_2F_1s and parents when the amplified product of STS marker RG136 was resolved in agarose gel 1.5 per cent. However, further digestion of the products using restriction enzyme Hinf1 generated polymorphic banding pattern (Plate 5, Table 14 and 15). The restriction digestion generated three amplicons of sizes 1277bp, 283bp and 212bp for the donor parent ISM and 1908bp, 283bp and 212bp for the recurrent parent Uma and other BC_2F_1s (Table 14 and 15). However, in BC_2F_1 Plant No. 8.2.9.10, restriction digestion generated four amplicons of sizes 1908bp, 1277bp, 283bp and 212bp corresponding to those produced in the donor parent (ISM) and recurrent parent (Uma). Being a co-dominant marker, STS marker RG136 helps differentiate a heterozygote from a homozygote. The result thus pointed out that the BC_2F_1 Plant No. 8.2.9.10 was heterozygous for the R-gene (*xa13)* under consideration.

The presence of *xa13* gene was further analysed using functional marker xa13promoter (Plate 6, Table 14 and 15). Amplicons of sizes 292bp and 67 bp were respectively produced in the donor parent ISM and the recurrent parent Uma. All the BC_2F_1s except Plant No. 8.3.9.10 recorded banding pattern similar to recurrent

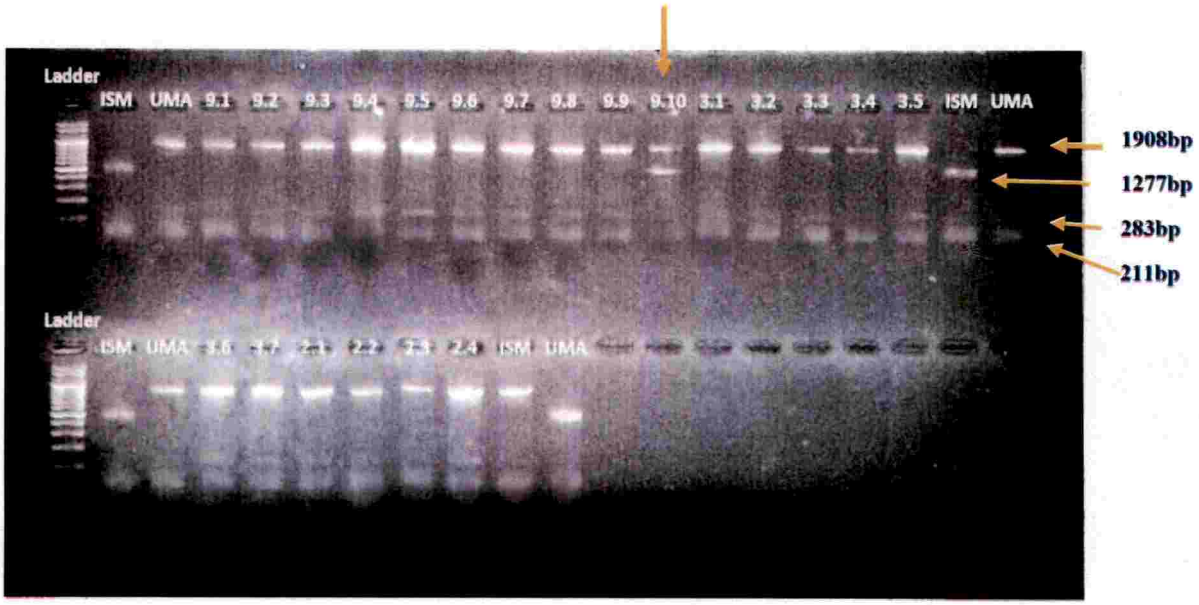


Plate 5. Specific amplicon polymorphism in BC_2F_1s on restriction digestion of PCR product of *xa13* linked STS marker RG136

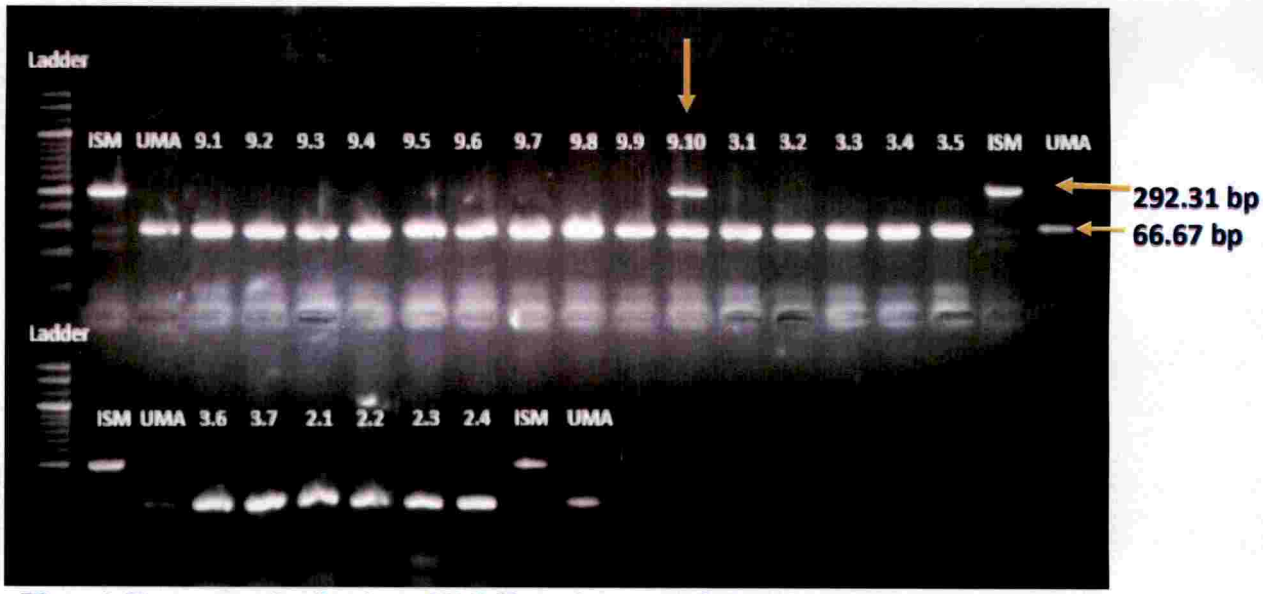


Plate 6. Foreground selection of BC_2F_1s using *xa13* linked functional marker xa13 promoter

ISM: Donor parent
Uma: Recurrent parent

9.1 – 9.10: BC_2F_1 Plant No. 8.3.9.1 to 8.3.9.10
3.1 – 3.7: BC_2F_1 Plant No. 8.3.3.1 to 8.3.3.7
2.1 – 2.4: BC_2F_1 Plant No. 8.3.2.1 to 8.3.2.4

parent. The plant possessed bands of sizes corresponding to both Uma and ISM. Thus, the heterozygous nature of the R-gene *xa13* present in Plant No. 8.3.9.10 was confirmed. The result indicated that out of the 21 BC_2F_1s screened, only one plant *i.e.,* BC_2F_1 Plant No. 8.2.9.10 was a 2-R- gene pyramid (*xa5* + *xa13*) while all the others possessed only a single R-gene (*xa5*).

The gene *xa13* is a recessive gene and hence, resistance conferred is only expressed in homozygous condition. As the identified BC_2F_1 Plant No. 8.2.9.10 was heterozygous at *xa13* loci, it would exhibit susceptibility to BB pathogen if subjected to pathotyping since the resistance expression conferred by *xa13* would be supressed by its dominant allele. Hence, MAS helps in identifying the presence of heterozygous loci for the recessive gene which may go unnoticed during conventional screening programes. Recessively inherited R-genes like *xa5* and *xa13* cannot be efficiently identified by conventional breeding methods (Singh *et al.*, 2015, Sundaram *et al.,* 2008). According to Pradhan *et al.* (2015a) and Arunakumari *et al.* (2016), MABB helps in circumventing the recombinant issue by getting rid of false positives unlike the conventional breeding system. Similar to the study, they had also recovered backcross individuals heterozygous for *xa13* locus.

In contrast to *xa5*, Li *et al.* (2001) reported that *xa13* being recessive in nature has no residual effects against virulent races of BB pathogen and exhibits pronounced race specificity and is considerably effective against many Indian races of *Xoo.* The superiority of gene pyramids over single R-genes in conferring resistance has been proved. Breakdown of resistance in varieties possessing single R-genes has been attributed to shifts in frequency of pathotypes or the emergence of new ones through mutation or other mechanisms. Therefore, multiple genes confer durable spectrum resistance through synergistic and complementary gene action to a wide range of races compared one, two gene combinationa (Dokku *et al.*, 2013a, 2013b; Das *et al.,* 2015; Das *et al.,* 2018; Mallikarjuna and Kotasthane, 2018)

4.1.2.3. Foreground selection for R-gene *Xa21*

STS marker pTA248 is reported to be tightly linked to dominant gene *Xa21* at a distance of 0.2cM (Dokku *et al.*, 2013a). Among the 21 BC_2F_1s evaluated one individual (Plant No. 8.3.9.10) exhibited amplicons of sizes 472bp and 300bp corresponding to parents ISM and Uma respectively while all others possessed only one amplicon (300bp) corresponding to the recurrent parent Uma (Plate 7, Table 14 and 15). This pointed out that the R-gene *Xa21* was present in the heterozygous condition in BC_2F_1 Plant No. 8.3.9.10, while in the other BC_2F_1s the resistance allele of gene *Xa21* was absent.

Broad spectrum resistance conferred by *Xa 21* has been well established (Huang *et al.*, 1997; Sanchez *et al.*, 2000; Singh *et al.*, 2001; Lee *et al.*, 2009; Antony *et al.*, 2010; Pradhan *et al.*, 2016; Sagar *et al.*, 2018). According to them, broad spectrum resistance of *Xa 21* and *xa13* is due to their synergistic effect in combating the BB pathogen and thus these are most widely used gene combination for marker assisted introgression for BB resistance in rice. Sanchez *et al.* (2000) opined that quantitative complementation of between the R-genes may result in enhanced levels of resitance to BB pathogen. However, according to Baliyan *et al.* (2018), *xa5* was most effective in conferring resistance to BB pathogen, followed by *Xa 21* and lines with *xa13* were susceptible and therefore gene in combinations were more effective against pathogen than a single gene.

4.1.2.4. Summary of foreground selection

Results of foreground selection revealed that among the 21 BC_2F_1s evaluated only one BC_2F_1 individual *i.e.*, Plant No. 8.3.9.10 was introgressed with all the three BB R-genes *xa5, xa13* and *Xa21* from donor parent ISM. The R-gene *xa5* was endogenously present in both the parents and therefore inherited by all BC_2F_1s, while R-genes *xa13* and *Xa21* were identified only in Plant No. 8.3.9.10. These were found to be introgressed in the heterozygous state. Foreground selection of 21 BC_2F_1s therefore revealed that BC_2F_1 Plant No. 8.3.9.10 was a 3-Rgene pyramid with the gene combination of *xa5xa5* +*Xa13 xa13* + *Xa21xa21*.

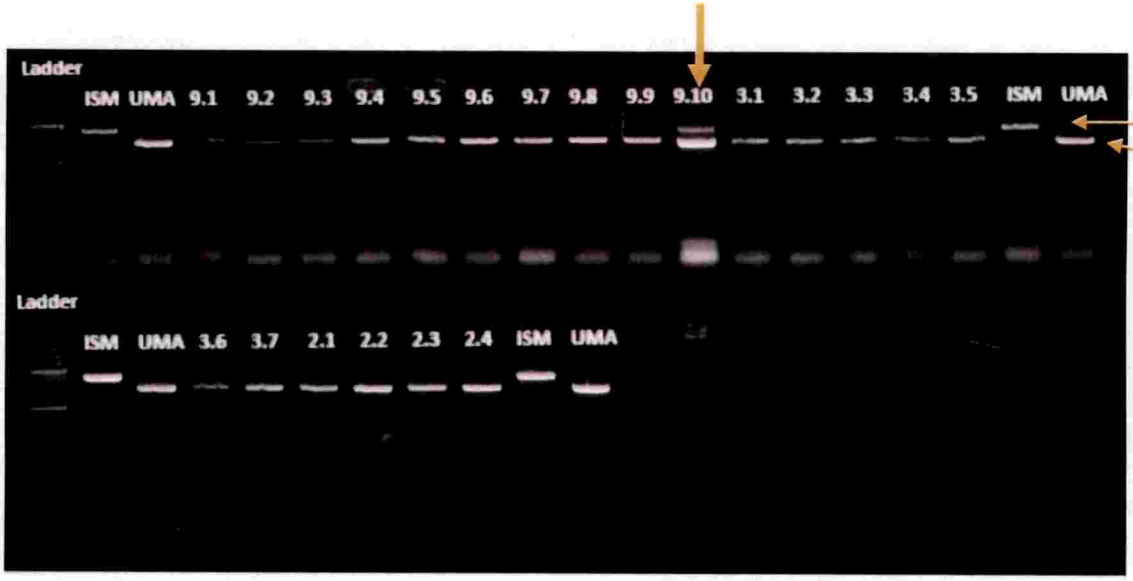


Plate 7. Foreground selection of BC_2F_1s using *Xa21* using linked STS marker pTA 248

ISM: Donor parent
Uma: Recurrent parent

9.1 – 9.10: BC_2F_1 Plant No. 8.3.9.1 to 8.3.9.10
3.1 – 3.7 : BC_2F_1 Plant No. 8.3.3.1 to 8.3.3.7
2.1 – 2.4 : BC_2F_1 Plant No. 8.3.2.1 to 8.3.2.4

Table 14. Distribution of alleles of PCR marker loci linked to BB resistance (R-genes) in the BC_2F_1s and parents

Target genes	*xa5*		*xa13*		*Xa21*
Marker	**RG 556**	**Xa5 SR**	**RG 136**	**Xa13 pro**	**pTA 248**
Donor parent (ISM)	A	A	A	A	A
Recurrent parent (Uma)	A	A	B	B	B
BC_2F_1 Plant No. 8.3.2					
1	A	A	B	B	B
2	A	A	B	B	B
3	A	A	B	B	B
4	A	A	B	B	B
BC_2F_1 Plant No. 8.3.3					
1	A	A	B	B	B
2	A	A	B	B	B
3	A	A	B	B	B
4	A	A	B	B	B
5	A	A	B	B	B
6	A	A	B	B	B
7	A	A	B	B	B
BC_2F_1 Plant No. 8.3.9					
1	A	A	B	B	B
2	A	A	B	B	B
3	A	A	B	B	B
4	A	A	B	B	B
5	A	A	B	B	B
6	A	A	B	B	B
7	A	A	B	B	B
8	A	A	B	B	B
9	A	A	B	B	B
10	A	A	H	H	H

A –Allele similar to donor parent **B – Allele similar to recurrent parent**

H – Heterozygous loci

Table 15. Segregation of molecular markers during foreground selection in 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 and parents

Sl. No.	Markers	Nature of amplification	Number of amplicon	Size of amplicon (bp)		
				Polymorphic		
				Donor parent (ISM)	BC2F1 Plant No. 8.3.9.10	Recurrent parent (Uma)
Markers employed in the foreground selection						
1	xa5 SR	Monomorphic	1	200		
2	RG 556	Monomorphic	4	460, 318, 267 and 216		
3	xa13 Pro	Polymorphic	2	292	292 and 66.7	66.7
4	RG 136	Polymorphic	3	1277, 283 and 211	1908, 1277, 283 and 211	1908, 283 and 211
5	pTA 248	Polymorphic	2	472	472 and 300	300

Owing to gene segregation and independent assortment, recovery of single R-gene introgression or two or three R-gene pyramids or various combinations of R-genes or gene combinations in heterozygous or homozygous state are not uncommon in a backcross programme. Arunakumari *et al.* (2016) had attempted to pyramid two R- genes (*Xa21* and *xa13*) and a major gene for blast resistance (*Pi54*) into an Indian rice variety MTU1010 through MABB. Among the 293 BC_1F_1 plants generated, only 55 were identified to be positive for *Xa21*, 68 were positive for *xa13* and 8 were double positive for both *Xa21* and *xa13*. Similar to the study, variable recovery of R-gene combinations has also been reported (Guvalla *et al.*, 2013; Nayak *et al.*, 2015; Pradhan *et al.*, 2016; Yap *et al.*, 2016; Das *et al.*, 2018; Sagar *et al.*, 2018)

The alleles of *xa13* and *Xa21* being in heterozygous condition however, will hinder the complete resistance expression of the R-gene pyramided plant. Hence, further selfing of the identified BC_2F_1 Plant No. 8.3.9.10 was recommended for attaining homozygosity for the alleles concerned.

4.1.2. Background selection

According to Joseph *et al.* (2004), the efficiency of MABB can be greatly enhanced through background selection, thus helping in the early release of BB resistant cultivar.

4.1.2.1. Allele distribution in R-gene introgressed BC_2F_1 Plant No. 8.3.9.10

Since the study aimed to improve the elite cultivar Uma by incorporating R-genes (*xa5*, *xa13* and *Xa21*) conferring resistance to BB pathogen, it is imperative that the linkage drag from the donor is minimum. This warranted a thorough understanding of recurrent parent genome recovery in the R-gene introgressed BC_2F_1 Plant No. 8.3.9.10. Hence, background selection was carried out along with the parents using 22 rice microsatellite markers that have been reported to be

polymorphic between the parents Uma and ISM in the earlier studies. The results are detailed in Plate No. 8(a) to 8(e) and Tables 16 and 17

SSR marker RM 1, RM 16, RM 205, RM 214, RM 254, RM 307, RM 5586, RM 10871, RM 14725, RM 15026, RM 15303, RM 17182, RM 24616 used for background selection, amplified a fragment of 481bp, 698bp, 487bp, 299bp, 487bp, 370bp, 206bp, 393bp, 188bp, 443bp, 1246bp, 284bp, 476bp respectively in both recurrent parent Uma and R-gene introgressed BC_2F_1 Plant No. 8.3.9.10. The above result indicated that BC_2F_1 Plant No. 8.3.9.10 possessed the same allele as that of the recurrent parent at these marker loci.

Presence of same allele in both the donor parent and R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 was evident on using RM 252, RM11554, RM 15583, RM 28267 markers as amplicon of size 786bp, 166bp, 272bp and 524bp were respectively produced in both the donor parent ISM and R-gene introgressed BC_2F_1 Plant No. 8.3.9.10.

However, the markers RM 13910 (254bp and 393bp), RM 15561 (284bp and 357bp), RM 19199 (476bp and 532 bp), RM 26213 (458bp and 504bp) and RM 26868 (410bp and 443bp) amplified the alleles of both the parents in the R-gene introgressed BC_2F_1 Plant No. 8.3.9.10. This pointed to the heterozygous nature of these marker loci in BC_2F_1 Plant No. 8.3.9.10. Results of background selection thus revealed that the R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 was similar to recurrent parent Uma at 13 out of 22 rice microsatellite marker loci while, it was similar to donor parent at four marker loci. Plant No. 8.3.9.10 was also found to be heterozygous at five marker loci. Such results are expected due to segregation and independent assortment of genes in the early backcross generations. Similarity between donor and R-gene introgressed backcross individuals and heterozygosity at marker loci as found in the study have been reported earlier (Sundaram *et al.*, 2008; Guvvala *et al.*, 2013, Nayak *et al.*, 2015; Pradhan *et al.*, 2015a and 2015b; Pradhan *et al.*, 2016; Baliyan *et al.*, 2018; Das *et al.*, 2018; Sagar *et al.*, 2018),

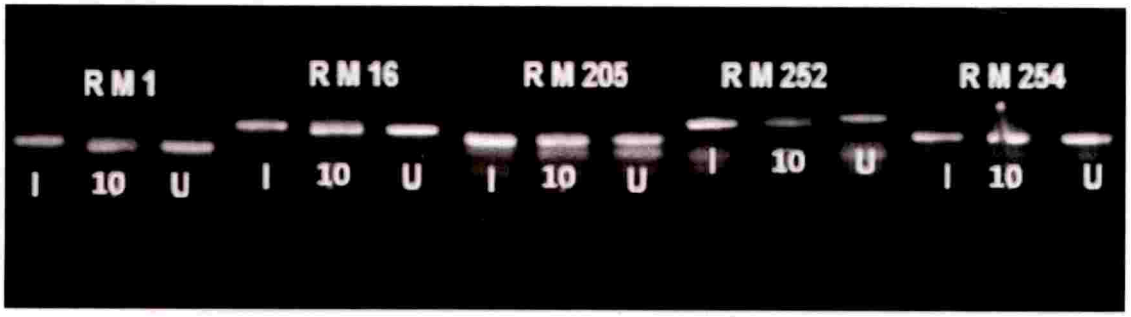


Plate 8(a). Background selection of the 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 using RM markers-I

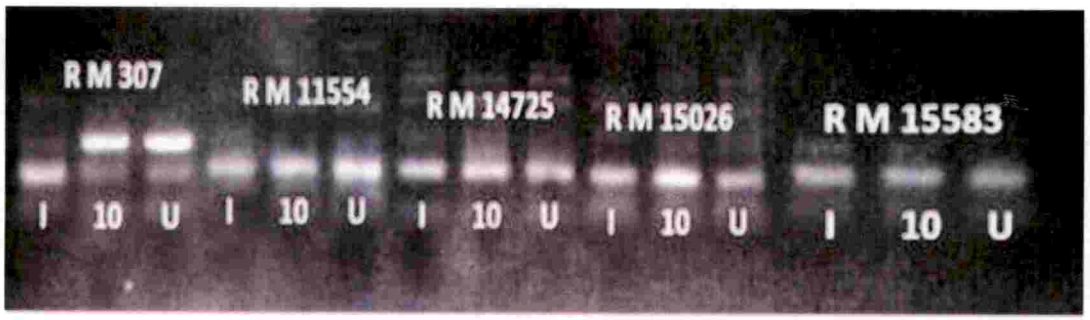


Plate 8(b). Background selection of the 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 using RM markers-II

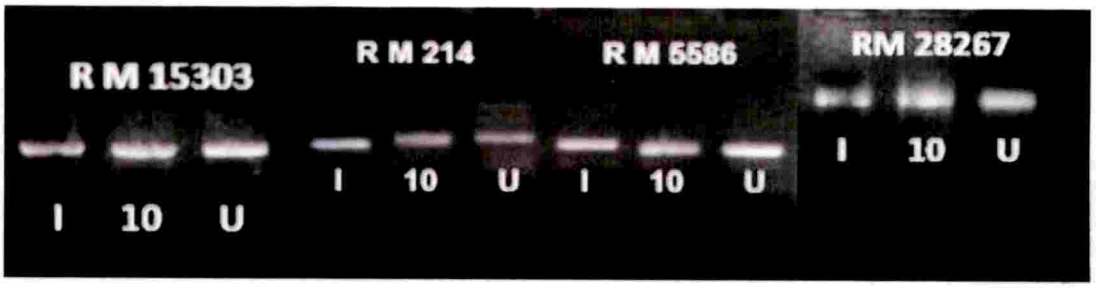


Plate 8(c). Background selection of the 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 using RM markers-III

I: ISM **U: Uma** **10: Plant No. BC2F1 8.3.9.10**

Plate 8(d). Background selection of the 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 using RM markers-IV

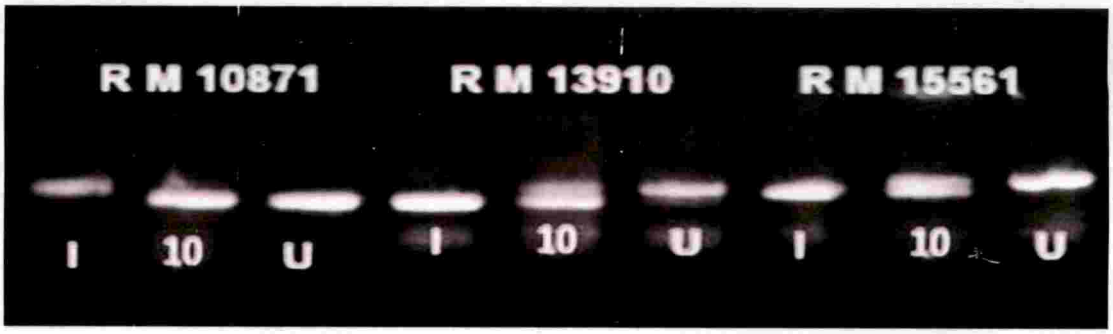


Plate 8(e). Background selection of the 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 using RM markers- V

I: ISM **U: Uma** **10: Plant No. BC2F1 8.3.9.10**

Table 16. Distribution of alleles of marker loci used for background selection in the 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 and parents

Sl. No.	RM marker	Chromosome No.	Recurrent parent	BC_2F_1 8.3.9.10	Donor parent
1	RM 1	1	A	A	B
2	RM16	3	A	A	B
3	RM 205	9	A	A	B
4	RM 214	4	A	A	B
5	RM 252	4	A	B	B
6	RM 254	11	A	A	B
7	RM 307	4	A	A	B
8	RM 5586	4	A	A	B
9	RM 10871	1	A	A	B
10	RM 11554	1	A	B	B
11	RM 13910	2	A	H	B
12	RM 14725	3	A	A	B
13	RM 15026	3	A	A	B
14	RM 15303	3	A	A	B
15	RM 15561	3	A	H	B
16	RM 15583	3	A	B	B
17	RM 17182	4	A	A	B
18	RM 19199	5	A	H	B
19	RM 24616	9	A	A	B
20	RM 26213	11	A	H	B
21	RM 26868	11	A	H	H
22	RM 28267	12	A	B	B

A –Allele similar to donor parent **B – Allele similar to recurrent parent**

H – Heterozygous loci

Table 17. Segregation of markers during background selection in 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 and parents

Sl. No.	Markers	Nature of amplification	Number of amplicon	Size of amplicon (bp)		
				Polymorphic		
				Donor parent (ISM)	BC2F1 Plant No. 8.3.9.10	Recurrent parent (Uma)
1	RM 1	Polymorphic	2	500	481	481
2	RM16	Polymorphic	2	834	698	698
3	RM 205	Polymorphic	2	494	487	487
4	RM 214	Polymorphic	2	206	299	299
5	RM 252	Polymorphic	2	786	786	786
6	RM 254	Polymorphic	2	487	487	487
7	RM 307	Polymorphic	2	166	370	370
8	RM 5586	Polymorphic	2	327	206	206
9	RM 10871	Polymorphic	2	409	393	393
10	RM 11554	Polymorphic	2	166	166	188
11	RM 13910	Polymorphic	2	254	254 and 393	393

(Contd....)

....Contd.

Table 17. Segregation of markers during background selection in 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 and parents

Sl. No.	Markers	Nature of amplification	Number of amplicon	Size of amplicon (bp)		
				Polymorphic		
				Donor parent (ISM)	BC2F1 Plant No. 8.3.9.10	Recurrent parent (Uma)
12	RM 14725	Polymorphic	2	200	188	188
13	RM 15026	Polymorphic	2	528	443	443
14	RM 15303	Polymorphic	2	1258	1246	1246
15	RM 15561	Polymorphic	2	284	284 and 357	357
16	RM 15583	Polymorphic	2	272	272	257
17	RM 17182	Polymorphic	2	357	284	284
18	RM 19199	Polymorphic	2	476	476 and 532	532
19	RM 24616	Polymorphic	2	524	476	476
20	RM 26213	Polymorphic	2	458	458 and 504	504
21	RM 26868	Polymorphic	2	410	410 and 443	443
22	RM 28267	Polymorphic	2	524	524	504

Incidentally, Joseph (2016) had reported that BC_1F_1 Plant No. 8.3.9 from which the present 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 has been developed, was similar to the donor at 21 out of 22 marker loci. This confirmed that the chance for recurrent parent genome recovery increases during each subsequent backcrosses. Singh *et al.* (2001) had pointed out that with each generation of backcrossing, the contribution of recurrent parent increases by one-half, while the undesirable donor allele contribution reduces considerably. The speedy recovery of recurrent parent genome can be achieved through repeated backcrossing followed by rigorous background selection through MAS. Later generations of backcrossing *viz.*, BC_2 and further generations have reported higher recurrent parent genome recovery (Sundaram *et al.*, 2008; Salgotra *et al.*, 2012; Suh *et al.*, 2013).

The result of background selection thus indicated a greater similarity between Plant No. 8.3.9.10 and recurrent parent genome. Unlike the results of present study, Rajpurohit *et al.* (2011) had reported reduced background recovery owing to higher linkage drag from the donor parent.

4.1.2.2. Recovery of recurrent parent (RP) genome in 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10

Results of the Graphical genotyping software GGT version 2.0 revealed that the per cent recovery of recurrent parent genome in the 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 was 81.82 (Fig. 2, Fig. 3 and Table 18) as against the expected recovery of 87.5 per cent. Meksem *et al.* (2009) had reported a lower recovery of recurrent parent genome than expected (75%) in BC_1F_1 generation studied and suggested that further backcrossing to recurrent parent may result in the recovery of 100 per cent recurrent parent genome. Similar to the findings of the present study, Sundaram *et al.*, (2008) found that the recurrent parent genome contribution in the backcross progeny was less than the expected for a cross between Samba Mahsuri and SS1113. They attributed the phenomenon to the

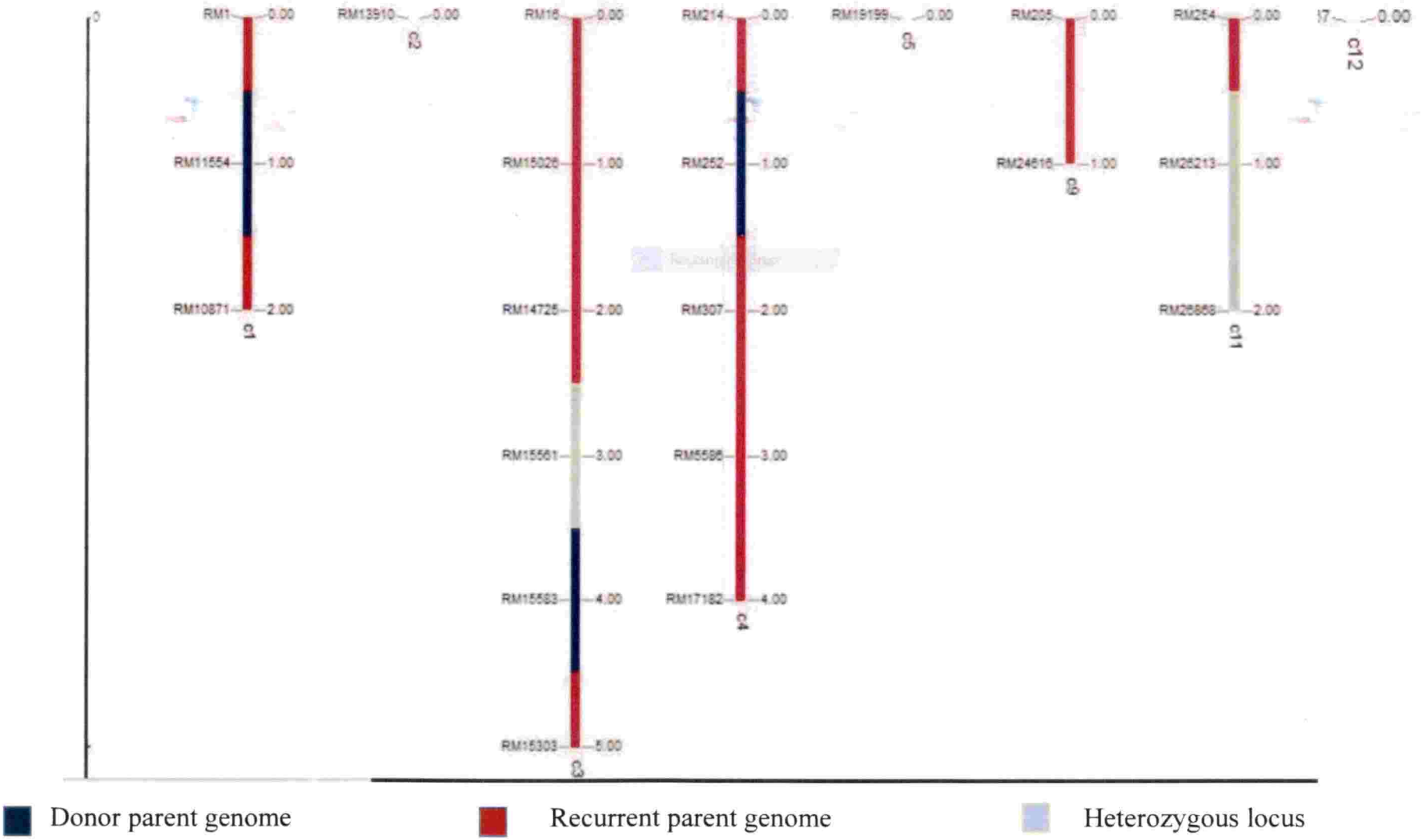


Figure 2. Graphical genotyping of 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10

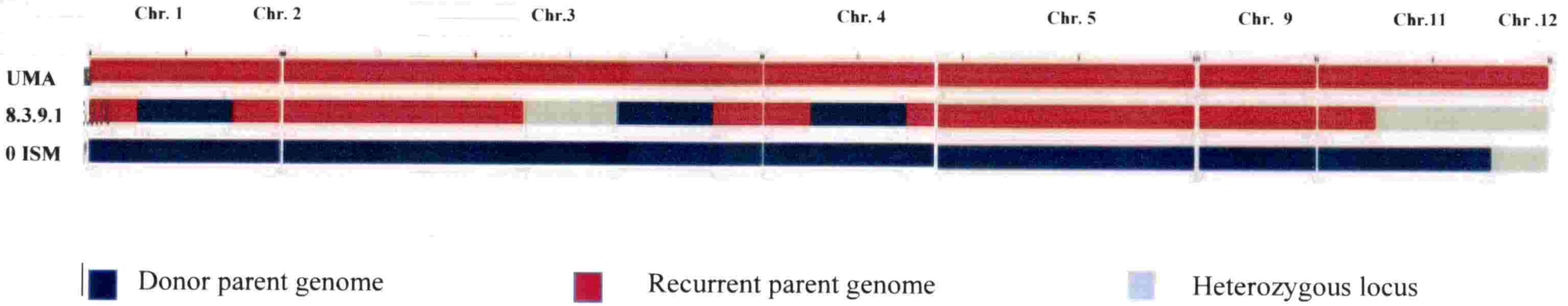


Figure 3. Recovery of recurrent parent genome in 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10

presence of some unknown mechanism resulting in the inheritance of undesirable donor parent loci in the backcross progenies.

Table 18. Contribution of recurrent parent genome (Uma) in 3-R-gene introgressed plants

Sl.No.	Details of genome	Plant No.	
		**BC_1F_1 Plant No.8.3.9	BC_2F_1 Plant No. 8.3.9.10
1	Per cent recovery of parent genome*	21.80	81.82
2	Similarity coefficient based on marker data (Dendrogram)	10.05	60.00

*Computed as per Sundaram *et al.* (2008)

**Joseph, 2016

The lower magnitude of recovery of RP genome in the present study may be the attributed to the low RP genome (21.80%) of the corresponding 3-R-gene introgressed BC_1F_1 parent plant (BC_1F_1 Plant No.8.3.9) as reported by Joseph (2016). Further, considering the size of rice genome (400-450Mbp), the use of extremely low number of markers (only 22) for background selection may alter the estimate of RP genome recovery in the R-gene introgressed plant. Several reports of variable extent of recovery of RP genome background in the R-gene introgressed lines obtained through MAS has been reported (Rajpurohit *et al.,* 2010; Dokku *et al.,* 2013a and 2013b; Nayak *et al.*, 2015; Arunakumari *et al.,* 2016; Kumar *et al.*, 2016; Sagar *et al.*, 2018)

The dendrogram (Fig.4) grouped the individuals (parents and Plant No. 8.3.9.10) into two clusters; cluster 1 being monogenic with only the donor parent ISM and cluster 2 comprised of the recurrent parent Uma and Plant No. 8.3.9.10, further suggesting that the 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 is more similar to the recurrent parent Uma. As in the present study, Dokku *et al.* (2013a) generated a dendrogram, which grouped 10 pyramided lines and two parents into two clusters. However, donor parent IRBB60 and four pyramided lines was grouped

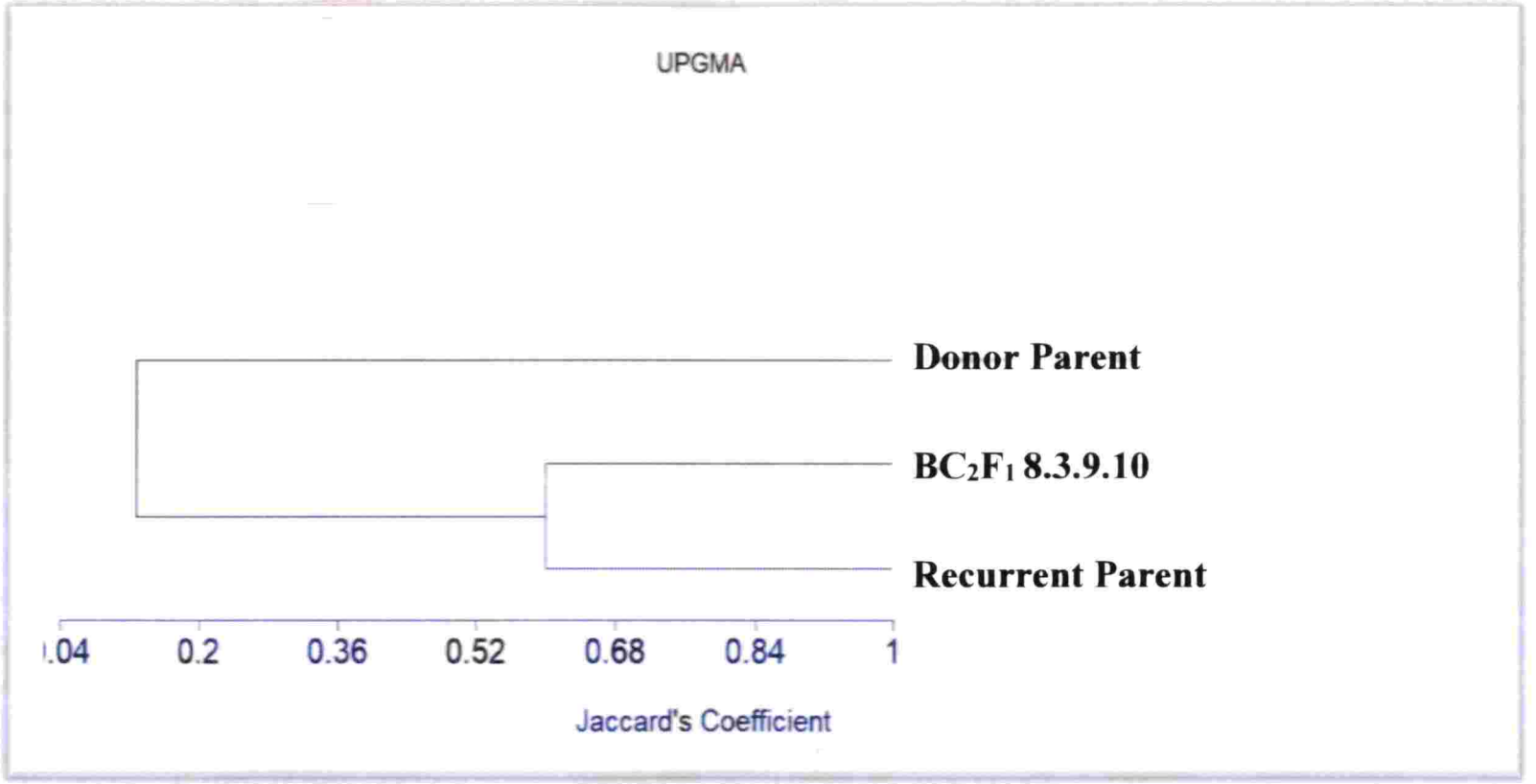


Figure 4. Clustering of 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 and parents based on marker segregation

as cluster I, while the remaining lines and the recurrent parent Tapaswini grouped under cluster II.

The availability of closely linked markers to the target locus and/or flanking markers, the size of the population, the number of backcrosses, and the position as well as the number of background markers determines the effectiveness of marker assisted breeding (Frisch and Melchinger; 2005; Hasan *et al.,* 2015; Deshmukh *et al.,* 2017). According to Rajpurohit *et al.* (2011) and Suh *et al.* (2013), the best strategy to pyramid or introduce multiple genes and recover a maximum recurrent parent background effect in the shortest time will be to take up the transfer of genes simultaneously, generate a large backcross population and select the target genes through foreground selection and flanking marker analysis to reduce the persistent linkage drag. Hence, it is advisable to grow a large back cross population to recover multi-gene pyramids.

Advancing the backcross generations has been opined to integrate higher recurrent parent genome contribution (Pradhan *et al.*, 2015a; Cuc *et al.*, 2012; Dash *et al.*, 2016).

4.2 Production of BC_3F_1s

Reduced linkage drag for the resistance genes (*xa5, xa13* and *Xa21*) results in higher recovery of the recurrent parent genome in the later backcross generations (Pradhan *et al.*, 2015a; Suh *et al.*, 2013; Dokku *et al.*, 2013a). Hence, further backcrossing of the 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 will result in a cultivar with durable resistance to BB pathogen.

Considering the above, backcrossing of the R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 to the RP (Uma) has resulted in the production of 15 BC_3F_1s (Table 19). These BC_3F_1s may be further subjected to foreground and background profiling to identify and recover the 3-R-gene pyramided lines with maximum recovery of recurrent parent.

4.3 Production of BC_2F_2s

Homozygosity at the various bacterial blight gene loci in a heterozygous individual can be achieved by selfing. Joseph *et al.* (2004) resorted to combined phenotypic selection along MAS after selfing, resulting in the recovery of 80.40 to 86.70 per cent recurrent parent genome of Pusa Basmati 1 in BC_1F_3 generation with two BB resistant genes, *xa13* and *Xa21* of along with the grain and cooking quality characteristics and desirable agronomic features.

In lieu with the above, to attain homozygosity for the three R-gene loci, selfing of BC_2F_1 Plant No. 8.3.9.10 was attempted. This resulted in the production of 28 BC_2F_2s (Table 19). The reduced seed set observed on selfing of BC_2F_1 may be due to the result of high temperature prevailing (25-36^{0}C) during the flowering time (March-April). Floral abnormalities induced by heat stress (i.e. stamen hypoplasia and pistil hyperplasia), leading to spikelet sterility causes reduction in rice production (Takeoka *et al.*, 1991) The seeds obtained are to be subjected to foreground and background selection to identify the presence of stable 3-R-gene pyramided lines for production of BB resistant individuals.

Table 19. BC_3F_1 and BC_1F_3 progenies of the 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10

Sl. No.	Progenies of Plant No. 8.3.9.10	Seeds obtained (No.)
1	BC_3F_1s	15
2	BC_2F_2s	28

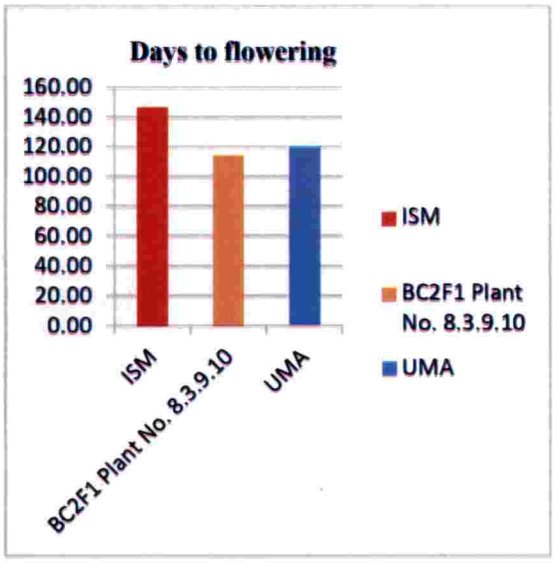
4.4 Morphological characterization of BC_2F_1s

Backcross breeding programme aims at integration of one or few desirable traits into an otherwise desirable cultivar. In the present study, introgression of BB resistance was envisaged into the elite rice variety Uma (Mo 16) cultivated to a very large extent in Kerala. It is desirable that the R-gene introgressed lines resemble the RP parent at both morphological and genic level. In order to analyse the resemblance of the BC_2F_1s to the parents, morphological characterization was conducted.

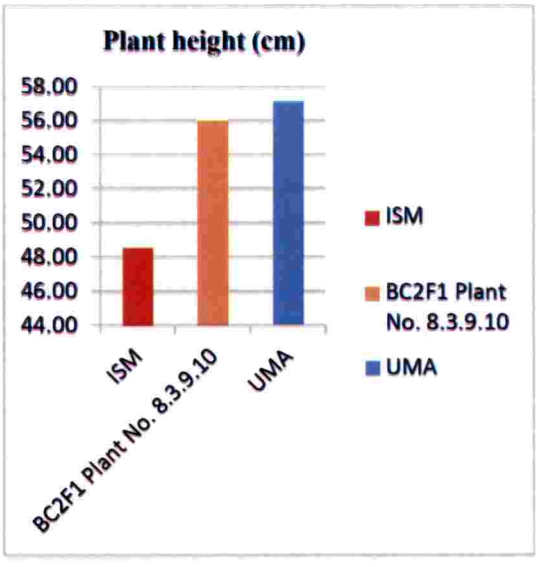
Results [Tables 20, 21, 22 and Appendices III, IV, V and VI] pointed to existence of wide variability in the BC_2F_1s.

Plant height of recurrent parent (RP) was 57.17 cm while it was 48.62 cm in donor. Flowering occurred very late in the donor parent (DP) (148 days) compared to RP (120 days). The leaves were longer (RP: 31.10 cm; DP: 36.97 cm) and thinner in the donor parent (RP: 0.94 cm; DP: 0.91 cm). Productive tiller production was comparatively higher in RP (14 Nos.) than in donor (8.00). However, panicle length (RP: 16.37 cm; DP: 21.94 cm) and number of spikelets per panicle (RP: 86; DP: 94) was higher in donor than in RP. The length of grain (RP: 6.47 mm; DP: 6.49 mm) and decorticated grain (RP: 5.80 mm; DP: 5.47 mm) was near similar in both the parents. But, the width of both grain (RP: 2.81 mm; DP: 1.74 mm) and decorticated grain (RP: 2.34 mm; DP: 1.45 mm) in donor was slender in comparison to the recurrent parent.

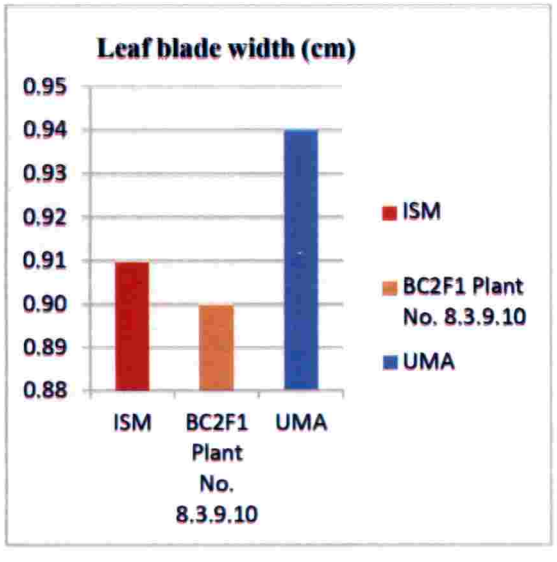
Comparison of the 3-R-gene pyramid (BC_2F_1 Plant No. 8.3.9.10) with the recurrent parent (Uma) and donor parent (ISM) [Fig. No. 5(a) to 5(k)] revealed greater similarity between the gene pyramid and recurrent parent (Uma) with respect to days to flowering (115 days), plant height (56.10 cm), length and width of leaf blade (28.50 cm and 0.90 cm respectively), panicle length (13.46 cm), length of decorticated grain (6.00mm) and kernel colour (red). Baliyan *et al.* (2018) observed that most three gene pyramids in their study were similar or superior to the recurrent parent CSR-30 for the agronomic traits.



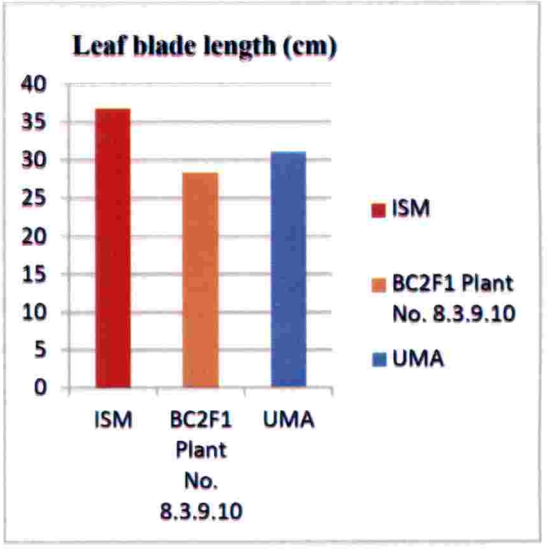
(a) Days to flowering



(b) Plant height

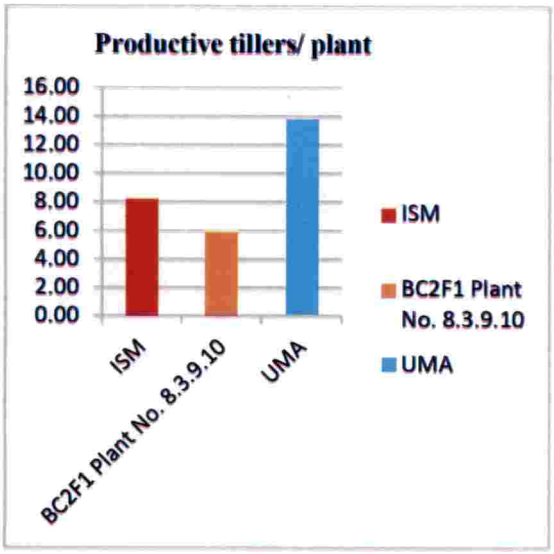


(c) Leaf blade width (cm)

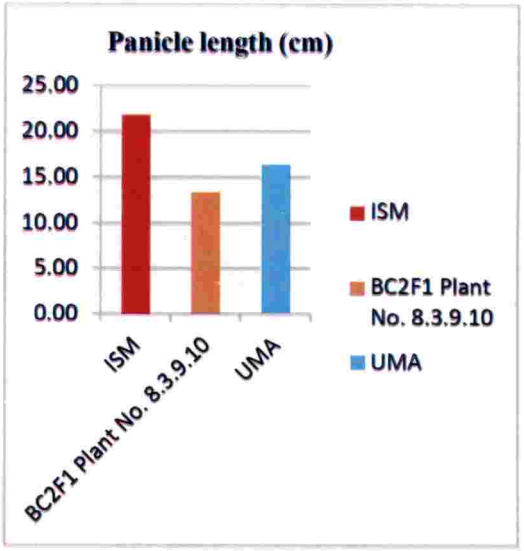


(d) Leaf blade length (cm)

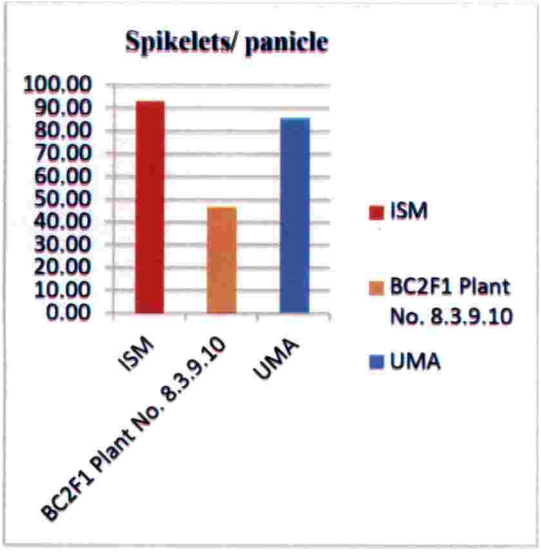
Figure 5 (a) to 5 (d). Morphological characteristics of 3-R-genes introgressed BC_2F_1 Plant No. 8.3.9.10 and parents



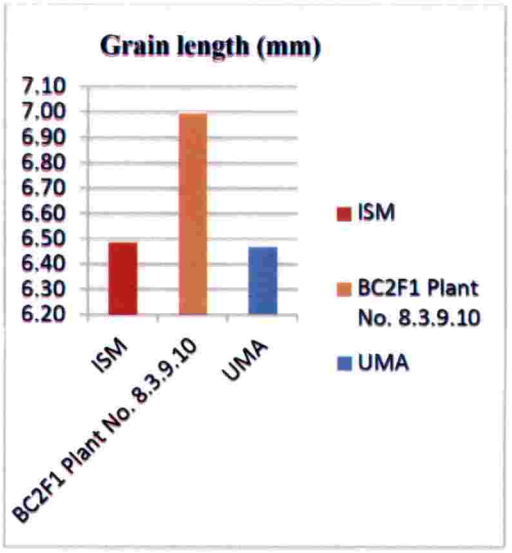
(e) Productive tillers/plant



(f) Panicle length (cm)

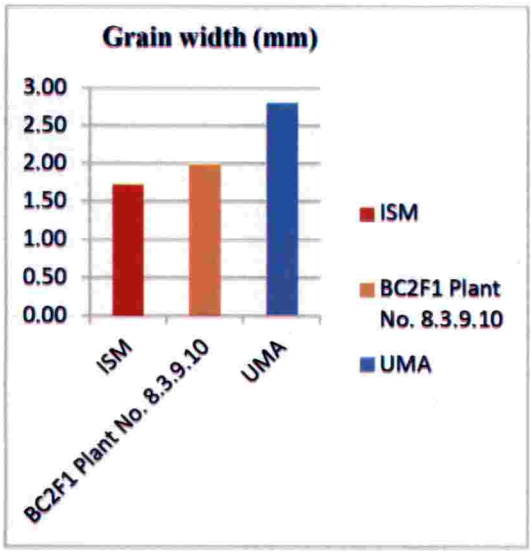


(g) Spikelets/panicle

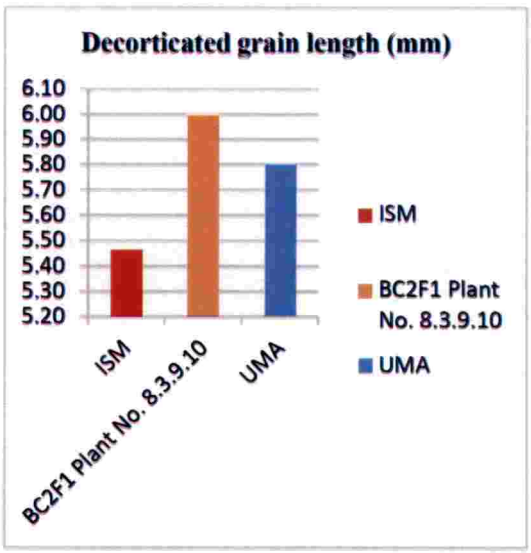


h) Grain length (mm)

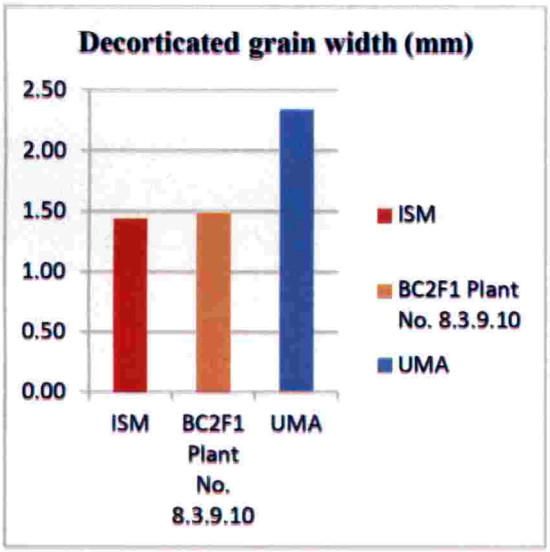
Figure 5 (e) to 5 (h). Morphological characteristics of 3-R-genes introgressed BC_2F_1 Plant No. 8.3.9.10 and parents



(i) Grain width (mm)



(j) Decorticated grain length (mm)



(k) Decorticated grain width (mm)

Figure 5 (i) to 5 (k). Morphological characteristics of 3-R-genes introgressed BC_2F_1 Plant No. 8.3.9.10 and parents

Table 20. Variability in morphological characteristics among BC_2F_1s derived from BC_1F_1 Plant No.8.3.2 and parents

SI. No	Plant character	Recurrent parent (Uma)			Donor parent (ISM)			BC_2F_1s of 8.3.2			BC_1F_1 Plant No.8.3.2 (Parental genotype)
		Range		Mean	Range		Mean	Range		Mean	
		Minimum	Maximum		Minimum	Maximum		Minimum	Maximum		
1	Days to Flowering	115	125	120	143	152	148	115	132	126	234
2	Plant height (cm)	52.40	62.10	57.17	45.10	52.52	48.62	37.50	48.90	42.00	48.97
3	Leaf width (cm)	0.83	1.06	0.94	0.83	0.99	0.91	0.70	0.87	0.77	1.40
4	Leaf blade length (cm)	24.00	37.30	31.10	30.16	40.46	36.97	21.80	30.40	25.87	44.88
5	Productive tillers / plant	11	16	14	7	10	8	6	10	8	7
6	Panicle length (cm)	14.70	18.10	16.37	21.10	23.10	21.94	13.90	18.56	16.46	20.38
7	Spikelets / panicle	70	98	86	85	98	94	66	74	69	88
8	Grain length (mm)	6.10	6.92	6.47	6.30	6.80	6.49	6.00	7.50	7.00	6.12
9	Grain width (mm)	2.71	2.92	2.81	1.50	1.90	1.74	2.00	2.00	2.00	2.76
10	Decorticated grain length(mm)	5.33	6.01	5.80	4.90	5.90	5.47	5.00	5.50	5.12	5.81
11	Decorticated grain width (mm)	2.23	2.46	2.34	1.30	1.60	1.45	1.50	2.00	1.87	2.21

Table 21. Variability in morphological characteristics among BC_2F_1s of BC_1F_1 Plant No.8.3.3 and parents

SI. No	Plant character	Recurrent parent (Uma)			Donor parent (ISM)			BC_2F_1s of 8.3.3			BC_1F_1 Plant No.8.3.3 (Parental genotype)
		Range		Mean	Range		Mean	Range		Mean	
		Minimum	Maximum		Minimum	Maximum		Minimum	Maximum		
1	Days to Flowering	115	125	120	143	152	148	115	135	125	228
2	Plant height (cm)	52.40	62.10	57.17	45.10	52.52	48.62	36.00	62.00	45.73	51.12
3	Leaf width (cm)	0.83	1.06	0.94	0.83	0.99	0.91	0.70	0.90	0.77	1.6
4	Leaf blade length (cm)	24.00	37.30	31.10	30.16	40.46	36.97	20.50	36.90	27.64	43.96
5	Productive tillers / plant	11	16	14	7	10	8	3	10	7	12
6	Panicle length (cm)	14.70	18.10	16.37	21.10	23.10	21.94	9.60	18.13	13.57	21.63
7	Spike lets / panicle	70	98	86	85	98	94	20	81	55	88
8	Grain length (mm)	6.10	6.92	6.47	6.30	6.80	6.49	6.00	7.50	6.93	6.18
9	Grain width (mm)	2.71	2.92	2.81	1.50	1.90	1.74	2.00	2.00	2.00	2.8
10	Decorticated grain length (mm)	5.33	6.01	5.80	4.90	5.90	5.47	4.00	5.00	4.57	5.78
11	Decorticated grain width (mm)	2.23	2.46	2.34	1.30	1.60	1.45	1.50	2.00	1.79	2.22

Table 22. Variability in morphological characteristics among BC_2F_1s of BC_1F_1 Plant No.8.3.9 and parents

SI. No	Plant character	Recurrent parent (Uma)			Donor parent (ISM)			BC_2F_1s of 8.3.9			BC_1F_1 Plant No. 8.3.9 (Parental genotype)
		Range		Mean	Range		Mean	Range		Mean	
		Minimum	Maximum		Minimum	Maximum		Minimum	Maximum		
1	Days to Flowering	115	125	120	143	152	148	115	155	126	158
2	Plant height (cm)	52.40	62.10	57.17	45.10	52.52	48.62	33.00	59.00	45.22	43.21
3	Leaf width (cm)	0.83	1.06	0.94	0.83	0.99	0.91	0.70	1.00	0.86	1.10
4	Leaf blade length (cm)	24.00	37.30	31.10	30.16	40.46	36.97	17.90	33.00	25.49	37.51
5	Productive tillers / plant	11	16	14	7	10	8	4	17	8	9
6	Panicle length (cm)	14.70	18.10	16.37	21.10	23.10	21.94	11.97	17.53	14.88	19.98
7	Spike lets / panicle	70	98	86	85	98	94	22	80	60	90
8	Grain length (mm)	6.10	6.92	6.47	6.30	6.80	6.49	6.00	7.00	6.50	6.2
9	Grain width (mm)	2.71	2.92	2.81	1.50	1.90	1.74	1.50	2.00	1.90	2.75
10	Decorticated grain length (mm)	5.33	6.01	5.80	4.90	5.90	5.47	4.00	6.00	4.90	5.79
11	Decorticated grain width (mm)	2.23	2.46	2.34	1.30	1.60	1.45	1.50	2.00	1.70	2.31

The pyramid was found to exhibit greater similarity to the donor parent with respect to number of productive tillers/ plant (6 Nos.), width of grain (2.00 mm) and decorticated grain (1.50 mm). However, the length of grain (7.00 mm) as well as decorticated grain (6.00 mm) in the gene pyramid was found to be greater than that of the parents. Classification of grain [Plate 9(a)] (IRRI, 1996) pointed out that the R-gene pyramid produced long slender grains while it was medium in recurrent parent and medium slender in the donor parent. Unlike the grain, the kernel of the pyramid as well as recurrent parent was red and long in comparison to medium white kernels of the donor [Plate 9(b)]. Joseph (2016) had also reported similarity in kernel colour and shape between the R-gene pyramided BC_1F_1 Plant No. 8.3.9 and the recurrent parent Uma.

Resorting to phenotype-based selection coupled with marker-assisted selection, Arunakumari *et al.* (2016) could recover the desirable plant and grain type in improved version of MTU1010. Similarly, Sagar *et al.* (2018) identified novel segregants with basmati grain qualities in backcross population through MAS aiming to introgress resistance to both BB and blast disease in early maturing basmati rice variety PB 1509. Earlier, Pradhan *et al.* (2015b) had reported complete recovery of yield and grain quality traits in Jalamagna alongwith transfer of three BB resistance genes through MAS.

4.4.2. Clustering of parents and BC_2F_1s based on morphological characters

Based on morphological characters, clustering of parents and the BC_2F_1s was done. At 80 per cent similarity coefficient, the BC_2F_1s and parents grouped into 10 major clusters (Fig. 6). Donor parent ISM formed a monogenic cluster. The 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 exhibited 45.39 per cent similarity to the recurrent parent Uma and 26.86 per cent similarity to the donor parent ISM. Suh *et al.* (2013) had reported that the insertion of even a small chromosome segment may greatly affect the phenotype of the plant thus even though the R-gene introgressed individual exhibit high RP genome recovery. The pyramids may not

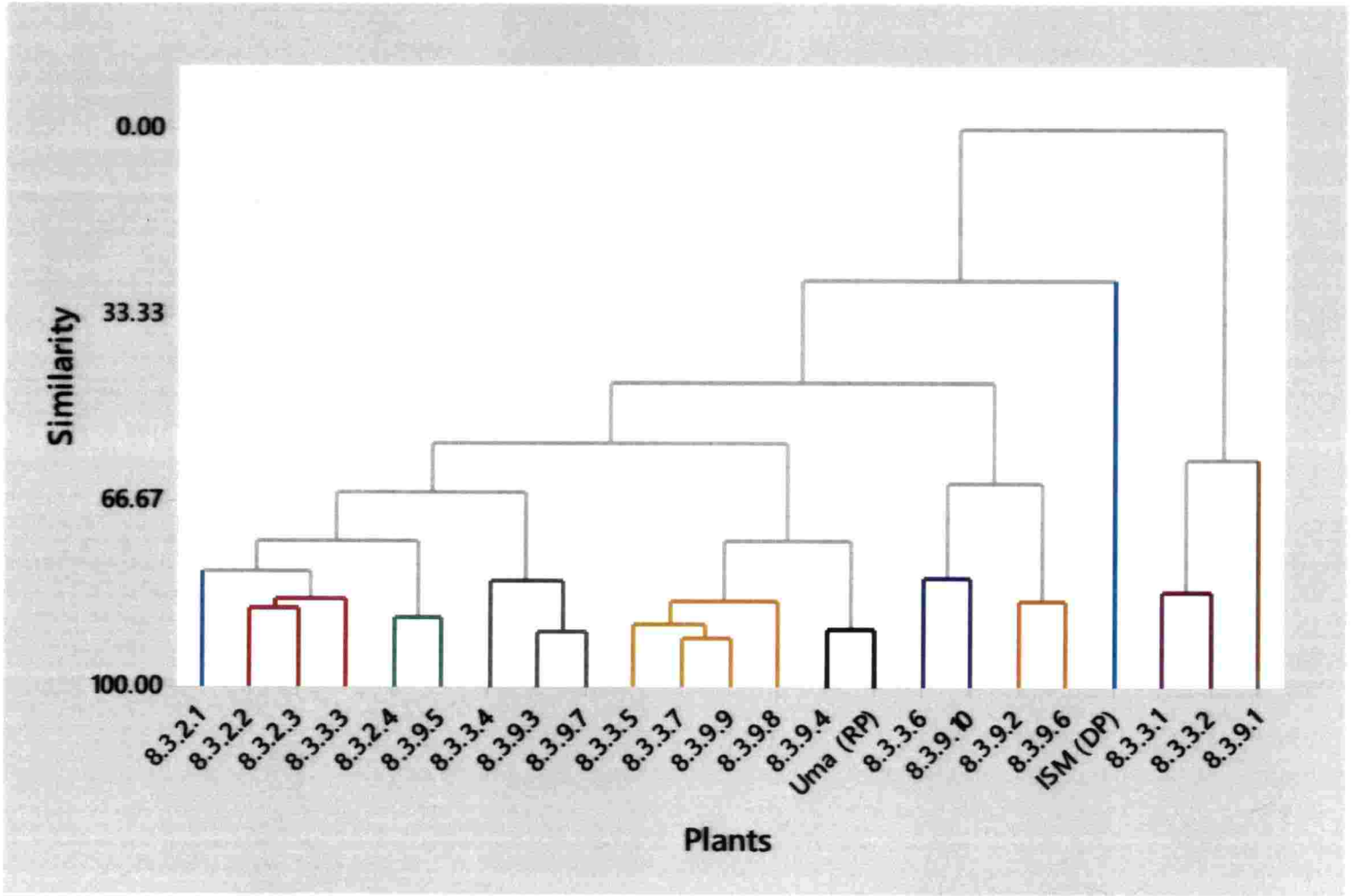


Figure 6. Clustering of BC_2F_1s Plant No. 8.3.9.10 and parents based on morphological traits

Plate 9(a). Grains of parents and 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10

Plate 9(b). Decorticated grains of parents and 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10

resemble the phenotype of the recurrent parent owing to the internal and external environment.

Similar to the clustering of genotypes based on molecular data, the clustering based on morphological data also placed the 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 closer to the recurrent parent than the donor parent [Plate 10(a), 10(b) and 10(c)]. Hence, it may be inferred that combining agro-morphological evaluation of genotypes and molecular genotyping would be more advantageous in rapid recovery of desirable genotypes with higher recurrent parent genome.

4.5. Bioassay of BC_1F_2s

Pathotyping of BC_1F_2s derived from the 3-R-gene pyramided BC_1F_1s (Plant No. 8.3.2, Plant No. 8.3.3 and Plant No. 8.3.9) in the earlier experiment (Joseph, 2016) was conducted in two stages.

4.5.1. Pathotyping –Set I

Initially, 450 BC_1F_2s (150 each from BC_1F_1 Plant No. 8.3.2, Plant No. 8.3.3 and Plant No. 8.3.9) were raised [Table 23, Plate 11] to assess their resistance to BB pathogen. Poor seedling emergence was observed (Plant No. 8.3.2: 150 Nos., Plant No. 8.3.3: 9 Nos. and Plant No. 8.3.9: 150 Nos.). Natural incidence of BB pathogen produced pronounced *kresek* symptom [Plate 11] in the seedling stage (14 days after sowing; DAS). Occurrence of BB coupled with incidence of blast disease within 14 DAS lead to severe mortality. None of the BC_1F_1s survived.

Table 23. Pathotyping of BC_1F_2s- Set I

Sl.No.	BC_1F_2 progenies of Plant No.	Number of seeds sown	Number of seeds germinated and screened for BB resistance
1	8.3.2	150	125
2	8.3.3	150	9
3	8.3.9	150	138
Total		**450**	**272**

Plate 10(a). Donor parent (ISM)

Plate 10(b). Recurrent parent (Uma)

Plate 10(c). 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10

4.5.2. Pathotyping –Set II

Of the remaining 400 BC_1F_2 (Plant No. 8.3.2: 120 Nos., Plant No. 8.3.3: 5 Nos. and Plant No. 8.3.9: 74 Nos.) only 83 and 23 seeds of 8.3.2 and 8.3.9 respectively germinated (Table 24 and Plate 12). No germination was observed in seeds of Plant No. 8.3.3. Hence, evaluation of resistance against BB pathogen through leaf clipping method was done in the 106 BC_1F_2s that survived.

Table 24. Pathotyping of BC_1F_2s- Set II

Sl. No.	BC1F2 progenies of Plant No.	Number of seeds sown	Number of seeds germinated	Number of seeds germinated and screened for BB resistance
1	8.3.2	123	120	83
2	8.3.3	134	5	0
3	8.3.9	143	74	23
Total		**400**	**199**	**106**

Inoculation of BC_1F_2s with virulent *Xoo* isolate (Plate No.13 and 14) from Kerala was done at maximum tillering stage (45-59 days) during May 2018 under field conditions. The pre- monsoon showers provided an ideal condition for disease spread. The severity of lesions was scored 15 days after inoculation based on the per cent of diseased leaf area (DLA) in accordance with the Standard Evaluation System (SES) [Plate 16] for rice (IRRI, 2002).

Results (Table 25 and 26, Plates 15,17,18 and 19, Appendix VII) indicated that out of 83 BC_1F_2 progenies of BC_1F_1 Plant No. 8.3.2., 55.41 per cent exhibited resistance reaction to BB infection, while, 16.87 per cent were moderately resistant, 10.84 per cent moderately susceptible, 4.82 per cent susceptible and 12.4 per cent highly susceptible.

Plate 11. Seedlings of BC_1F_2s exhibiting '*kresek*' symptom: Pathotyping Set-I

Plate 12. BC_1F_2s raised for bioassay: Pathotyping Set-II

Plate 13. Inoculation of BC_1F_2s with BB pathogen

Plate 14. Incubation of inoculated BC_1F_2s during pathotyping

Plate 15. Bioassay of BC_1F_2s for resistance to BB pathogen

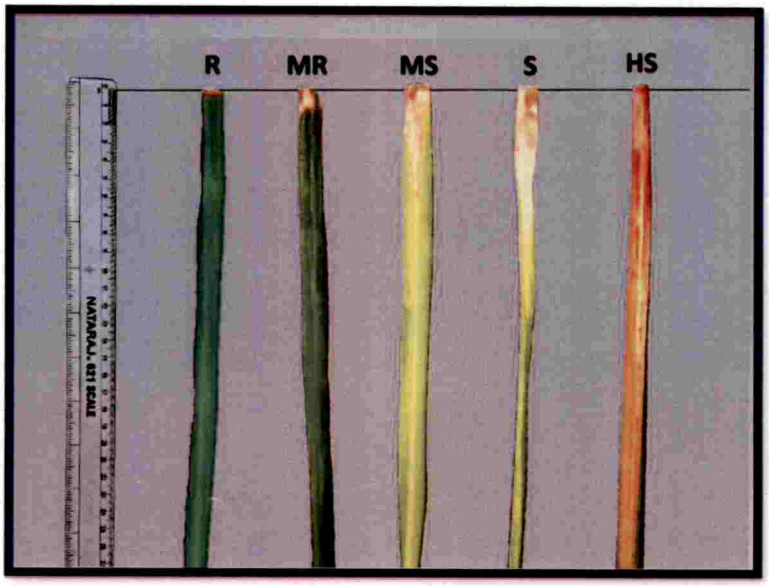


Plate 16. Scoring of BC_1F_2 leaves based on SES

Uma **ISM**

Plate 17. Reaction of parents to BB infection during pathotyping

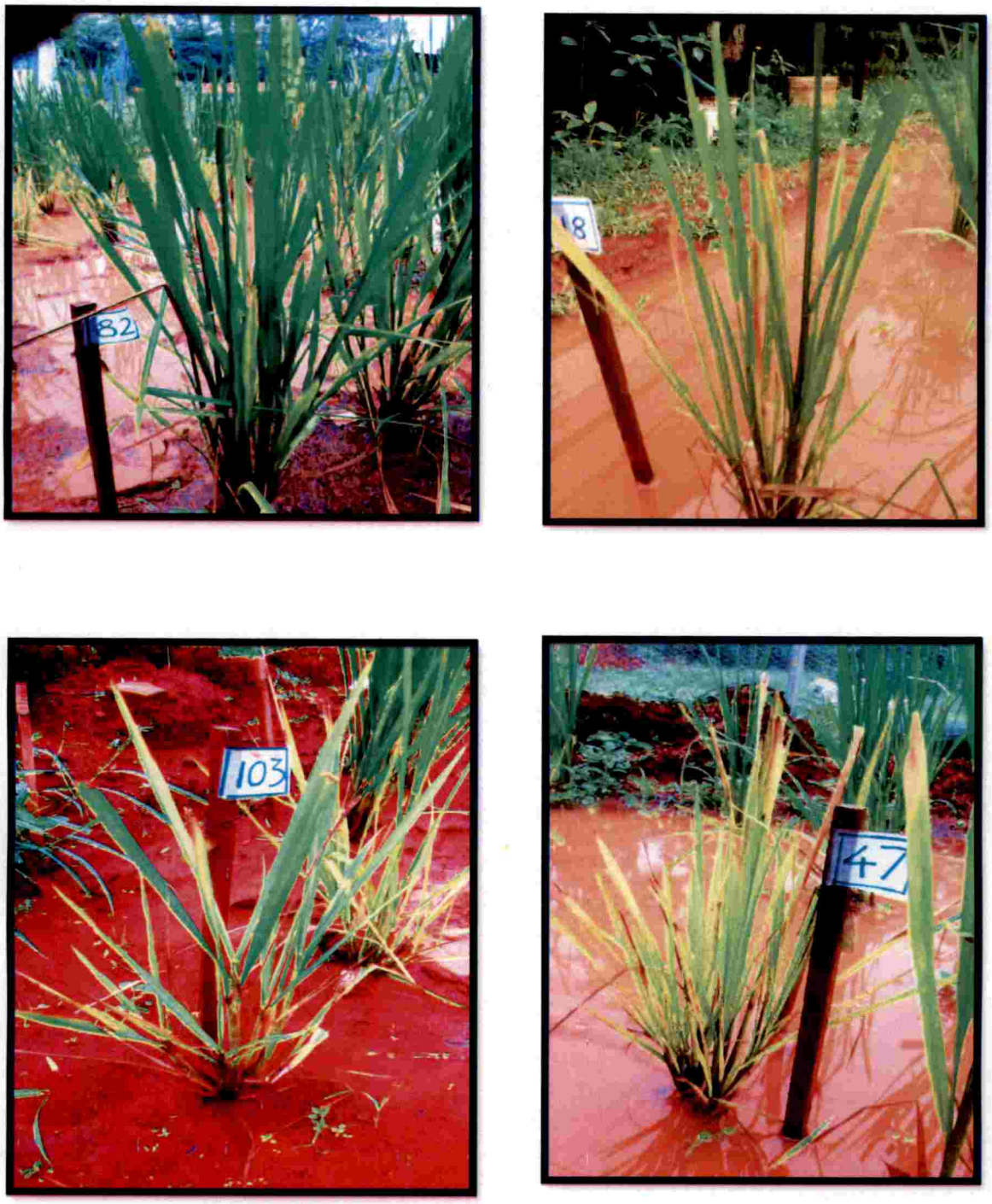


Plate 18. Reaction of BC_1F_2s during pathotyping

Plate 19. BC_1F_2 genotype exhibiting high susceptibility to BB infection

Similarly, over half (52.17%) the BC_1F_2 progenies derived from BC_1F_1 Plant No. 8.3.9 exhibited resistance to BB infection. However, unlike in the earlier case, 34.78 per cent were moderately resistant, 8.69 per cent moderately susceptible, 0.00 per cent susceptible and 4.34 per cent highly susceptible.

Pathotyping thus revealed that in general, higher per cent of BC_1F_2 individuals derived from BC_1F_1 Plant No. 8.3.9 showed resistance to moderate resistance to BB pathogen (Fig.7) in comparison to those derived from BC_1F_1 Plant No. 8.3.2.

This results indirectly indicated the presence of appropriate R-gene combinations in the BC_1F_2 individuals that exhibited resistant to moderately resistant reaction to BB pathogen. It may be expected that the resistant individuals may possess the gene combination *xa5xa5* + *xa13xa13* + *Xa21Xa21*. However, further confirmation through molecular assay and pathotyping is required to decisively conclude the order and nature of gene combinations that had elucidated resistance reaction to BB infection.

The bioassay against BB also revealed the presence of high levels of resistance in the BC_1F_2 individuals compared to recurrent parent Uma. Similar instances of improvement in resistance to BB pathogen in cultivars introgressed with various combinations R-genes through MAS have been reported (Zhang *et al.*, 2001; Sanchez *et al.*, 2000, Singh *et al.*, 2001; Guvvala *et al.*, 2013; Das *et al.*, 2015; Pradhan *et al.*, 2015a and 2015b; Luo *et al.*, 2016; Mubassir *et al.*, 2016; Baliyan *et al.*, 2018; Das *et al.*, 2018)

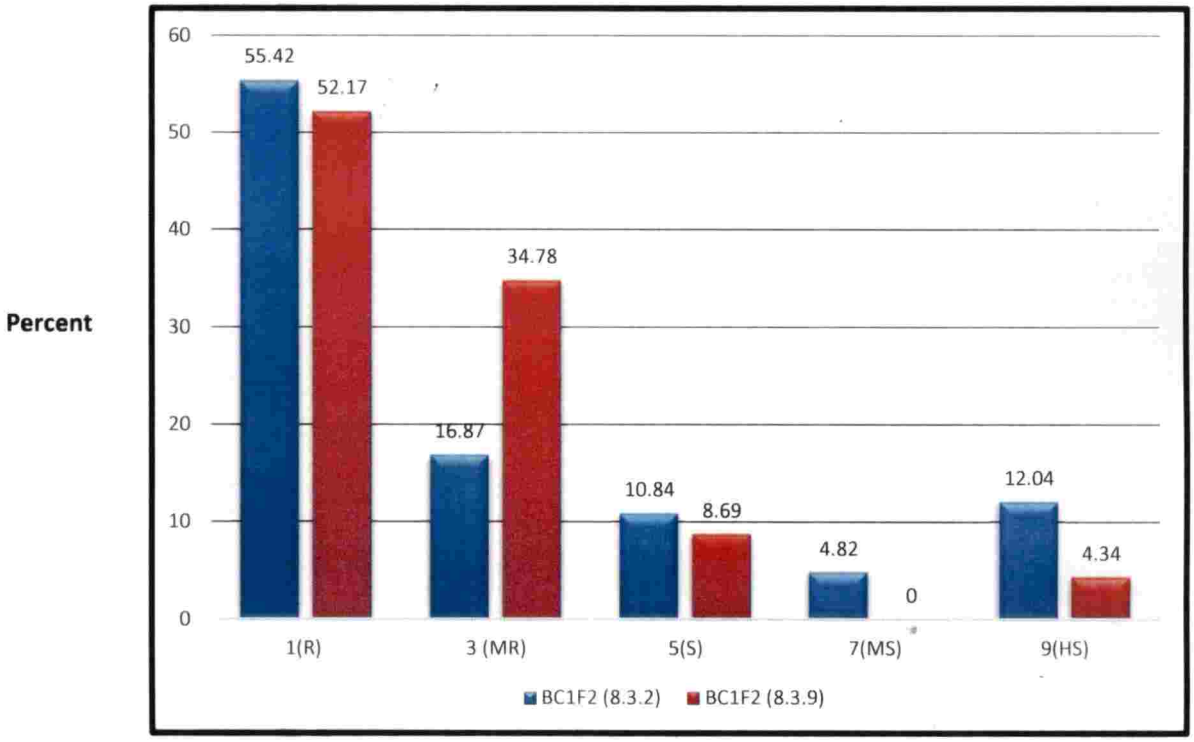


Figure 7. Grouping of BC_1F_2s based on BB incidence

Table 25. Grouping of BC_1F_2 progenies of BC_1F_1 Plant. No. 8.3.2 and Plant. No. 8.3.9 based on diseased leaf area (DLA)

Diseased Leaf Area per cent	Scale	Description	BC1F2 (8.3.2)	BC1F2 (8.3.9)
1-5	1	Resistant (R)	46	12
6 – 12	3	Moderately resistant (MR)	14	8
13 – 25	5	Moderately susceptible (MS)	9	2
26 – 50	7	Susceptible (S)	4	0
>50 %	9	Highly susceptible (HS)	10	1
Total			**83**	**23**

Table 26. Grouping of BC_1F_2 progenies of BC_1F_1 Plant. No. 8.3.2 and Plant. No. 8.3.9 based on reaction to BB infection

Diseased Leaf area (%)	Scale	Description	Individuals (%)			
			BC1F2 progeny of BC1F1 Plant No. 8.3.2	BC1F2 progeny of BC1F1 Plant No. 8.3.9	ISM	Uma
1-5	1	Resistant (R)	55.42	52.17	100	0.00
6 – 12	3	Moderately resistant (MR)	16.87	34.78	0.00	0.00
13 – 25	5	Moderately susceptible (MS)	10.84	8.69	0.00	0.00
26 – 50	7	Susceptible (S)	4.82	0.00	0.00	0.00
>50 %	9	Highly susceptible (HS)	12.04	4.34	0.00	100

4.6. Production of BC_1F_3s

Selfing of the BC_1F_2 individuals that exhibited resistance to moderate resistance reaction to BB infection was done to obtain 725 BC_1F_3 seeds. These are to be evaluated further through MAS well as agro-morphological screening to isolate novel genotypes with BB resistance.

To conclude, conferring broad spectrum resistance against BB to elite cultivar Uma widely grown across the rice belts of Kerala is a major challenge owing to a number of genetically distinct virulent *Xoo* strains in the different rice growing eco-systems in the state. Pyramided lines carrying two, three or four BB resistance R-genes are found to confer broad spectrum resistance than lines with single R-genes. The R-gene combination of *xa5* + *xa13* + *Xa21* is reported to impart resistance to Kerala isolates of *Xoo*. MAS has helped in the successful integration of the 3-R genes in the backcross of Uma. The foreground and background selection in the study has enabled identification of a 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10. (*xa5xa5* + *Xa13xa13* + *Xa21xa21*), with high recovery of recurrent parent background in the early backcross generations. Isolation of individuals with recessive R-gene in heterozygous combinations is impossible through conventional approach. Further backcrossing of the R-gene introgressed pyramids and their evaluation through a combination of MAS and phenotypic evaluation will lead to development of BB resistant cultivar in the background of cultivar Uma. The novel gene combinations arising in the advanced breeding lines developed from the backcross generations of R-gene pyramids can serve as base population for future breeding programmes.

Summary

V. Summary

The research programme 'Marker assisted backcross breeding for pyramiding genes conferring resistance to bacterial blight in rice variety Uma (Mo16),' was carried out between 2016 and 2018 in the Department of Plant Breeding and Genetics, College of Horticulture, Kerala Agricultural University, Vellanikkara, Thrissur.

Uma is among the most widely cultivated high yielding rice varieties in Kerala. However, yield loss due to occurrence of bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in the crop, is a recurring phenomenon. Twenty-one BC_2F_1s and 850 BC_1F_2s produced through an earlier MABB involving Uma as the recurrent parent and Improved Samba Mahsuri (ISM) as the donor parent served as the experimental population. The objective of the study was to elucidate the R-gene pyramids (*xa5*, *xa13* and *Xa21*) from among the BC_2F_1 population through MAS and to generate backcross progenies from the identified gene pyramids. Pathotyping the BC_1F_2s to assess their response to BB pathogen was also aimed at. The research programme comprised of four experiments *viz.*, I) Genotyping of BC_2F_1 population, II) Morphological characterisation of pyramided lines of BC_2F_1 population, III) Production of BC_3F_1's and BC_2F_2's and IV) Morphological characterisation and pathotyping of BC_1F_2 population, and production of BC_1F_3 population. The salient findings of the study are summarized below.

Foreground selection of BC_2F_1s

1. Adequate quantity of good quality total genomic DNA was extracted from 21 BC_2F_1s and the parents (Donor parent: Improved Samba Mahsuri (ISM); Recurrent parent:Uma).
2. The foreground analysis with the STS marker RG 556 that is tightly linked to R-gene *xa5* did not produce any polymorphism in the BC_2F_1s and the parents.
3. Restriction digestion of the RG 556 amplified PCR product with restriction enzyme Dra1 produced alleles of size 216bp, 267bp, 318bp

and 460bp in the BC_2F_1s and the parents, indicating the presence of R-gene *xa5* in the backcross population as well as the parents.

4. Functional marker xa5 SR assay of the 21 BC_2F_1s and the parents also revealed monomorphic banding pattern. Amplicon of size 200bp was resolved in all the individuals studied. This reconfirmed the endogenous presence of R-gene *xa5* in both the backcross population as well as parents; the R-gene being in the homozygous state.
5. The PCR product of STS marker RG 136 when digested with restriction enzyme Hinf1 produced amplicons of size 211bp, 283bp and 1908bp in the susceptible parent as well as the BC_2F_1s. This pointed out the presence of susceptible allele in all backcross individuals.
6. In BC_2F_1 Plant No.8.3.9.10, in addition to the susceptible allele an additional amplicon of size 1277bp associated with the resistant allele of R-gene *xa13* was also found. This pointed out that the BC_2F_1 Plant No.8.3.9.10 was heterozygous at the R-gene *xa13* locus. Conversely, the result also indicated the absence of resistant allele of R-gene *xa13* in all the other backcross individuals.
7. PCR amplification of DNA of BC_2F_1s with functional marker *xa13* promoter further confirmed the heterozygous nature of R-gene *xa13* locus. It also confirmed that the remaining 20 BC_2F_1 individuals screened were not introgressed with the R-gene *xa13*. Thus, it was inferred that the BC_2F_1 Plant No.8.3.9.10 was a 2-R-gene pyramid (*xa5xa5* + *Xa13xa13*).
8. Out of the 21 BC_2F_1sscored with the STS marker pTA 248 that is tightly linked to dominant R-gene *Xa21,* the 2-R-gene pyramid *i.e,* BC_2F_1 Plant No.8.3.9.10 was the only one found to possess the resistance allele (472bp) similar to that found in the donor parent ISM. It also had the susceptible allele (300bp) as found in Uma. However, all the other BC_2F_1s possessed the allele similar to susceptible parent.

   This indicated the presence of R-gene *Xa21*in the heterozygous state in BC_2F_1 Plant No.8.3.9.10.

9. From the results of foreground selection, it can be inferred that, among the 21 BC_2F_1s studied, BC_2F_1 Plant No.8.3.9.10 was the only 3-R-gene introgressed BC_2F_1individual. All the other individuals possessed only a single R-gene (*xa5*).
10. The BC_2F_1 Plant No.8.3.9.10 possessed R-gene *xa5* in the homozygous state while, the other two R-genes were heterozygous in nature (*xa5xa5* + *Xa13xa13* + *Xa21xa21*)

Background selection of the 3-R-gene introgressed BC_2F_1s

1. Background profiling of the 3-R-gene introgressed BC_2F_1 Plant No.8.3.9.10 along with parents revealed the presence of alleles similar to that found in the recurrent parent Uma in 13 out of 22 rice microsatellite markers used.
2. The 3-R-gene pyramid was monomorphic to donor parent with respect to four markers and heterozygous at five other marker loci.
3. The recovery of recurrent parent genome in the 3-R-gene pyramid was 81.82 per cent. Graphical representation of the results of genotyping of the pyramided plant done using the GGT software also confirmed higher recovery of the genetic background of recurrent parent Uma.
4. The dendrogram generated using the marker data resulted in two clusters at 60 per cent similarity coefficient. Donor parent ISM formed a monogenic cluster while, the R-gene pyramid (BC_2F_1 Plant No. 8.3.9.10) clustered with the recurrent parent Uma. This indicated that the 3-R-gene introgressed plant was genetically more similar to the recurrent parent Uma

Morphological characterization of BC_2F_1s

1. Presence of wide variability in morphological characteristics was observed among the BC_2F_1 population.

2. The 3-R-gene pyramid (BC_2F_1 Plant No. 8.3.9.10) exhibited greater similarity to recurrent parent (Uma) with respect to days to flowering, plant height, length and width of leaf blade, panicle length, length of decorticated grain and kernel colour (red).

3. The pyramid was found to exhibit greater similarity to the donor parent with respect to number of productive tillers/ plant (6 Nos.), width of grain (2.00 mm) and decorticated grain (1.50 mm).

4. However, the length of grain (7.00 mm) as well as decorticated grain (6.00 mm) in the gene pyramid was found to be greater than that of the parents.

5. Classification of grain (IRRI, 1996) pointed out that the grains of Rgene pyramid produced long slender while it was medium in recurrent parent and medium slender in the donor parent. Unlike the grain, the kernel of the pyramid as well as recurrent parent was red and long in comparison to medium white kernels of the donor

6. At 80 per cent similarity coefficient, the BC_2F_1 and parents grouped into 10 major clusters. Donor parent ISM formed a monogenic cluster.

7. The R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 exhibited 45.39 per cent similarity to the recurrent parent Uma and 26.86 per cent similarity to the donor parent ISM.

8. Similar to the clustering of genotypes based on molecular data, the clustering based on morphological data also placed the R-gene

introgressed BC_2F_1 Plant No. 8.3.9.10 closer to the recurrent parent than the donor parent.

Production of BC_3F_1s and BC_1F_3s

1. Backcrossing of the R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 to the recurrent parent (Uma) has resulted in the production of 15 BC_3F_1s.

2. Simultaneously selfing of the 3-R-gene pyramid produced 28 BC_1F_3 seeds.

Bioassay of BC_1F_2s

1. Pathotyping of BC_1F_2s derived from the R-gene pyramided BC_1F_1s (Plant No. 8.3.2, Plant No. 8.3.3 and Plant No. 8.3.9) in the earlier experiment (Joseph, 2016) was conducted in two stages.
2. Initially, 450 BC_1F_2s seeds (150 each from BC_1F_1 Plant No. 8.3.2, Plant No. 8.3.3 and Plant No. 8.3.9) were raised to assess their resistance to BB pathogen. Natural occurrence of BB coupled with incidence of blast disease within 14 DAS lead to severe mortality. None of the BC_1F_1s survived.
3. Evaluation of resistance against BB pathogen through leaf clipping method was done in the 106 BC_1F_2s that survived (Pathotyping set-II).
4. On evaluation of the 83 BC_1F_2 individuals derived from BC_1F_1 Plant No. 8.3.2, 55.41 per cent exhibited resistance reaction to BB infection, while, 16.87 per cent were moderately resistant, 10.84 per cent moderately susceptible, 4.82 per cent susceptible and 12.4 per cent highly susceptible.
5. Similarly, over half (52.17%) the BC_1F_2 progenies derived from BC_1F_1 Plant No. 8.3.9, exhibited resistance to BB infection. However, unlike in the earlier case, 34.78 per cent were moderately resistant, 8.69 per cent moderately susceptible, 0.00 per cent susceptible and

4.34 per cent highly susceptible.

6. Pathotyping thus revealed that in general, higher per cent of BC_1F_2 individuals derived from BC_1F_1 Plant No. 8.3.9 showed resistance to moderate resistance to BB pathogen in comparison to those derived from BC_1F_1Plant No. 8.3.2.

Production of BC_1F_3s

1. Selfing of the BC_1F_2 individuals that exhibited resistance to moderate resistance reaction to BB infection was done to obtain 725 BC_1F_3 seeds.
2. These are to be evaluated further through MAS well as agromorphological screening to isolate novel genotypes with BB resistance.

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Appendices

Appendix I. Quantity and quality of DNA of the BC_2F_1s

Progeny No.	Quantity (µg/ml)	Quality
BC_2F_1 8.3.2		
1	503.60	1.73
2	7.70	2.11
3	242.50	1.83
4	221.10	1.84
BC_2F_1 8.3.3		
1	415.80	1.89
2	97.60	2.01
3	195.60	1.94
4	83.20	1.78
5	1341.60	1.83
6	99.40	1.88
7	50.30	2.01
BC_1F_2 8.3.9		
1	410.30	1.75
2	315.70	1.80
3	51.80	2.00
4	86.50	1.78
5	73.00	1.73
6	426.70	2.05
7	34.50	2.08
8	83.40	1.90
9	302.20	1.86
10	80.30	1.96

Appendix II. Quantity and quality of DNA of parents

Genotype	Quantity (µg/ml)	Quality
Uma		
1	122.80	1.90
2	15.80	1.91
3	34.50	1.94
4	145.30	1.81
ISM		
1	261.00	1.90
2	253.50	1.89
3	281.90	1.87
4	233.50	1.92

Appendix III. Morphological characterization of BC_2F_1 progenies of BC_1F_1 Plant No. 8.3.2 and BC_1F_1 Plant No. 8.3.3

Progeny of Plant No.	Days to Flowering	Plant height (cm)	Leaf width (cm)	Leaf blade length (cm)	Productive tillers / plant	Panicle length (cm)	Spikelets / panicle	Grain length (mm)	Grain width (mm)	Decorticated grain length (mm)	Decorticated grain width (mm)
BC_2F_1 8.3.2											
1	132.00	48.90	0.87	30.40	8.00	13.90	68.00	7.50	2.00	5.00	2.00
2	132.00	37.50	0.83	28.70	6.00	16.73	66.00	7.50	2.00	5.00	1.50
3	124.00	38.00	0.70	21.80	6.00	18.56	69.67	6.00	2.00	5.00	2.00
4	115.00	43.60	0.70	22.60	10.00	16.67	73.67	7.00	2.00	5.50	2.00
BC_2F_1 8.3.3											
1	128.00	36.00	0.70	21.30	3.00	9.60	20.00	7.00	2.00	4.00	1.50
2	125.00	37.90	0.70	23.30	4.00	10.40	33.00	6.00	2.00	4.00	2.00
3	128.00	39.20	0.70	20.50	10.00	14.37	59.33	7.00	2.00	5.00	2.00
4	135.00	43.50	0.70	24.90	7.00	17.27	81.33	7.50	2.00	5.00	2.00
5	123.00	50.50	0.90	34.10	8.00	11.40	69.00	7.00	2.00	4.00	1.50
6	115.00	62.00	0.90	36.90	9.00	13.80	59.00	7.00	2.00	5.00	1.50
7	119.00	51.00	0.77	32.50	9.00	18.13	66.33	7.00	2.00	5.00	2.00

Appendix IV. Morphological characterization of BC_2F_1 progenies of BC_1F_1 Plant No. 8.3.9

Progeny of Plant No.	Days to Flowering	Plant height (cm)	Leaf width (cm)	Leaf blade length (cm)	Productive tillers / plant	Panicle length (cm)	Spikelets / panicle	Grain length (mm)	Grain width (mm)	Decorticated grain length (mm)	Decorticated grain width (mm)
BC_2F_1 8.3.9											
1	155.00	33.00	0.77	19.30	5.00	14.20	22.33	6.50	2.00	5.00	2.00
2	119.00	43.60	0.70	17.90	5.00	13.50	47.67	6.50	2.00	4.00	1.50
3	138.00	34.50	0.80	22.20	4.00	11.97	71.33	6.00	2.00	5.00	2.00
4	117.00	54.00	0.93	29.50	10.00	16.80	80.00	7.00	2.00	5.00	2.00
5	115.00	42.00	0.83	24.90	17.00	15.33	67.00	6.00	1.50	5.00	1.50
6	124.00	41.00	0.90	28.30	9.00	13.53	46.33	6.00	2.00	5.00	2.00
7	136.00	35.00	0.77	20.00	9.00	15.20	75.67	6.50	1.50	5.00	1.50
8	120.00	59.00	0.97	31.30	10.00	17.30	74.00	6.50	2.00	5.00	1.50
9	119.00	54.00	1.00	33.00	5.00	17.53	71.33	7.00	2.00	4.00	1.50
10	115.00	56.10	0.90	28.50	6.00	13.46	47.33	7.00	2.00	6.00	1.50

150

Appendix V. Morphological characterization of recurrent parent (Uma)

Plants	Days to Flowering	Plant height (cm)	Leaf width (cm)	Leaf blade length (cm)	Productive tillers / plant	Panicle length (cm)	Spikelets / panicle	Grain length (mm)	Grain width (mm)	Decorticated grain length (mm)	Decorticated grain width (mm)
1	120.00	54.00	0.90	25.10	11.00	15.00	69.67	6.21	2.73	5.33	2.46
2	118.00	55.80	0.83	24.00	13.00	16.03	86.33	6.47	2.71	5.69	2.23
3	115.00	59.10	1.06	37.30	15.00	18.10	84.33	6.58	2.75	5.72	2.30
4	118.00	60.70	1.00	34.50	15.00	17.67	98.33	6.10	2.92	5.91	2.46
5	123.00	57.90	1.03	33.50	14.00	16.73	90.33	6.91	2.81	5.82	2.31
6	124.00	53.30	0.83	25.90	11.00	15.70	82.33	6.24	2.79	5.92	2.34
7	125.00	60.10	0.96	36.50	15.00	16.70	82.33	6.35	2.80	5.97	2.25
8	118.00	62.10	1.03	36.80	16.00	17.80	92.33	6.92	2.85	5.83	2.35
9	125.00	52.40	0.93	28.10	14.00	15.23	91.00	6.58	2.83	6.01	2.34
10	115.00	56.30	0.83	29.30	14.00	14.70	81.67	6.35	2.87	5.82	2.35

Appendix VI. Morphological characterization of donor parent (ISM)

Plants	Days to Flowering	Plant height (cm)	Leaf width (cm)	Leaf blade length (cm)	Productive tillers / plant	Panicle length (cm)	Spikelets / panicle	Grain length (mm)	Grain width (mm)	Decorticated grain length (mm)	Decorticated grain width (mm)
1	148.00	46.24	0.86	30.16	8.00	21.20	89.60	6.30	1.80	5.30	1.50
2	150.00	48.39	0.91	37.90	10.00	22.03	97.60	6.50	1.80	5.10	1.50
3	146.00	52.52	0.92	39.30	8.00	22.30	95.30	6.30	1.80	5.20	1.50
4	143.00	45.96	0.83	31.84	8.00	23.10	98.30	6.40	1.90	5.60	1.60
5	149.00	50.71	0.90	36.96	8.00	22.80	93.70	6.30	1.90	5.80	1.60
6	149.00	51.32	0.88	37.40	9.00	21.10	85.30	6.40	1.70	5.30	1.40
7	152.00	48.90	0.89	37.96	8.00	21.50	90.30	6.60	1.60	4.90	1.30
8	147.00	48.56	0.95	39.93	9.00	21.30	95.00	6.60	1.50	5.70	1.40
9	148.00	48.53	0.97	40.46	8.00	21.30	97.30	6.80	1.70	5.90	1.30
10	146.00	45.10	0.99	37.77	7.00	22.80	93.70	6.70	1.70	5.90	1.40

Appendix VII. Grouping of BC_1F_2s based on their response to BB infection

Sl. No.	Disease reaction score of BC_1F_2 progenies of BC_1F_1 Plant. No. 8.3.2					Sl. No.	Disease reaction score of BC_1F_2 progenies of BC_1F_1 Plant. No. 8.3.9				
	Progeny No.						Progeny No.				
	1 (R)	3 (MR)	5 (MS)	7 (R)	9 (HS)		1 (R)	3 (MR)	5 (MS)	7(R)	9 (HS)
1	1	8	15	24	14	1	20	18	19		91
2	2	9	22	25	26	2	54	21	67		
3	3	31	23	37	33	3	55	68			
4	4	32	43	63	34	4	65	69			
5	5	42	46		35	5	66	70			
6	6	48	53		41	6	71	82			
7	7	57	56		45	7	80	84			
8	10	61	77		52	8	81	85			
9	11	64	106		60	9	83				
10	12	73			86	10	89				
11	13	75				11	90				
12	16	98				12	92				
13	17	100									
14	27	104									
15	28										
16	29										
17	30										
18	36										

Marker assisted backcross breeding for pyramiding genes conferring resistance to bacterial blight in rice variety Uma

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ABSTRACT OF THE THESIS

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ABSTRACT

As in other rice growing locales around the world, bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) assumes a huge role in deciding rice profitability in Kerala. The elite rice varieties of Kerala, both PTB 39 (Jyothi) and Mo 16 (Uma), are found to be extremely susceptible to bacterial blight. Since both the major rice cropping seasons (*virippu* and *mundakan*) in the state coincide with monsoons, the control of the disease through chemicals or biological agents proves inadequate owing to the washing-off of the applied materials. Host-plant resistance is advocated as the most effective breeding strategy to combat the bacterial blight (BB) pathogen.

Considering the impact of the disease on food security and sustainability, efforts were taken to introgress three R-genes (*xa5, xa13* and *Xa21*) into the variety Uma from donor parent Improved Samba Mahsuri (ISM) through Marker Assisted Selection (MAS). Further, backcrossing to Uma (recurrent parent) and advancing the resultant BC_1F_1s have resulted in production of BC_2F_1 generation (21 Nos.). The present study aimed to identify BC_2F_1 plants pyramided with genes (*xa5*, *xa13* and *Xa*21) conferring resistance to bacterial blight using functional markers. In addition, advancing the R-gene introgressed BC_2F_1s to BC_3F_1 and BC_2F_2 generation was envisaged. Pathotyping of BC_1F_2s (850 Nos.) and generating BC_1F_3s from the plants exhibiting resistance to BB pathogen was also aimed at.

Foreground selection of the BC_2F_1 individuals using the *xa5* gene linked STS marker RG 556 and functional marker xa5 SR confirmed the presence of the R-gene in the parents as well as the 21 BC_2F_1 individuals. Screening of BC_2F_1 individuals with STS marker RG 136 linked to R-gene *xa13* and functional marker *xa*13 promoter revealed that the BC_2F_1 Plant No. 8.3.9.10 was heterozygous at *xa13* locus while, all other BC_2F_1 individuals possessed alleles similar to that of the recurrent parent (RP) Uma. The result also pointed out that BC_2F_1 Plant No. 8.3.9.10 was a 2-R-gene pyramid (*xa5xa5* + *Xa13xa13*). Foreground selection with STS marker pTA 248 to detect the presence of *Xa21* gene revealed that none of the BC_2F_1 plants analysed except BC_2F_1 Plant No. 8.3.9.10, possessed the resistant allele of R-gene *Xa21*. However, presence of alleles of both the parents in Plant No. 8.3.9.10 indicated that it was heterozygous at *Xa21* locus. Results obtained thus revealed that, of the 21 BC_2F_1s subjected to foreground selection, BC_2F_1 Plant No. 8.3.9.10 was the only 3-R-gene introgressed pyramid (*xa5xa5* + *Xa13xa13* + *Xa21xa21*).

In addition, the 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 was subjected to background screening using 22 rice microsatellite (RM) markers. Background profiling

revealed that the banding pattern in Plant No. 8.3.9.10 was similar to recurrent parent in case of thirteen RM markers. The plant was found to be heterozygous at five other marker loci.

Considering the segregation of the 22 markers, the magnitude of recovery of recurrent parent genome in 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 was found to be 81.82 per cent. The dendrogram based on molecular data grouped the individuals into two major clusters. Cluster 1 was monogenic with only the donor parent ISM and cluster 2 comprised of the recurrent parent Uma and Plant No.8.3.9.10, further suggesting that the 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 was more similar to the recurrent parent Uma.

Wide variability was observed among the BC_2F_1 individuals for various morphological traits. The 3-R-gene introgressed BC_2F_1 Plant No. 8.3.9.10 was shorter in duration than both the parents and also possessed red kernels similar to the recurrent parent Uma. The dendrogram generated based on the morphological characters also indicated greater similarity between the 3-R-gene pyramid and recurrent parent Uma.

The identified 3-R-gene pyramid (BC_2F_1 Plant No. 8.3.9.10) was backcrossed to both recurrent parent Uma as well as selfed resulting in production of BC_3F_1s (15 Nos.) and BC_2F_2s (28 Nos.) respectively.

Bioassay of BC_1F_2 population (106 Nos.) through leaf clipping method of pathotyping suggested by IRRI (1991) revealed that more than half the BC_1F_2 individuals screened exhibited resistance to BB pathogen. The plants that exhibited moderate reaction to BB infection ranged between16.87 per cent and 34.78 per cent in progeny of BC_1F_2 Plant No. 8.3.2 and BC_1F_2 Plant No. 8.3.9 respectively. Selfing of the BC_1F_2 individuals exhibiting resistance and moderate resistance to BB pathogen resulted in production of 725 BC_1F_3 seeds.

Modern molecular techniques make it possible to use markers and probes to track the simultaneous introgression of several R-genes into a single cultivar during a crossing programme. Foreground and background profiling of backcross generations can ensure precise identification of R-gene introgressed genotypes that resemble the recurrent parent Uma.