PYRAMIDING OF BACTERIAL LEAF BLIGHT RESISTANCE GENES IN RICE VARIETY JYOTHI (PTB 39) THROUGH MARKER ASSISTED SELECTION

By

KABADE PRAMOD GORAKHANATH

(2015-11-101)

THESIS

Submitted in partial fulfillment of the requirement for the degree of

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CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

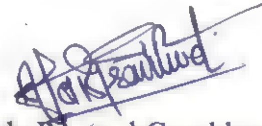
COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR – 680656

KERALA, INDIA

DECLARATION

I, hereby declare that the thesis entitled **'Pyramiding of bacterial leaf blight resistance genes in rice variety Jyothi (PTB 39) through Marker Assisted Selection,'** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.



Place: Vellanikkara
Date: 29|08|2017

Kabade Pramod Gorakhanath
(2015-11-101)

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Certified that the thesis entitled **'Pyramiding of bacterial leaf blight resistance genes in rice variety Jyothi (PTB 39) through Marker Assisted Selection,'** is a record of research work done independently by **Mr. Kabade Pramod Gorakhanath** 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 him.

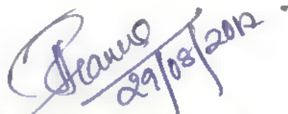
Place: Vellanikkara

Date : 29/08/2017

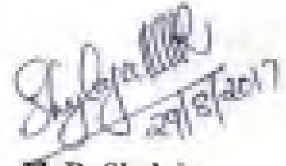
Dr. Rose Mary Francies
(Major Advisor, Advisory Committee)
Professor and Head
Department of Seed Science and Technology
College of Horticulture
Vellanikkara, Thrissur

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Mr. Kabade Pramod Gorakhanath (2015-11-101)**, a candidate for the degree of **Master of Science in Agriculture** with Major field in **Plant Biotechnology**, agree that the thesis entitled **'Pyramiding of bacterial leaf blight resistance genes in rice variety Jyothi (PTB 39) through Marker Assisted Selection,'** may be submitted by **Mr. Kabade Pramod Gorakhanath,** in partial fulfilment of the requirement for the degree.



Dr. Rose Mary Francies
(Major Advisor, Advisory Committee)
Professor (Pl. Br. & Gen.) and Head
Dept. of Seed Science and Technology
College of Horticulture, Vellanikkara.



Dr. M. R. Shylaja
(Member, Advisory Committee)
Professor (Hort.) and Head
Centre for Pl. Biotech. and Mol. Biology
College of Horticulture, Vellanikkara

Dr. P. S. Abida
(Member, Advisory Committee)
Professor (Pl. Physiol.) and Head
Dept. of Plant Breeding and Genetics
RARS, Pattambi.

Dr. Jiji Joseph
(Member, Advisory Committee)
Professor (Pl. Br. & Gen.) and Head
Dept. of Plant Breeding and Genetics
College of Horticulture, Vellanikkara

Dr. Rekha. K.
(External Examiner)
Senior Scientist
Biotechnology Division
Rubber Research Institute of India
Kottayam, 686009.

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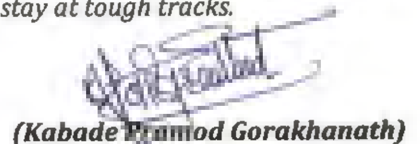

(Kabade Pramod Gorakhanath)

Dedicated to my Uncle &
Parents

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LIST OF ABBREVIATIONS

%	Per cent
µl	Microlitre
µM	Micro molar
°C	Degree Celsius
avr	Avirulence
BB	Bacterial blight
cm	Centimetre
cM	centiMorgan
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxy ribonucleic acid
dNTP	Deoxy ribonucleoside triphosphate
EDTA	Ethylene diamine tetra acetic acid
g	Grams
HCl	Hydrochloric acid
ISM	Improved Samba Mahsuri
KCl	Potassium Chloride
kg/ha	Kilograms per hectare
M	Molar
MAS	Marker assisted selection

$MgCl_2$	Magnesium Chloride
min	Minute
ml	Milliliter
mm	Millimetre
mM	Milimolar
NaCl	Sodium chloride
PCR	Polymerase chain reaction
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNA
RNase A	Ribonuclease A
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
SSR	Simple sequence repeat
STS	Sequence tagged sites
Taq	*Thermus aquaticus*
TE	Tris EDTA
Tm	Melting temperature
V	Voltage
w/v	Weight/volume

Introduction

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) with chromosome number 2n=24 and genome size of approximately 400 to 430 Mb, belongs to F: Poaceae (Arumuganathan and Earle, 1991). Over 90 per cent of world's rice is grown and consumed in Asia and approximately 60 to 70 per cent of the caloric requisite of the Asian population is gained from rice. Owing to this, rice has become a paramount element of food security (Nanda and Agarwal, 2006). A quantum leap in rice production has occurred since 1960s. Global production of rice has increased from 220.61 million tonnes in 1960-61 to 717.83 million tonnes during 2016-17, the production being from an area of 120.138 million hectares and 161.83 million hectares respectively (www.irri.org). Notwithstanding a two-fold increase in rice production world over, in the past few decades, the production is deficient to cope with the spiraling demand by the exploding population. This demand is expected to grow at the rate of about 1 per cent per annum in the years to come (Khush, 1997).

Among the rice growing countries in the world, India has the largest area under the crop and ranks second in production next to China. According to Ministry of Agriculture and Farmers Welfare, GOI, total production of food grains in India has increased from 51 million tonnes in 1950-51 to 252 million tonnes in 2015-16, with wheat and rice accounting for 78 per cent of the food grains produced in the country (www.agricoop.nic.in). During 2015-16, India produced 159.77 million tonnes of rice from 44.50 million hectares; the average productivity reaching 3590 kg/ha (www.irri.org). In the same year, Kerala registered an average productivity of 2834 kg/ha, producing 0.56 million tonnes of rice from a meagre area of 0.20 million hectares (DES, 2016).

As found elsewhere, in Kerala too, the production and demand scenario of rice is not balanced. Hence, guaranteeing food security in future, demands a quantum jump in production and productivity. This increase in crop productivity is to be achieved in face of challenges from the diminishing rice cropped area owing to urbanization and unscientific land conversions, labour and water shortages, lowered profitability and a constant threat from biotic and abiotic stresses.

The humid environment prevailing in Kerala favors occurrence of both insect pests and pathogens throughout the rice cropping period. Among the various diseases affecting the rice crop in the state, bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae (Xoo)* has been found to be the most obliterating one. Drastic reduction in production and productivity of the popular elite varieties Uma and Jyothi grown in the state has been reported by the recurrent occurrence of bacterial blight. Hence, efficient and effective management of BB disease recurrence in these elite cultivars is the need of the hour.

Cultural and chemical control measures have been advocated for the management of BB pathogen. However, incessant rains during the cropping period and non-availability of adequate labor, stand in the way of effective control of the disease, usually resulting in huge crop loss. Under these circumstances, it was realized that the most efficient way for BB disease control, is to exploit the plant's ability to resist damaging pathogen invasions, the resistance being typically conferred by the major R-genes.

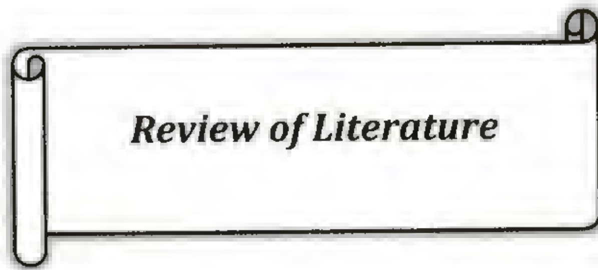
The R-gene family which confers resistance to the *Xanthomonas* is reported to be a multigene family with genes located throughout the rice genome at multiple loci. Presently, about 42 resistance (R) genes have been distinguished which provide host resistance against different strains of *Xoo*. These major genes have been designated in series from *Xa1* to *Xa42* and include 27 dominants and 14 recessive genes (*xa5*, *xa8*, *xa13*, *xa15*, *xa19*, *xa20*, *xa24*, *xa25*, *xa26b*, *xa28*, *xa31*, *xa32*, *xa33*, and *xa34*) in the series from *Xa1* to *Xa42* (Vikal and Bhatia, 2017). 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. Studies conducted at Regional Agricultural Research Station, Kerala Agricultural University, Pattambi revealed that the R-gene combination *Xa4 + xa13 + Xa21, xa5 + xa13 + Xa21 and Xa4 + xa5 + xa13 + Xa21,* confers broad spectrum resistance to Kerala isolate of *Xoo* (DRR, 2015). However, breakdown of resistance of cultivars with *Xa4* has been reported earlier by Mew *et al.* (1992).

Introgression of multiple resistance genes into rice genotypes has been accepted as an effective methodology to confer durable resistance against BB pathogen. In conventional breeding, the selection of plants with multiple resistance genes merely depends on phenotype. However, high influence of environment as well as gene interactions may mislead the selection process (Bharani *et al.*, 2010). Marker-assisted backcross (MAB) breeding approach has been

effectively used to incorporate multiple R-genes into the rice crop to confer resistance to bacterial blight resistance genes. This procedure not only guarantees identification of genotypes having the genes of interest, but also ensures that the resultant genotype resembles recurrent parent in all aspects with the exception of the resistance. Molecular markers tightly linked to the genes conferring resistance to *Xoo* isolates (*Xa1* to *Xa42* genes) have been developed. These include functional markers that are superior to DNA markers such as RFLPs, SSRs and AFLPs because of tight linkage with the trait locus.

Considering the above, an attempt was made to pyramid three bacterial blight resistant genes *viz.*, *xa5*+ *xa13* + *Xa21* into elite cultivar Jyothi (PTB 39) at the College of Horticulture, Vellanikkara. The BC_2F_4 population developed from the cross between high yielding rice variety Jyothi (PTB 39) and donor Improved Samba Mahsuri (ISM) formed the basis of the present study. The study was formulated with the following objectives:

1. To identify BC_2F_4 lines pyramided with bacterial leaf blight resistant genes (*xa5*, *xa13* and *Xa21*) using molecular markers.
2. To generate backcross population (BC_3F_1 and BC_2F_5) introgressed with the resistance genes.


Review of Literature

CHAPTER II

REVIEW OF LITERATURE

As in other rice growing locales around the world, in Kerala too, various climatic, edaphic, biological, physical, physiological and socio-economic variables impact the area, production and productivity of the rice. Bacterial blight (BB), an important biotic stress assumes a huge role in deciding rice profitability in Kerala.

PTB 39 (Jyothi) and Mo 16 (Uma) are both elite rice varieties of Kerala but extremely susceptible to the bacterial blight. The drastic decrease in their yield due to the disease outbreak is a common occurrence in the state. Apart from cultural and chemical control measures, exploiting host plant resistance to combat this biotic stress is being stressed off late. To introduce a resistance breeding project a thorough comprehension of the pathogen, its manifestation and management strategies is vital. The concise review of literature available in rice is detailed under the following sections.

2.1. Etiology of bacterial blight disease in rice

- 2.1.1 Ecology of the pathogen
- 2.1.2 Epidemiology and disease cycle
- 2.1.3 Symptoms on rice plant
- 2.1.4 Disease Management
    - 2.1.4.1 Host plant resistance
    - 2.1.4.2 Exploiting host plant resistance to combat BB pathogen
        - 2.1.4.2.1 The R-gene family conferring resistance to BB pathogen
        - 2.1.4.2.2 Mechanism of BB resistance

2.2. Breeding for resistance to BB pathogen

- 2.2.1 Building resistance through conventional approach
- 2.2.2 Building resistance through marker-assisted selection

2.1. Etiology of bacterial blight disease in rice

Bacterial leaf blight disease of rice, usually referred to as bacterial blight (BB), is caused by the gram-negative bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) from the family Xanthomonadaceae. It is a devastating disease causing extensive damage to the rice crop world

over. Cultivated rice as well as most of the wild rice species are susceptible to the *Xoo pathogen* (Sonti, 1998).

The BB pathogen was first noticed in the dew drops on rice leaves by Takaishi in 1908 (Mizukami and Wakimoto, 1969). Bokura in 1911 studied the physiology and morphology of the bacterium isolated from the rice leaves and named it *Bacillus oryzae* (Hori and Bokura). Ishiyama later renamed the BB pathogen as *Pseudomonas oryzae* (Uyeda and Ishiyama). It was further redesignated as *Xanthomonas oryzae*. The BB pathogen was raised to the status of species and was renamed as *Xanthomonas oryzae* pv. *oryzae* in 1990 (Swings *et al.*, 1990). Manifestation of the disease in India was first reported from the southern states during 1959 (Parthasarathy *et al.*, 2014). Since then the disease has assumed alarming proportions in the rice growing states of the nation.

Xanthomonas oryzae pv. *oryzae* is a rod-shaped, yellow, slime producing, gram-negative bacterium. It is an obligate aerobic pathogen that usually does not sporulate. The size of bacterial cells differs in length from around 0.7 - 2.0 μm and width 0.4 - 0.7 μm. The bacterial cells transports itself with the assistance of flagellum. The cells are protected by a capsule of carbohydrates comprising of galactose, glucose, xylose, and uronic acid. *Xoo* cells produce considerable amount of the capsular extracellular polysaccharides, leading to the development of strands or beads of bacterial exudates from infected leaves. These polysaccharides insure the *Xoo* cells from desiccation and help in its spread through wind and rain (Swings *et al.*, 1990).

2.1.1. Ecology of the pathogen

Host range of *Xanthomonas oryzae pv. oryzae* includes rice and several members of family Poaceae. Other weedy members of this family especially *Leersia oryzoides* var. *japonica, L. oryzoides* var. *zizania latifolia,* and *Pharlaris arundinacea* are extremely susceptible to the *Xoo* pathogen, in comparison to *L. japonica, Phragmites communis,* and *Lsachne globosa* (Goto *et al.*, 1953). Mizukami (1961) observed that the pathogen can persists in soil of the infected area, seeds, and stubbles. Rice straw was also observed to be harbouring the pathogen. They persist in their latent form and when the surroundings become conducive, becomes activated and infest the rice plants usually through the rice roots.

According to Tagami *et al.* (1964), rice plants get infected with *Xoo* pathogen from the inoculum surviving in the seeds, tillers or roots that are abandoned at harvest, and additionally from alternative weed hosts.

2.1.2. Epidemiology and disease cycle

The pathogen infects the plants through the injuries and openings like the hydathodes found along the edges of the leaf (Ou, 1985). It reproduces inside the xylem to cause widespread infection. The bacteria ooze out on to the leaf exteriors as droplets. Wind, rain-water and irrigation water assists in spreading of the pathogen inoculum in the ooze to non-infected parts or plants. It is observed that cutting off the seedling tips while transplanting encourages disease spread (Ou, 1985). The pathogen that persists on seeds and dead plants, will possibly be transferred from one plant to another by wind or water system.

Epidemics of bacterial leaf blight are common during the rainy seasons (June to September) in South East Asia and India (Liu *et al.*, 2004). The amount and pattern of rainfall in rainy season fundamentally decides the disease spread. The high rate of rainfall and relative humidity (90% for 12-14 hours per day) support quick disease development. Temperatures between 23^{0}C - 31^{0}C highly favours disease occurrence during the wet season. Occurrence of kresek (seedling blight) increases when the day time temperature fluctuates between 28^{0}C and 35^{0}C day (Liu *et al.*, 2004).

Bacterial inoculum in rice leaves is observed to be extreme between May to July months and minimum in August. According to Tagami *et al.* (1964), the population rises incrementally from maximum tillering and reaches the maximum as the crop reaches the panicle initiation stage. Environmental factors like temperature, humidity play a major role in the intensity of disease incidence. Compared to the upper leaf surface, the lower leaf surface of rice is found to contain more bacterial inoculum at early stages of invasion. However, the population diminishes with a reduction in metabolic activity of the leaves.

Field operations like nursery bed preparation and compost application, and choice of rice variety also add to the severity of disease (Mizukami and Takemoto, 1969). Rice seedlings raised in submerged nurseries are at a higher risk of being infected with the bacterium than those from dry nurseries. The yield loss was found to be higher in rice crop raised from wet nurseries than from the seedlings obtained from semi-irrigated or upland nurseries.

Higher use of nitrogen fertilizer application also encourages disease improvement. This aids lesion development, increases vegetative growth in plants, all of which creates a positive micro-atmosphere for the pathogen multiplication. Reddy *et al.* (1979) reported that higher application of N was linked with increased disease severity and henceforth lowered grain yield.

They concluded that the full potential of a BB vulnerable high yielding varieties can be exploited, only when it is protected with bactericide or cultivated in the disease-free seasons.

The kind of soils are also crucial in disease development. Clay or clay loam alluvium, sandy loam soils favours disease development and the infection is found to be rather low in the sandy soil of dunes. Acidic soils with poor drainage were observed to favour disease spread (Tagami *et al.*, 1966).

In temperate zone, *Xoo* pathogen is found to persist over winter in the rhizosphere of weedy plants like *Leersia* and *Zizania* species. The pathogen was also stated to persist on root and base of the tillers of rice debris (Mizukami and Wakimoto, 1969). Depending on the soil acidity and humidity, *Xoo* pathogen survives in the soil from 1 to 4 months. A significant amount of *Xoo* inoculum is found to hibernate in piled straw too. This source of inoculum could gain significance in regions where weedy hosts are absent or are very few (Ou, 1985).

2.1.3. Symptoms of the disease on rice plant.

Manifestations of the disease occur fundamentally on leaf sheaths, leaf blades, and occasionally on grains. They are described as a seedling blight (Kresek) and leaf blight as per the phase of infection. Kresek symptoms appears at the seedling growth phase on transplanting them from nursery to main field (Nino-Liu *et al.*, 2006). Little water-soaked spots usually develop along the edges of the older leaves late in the nursery stage. These tiny spots develop and subsequently turn yellow. In the wake of transplanting, disease manifestations occur by the third or fourth week and spreads as the plant grows. The lesions more often appear on the adaxial side of the leaf edges where water pores are more recurrently distributed aiding easy bacterial dispersal (Mew, 1987). In two or three days, enlarged yellow coloured lesions appear in the veins later turning white to greyish white.

Besides these, the leaves are found to roll and wither following infection. Either, only the upper half, or sometimes, the entire leaf quickly dries and turns pale white before withering, in the diseased plants. Manifestation of symptoms on infection largely depends on the variety, the physiological condition of the plant, prevailing climate and virulence of the pathogen (Tagami *et al.*, 1964). The seedlings with kresek symptoms become stunted and usually die within one to six weeks subsequent to transplanting (Mew, 1987).

Unlike kresek, the symptoms of leaf blight begin from the leaf blades, grow basipetaly and later, stretch out through the midribs to sheath. On infection, the leaf sheath become

discoloured and decays. Discoloured water-soaked lesions generally show up on the glumes of young spikelet's which may become conspicuous on young grains. On matured grains the lesions turn grey to yellowish white in the centre surrounded by an ill-defined margin (Yoshimura, 1960).

According to Gnanamanickam *et al.* (1999), the disease is found to manifest in all phases crop growth. It generally appears at the maximum tillering stage extending up to crop maturity. Intense damage occurs when Kresek precedes leaf blight. Although grain development becomes seriously affected and increased sterility occurs when the infection occurs in the vegetative stages or at panicle initiation phase, the crop yield is less compromised in the event of post flowering infections.

2.1.4. Disease management

The severity of damage from BB disease necessitates the adoption of appropriate management practices. Practices like disease estimation (forecasting), organic and chemical control strategies and host gene resistance are adopted. By and large, the vast majority of these strategies are utilized alone or in combination with each other for battling the pathogen.

An economic and effective chemical control strategy of BB pathogen is unavailable mainly owing to high variability among the *Xoo* populance. This variability in turn leads to high variation in their sensitivity to the antibiotics utilized. Additionally, the evolution and presence of drug-resistant strains impairs the effectiveness of the adopted disease management technique (Gnanamanickam *et al.*, 1999). Hence, in spite of resorting to disease forecasting, biological, chemical, cultural strategies for the management of BB pathogen, a total check of the pathogen on rice crop is not accomplished. The fact that both the major rice cropping seasons (*virippu* and *mundakan*) in the state coincides with monsoons, the control of the disease through chemicals or biological agents proves inadequate owing to the washing off of the applied materials.

Peng *et al.* (2015) revealed that in addition to the ecological and food safety issues that the chemical pesticides and biocontrol agents (plant extracts and antagonistic organisms) may produce, their effectiveness and efficiency considerably decreases on continuous application. Thus, in the long run, exploiting durable host plant resistance governed by R-genes would prove to be more reliable and beneficial.

2.1.4.1. Host plant resistance

The interaction between a specific strain of a plant pathogen and its host plant can result in either resistance or susceptibility. The pioneering work of Biffen (1905) on stripe rust of wheat (*Puccinia striiformis*) uncovered that the resistance of the host against the pathogen is under genetic control and is heritable. Flor, in 1956 studied the genetics of flax cultivars and the isolates of rust pathogen *Melamposara lini.* A clear genetic relationship between the host and the pathogen was delineated and subsequently he defined the well-known 'gene-for-gene hypothesis'. According to this hypothesis, for each gene contributing to disease resistance in host plant, there exists a complementary gene in the pathogen which decides virulence. The specific interaction between a resistance gene product and an elicitor produced by an avirulence gene in the pathogen triggers host plant resistance (Flor, 1971).

In view of the number of genes representing resistance, disease resistance is categorised into two; vertical and horizontal resistance. Vertical resistance (qualitative resistance) is governed by a solitary major gene which might be dominant or recessive. It restrains the development of the pathogen and the resistance is race specific in nature.

On the other hand, horizontal resistance (quantitative resistance) is conferred by numerous genes, each having small effects. It is a low-level resistance and exhibits no pathogen race specificity. This kind of resistance is of interest since it prevents breakdown of varietal resistance in a breeding system.

2.1.4.2. Exploiting host plant resistance to combat BB pathogen

Washio *et al*. (1966) first reported quantitative inheritance of BB resistance. Yamada (1984) inferred that cultivar IR 28 showed both qualitative and quantitative resistance to *Xoo* pathogen. Horino *et al.* (1982), Wasano (1982) and Yamada (1986) have likewise distinguished varieties demonstrating polygenic inheritance for BB resistance.

Depending on the race of the *Xoo* pathogen, the host plant shows various levels of resistance. According to Kelemu and Leach (1990), race particular interaction to bacterial blight in rice follows the gene for gene model. Plant disease resistance genes produces particular signal molecules which enables the host to perceive pathogens. In turn, avirulence (*avr*) genes controls production of signal molecules within the pathogen. The interaction between R-genes and *avr* genes trigger the rapid induction of host defence mechanism and finally result in physical manifestation of resistance (Kunnel, 1996). Disease susceptibility is

the result of either the plant R-gene or the pathogen avirulence gene being absent from the interacting organisms.

Hopkins *et al.* (1992) recognized three independent clones of *Xoo* pathogen, each of which contained avirulence genes (*avr xa5*, *avr Xa7*, and *avr Xa10*) that combat the defence machinery in rice cultivars containing the *xa5, Xa7* and *Xa10* resistance genes. It was established that aviruvelence genes in *Xoo* pathogen are highly similar to *avr Bs3*, a gene from *X. campestris pv vasicatoria* that elicits disease resistance response in pepper cultivars carrying the resistance gene *Bs3* (Bonas *et al.*, 1989). The *avr Bs3* encodes a 122-kDa protein, the internal portion of which contains a 34-amino acid motif which is repeated 17 times in tandem (Knoop *et al.*, 1991). In *Xoo, avr Xa7* and *avr Xa10* contains 15 and 25 repeats, respectively, and deletion of these repeats results in either a compatible reaction or altered race specificity (White *et al.*, 1995).

According to Horgan and Henderson (2015) the resistance to BB pathogen is not confined to a definite locus or a area or chromosome, but is the result of the dynamic interaction between the R-genes and the host genome. On infecting the rice plant, *Xoo* pathogen delivers certain race particular effectors which triggers both host resistance as well as infection. The effectors target the host cell nucleus, pair with the host resistance or susceptible genes and trigger infection or instigate resistance individually.

2.1.4.2.1 The gene family conferring resistance to BB pathogen

The R-gene family conferring resistance to the *Xanthomonas* is reported to be a multigene family with genes distributed throughout the rice genome. Presently, about 42 resistance (R) genes have been distinguished which provide host resistance against different strains of *Xoo*. These major genes have been designated in series from *Xa1* to *Xa42* and include 27 dominants and 14 recessive genes (*xa5, xa8, xa13, xa15, xa19, xa20, xa24, xa25, xa26b, xa28, xa31, xa32, xa33*, and *xa34*) in the series from *Xa1* to *Xa42* (Vikal and Bhatia, 2017). *Xa21* was the first R-gene to be cloned providing broad spectrum resistance and is therefore the most abundantly used R-gene in rice breeding programs. The R-genes are named with '*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).

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. Studies conducted at Regional Agricultural Research Station, Kerala Agricultural University, Pattambi revealed that the R-gene combination *Xa4 + xa13 + Xa21, xa5 + xa13 + Xa21 and Xa4 + xa5 + xa13 + Xa21,* confers broad spectrum resistance to Kerala isolate of *Xoo* (DRR, 2015). However, breakdown of resistance of cultivars with *Xa4* has been reported earlier by Mew *et al.* (1992).

About 42 resistant genes (Table 1) have been identified till date (Khan *et al.*, 2014; Zhang *et al.*, 2014; Busungu *et al.*, 2016).

Table 1. Resistant (R) genes and linked markers identified for BB resistance

Gene	Location (Chromosome No.)	Linked markers	Distance (cM)	Conferring resistance to	Reference
Xa1	4	XNpb235	1.5	Japanese races	Yoshimura *et al.* (1996)
		XNpb264	1.5		
		c600	1.5		
		U08750	1.5		
		U03700	-		
Xa2	4	HZR950-5	0.19	Vietnam races	He *et al.* (2006)
		HZR970-4	0.19		Yoshimura *et al.* (1995)
		Npb197	-		Yoshimura *et al.* (1995)
		Y03700	2		Yoshimura *et al.*(1992)
Xa3/Xa26	11	XNpbl81	-	Japan races	Yoshimura *et al.* (1995)
		XNpb78	2.3		Yoshimura *et al.* (1992)
		RG303, RG2	-		Yoshimura *et al.* (1992)
		CD0520, CD0365	-		Yoshimura *et al.* (1992)

Xa4	11	Npb181	1.7	Philippine race-I	Yoshimura *et al.* (1995)
		Npb78	1.7	India races	McCouch *et al.* (1991)
		RG53	1.7		
		RM254	3.4	Philippine race-I	Chen *et al.*(1997)
		RG55	0-1	Bangladesh races	Mc Couch *et al.* (1991)
		RG207	0-1		Panaud *et al.* (1996)
xa5	5	RM13	2.4		Panaud *et al.* (1996)
		RM12	2.1		Blair and McCouch, (1997)
		RM-390	1.7		Yoshimura *et al.*(1995)
Xa6/Xa 3/Xa8	11	-	-	USA races	Sidhu *et al.* (1978)
Xa7	6	M3	0.5	Philippine races	Porter *et al.*(2003)
		M5	0.16		
		M7	1.8		
xa8	7			Philippine races	Vikal *et al.*(2014)
Xa9	11			Philippine races	Singh et al.(1983)
Xa10	10	CD0365	16.2	Philippine and Japanese races	Yoshimura *et al.* (1995)
Xa11	-	L19	1.1	Japanese races IB, II, IIIA, V	Yoshimura *et al.* (1996)
Xa12	4	-	-	Indonesian race V	Ogawa et al. (1978)
xa13	8	RG136	3.8	Philippine race 6	Zhang *et al.* (1996)
		S14003	0.4		Chu *et al.* (2006)
		OPAC 05-900	5.3	Philippine race 6	Zhang *et al.* (1996)
		R2027	-		Sanchez *et al.* (2000)

xa13	8	RM230	2.3	Philippine race 6	Chen *et al*. (1997)
	11	RM21			Panaud *et al*. (1996)
	6	RM190			Temenykh *et al*.(2000)
Xa14	4	-	-	Philippine races	Taura *et al*.(1987)
xa15	-	-		Japanese races	Nakai *et al*.(1998)
Xa16	-	-	-	Japanese races (Tetep)	Noda *et al*.(1989)
Xa17	-	-	-	Japanese races (Asominori)	Ogawa *et al*. (1978)
Xa18	-	-	-	Burmese races (IR24, Miyang23, Toyonishiki)	Ogawa *et al*. (1978)
xa19	-	-	-	Japanese races [XM5 (mutant of IR24)]	Ogawa *et al*. (1978)
xa20	-	-	-	Japanese races [XM5 (mutant of IR24)]	Ogawa *et al*. (1978)
Xa21	11	RG103 (pTA248)	0-1	Philippine and Japanese races	Ronald *et al*.(1992)
Xa22	11	-	-	Chinese races(Zhachan glong)	Lin *et al*. (1996) Wang *et al*.(1998)
Xa23	11	-	-	Indonesian races *O*. [*rufipogon* (CBB23)]	Zhang *et al*.(1998)
xa24(t)	2	-	-	Philippine and Chinese races (DV86)	Mir *et al*. (1990)
xa25/ Xa25(t) Xa25	12	-	-	Chinese and Philippine races	Amante-Bordeos *et al*. (1992)

Xa26	11	RM224	0.21	Philippine races	Yang *et al.*(2003)
Xa27	6	M1059	0.31	Chinese strains and Philippine race 2 to 6	Gu *et al.* (2004)
		M336	1.8		
		M1081	0.97		
		M631/M1230	-		
xa28(t)	-	-	-	Philippine race2(Lota sail)	Lee *et al.* (2003)
Xa29	1	C904	1.3	Chinese races	Tan *et al.* (2004)
Xa30(t)	11	-	-	Indonesian races	Jin *et al.* (2007)
Xa31(t)	4	G235	0.2	Chinese races	Wang *et al.* (2009)
		C600			
Xa32(t)	12	RM8216	6.9	Philippine races	Hui-hui1 *et al.* (2008)
		RM20A	1.7		
xa33(t) *Xa33(t)*	6	-	-	Thai races	Korinsak *et al.* (2009)
Xa34(t)	11	RM224	-	Thai races	Korinsak *et al.* (2009)
Xa35(t)	11	-	-	Philippine races	Guo *et al.*(2010)
Xa36(t)	-	-	-	Philippine races	Miao *et al.* (2010)
Xa38	-	-	-	Indian Punjab races(*O. nivara* IRGC8 1825)	Cheema *et al.* (2008)
Xa39	11	-	-	Chinese and Philippines races	Zhang *et al.* (2014)
Xa40(t)	11	-	-	Korean race	Kim *et al.* (2015)
xa41(t)	-	-	-	Various *Xoo* strains	Hutin *et al.* (2015)

Xa42	3	-	-	Japanese races	Busungu *et al.* (2016)

Majority of the genes conferring resistance to *Xoo* pathogen are dominant while a few are recessive. Some widely studied R-genes are:

_Xa1_ gene

Sakaguchi (1967) first reported that R-gene *Xa1* exhibiting resistance to Japanese race I of *Xoo*. The *Xa1* gene locus was mapped onto chromosome 4 with RFLP marker XNpb235 (Yoshimura *et al*., 1996). In Japan, positional cloning of the gene as a part of the rice genome project confirmed that *Xa1* gene was conveyed by a 340-kb YAC clone (Y5212). It became obvious that *Xa1* gene encoded a nucleotide-restricting site-leucine-rich repeat (NBS-LRR) kind of protein (Yoshimura *et al*., 1998).

_Xa21_ gene

Xa21 confers broad spectrum resistance to BB pathogen. The resistance gene was introgressed into *O. sativa* from the wild species *O. longistaminata* (Khush *et al*., 1989). RAPD marker RAPD 248 was used for tagging the gene (Ronald *et al*., 1992). They also observed that the marker RG103 was linked to *Xa21* at a distance of 1.2 cM. The STS marker pTA248 was designed based on these markers and has proved highly efficient in identifying *Xa21* introgressed genotypes in marker-assisted breeding programmes. Gene sequencing uncovered that *Xa21* coded for receptor kinase domain with serine–threonine specificity.

_xa5_ gene

Petpisit *et al.* (1977) first reported *xa5* gene. Garris *et al.* (2003) found that *xa5* is recessively inherited and it can provide race-specific resistance. The R-gene *xa5* was tagged with RFLP markers RG556 and RZ390 and rice microsatellites RM122 and RM390 and mapped onto chromosome 5 (Blair and McCouch, 1997). Utilizing the RFLP marker RG556, a PCR-based STS marker was designed, to help in marker-assisted breeding programs (Huang

et al., 1997). The STS marker on PCR amplification was found to display a monomorphic banding pattern among the resistant and susceptible plants. It was recommended that the PCR product are to be digested with a restriction enzyme *Dra*I to produce Specific Amplicon Polymorphism (SAP). In 2007, Iyer and McCouch developed functional markers for the gene.

***xa13* gene**

The recessive gene *xa13* conferred resistance to the Philippines race 6 of BB pathogen. The gene was mapped onto chromosome 8 (Zhang *et al.*, 1996) and was tagged with RFLP marker RG136 and RAPD marker OPAC05 900. The RFLP marker RG136 prompted the development of a PCR-based STS marker. Like RG556 linked to *xa5*, RG136 also produced monomorphic banding design among the resistant and susceptible plants. It was, therefore, necessary to digest the PCR product with restriction enzyme HinfI to generate specific amplicon polymorphism.

2.1.4.2.2. Mechanism of BB resistance

Studies indicated that incompatible reactions are responsible for the bacteriostasis. In incompatible reactions, early accumulation of bright yellow green fluorescent compound and host cell death leads to bacteriostasis. (Reimers and Leach, 1991). Lignin-like polymers are formed rapidly during incompatible reactions whereas these polymers were not found deposited in compatible reactions. Rise in the activity of extracellular peroxidases during incompatible reactions were associated with bacteriostasis and lignin-like polymer deposition. (Reimers *et al.*, 1992). Oxidation of cinnamyl alcohol into free radical intermediates which are polymerized into lignin is last enzymatic step of lignin biosynthetic pathway leads to the formation of peroxidases. It prevents *Xoo* pathogen from invading the host cells because peroxidases mostly accumulate in the vascular tissues and acts as a physical barrier to the pathogen (Rao *et al.*, 2002).

The disease resistance (R) genes predicted products can be classified into five groups: kinases, NBS/LRR proteins, detoxifying enzymes, extracellular receptors and receptor kinases. The fifth class of disease resistance genes encodes a putative receptor kinase eg. *Xa21* resistance gene. The *Xa21* receptor kinase-like protein carries serine–threonine specificity in the kinase domain (Ronald, 1997). Upon ligand binding (pathogen produced peptide) to the receptor kinase domain, a signal is transduced across the transmembrane domain activating the

intracellular kinase domain. Upon activation, a phosphorylation cascade transduces the signal to defense molecules which restrict pathogen growth. Similar results were obtained in case of bacterial blight resistance gene *Xa1* conferring resistance to *Xoo* race I. The gene encoded the LRR protein (Yoshimura *et al.*, 1996).

Xoo like most of the phytopathogenic *Xanthomonas* secrets type III effectors (T3Es) directly into the rice cell via a specialized secretary channel referred as type III secretion systems (T3SS). The pathogen utilizes T3E weapons to suppress innate immune responses in rice that are collectively referred to as PAMP-triggered immunity (PTI) (Jones and Dangl, 2004; White, 2009). Several transcriptions activator-like (TAL) effectors are encoded in the genome of *Xoo* pathogen. These effectors are proteins which are injected by *Xoo* pathogen into the plant cells upon infection (Verdier *et al.* 2012; Khan *et al.*, 2014). There are broadly two families of effectors, *Xanthomonas* outer protein (Xop) and transcription activator like (TAL) domain. These T3Es compositions vary from one strain to other strain (Sattari *et al.*, 2014).

The two main R-gene classes identified with disease development in rice are the receptor-like kinase (RLK) class and nucleotide binding site-leucine-rich repeat (NBS)- LRR. The R-gene *Xa*21 having a place with the RLK class was the first to be replicated to initiate wide range resistance against BB pathogens. Sequence examination of *Xa21* demonstrated that a putative receptor kinase with an extracellular domain that contains a few Leucine-Rich Repeats (LRRs) is encoded by this gene. The extracellular domain of *Xa*21 may be associated with an elicitor (possibly a peptide) that is released by the pathogen. This interaction enacts the kinase domain of the protein which then starts a signal cascade that prompts resistance (Staskawicz *et al.*, 1995). The second class (NBS)-LRR is the largest R-gene class conferring resistance against bacteria, fungi and viruses (Hulbert *et al.* 2001). *Xa1* represents this major class of R-genes encoding a nucleotide binding-LRR protein (Yoshimura *et al.* 1998). *Xa27* is unique in a sense that it is only expressed upon inoculation with *Xoo* strains harbouring TAL effector gene *avr Xa27*.

The rice *xa5* gene for disease resistance to *Xanthomonas oryzae pv. oryzae* has been positionally cloned and found to encode the gamma subunit of transcription factor IIA (TFIIAγ). TFIIAγ is a general eukaryotic transcription factor with no previously known role in disease resistance. Gene *xa5* is unusual in that it is recessive and does not conform to one of the typical resistance gene structural classes. Sequencing of TFIIAγ in resistant and susceptible

isolines revealed two nucleotide substitutions resulting in an amino acid change between resistant and susceptible cultivars. This association was conserved across 27 resistant and nine susceptible rice lines in the Aus-Boro group. (Rao *et al.*, 2002; White and Yang 2009; Verdier *et al.* 2012)

Cloning and sequence analysis of the recessive gene *xa13* gene (protein transcribed: sugar transporter sweet11) showed that it is a novel type of R-gene that shows no sequence similarity with any known plant R-genes (Zhaohui *et al.*, 2005). The *xa13*-mediated resistance is race-specific resistance, has been defeated by some strains of *X. oryzae* pv. *oryzae* (Lee *et al.*, 2003; Chu *et al.*, 2006). Yang and White (2004) reported that PthXo1 is one of four known TAL effectors from different strains of *X. oryzae* pv. *oryzae* that have major contributions to virulence. These major TAL effectors also include *AvrXa7*, *PthXo2*, and *PthXo3*, and each contain unique repetitive regions. *AvrXa7* further differs among the four as the cognate effector for the dominant R-gene *Xa7*. Furthermore, the three alternate major TAL effectors were identified in races of the pathogen that are compatible on rice lines containing *xa13*, and it was demonstrated that introduction of the gene *avrXa7* into PXO99A was sufficient to overcome *xa13*-mediated resistance (Yang *et al.*, 2006). Gene *xa13* have been characterized in rice to encode a novel plasma membrane protein (White and Yang 2009; Verdier *et al.* 2012)

2.2. Breeding for resistance to BB pathogen

Breeders have attempted to introgress disease resistance genes into rice cultivars to impart BB resistance. Resistant varieties are more reliable, cost-effective and environmental safe approach for disease management (Gnanamanickam *et al.*, 1999).

2.2.1 Building resistance through conventional approach

The resistance gene *Xa4* have been incorporated in rice varieties through conventional backcross breeding to build immunity against *Xoo* pathogen (Khush *et al.*, 1989). In India, TKM6 was used as a donor of bacterial blight resistance and several varieties (e.g. ABT 32, Govind, UR 20, IR 36, Karjat, Radha and Ramakrishna) were developed. The genotype Sigadis was used as the BB resistant parent for developing varieties Ratnagiri and 68-1 while BJI was used as a donor for BB genes to develop varieties PR 4141 and IET 8585 (Ajaya).

Prolonged cultivation of varieties with *Xa4* resulted in the appearance of newer races of the pathogen that broke down the resistance of rice varieties. The probability of simultaneous pathogen mutations for virulence to defeat two or more effective genes is much lower than the probability of breakdown of a single resistance gene (Mundt, 1990). Hence, to avoid such breakdown, introgression of more than one resistance gene was observed to be a more viable strategy.

Yoshimura *et al.* (1995a, b) combined resistance genes in pairs *Xa4/xa5* and *xa5/Xa10* and showed that plants with two genes can have a higher level of resistance to *Xoo* than would be expected from the sum of the parental levels.

Under rice breeding programs at IRRI, India, and Indonesia resistance genes *Xa4, xa5, Xa7,* and *Xa21* have being introgressed in to economically essential rice varieties (Nelson *et al.*, 1996).

The convergence breeding technique was adopted by Xu *et al.* (1996) in China to combine the early Japonica line G38 carrying the gene *xa5* with the recurrent parent Xiushui 11, which has adult plant resistance conferred by *Xa3* gene. As a result of successive back crosses, three late Japonica lines were bred with resistance at all growth stages.

This approach of convergence breeding however, can be very difficult or impossible when there is epistasis or masking effect of genes, particularly when a breeding line already harbors a gene like *Xa21* which shows resistance to all known *Xoo* races. With the conventional approach, breeding lines with *Xa21* alone cannot the distinguished from breeding lines with *Xa21* plus other genes. However, if DNA markers are available for each resistance gene, the identification of plants with multiple genes would become easy (Rao *et al.*, 2002).

Resorting to coupling of conventional breeding and MAS pyramiding of multiple genes in different rice cultivars a greater resistance to BB pathogen can be obtained (Khush, 1990; Huang, 1997).

2.2.2 Building resistance through Marker assisted selection (MAS)

In the conventional breeding approach, it is extremely difficult to identify segregants harboring recessive genes like *xa13*, which is expressed only when homozygous. Through tagging of R-genes with molecular markers detection of individuals carrying the genes turns out to be simpler and precise (Joshi *et al.*, 1999).

Plant improvement depends upon creating, evaluating and selecting the correct blend of alleles. With the utilization of molecular markers, it has turned out to be conceivable to follow significant alleles in a segregating population and mapping them. These markers once mapped and tagged to genes of interest, provide the breeders with a precise tool to ensure incorporation of the genes of interest into desired backgrounds thereby increase the efficiency of selection of desirable genotypes. MAS is dependent on the concept that the marker is firmly linked to the gene of interest. The marker assay can prove advantageous in backcross breeding as a part of the recurrent selection for foreground selection and background selection (Edwards and Johnson., 1994; Openshaw *et al*., 1994; Hospital and Charcosset, 1997).

Abenes *et al.* (1993) utilized MAS for the first time to pyramid gene(s) for BB resistance. Huang *et al*. (1997) effectively utilized MAS for pyramiding four BLB resistance genes, *Xa4, xa5, xa13* and *Xa21* utilizing RFLP and PCR based markers. The pyramided lines exhibited increased resistance to BB pathogen when compared to lines with just a single gene.

Chen *et al*. (2000) effectively incorporated *Xa21* gene into 'Mingui 63', an elite restorer line of hybrid rice through MAS. Using 158 polymorphic RFLP markers distributed throughout the 12 chromosomes, they reconstituted the Mingui 63 plant type within 3 generation of backcrossing.

Chen *et al*. (2001) utilized 129 polymorphic AFLP markers to recover 98.8 per cent of the recurrent parent '6078' elite rice restorer line genome in the BC_3F_2 generation. Joseph *et al.* (2004) used AFLP markers for choosing desirable recombinants in a BC_1F_2 generation. In a cross between Pusa Basmati 1 and IRBB55, they recognized a few promising recombinants with *Xa21* and *xa13*.

Gopalakrishnan *et al.* (2008) consolidated BB resistance genes *Xa21* and *xa13* from IRBB55 with basmati quality traits of Pusa Basmati 1 and a few recombinant predominant lines were developed. One of the lines named Pusa 1460 now commercially released as a variety, is India's first rice variety developed through MAS.

Basavaraj *et al*. (2010) introgressed BB resistance genes *xa13* and *Xa21* into parental lines (Pusa 6B and PRR 78) of Pusa RH10, the superfine grain aromatic rice hybrid. This is the first successful case of pyramiding BB resistance genes in both the parental lines of a broadly grown rice hybrid in India.

Few successful examples of developing BB resistance rice cultivars through marker-assisted breeding (MAB) are cited in Table 2.

Table 2. Commercially released rice cultivars with BB resistance in Asia.

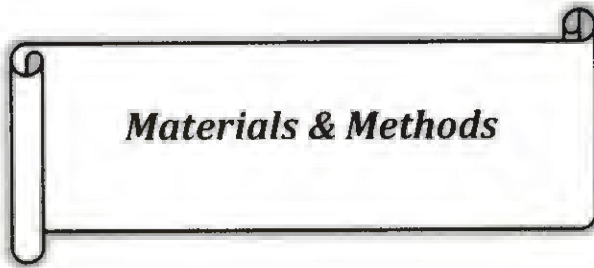
Variety conferred resistance	Gene combination	Country	Release year/reference
Angke	*Xa4* and *xa5*	Indonesia	2002
Conde	*Xa4* and *Xa7*	Indonesia	2002
NSIC Rc154 (Tubigan 11)	*Xa4* and *Xa21*	Philippines	2006
Guodao 1	*Xa4* and *Xa21*	China	2004
Improved Pusa Basmati-1	*xa5, xa13* and *Xa21*	India	Singh *et al.* (2007)
PR106	*xa5, xa13* and *Xa21*		Singh *et al.* (2001)
Samba Mahsuri	*xa5, xa13* and *Xa21*	India	Raman *et al.* (2008)
Type 3 Basmati	*Xa21, xa13, sd-1*	India	Rajpurohit *et al.* (2011)
RD6	*xa5*/Blast *R*	Thailand	Pinta *et al.* (2013)
Mahsuri	*Xa4, xa5, xa13* and *Xa21*	India	Guvvala *et al.* (2013)
MTU1010	*Xa21, xa13* and blast resistance (*Pi54*)	India	Arunakumari *et al.* (2016)

In addition to the above, attempts to introgress genes through marker assisted backcross breeding (MABB) to impart BB resistance to the rice crop has been attempted by several workers. An insight into the R-avrgenes introgressed through this approach is further detailed in Table 3.

Table 3: Bacterial blight resistance genes introgressed in rice through marker assisted selection

Gene introgressed	Marker used for selection of resistance progeny		Reference
	Foreground	Background	
xa5, *xa13*, and *Xa21*	STS	SSR	Deshmukh *et al.* (2017)
xa5, *xa13*, and *Xa21*	STS	SSR	Ramalingam *et al.* (2017)
Xa21, *xa13*,*blast* resistance *(Pi54)*	Microsatellite markers, STS	SSR	Arunakumari *et al.* (2016)
xa-5, *xa13 and Xa21*	STS	SSR	Pha *et al.*(2016)
xa5, *xa13*, *Xa21*	STS	SSR	Pradhan *et al.* (2015)
Xa21, *xa13*, *xa5*, *Xa4*, *and Xa2*	STS	SSR	Singh *et al.* (2015)
Xa4, *xa5*, *Xa21*	STS	SSR	Suh *et al.* 2013
Xa21, *xa13 and xa5*	STS	SSR	Bharani *et al.* (2010)
Xa4, *xa5*, *xa13* and *Xa21*	STS	STMS	Shanti *et al.* (2010)
xa5, *xa13* and *Xa21*	STS	SSR	Perumalsamy *et al.* (2010)
xa13 and *Xa21*	STS	Conventional breeding	Basavaraj *et al.* (2010)
xa5, *xa13* and *Xa21*	STS	SSR	Sundaram *et al.* (2009); Sundaram *et al.* (2008)
xa13 and *Xa21*	STS	SSR	Gopalakrishnan *et al.* (2008)
Xa4, *Xa7* and *Xa21*	STS	SSR	Perez *et al.* (2008)
Xa4, *xa5*, *xa13* and *Xa21*	STS	NP	Bharatkumar *et al.* (2008)
Xa4, *xa5*, *xa13* and *Xa21*	STS	NP	Bharatkumar *et al.* (2008)
Xa4, *and xa13*	SSR	SSR	Phuc *et al.* (2005)
xa5, *xa13 and Xa21*	STS	SSR	Pha *et al.* (2004)
Xa4 and *Xa21*	STS	SSR and RAPD	Huang *et al.* (2003)
Xa21	STS	AFLP	Chen *et al.* (2001)
xa5, *xa13* and *Xa21*	STS	NP	Singh *et al.* (2001)
Xa21	STS	RFLP	Chen *et al.* (2000)

xa5, *xa13* and *Xa21*	STS	NP	Sanchez *et al*. (2000)
xa5, *xa13* and *Xa21*	STS	NP	Sanchez *et al*. (2000)


Materials & Methods

CHAPTER III

MATERIALS AND METHODS

Considering the efficiency of marker assisted backcross breeding (MABB) programme for incorporation of multiple R-genes, an attempt was made to pyramid three R-genes (*xa5, xa13* and *Xa21*) into rice variety Jyothi (PTB 39) from donor 'Improved Samba Mahsuri' (ISM) through Marker Assisted Selection under the DBT project: 'Rice-Gene pyramiding to develop cultivars with durable resistance to Bacterial Leaf Blight through Marker Assisted Selection'. The present study conducted during 2015-2017, aimed to identify the R-gene pyramids from among the BC_2F_4 population developed in the project and generate further backcross progenies. The research programme was categorised under four major experiments *viz.*, I) Genotyping of BC_2F_4 population, II) Morphological characterization of pyramided lines (BC_2F_4 population) and parents, III) Production of BC_3F_1 population and IV) Production of BC_2F_5 population. The materials and methods employed during the course of investigation are described in this chapter.

3.1 Location

Raising of BC_2F_4 population along with parents and subsequent backcrossing were carried out in the field facility of Department of Seed Science and Technology, Kerala Agricultural University (KAU), Thrissur 680 656, between 2015 and 2017. KAU experiences humid tropical climate and is located, 40 m above MSL between 10° 31'N latitude and 76° 13'E longitude. The laboratory facilities under Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, Thrissur 680 656 were used for the study.

3.2 Experimental material

Fifty-one BC_2F_4 plants derived from the cross: Jyothi (PTB 39) x Improved Samba Mahsuri (ISM), along with parents [Recurrent parent: Jyothi (PTB 39) and Donor parent: Improved Samba Mahsuri (ISM)] comprised the study material (Table 4). Improved Samba Mahsuri released from Indian Institute of Rice Research, Hyderabad (formerly Directorate of Rice Research (DRR), Hyderabad), is an essentially derived variety (EDV) derived from the

popular rice variety Samba Mahsuri. It was developed through MABB programme and contains the three bacterial blight resistance genes *xa5, xa13* and *Xa21*. The recurrent parent Jyothi is one of the most widely grown red kernelled rice variety in Kerala.

Table 4: Salient features of parents Jyothi and Improved Samba Mahsuri (ISM)

Variety	Pedigree	Year of release	Salient features
Recurrent parent: Jyothi **(PTB 39)**	Ptb10 × IR8 (HS)	1974	Medium duration (110-125 days) rice variety with red long bold grains and a yield potential of 5.5 – 6.0 tonnes /ha
Donor parent: Improved Samba Mahsuri (ISM) **(RP Bio-226)**	Samba Mahsuri × 4/ SS1113	2008	Long duration (135-140 days) rice variety with white medium slender grains and a yield potential of 4.75 to 5.0 tonnes/ha. ISM yields 15-30 per cent more than the bacterial blight susceptible variety Samba Mahsuri.

3.3 Methods

3.3.1. Experiment I: Genotyping of BC_2F_4 population

Seedlings of BC_2F_4s (90 nos.) were raised in sterile sand beds during August, 2016-Jan, 2017. Staggered sowing of the parents (Jyothi and ISM) was also done at weekly intervals starting from 16th August, 2016 to 30th September, 2017 to provide sufficient pollen load for production of next backcross generation. The germinated BC_2F_4s (51 nos.) and parents were transplanted into pots of 30 cm height and 25 cm diameter on the 21st day after sowing. Three plants were maintained in each pot. Standard field practices in accordance with the guidelines of the package of practices (POP), Kerala Agricultural University, 2011 were followed during the crop growth period. Subsequently, genotyping of BC_2F_4 plants was carried out using three STS markers, RG556, RG136 and pTA248, closely linked to the BB resistance genes *xa5, xa13 and Xa21*, respectively to confirm the presence of the resistance allele of each gene in the backcross generation. In addition, the functional marker *xa13* promoter and *xa5*SR were also used (Sundaram *et al.,* 2008) for foreground selection. A set of 50 Rice Microsatellite (RM) (SSRs markers) accessible at Rice marker database www.gramene.org. which were found to be polymorphic between the parents in earlier studies were selected for background selection. It was ensured that the markers selected for background selection covered all the 12 linkage groups in rice. The markers used for foreground and background selection are detailed in following Table 5 and Table 6 respectively.

Table 5. List of markers used for foreground selection

Gene	Primer name	Primer sequence	Marker distance (cM)	PCR product size (bp)	Reference
xa5	*xa5*SR F	AGC TCG CCA TTC AAG TTC TTG AG	0.0	410, 310, 180	Petpisit *et al.* (1977)
	*xa5*SR R	TGA CTT GGT TCT CCA AGG CTT			
	RG 556 F	ATA CTG TCA CAC ACT TCA CGG	0.1		
	RG 556 R	GAA TAT TTC AGT			

		GTG TGC ATC		440, 410	
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 promoter F	GGC CAT GGC TCA GTG TTT AT	0.7	500	
	xa13 promoter 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			

Table 6. Rice Microsatellite markers used for background selection

Serial No.	Marker name	Chromosome No.	cM position	Forward primer sequence	Reverse primer sequence	Annealing temp.	PCR product size
1	RM1	1	29.70	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC		113
2	RM6340	1	3.10	AACGGAGATCGAGATCGATG	TGCTTCCTCATCTCCCTCAC		149
3	RM10871	1	14.05	TGAGGCTGTAACGTAGACGATGAACC	AAGCCTGCTAGAGAGGCCCAACC		234
4	RM11069	1	19.03	GGTACAATGAAGCTTGGCAACG	CGGTGGAGTAGAACCACGAAGC		279
5	RM11313	1	24.01	TGAGGCTGATAGAAAGCAGAATGC	CCCGTTTCTTCCATATCATGTCG		388
6	RM11342	1	14.05	CCATCCATGCACATTTAGGAGTAGG	TGTACAATCACGTCGCTCTACACG		288
7	RM12038	1	39.07	AGACGCACGCAACATCACTCG	GGGTATACCATACTCCCTCCGTTGC		384
8	RM482	2	187.50	TCTGAAAGCCTGACTCATCG	GTCAATTGCAGTGCCCTTTC		188
9	RM3340	2	0.38	TCTTGGCAAGCTCTCCTCTC	CCATCATCTCGATCTTGACG		117
10	RM12941	2	9.03	TTATGCCATGTGGTCCAATCAGC	ATTTGAACCATTTGGGCCTTGG	55^{0}C	186
11	RM13910	2	29.05	GAGCGAGCTATACCACCGTGACC	ATCGCGTCCAAGAAAGGTGTCG		188
12	RM16	3	131.50	CGCTAGGGCAGCATCTAAA	AACACAGCAGGTACGCGC		181
13	RM49	3	161.10	TTCGGAAGTTGGTTACTGATCA	TTGGAGCGGATTCGGAGG		189
14	RM85	3	231.00	CCAAAGATGAAACCTGGATTG	GCACAAGGTGAGCAGTCC		107
15	RM251	3	79.10	GAATGGCAATGGCGCTAG	ATGCGGTTCAAGATTCGATC		147
16	RM15583	3	24.03	CCCAAATAGTCACCAGCATTATCG	TTGCCTGTGCAACCTTATGAACC		174
17	RM307	4	0.00	GTACTACCGACCTACCGTTCAC	CTGCTATGCATGAACTGCTC		174
18	RM5586	4	19.22	CTCCATAATCAAGGAAGCTA	ATGAGTTCTTTCGTCAGTGT		134
19	RM6089	4	29.12	CCACCGAATCGAATAACCAC	ATGGCCAGCGTGATCTCC		170

20	RM6679	4	19.22	TTTAGGCCGTAAGAGCGAAC	GAATTTGAGTAGCTGGCTCC	55^{0}C	141
21	RM17377	4	29.12	ATATTACTTCGACGCTGGATCAGG	GTCAGTTCGTCAGGCACAACG		168
23	RM18225	5	9.05	CGACAGGAGGGAGAGAGGAAGG	GGTTTGACCGTGGTTTGACTAACG		456
24	RM18919	5	24.02	AGGAGTTCAGTTTCTGCAAGTCAGG	CAGCATGCCGTAGTTCACACC		495
25	RM19218	5	28.99	CGGAGGGAGTAGGTACGTAGGG	CCCATTCCATTCTACACTGACG		169
26	RM217	6	38.70	ATCGCAGCAATGCCTCGT	GGGTGTGAACAAAGACAC		133
27	RM214	7	34.70	CTGATGATAGAAACCTCTTCTC	AAGAACAGCTGACTTCACAA		122
28	RM248	7	116.60	TCCTTGTGAAATCTGGTCCC	GTAGCCTAGCATGGTGCATG		102
29	RM295	7	0.00	CGAGACGAGCATCGGATAAG	GATCTGGTGGAGGGGGAGG		180
30	RM20833	7	0.05	GATATGGTTCCACTTCACCATGC	TTAGAAACTCGCCTTCAGAACTGC		266
31	RM21345	7	8.81	GCATGCTAAGCTGTAGAAGTTAGTGG	GCTACATGTCACCGATCAGACC		388
32	RM72	8	60.90	CCGGCGATAAAACAATGAG	GCATCGGTCCTAACTAAGGG		65
33	RM5545	8	0.00	CAGCACTCCTCCCCTACCAG	GGCTAAGTCAGCGTGAGACC		156
34	RM5556	8	0.00	ATCTCCCTCCCTCTCCTCAC	TCCACACCTTCACAGTTGAC		102
35	RM23087	8	19.06	GATATTAGCTAGACATGGCACTCTGC	GTACATCCGCATGAATAGAGTGG		446
36	RM107	9	82.40	AGATCGAAGCATCGCGCCCGAG	ACTGCGTCCTCTGGGTTCCCGG		189
37	RM205	9	114.70	CTGGTTCTGTATGGGAGCAG	CTGGCCCTTCACGTTTCAGTG		122
38	RM524	9	13.20	TGAAGAGCAGGAACCGTAGG	TCTGATATCGGTTCCTTCGG		198
39	RM23998	9	8.90	CTGCACGTACGGTCAAGTCTACC	GCATTGCAAGGGTTGAAGTGG		249
40	RM304	10	73.00	TCAAACCGGCACATATAAGAC	GATAGGGAGCTGAAGGAGATG		160
41	RM7545	10	0.00	GTATCCGCTCCGTTTTCATC	GAGGGGGGGGTGTAGAATAG		225
42	RM202	11	54.00	CAGATTGGAGATGAAGTCCTCC	CCAGCAAGCATGTCAATGTA		189

43	RM224	11	120.10	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG		157
44	RM26213	11	4.10	GCCACAGGAGACAGCAAGAACC	CGATCCAATTCCAGCCTAGATAGC		345
45	RM26868	11	19.04	CAACTGTACTGTGCTGACCATCG	AGTAGGGACGAGGATTTCATGG		168
46	RM17	12	109.10	TGCCCTGTTATTTTCTTCTCTC	GGTGATCCTTTCCCATTTCA		63
47	RM19	12	209.00	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA	55ºC	226
48	RM247	12	32.30	TAGTGCCGATCGATGTAACG	CATATGGTTTTGACAAAGCG		131
49	RM260	12	61.70	ACTCCACTATGACCCAGAG	GAACAATCCCTTCTACGATCG		111
50	RM28277	12	19.06	TGCACCACCTATTTCAATCCACTCC	CCTTCCTCAAGGGAAATCACAGAAGC		296

3.3.1.1. DNA isolation, quality and quantity of extracted DNA

DNA (deoxy ribonucleic acid) of parents and backcross population (BC_2F_4s) was extracted as per the Modified CTAB method and checked for quality and quantity of isolated DNA samples.

3.3.1.1.1. Extraction of genomic DNA

Fresh leaves from 25 days old seedlings were collected early in the morning, and wrapped in aluminum foil before placing in ice-box. The samples were washed with sterile distilled water and the leaf surface was wiped with 70 per cent ethanol. The cleansed leaves were transferred to -80^{0}C deep freezer without further delay in order to prevent deterioration. It was stored thus until being used for DNA extraction. Modified CTAB method advocated by Dellaporta *et al.*, (1983) was used for the isolation of good quality DNA.

Reagents used

1. CTAB buffer

Table 7. Composition of CTAB buffer

Materials	Concentration	Quantity for 500 ml
CTAB (W/V)	2%	10g
NaCl	1.4M	40.6g
EDTA (pH 8)	20mM	3.7g
Tris HCL (pH 8)	100mM	6.07
Polyvinyl pyrrolidine (PVP)	1%	5g
β-mercaptoethanol	10mM	0.5ml
Distilled water	-	500ml

2. Chloroform – Isoamyl alcohol (24:1)
3. 70% ethanol

70ml – Ethanol

30ml – Distilled water

4. Chilled isopropanol (100%)

Procedure

1. Weighed 0.1g of leaf sample and surface sterilized with 70 per cent ethanol.
2. Plant samples were cryogenically ground in a mortar and pestle after imposing chilling treatment with liquid nitrogen.
3. CTAB extraction buffer @ 1000µl for each 100mg homogenized tissue was used to transfer the ground homogenate to a centrifuge tube.
4. The homogenate was incubated at 65^{0}C for 30 to 60 minutes in a water bath ensuring intermittent shaking of the tubes at 5-minute intervals.
5. Equal volume of Chloroform: Isoamyl alcohol (24:1) was added to the homogenate and vortexed gently for 5 seconds before centrifugation at 15,000 rpm for 20 minutes resulting in separation of the phases and precipitation the DNA.

Aqueous top layer	-	DNA with small quantity of RNA
Mid layer	-	Protein and other cell debris
Lowest layer	-	Chloroform, pigments etc.

6. The upper aqueous phase was carefully transferred in to a new tube and the process repeated until the upper phase was clear.
7. To the above, 0.2 µl of RNase (10mg/ml) was added [Prepared (10mg/ml) stock by adding powdered RNase A in sodium acetate (10mM) and adjusted to pH 5.2 with addition of HCl or NaOH. Heated to 100^{0}C for 15 min, and then allowed to cool to room temperature and the pH adjusted to 7.4. It was noted that precipitation of RNA occurs only when RNase is boiled at neutral pH.].
8. After 30 min of RNase incubation, added 400-500 µl Chloroform: Isoamyl alcohol (24:1) and the sample centrifuged at 10000 rpm for 10 min.
9. The upper aqueous phase was then transferred to a 1.5ml fresh tube and the DNA precipitated by adding an equal volume of cold isopropanol and incubate at -200C for 2 to 24 hrs.

10. On incubation the sample was centrifuged at 10000 rpm for 10 min. The supernatant was decanted carefully without disturbance to the pellet.

11. The pelleted DNA was washed with 500 µl ice cold 70 per cent ethanol, centrifuged at 10,000 rpm at 4°C for 10 minutes.

12. The supernatant was carefully discarded after spin to retain only the pellet.

13. The DNA pellet was dried long enough such that there was no trace of alcohol.

14. DNA was then dissolved in 20-50 µl distilled water (the pellet may need to be warmed in order to dissolve) and stored at -20^{0}C.

3.3.1.1.2 Determination of quality and quantity of isolated DNA

3.3.1.1.2.1 Assessing quality by agarose gel electrophoresis

Agarose gel electrophoresis was used to visualize and quantify the isolated DNA samples with the help of a ladder.

Reagents used

1. Agarose (2%)
2. 50 X TAE buffer
3. 6X loading dye
4. Ethidium bromide (0.5µg /ml stock)
5. Tracking dye
6. Ethidium bromide (0.5µg /ml stock)

Table 8. Composition of 50X TAE buffer

Contents	50X stock concentration	
Tris base	242g	Final volume 1 litre
Glacial acetic acid	57.1 ml	
EDTA(p^H=8)	100 ml of 500mM	

In order to prepare 1X TAE from 50X TAE stock, 20 ml of stock was diluted with 980 ml of deionized water

Procedure

Agarose gel (2 per cent) was prepared by boiling 5g agarose in 250ml of 1X TAE buffer. After 5 to 10 min of melting agarose, ethidium bromide was added (0.5µg/ml) and mixed well. Care was taken to prevent the formation of air bubbles. While the agarose cooled, the gel casting tray and the combs were wiped with 70 per cent ethanol. The liquefied agarose gel was then poured into the casting tray after placing the comb and allowed to stand for 30 minutes to solidify. The comb was then removed and the tray kept in the electrophoresis unit. 1X TAE buffer was added to the electrophoresis unit.

DNA sample was diluted with Millipore water in 1:9 ratios. The DNA sample (5µl) along with 2µl, 6X loading dye was added to the wells using a micropipette. 100bp molecular weight DNA ladder was loaded in one of the wells as a standard marker for easy detection of the molecular weight of PCR product and interpretation of PCR results. The electrophoresis unit was then run for 90-120 minutes at 80-90 Volts until the loading dye reached $2/3^{rd}$ of the gel. After electrophoresis was complete the gel was carefully moved to gel documentation unit (Gel Doc Bio-Rad) and observed under UV light. In the presence of UV light presence of highly resolved high molecular weight thick bands near the wells indicated the presence of DNA. RNA contamination if present can be visualized as a thick smear around 100bp region while a thick white patch observed inside the well indicated the presence of protein. Presence of a white tail like patch in the lane pointed to presence of phenol. Search results in Quantity One® 1-D Analysis Software provided by Bio-Rad, California was used to analyze the electrophoresed agarose gel.

3.3.1.1.2.2 Quantifying quality and quantity using Nanodrop

Further confirmation of the quality and quantity of the DNA isolated was analysed using Nanometer (JH Bioinovations, India). The maximum absorbance of nucleic acids and proteins occurs at 260 nm and 280nm respectively. The absorbance of the samples (1µl) was measured at wavelengths 260 nm and 280 nm. The purity of DNA was assessed based on the A_{260}/A_{280} ratio. A ratio of 1.8 to 2.0 indicated pure DNA. Protein contamination was inferred if the ratio was less than 1.8 and RNA contamination was inferred if the ratio was greater

than 2.0. The quantity of DNA was calculated based on the relation that Optical density (OD) for a DNA sample with a concentration of 50 μg/ml (double stranded) at 260 nm equals one.

1 OD260 = 50 μg/ml (ds)

Therefore, quantity of DNA (μg/ml) = Absorbance at OD260 ×50

3.3.1.1.3. Dilution of DNA for PCR

The recorded OD values and DNA quantity (ng/μl) values were used for further dilution of the DNA. The DNA with a concentration of 50ng/μl is apt for PCR reactions. Therefore, the obtained concentration of DNA was diluted to obtain 100μl, 50ng DNA per sample using the formula $V_1N_1 = V_2N_2$.

3.3.1.2. Polymerase Chain Reaction (PCR)

The good quality DNA isolated from the leaf samples were further diluted to make a concentration of 50 ng/μl and were used for polymorphism study. The DNA was amplified for both foreground and background selection using appropriate STS/ RM markers detailed in Tables 5 and 6 earlier. PCR amplification was performed using thermal cycler Applied Bio systems ProFlex PCR System (Thermofisher Scientific, catalog# 4484073).

3.3.1.2.1. Foreground selection

For confirming the presence of the resistance allele of each gene in the backcross generation, three STS markers RG556, RG136 and pTA248, closely linked to the BB resistance genes *xa5*, *xa13*, and *Xa*21, respectively were used. Restriction digestion of the STS markers RG556 with restriction enzyme *Dra*I and marker RG136 with enzyme *Hinf* I, was done after PCR amplification as advocated by Sundaram *et al.* (2008). In addition, the functional marker *xa5*SR and *xa13* promoter were also used to confirm the presence of R-genes *xa5* and *xa13* respectively.

3.3.1.2.1.1 Primer dilution and PCR

The primers were first diluted with distilled water. It was diluted to a ratio 1:9 using 10 μl of the primer and 90 μl distilled water. Amplification of the diluted genomic DNA was performed using 15 μl reaction mixture in 0.2 ml PCR tubes. Required number of PCR tubes was arranged and the PCR reaction mixture for each tube was prepared as detailed in Table 9. The tubes were then centrifuged for 1 minute at 4°C and placed in the thermal cycler as per the PCR reaction profile provided (Table 10) by Sundaram *et al.* (2008).

Table 9. Composition of PCR reaction mix

Aliquot	Quantity (µl)
10x Taq buffer	1.00
dNTPs mix	0.50
$MgCl_2$(25mM)	0.25
Taq DNA polymerase (1U)	0.40
Primers (Forward and Reverse)	1.00 each
DNA sample	3.00
Distilled water	2.85
Total	**10.00**

Table 10. PCR reaction profile followed

Stage	Temperature (°C)	Time (Minutes)	Number of cycles
Initial denaturation (hot start)	95	3.00	
Denaturation	94	1.00	35
Primer annealing	Varied as per Tm value of Primer	1.00	
Primer elongation	72	1.20	
Final extension	72	10.00	
Cold storage	4	Infinity	

3.3.1.2.1.2 Restriction digestion of PCR product

Restriction digestion of the PCR products was done in case of STS primers RG 556 and RG 136. Five µl of the PCR amplified product was used initially to detect the amplification of DNA. The remaining DNA was used for restriction digestion with enzymes Dra1 (Thermofisher Scientific catalog# ER0221) and *Hinf* 1 (Thermo fisher Scientific catalog# ER0801) respectively for the STS primers RG 556 and RG 136 as detailed in Tables 11 and 12.

Table 11. Reaction mixture for restriction digestion with *Hinf*I

Aliquot	**Quantity (µl)**
Nuclease-free water	18.00
10X- buffer R	2.00
PCR reaction mixture	0.50
*Hinf*I	1.00

Table 12. Reaction mixture for restriction digestion with *Dra*1

Aliquot	**Quantity (µl)**
Nuclease-free water	18.00
10X- buffer Tango	2.00
PCR reaction mixture	0.50
*Dra*1	1.00

In both case the reaction mixture was swirled gently and spinned down for a few seconds and incubated at 37^{0}C for 1-2 hours

3.3.1.2.1.3. Screening and analysis of PCR products

The restriction digested PCR products were separated by gel electrophoresis along with 100bp ladder (Genei, Banglore) on 1.5 per cent agarose gel stained with ethidium bromide. Banding pattern obtained was visualized using gel documentation unit (Gel Doc Bio-Rad, California) under UV exposure. The gel pictures were saved in image format for further scoring and detection of polymorphism among parents and BC_2F_4s.

3.3.1.2.2. Background selection

Fifty rice microsatellite (RM) markers [SSR (Simple Sequence Repeats) markers] (Table 12) reported to be polymorphism between the parental genotypes Jyothi and Improved Samba Mahsuri, were chosen to genotype BC_2F_4 generation, in order to determine the genotypic background of the pyramided lines with recurrent parent Jyothi. PCR products were separated by agarose gel electrophoresis. No restriction digestion of PCR product was done for markers used for background selection.

3.3.1.3 Analysis of DNA amplification pattern in parents and BC_2F_4s

The banding pattern of PCR bands was observed to be either monomorphic or polymorphic using UVITEC Fire Reader software (Cambridge, UK). The molecular weight of every PCR bands was measured by comparing with given known reference molecular weight marker ladder. Amplicons of the same size were recorded as monomorphic bands whereas bands of different sizes were recorded as polymorphic. Data were then processed with Graphical Genotyping Tool (GGT) *version* 2.0 (Van Berloo, 1999) software.

3.3.1.4. Observations recorded

3.3.1.4.1. Genotyping of BC_2F_4 population

3.3.1.4.1.1 Quality and quantity of DNA isolated

The purity of DNA was assessed using the A_{260}/A_{280}. A ratio between a range of 1.8 – 2.0 indicated pure DNA.

The concentration of nucleic acid in the sample was checked using Nano drop.

3.3.1.4.1.2 Nature of amplification

By using the image captured by the gel documentation system (UVITEC Fire Reader software), the banding pattern resolved on the gel for each marker was observed. The band amplified was recorded either as monomorphic or polymorphic.

3.3.1.4.1.3 Number of amplicons

By using the image captured by the gel documentation system (UVITEC Fire Reader software) the number of amplicons resolved on the gel for each marker was counted.

3.3.1.4.1.4 Size of amplicons

Estimation of the size of amplicons resolved on the gel for each marker in base pairs (bp) was done by using UVitec Fire Reader software (GeNei™ – UVITEC, Merck, UK).

3.3.1.5. Statistical analysis

The data generated from genotyping of BC_2F_4 population where analysed with Graphical GenoTypes (GGT) version 2.0 (Van Berloo, 1999) software. It was used for evaluating the genomic contribution of the parent in each recombinant based on the SSR data. The software generates similarity matrix as per Sneath and Sokal (1973) and clusters based on default similarity coefficient and dendrogram were generated. Mini-tab software was used for cluster analysis of parents and selected BC_2F_4s.

The proportion of genome of the recipient parent was estimated according to Sundaram *et al.* (2008) as follows:

$$G= [(X+1/2Y)\times 100]/N$$

where N = total number of parental polymorphic markers screened,

X = number of markers showing homozygosity for the recipient parent alleles,

Y = number of markers showing heterozygosity for the parental alleles.

3.3.2 Experiment II: Morphological characterization of pyramided lines (BC_2F_4 population) and parents

Morphological characterisation of all the 51 BC_2F_4 plants and parents (10 plants each) was done at appropriate growth stages growth stages as per IRRI (1996).

3.3.2.1 Observations recorded

1. **Plant height (cm)**

   Plant height was measured from the ground level to the tip of flag leaf at flowering and expressed in centimetre.

2. **Days to flowering**

   The number of days required from the date of sowing to the date of first panicle emergence in 50 per cent of the population was recorded.

3. **Leaf width (cm)**

Measured across the leaf lamina at the broadest point of ten random leaves at flowering and the average computed and expressed in centimetre.

4. **Leaf blade length (cm)**

The distance from the base to tip of ten representative leaves were measured at flowering and the average computed and expressed in centimetre.

5. **Productive tillers/ plants**

The total number of grain-bearing tillers per plant was counted at maturity stage and expressed as integer numbers.

6. **Panicle length (cm)**

Length of the main axis of panicle was measured from the panicle base to the tip at flowering and expressed in centimetre.

7. **Spikelets/panicle**

Number of spikelets in each panicle was counted on three randomly selected selfed panicles from each plant at flowering and the average computed.

8. **Grains /panicle**

Number of filled grains/panicle was counted at maturity on three randomly selected selfed panicles from each plant at maturity and the average computed.

9. 1000 grain weight (g)

A random sample of 100 well-developed, whole grains, was weighed after harvest and extrapolated to arrive at 1000 grain weight and expressed in grams.

10. Grain length (mm)

The length of grains was measured from ten randomly selected selfed seeds to obtain the grain length in millimetre.

11. Grain width (mm)

The width of grains was measured from ten randomly selected selfed seeds to obtain the grain length in millimetre.

12. Decorticated grain length (mm)

The seeds were decorticated and the length measured from ten randomly selected selfed and expressed in millimetre.

13. Decorticated grain width (mm)

The seeds were decorticated and the width measured from ten randomly selected selfed and expressed in millimetre.

14. Grain yield/ plant (g)

Grain yield from each plant was computed from the data on number of productive tillers per plant, number of grains per panicle and 1000 grain weight and expressed in grams.

$$\text{Grain yield/ plant (g)} = \frac{\text{Productive tillers/ plant x Grains/ panicle}}{\text{1000 grain weight (g)}}$$

15. Straw yield/ plant (g)

Vegetative growth or straw yield from each plant was weighed and the average value expressed in grams.

3.3.2.2. Statistical analysis

3.4.2.2.1. Parameters of variability

1. Mean

The mean value of each observation was calculated by dividing the total of individual values of each observation by equivalent number of the observation made:

$$\mathbf{X} = \frac{\sum X_i}{N}$$

Where,

X_i - any observation in i^{th} treatment

N - Total number of observations

2. Range

The range of each observation was noted as the lowest and highest values present in the observations of a sample.

3.4.2.2.2. Diversity analysis

Then construction of dendrogram was done based on morphological data based on similarity matrix as per Sneath and Sokal (1973).

3.3.3 Experiment III: Production of BC_3F_1s

The BC_2F_4s introgressed with BB resistance genes were backcrossed to the recurrent parent Jyothi to get BC_3F_1 seeds. Few BC_2F_4s were also backcrossed to donor parent ISM. During backcrossing, the florets of BC_2F_4s were emasculated before anthesis following the clipping method. The pollen collected from the male parent were then dusted on the emasculated spikelets to ensure seed set.

3.3.3.1. Emasculation

The panicles of female parent (BC_2F_4s) that exhibited fifty to sixty per cent emergence were selected for emasculation. To expose the spikelet's and for ease of emasculation, the leaf sheath was slightly detached from the panicle. Emasculation was done late in the afternoon (after 3 p.m.). Very young florets from the bottom of the panicle, in which the height of the anthers was less than half the floret, were cut away. Florets that were likely to open the next day (with the height of anthers being equal or more than half the florets) were selected for emasculation. Scissors were used to clip the top one-third of each floret selected for emasculation and expose the anthers. The anthers were then removed using the tip of the forceps prong by pressing them against the side of the floret and lifting out. The emasculated panicles were enclosed in butter paper bags, tagged and labeled. The butter paper bags were held securely in place by folding its bottom edge against the peduncle before tagging.

3.3.3.2. Pollination

Subsequent to emasculation, pollination was carried out at 8 a.m. the following day. The panicles from the male parent variety (Jyothi / ISM) that were about to dehisce, were selected and the spikelets enclosed in a petri dish. Collected pollen grains were gently tapped on the top of the petri dish. The collected pollen grains were then transferred to the stigma with the help of a thin camel brush. To avoid contamination by foreign pollen, the pollinated

panicles were re-bagged. The panicles were checked for seed set on the fifth-day after hybridization. Maximum seed set was observed when pollination was done on the day of emasculation, although the stigma remained receptive for three to seven days. The seeds were harvested at the time of maturity, dried to 13 per cent and stored.

3.3.4. Experiment IV: Production of BC_2F_5s

BC_2F_4s plant were also selfed to obtain BC_2F_5 seeds. The seeds were harvested at maturity and dried to 13 percent before storage.

Results

CHAPTER IV

RESULTS

Bacterial blight (BB) in rice, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is a devastating disease affecting rice crop and impacts rice production and productivity world over. The two most popular rice cultivars of Kerala; Jyothi and Uma, are highly susceptible to the *Xoo* pathogen. Considering the advantage of host-plant resistance over cultural practices or chemical means to check the pathogen, an attempt was made to pyramid three genes conferring resistance to bacterial blight pathogen (*xa*5, *xa*13, and *Xa*21) into Jyothi (PTB39) to develop durable resistance from Improved Samba Mahsuri (ISM) through marker assisted selection. Backcrossing of F_1s and the resultant backcross generation to Jyothi, had produced BC_2F_{1s} pyramided with the three R-genes. The BC_2F_4 individuals generated subsequently, served as the base population for the present study. Morphological and molecular characterization of the BC_2F_4 population to identify R-gene pyramids and development of BC_3F_1s and BC_2F_5s were also envisaged. The outcome of the attempt is detailed below.

1.1.Genotyping of BC_2F_4 population

4.1.1. Quantity and quality of genomic DNA of BC_2F_4 and parents

The quantity and quality of genomic DNA extracted from BC_2F_4s individuals and parents are given in Table 13 and Appendices 1 and 2.

The quantity of DNA extracted from BC_2F_4s individuals ranged from 667 ng/µl in Plant No. 40 to 1403 ng/µl in Plant No. 17. The average concentration of extracted DNA in the recurrent parent Jyothi and donor parent ISM was 959.3 ng/µl and 994.6 ng/µl respectively.

The A_{260}/A_{280} ratio of DNA extract from BC_2F_4s ranged from 1.7 to 1.9. It varied between 1.8 and 1.9 in the recurrent parent Jyothi as well as the donor parent ISM.

4.1.2 Foreground selection

4.1.2.1. Foreground selection for R-gene *xa5*

The result of foreground selection is presented in Table 14 and Plates 1 to 5. Individuals of BC_2F_4 population were genotyped with, STS marker RG556 and functional marker *xa5*SR to detect the presence of resistance allele of *xa5* gene. The PCR products of RG556 marker on

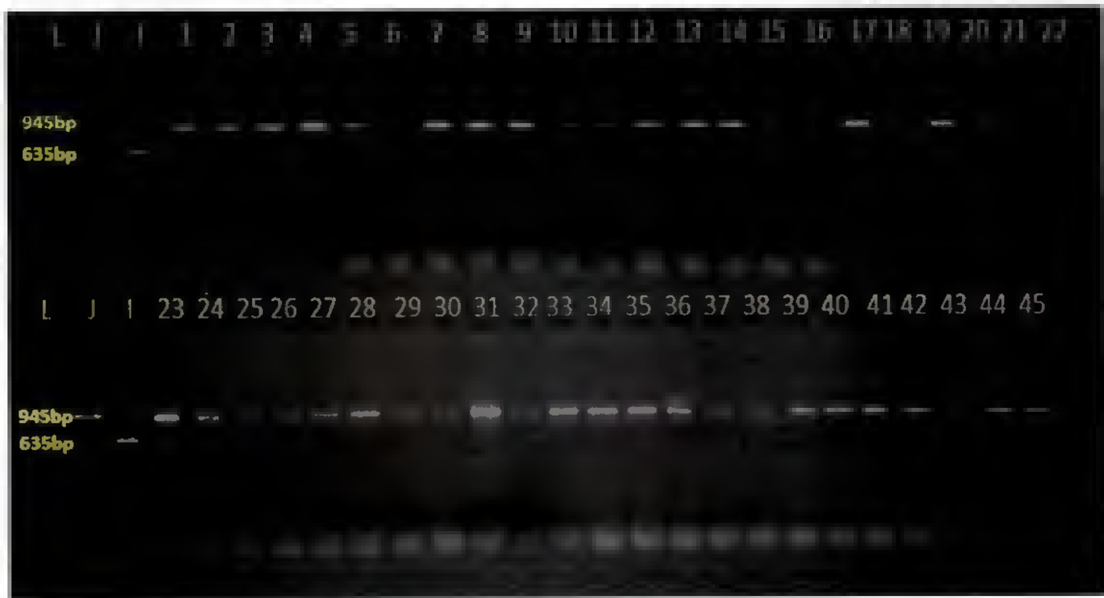
Plate 1. Foreground selection of BC_2F_4s using *xa5* linked STS marker RG556

L: 1 kb Ladder **J: Jyothi** **I: Improved Samba Mahsuri** **1-45: BC_2F_4**

Plate 2. Foreground selection of BC_2F_4s using *xa5* linked functional marker *xa5*SR

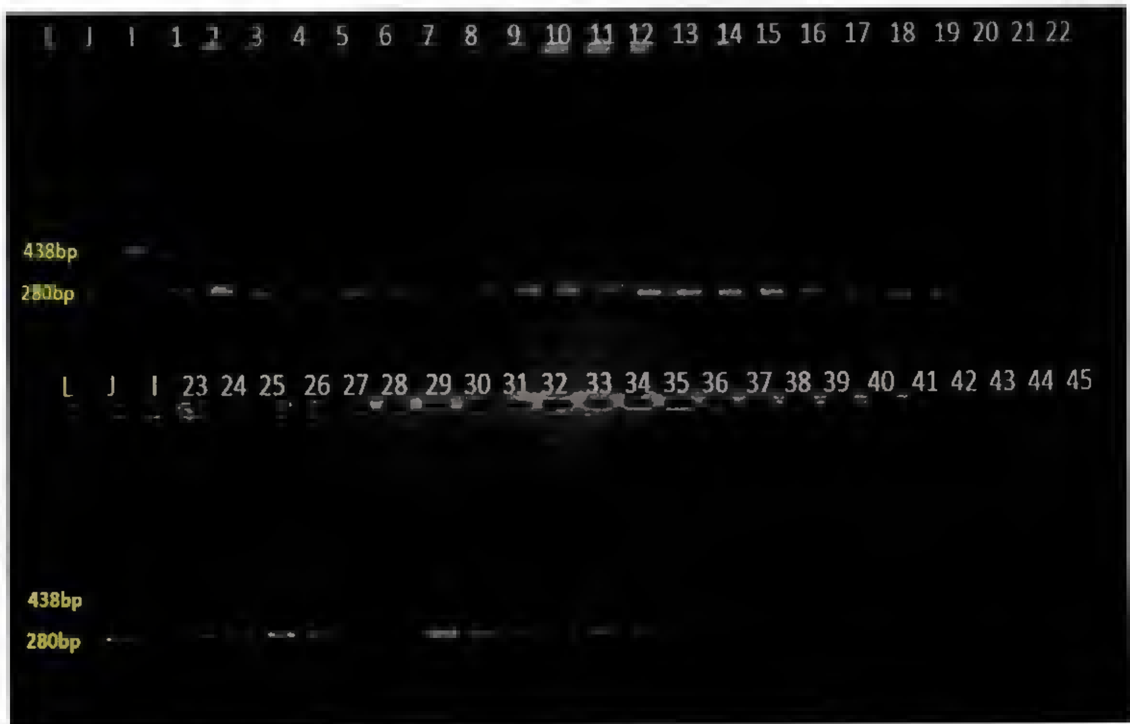
L: 1 kb Ladder **J: Jyothi** **I: Improved Samba Mahsuri** **1-45: BC_2F_4**

Plate 3. Foreground selection of BC_2F_4s using *xa13* linked STS marker RG136



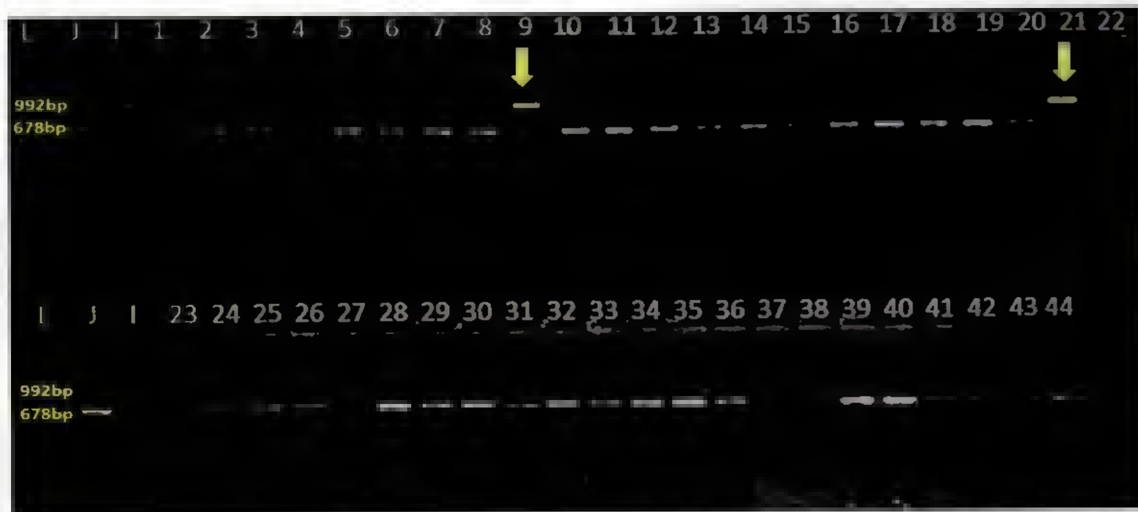
L: 1 kb Ladder J: Jyothi I: Improved Samba Mahsuri 1-45: BC_2F_4

Plate 4. Foreground selection of BC_2F_4s using *xa13* linked functional marker *xa13* promoter



L: 1 kb Ladder J: Jyothi I: Improved Samba Mahsuri 1-45: BC_2F_4

Plate 5. Foreground selection of BC_2F_{4}s using *Xa*21 linked STS marker pTA248



L: 1 kb Ladder J: Jyothi I: Improved Samba Mahsuri 1-44: BC_2F_4

Table 13. Quantity and quality of genomic DNA of parents and BC_2F_4s

Genotypes	Quantity of DNA (µg/ µl)			Quality of DNA (A_{260}/A_{280})		
	Mean	Range		Mean	Range	
		Max	Min.		Max.	Min.
Jyothi (Recurrent parent)	959.30	1102	703	1.82	1.9	1.8
ISM (Donor parent)	994.60	1123	915	1.83	1.9	1.8
BC_2F_4s	1035.13	1403	667	1.83	1.9	1.7

Table 14. Distribution of alleles of foreground marker loci linked to BB resistance genes in parents and BC_2F_4s

Target genes	*xa5*		*xa13*		*Xa21*
Marker	**RG 556**	***xa5*SR**	**RG 136**	***xa13* promoter**	**pTA 248**
Donor parent (ISM)	+	+	+	+	+
Recurrent parent (Jyothi)	+	+	-	-	-
BC_2F_4 plants					
1	+	+	-	-	-
2	+	+	-	-	-
3	+	+	-	-	-
4	+	+	-	-	-
5	+	+	-	-	-
6	+	+	-	-	-
7	+	+	-	-	+
8	+	+	-	-	-
9	+	+	-	-	+
10	+	+	-	-	-
11	+	+	-	-	-
12	+	+	-	-	-
13	+	+	-	-	-
14	+	+	-	-	-
15	+	+	-	-	-
16	+	+	-	-	-
17	+	+	-	-	-
18	+	+	-	-	-
19	+	+	-	-	-
20	+	+	-	-	-
21	+	+	-	-	+
22	+	+	-	-	-
23	+	+	-	-	-
24	+	+	-	-	-
25	+	+	-	-	-
26	+	+	-	-	-
27	+	+	-	-	-
28	+	+	-	-	-
29	+	+	-	-	-
30	+	+	-	-	-
31	+	+	-	-	-
32	+	+	-	-	-
33	+	+	-	-	-
34	+	+	-	-	-
35	+	+	-	-	-
36	+	+	-	-	-
37	+	+	-	-	-
38	+	+	-	-	-
39	+	+	-	-	-

40	+	+	-	-	-
41	+	+	-	-	-
42	+	+	-	-	-
43	+	+	-	-	-
44	+	+	-	-	-
45	+	+	-	-	-
46	+	+	-	-	-
47	+	+	-	-	-
48	+	+	-	-	-
49	+	+	-	-	-
50	+	+	-	-	-
51	+	+	-	-	-
+ indicates presence of the gene				**- indicates absence of the gene**	

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digestion with restriction enzyme *Dra*I (Plate 1) produced two fragment of size 238 bp and 438 bp associated with the resistant allele in all the BC_2F_4 plants as well as the parents.

Foreground selection with the functional marker *xa5*SR (Plate 2) also produced monomorphic bands of 190 bp in both the parents as well as the BC_2F_4 plants analyzed

4.1.2.2. Foreground selection for R-gene *xa13*

To confirm the presence of *xa13* gene-resistance alleles, STS marker RG136 and functional marker *xa13* promoter were used to genotype the individual of BC_2F_4 population. The PCR products were digested with restriction enzyme *Hinf*I and examined for specific amplicon polymorphism (SAP). On restriction digestion (Plate 3) fragments of size 635 bp and 945 bp was detected in ISM and the recurrent parent Jyothi respectively. All BC_2F_4 plants produced a 945 bp band similar to Jyothi.

Similarly, foreground selection with the functional marker *xa13* promoter, resulted in production of 280 bp amplicon in recurrent parent Jyothi and all BC_2F_4 individuals while an amplicon of size 428 bp was produced in donor parent ISM (Plate 4).

4.1.2.3. Foreground selection for R-gene *Xa21*

Foreground selection with STS marker pTA 248 to detect the presence of *Xa21* gene (Plate 5) revealed that BC_2F_4 Plant No. 9 and Plant No. 21 (Lane 9 and 21 respectively) exhibited amplicon of size 992 bp as in the donor parent ISM. The remaining 49 BC_2F_4 individuals exhibited a band of size 678 bp similar to that in the susceptible recurrent parent Jyothi.

4.1.3. Background selection

Out of the 51 BC_2F_4s subjected to foreground selection, two individuals (plants 9 and 21), were identified to possess *xa5* and *Xa21* R-genes under section 4.1.2. In addition, Plant No. 5, Plant No. 25 and Plant No. 27 that were near similar to recurrent parent Jyothi with respect to plant height and days to flowering were also selected for background screening. These plants had produced higher number of productive tillers/ plant, panicle length and spikelets/panicle. Fifty RM (Rice microsatellite) markers detailed in Table 6 listed under Chapter 2 were used for background selection. The results are detailed in Table 15 and Plates 6(i) to 6(x).

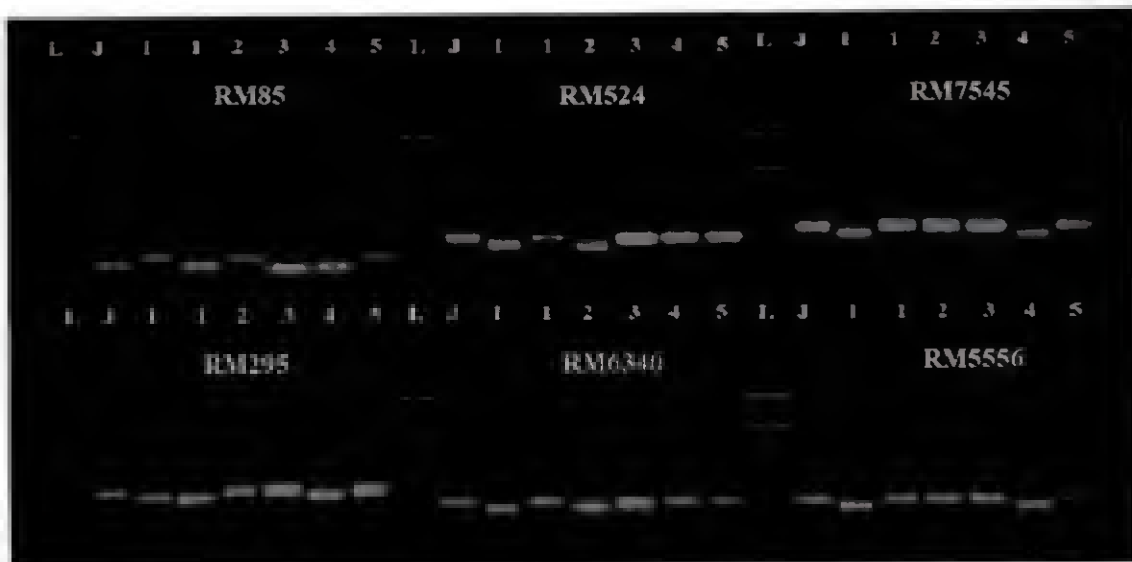


Plate 6 (i). Background selection of BC_2F_4 plants using microsatellite markers (Set-1)

L: 1kb Ladder	J: Jyothi	I: ISM	1: Plant No. 5
2: Plant No. 9	3: Plant No. 21	4: Plant No. 25	5: Plant No. 27

Plate 6 (ii). Background selection of BC_2F_4 plants using microsatellite markers (Set-2)

L: 1kb Ladder	J: Jyothi	I: ISM	1: Plant No. 5
2: Plant No. 9	3: Plant No. 21	4: Plant No. 25	5: Plant No. 27

Plate 6 (iii). Background selection of BC_2F_4 plants using microsatellite markers (Set-3)

L: 1kb Ladder	J: Jyothi	I: ISM	1: Plant No. 5
2: Plant No. 9	3: Plant No. 21	4: Plant No. 25	5: Plant No. 27

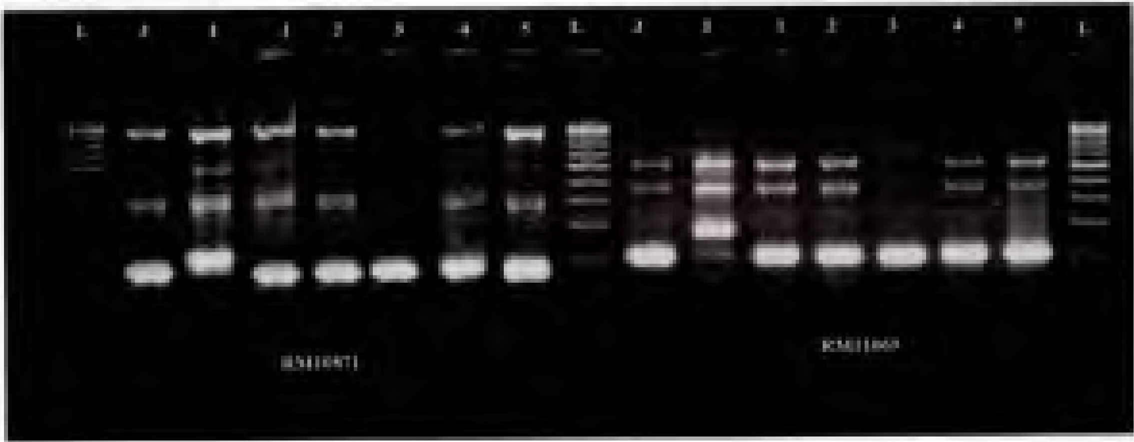


Plate 6 (iv). Background selection of BC_2F_4 plants using microsatellite markers (Set-4)

L: 1kb Ladder	J: Jyothi	I: ISM	1: Plant No. 5
2: Plant No. 9	3: Plant No. 21	4: Plant No. 25	5: Plant No. 27

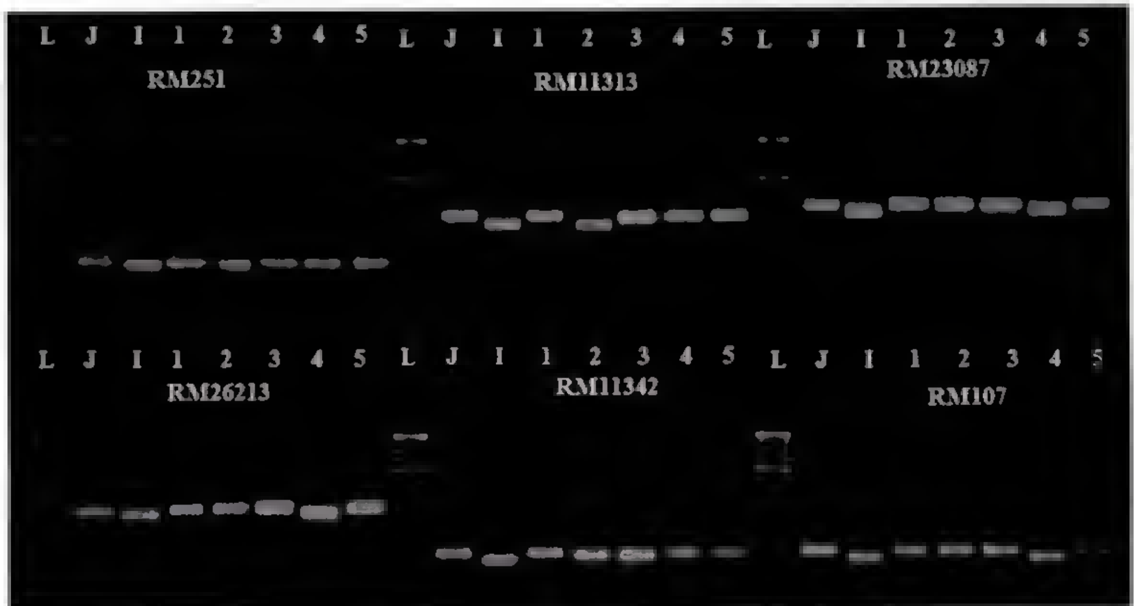


Plate 6 (v). Background selection of BC_2F_4 plants using microsatellite markers (Set-5)

L: 1kb Ladder	J: Jyothi	I: ISM	1: Plant No. 5
2: Plant No. 9	3: Plant No. 21	4: Plant No. 25	5: Plant No. 27

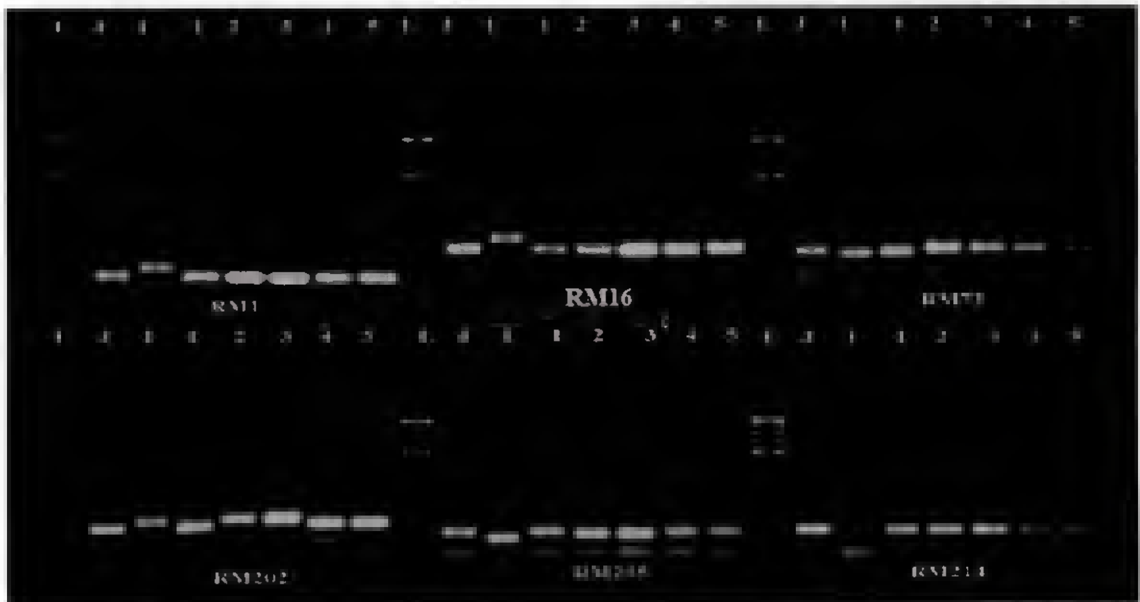


Plate 6 (vi). Background selection of BC_2F_4 plants using microsatellite markers (Set-6)

L: 1kb Ladder	J: Jyothi	I: ISM	1: Plant No. 5
2: Plant No. 9	3: Plant No. 21	4: Plant No. 25	5: Plant No. 27

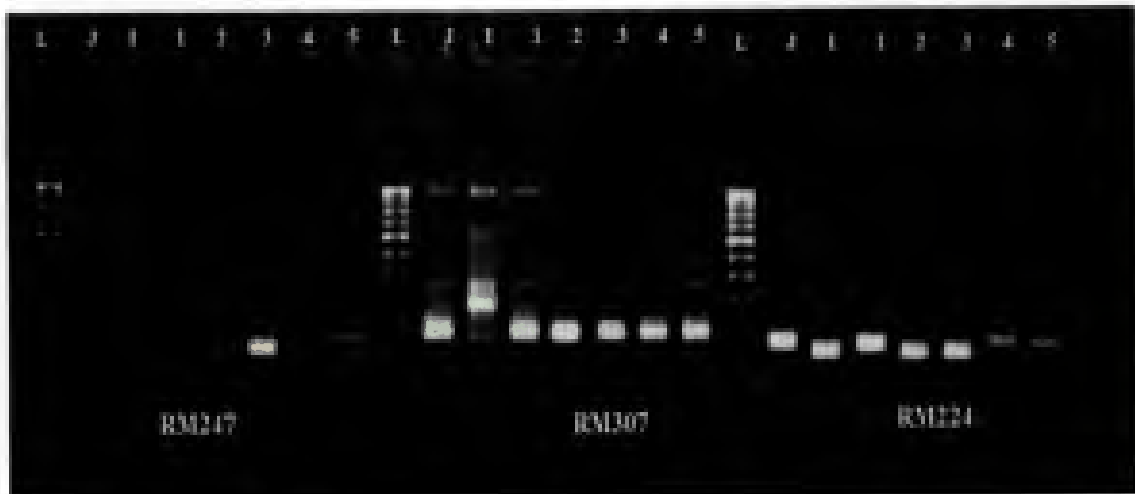


Plate 6 (vii). Background selection of BC_2F_4 plants using microsatellite markers (Set-7)

L: 1kb Ladder	J: Jyothi	I: ISM	1: Plant No. 5
2: Plant No. 9	3: Plant No. 21	4: Plant No. 25	5: Plant No. 27

Plate 6 (viii). Background selection of BC_2F_4 plants using microsatellite markers (Set-8)

L: 1kb Ladder	J: Jyothi	I: ISM	1: Plant No. 5
2: Plant No. 9	3: Plant No. 21	4: Plant No. 25	5: Plant No. 27

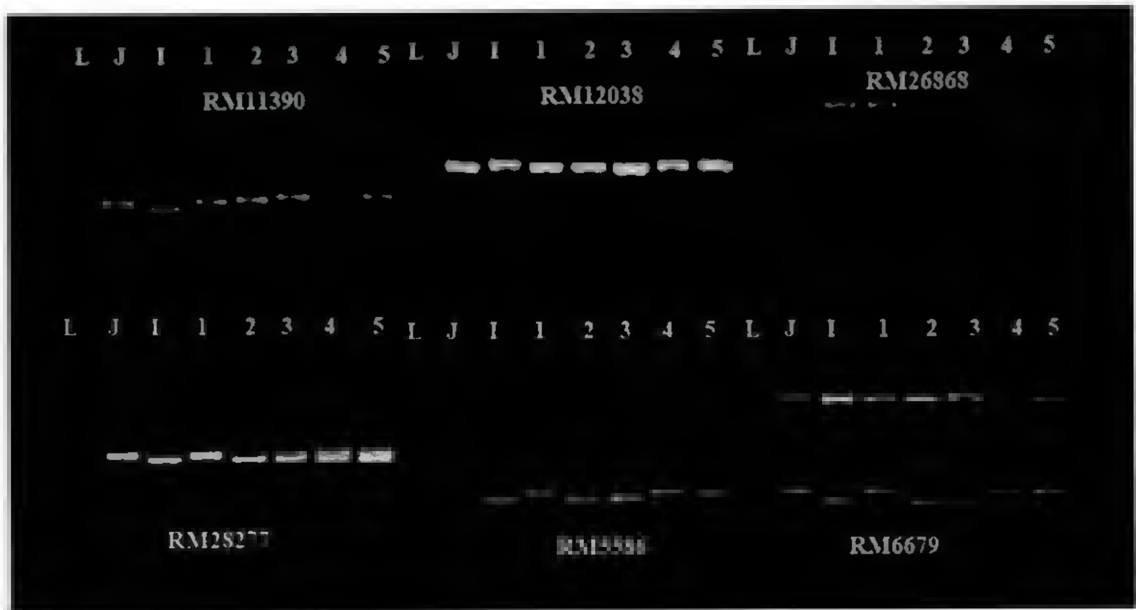


Plate 6 (ix). Background selection of BC_2F_4 plants using microsatellite markers (Set-9)

L: 1kb Ladder	J: Jyothi	I: ISM	1: Plant No. 5
2: Plant No. 9	3: Plant No. 21	4: Plant No. 25	5: Plant No. 27

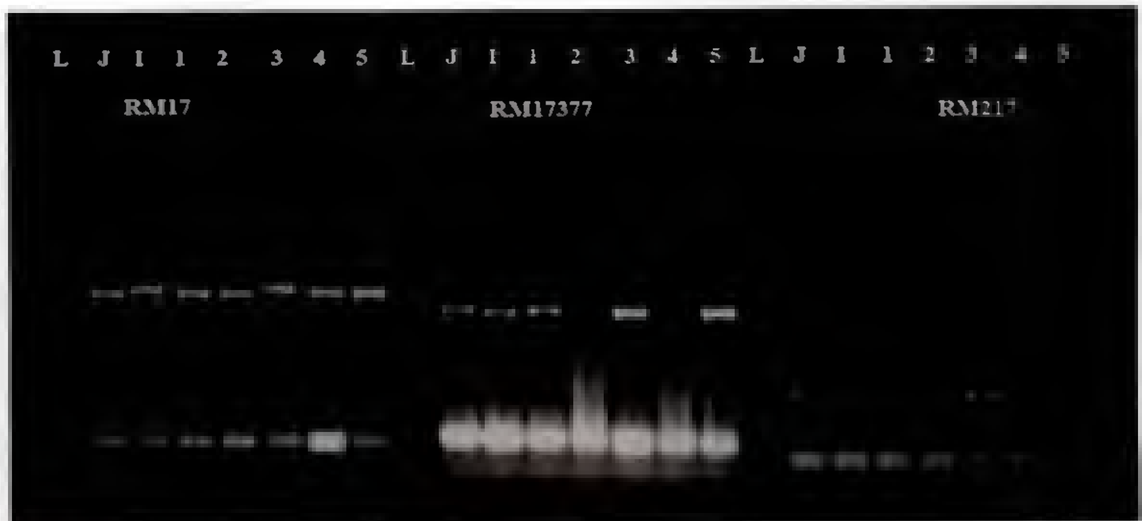


Plate 6 (x). Background selection of BC_2F_4 plants using microsatellite markers (Set-10)

L: 1kb Ladder	J: Jyothi	I: ISM	1: Plant No. 5
2: Plant No. 9	3: Plant No. 21	4: Plant No. 25	5: Plant No. 27

Table 15. Distribution of alleles of PCR marker loci used for background selection in selected BC_2F_4s and parents

Genotype	Jyothi (Recurrent parent)	BC_2F_4s					ISM (Donor parent)
		Plant No. 5	Plant No. 9	Plant No. 21	Plant No. 25	Plant No. 27	
Chromosome 1							
RM1	A	A	A	A	A	A	B
RM6340	A	A	B	H	A	A	B
RM10871	A	A	A	A	A	A	B
RM11069	A	A	A	A	A	A	B
RM11313	A	A	B	A	A	A	B
RM11342	A	A	A	A	A	A	B
RM12038	A	A	A	A	B	B	B
Chromosome 2							
RM482	A	A	A	B	A	A	B
RM3340	A	A	B	A	A	A	B
RM11390	A	A	A	A	A	A	B
RM12941	A	H	A	A	B	B	B
Chromosome 3							
RM16	A	A	A	A	A	A	B
RM49	A	H	H	H	B	A	B
RM85	A	A	B	A	A	B	B
RM251	A	A	B	A	A	A	B
RM15583	A	A	A	A	A	A	B
Chromosome 4							
RM307	A	A	A	A	A	A	B
RM5586	A	A	B	B	A	A	B
RM6089	A	A	B	A	A	A	B
RM6679	A	A	B	B	A	A	B
RM17377	A	A	B	B	B	B	B
Chromosome 5							
RM18225	A	A	B	A	A	A	B
RM18918	A	A	B	A	B	A	B

RM19218	A	A	A	A	B	A	B
Chromosome 6							
RM217	A	H	A	A	B	B	B
RM439	A	A	A	A	A	A	B
RM19255	A	A	A	A	A	A	B
RM19514	A	A	A	A	A	A	B
RM19793	A	A	A	A	A	A	B
RM20416	A	A	A	A	A	A	B
Chromosome 7							
RM214	A	A	A	A	A	A	B
RM248	A	A	A	A	A	A	B
RM295	A	B	A	A	B	A	B
RM20833	A	B	A	A	A	A	B
RM21345	A	A	H	H	B	A	B
Chromosome 8							
RM72	A	H	H	A	A	A	B
RM308	A	A	A	A	B	A	B
RM5545	A	A	A	A	A	A	B
RM5556	A	A	A	A	B	A	B
RM23087	A	A	A	A	B	A	B
Chromosome 9							
RM107	A	A	A	A	B	A	B
RM205	A	A	H	H	A	A	B
RM524	A	A	B	A	A	A	B
RM23998	A	A	B	B	A	A	B
Chromosome 10							
RM304	A	A	A	A	B	B	B
RM7545	A	A	A	A	B	A	B
Chromosome 11							
RM202	A	A	B	B	A	A	B
RM224	A	A	B	B	A	A	B
RM26213	A	A	A	A	B	A	B
RM26868	A	A	A	A	A	A	B

Chromosome 12							
RM17	A	A	A	B	A	A	B
RM19	A	A	B	B	A	A	B
RM247	A	A	B	B	A	A	B
RM260	A	A	B	A	A	A	B
RM28277	A	A	B	B	H	H	B

A: Allele of recurrent parent **B: Allele of donor parent** **H: Heterozygous locus**

There was no consistency in banding pattern among the selected BC_2F_4s on genotyping with the remaining 34 RM markers. In Plant No. 5, four RM markers (RM12941, RM 217, RM 49 and RM 72) registered heterozygous alleles while two markers (RM 295 and RM 20833) registered alleles similar to ISM.

In Plant No. 9, 17 markers deduced alleles similar to that of donor parent ISM while four markers (RM 49, RM 21345, RM 72 and RM 205) exhibited heterozygous banding pattern.

Marker RM 6340, RM 49, RM 21345 and RM 205 deduced heterozygous loci in Plant No. 21 whereas, 11 markers produced monomorphic bands between the plant and the donor parent ISM.

Monomorphic banding pattern was observed between Plant No. 25 and donor parent ISM with respect to 16 RM markers while marker RM 28277 deduced a heterozygous locus in this plant.

As in Plant No. 25, RM 28277 deduced a heterozygous locus in Plant No. 27 also. Six markers were found to be monomorphic between Plant No. 27 and donor parent ISM.

4.1.4. Recovery of recurrent parent genome

The recovery of the recurrent parent genome in the selected BC_2F_4s individuals (Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27) estimated from the results of the background profiling of these plants using GGT software is detailed in Table 16.

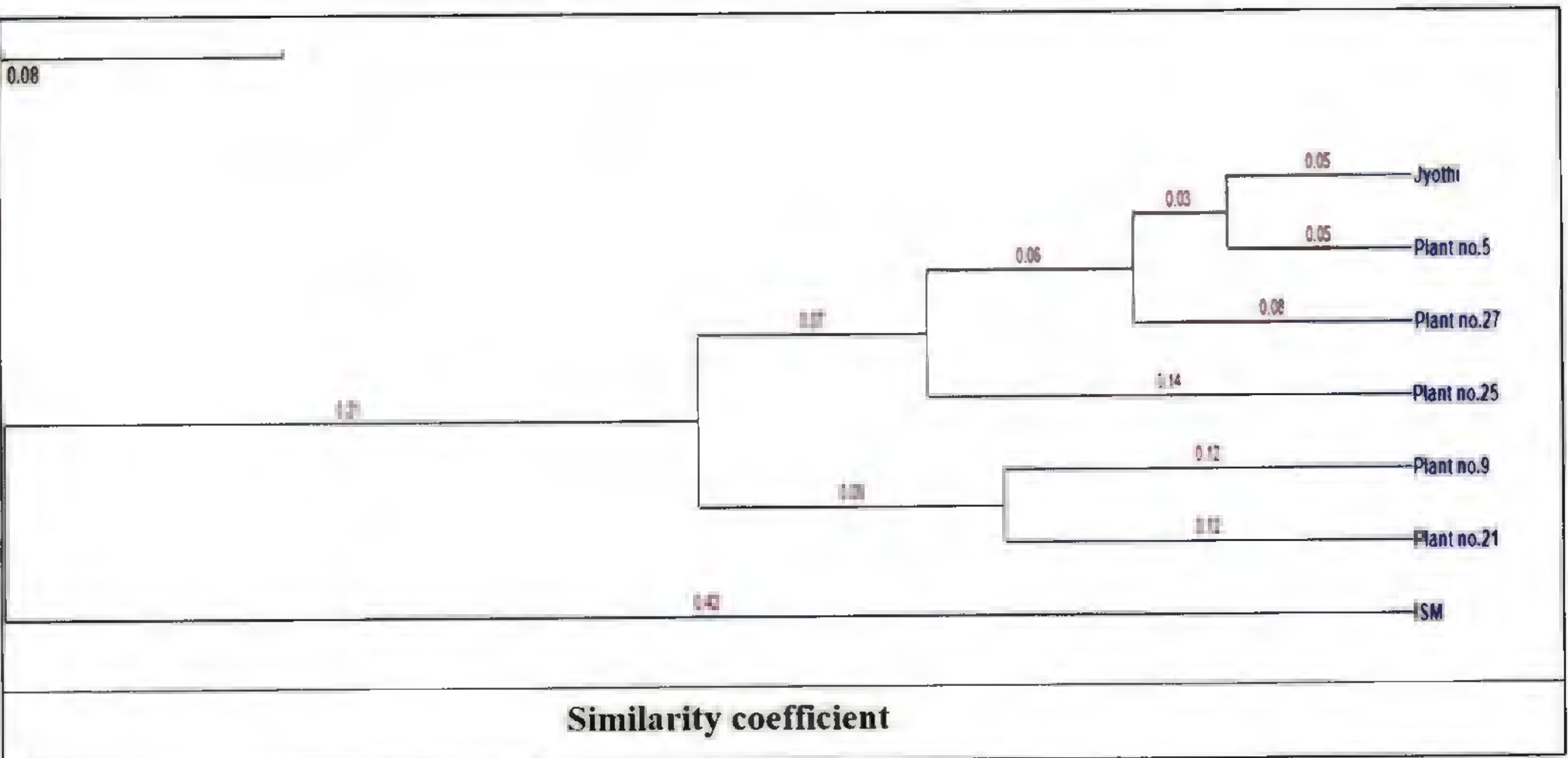
The results indicated that the per cent recovery of recurrent parent genome varied among the BC_2F_4s individuals. The magnitude of recovery of recurrent parent genome was found to be highest in Plant No. 5 (92.20%), followed by Plant No. 27 (91.60%) Plant No. 25 (76.40%) and Plant No. 21 (64.40%). It was found to be least in Plant No. 9 (58.80%).

Based on the marker data (allele sharing), similarity co-efficient was calculated and a dendrogram showing the genetic similarity between the parents and the pyramids was generated (Figure 1). The dendrogram grouped the individuals into two major clusters at 58 per cent similarity. Cluster 1 was monogenic with only the donor parent ISM while, cluster 2 comprised of the recurrent parent Jyothi and all the five BC_2F_4 individuals. Highest similarity (88%) was observed between the R-gene pyramids Plant No. 9 and Plant No. 21. These plants grouped into a separate sub-cluster farthest from recurrent parent Jyothi under cluster 2. Plant No. 5 showed maximum similarity with recurrent parent Jyothi.

Table 16. Contribution of recurrent parent genome in selected BC_2F_4s

<table>
<tr><th rowspan="2">Plant number</th><th colspan="2">Per cent recurrent parent genome in BC_2F_4s</th></tr>
<tr><th>Estimated recovery</th><th>Expected recovery in second backcross generation</th></tr>
<tr><td>Plant No. 5</td><td>92.20</td><td rowspan="5">87.50</td></tr>
<tr><td>Plant No. 9</td><td>58.80</td></tr>
<tr><td>Plant No. 21</td><td>64.40</td></tr>
<tr><td>Plant No. 25</td><td>76.40</td></tr>
<tr><td>Plant No. 27</td><td>91.60</td></tr>
</table>

Figure 1. Clustering of parents and selected BC_2F_{4S} based on molecular data.



4.2. Morphological characterization of BC_2F_4s and parents

The BC_2F_4s were evaluated for their agro-morphological characteristics along with parents. The results obtained are presented in Tables 17, 18a and 18b, Appendices 3 to 8, and detailed below:

4.2.1. Plant height

Plant height of the BC_2F_4 individuals varied between 50.33 cm and 72.68 cm with an average value of 63.59 cm. The height of the selected five BC_2F_4s *i.e.*, Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 was found to be 67.38cm, 50.33cm, 53.05cm, 64.50cm and 67.23cm respectively. The average plant height of parents Jyothi and ISM was 67.69 cm and 47.29 cm respectively.

4.2.2. Days to flowering

The average days to flowering, in the donor parent ISM was 116.44 days. The recurrent parent Jyothi flowered in 85.52 days. Among the BC_2F_4s, days to flowering varied between 86.00 days to 105.00 days. The days to flowering registered in BC_2F_4 Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 was 87.00, 105.00, 104.00, 92.00 and 87.00 days, respectively.

4.2.3. Leaf width

An average leaf width of 1.51 cm, 1.58 cm and 0.95 cm was recorded in the BC_2F_4s, Jyothi and ISM was respectively. Leaf width of the BC_2F_4s ranged from 1.03 cm to 1.98 cm. Leaf width was 1.66cm, 1.03cm, 1.06cm, 1.34cm and 1.96cm in BC_2F_4 Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 respectively.

4.2.4 Leaf blade length

Leaf blade length of the BC_2F_4s ranged from 28.06 cm to 39.4 cm with an average value of 35.49 cm. Leaf blade length of the selected BC_2F_4s *i.e.*, Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 was 29.83cm 35.32cm, 35.91cm, 34.46cm, and 36.67cm, respectively. The average leaf blade length of parents Jyothi and ISM was 37.95cm and 35.50cm respectively.

Table 17. Variability in morphological characteristics among parents and BC_2F_4 selected for background selection

Sl.no.	Plant characters	Recurrent parent			Donor parent(ism)			BC_2F_4s		
		Range			Range			Range		
		Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
1	Plant height	29.29	72.98	67.69	40.81	53.55	47.29	50.33	72.68	63.59
2	Days to flowering	80.00	91.00	85.52	110.00	122.00	116.44	86.00	105.00	93.76
3	Leaf width (cm)	1.05	1.95	1.58	0.09	1.10	0.95	1.03	1.98	1.51
4	Leaf blade length (cm)	37.00	38.72	37.95	29.21	43.22	35.50	28.06	39.40	35.49
5	Productive tillers/plant	1.00	9.00	7.48	6.00	9.00	7.92	6.00	9.00	8.04
6	Panicle length (cm)	20.05	21.25	20.99	21.37	29.03	22.89	26.88	39.68	36.82
7	Spikelet's / panicle	112.00	130.00	123.12	81.00	106.00	96.64	108.23	140.32	131.01
8	Grains / panicle	90.00	114.00	100.48	68.00	91.00	80.44	80.34	134.14	103.44
9	1000 grain weight (g)	24.41	27.90	26.37	9.34	11.78	10.54	18.25	24.95	22.13
10	Grain length (mm)	7.01	7.84	7.50	6.09	6.91	6.48	6.11	8.46	7.05
11	Grain width (mm)	1.10	1.40	1.29	1.59	1.94	1.75	1.14	1.96	1.62
12	Decorticated grain length (mm)	4.14	5.14	4.61	5.64	6.14	5.87	4.70	5.91	5.36
13	Decorticated grain width (mm)	0.58	1.01	0.84	1.36	1.76	1.56	0.84	1.09	0.93
14	Grain yield/ plant (g)	14.23	17.43	15.92	7.27	9.46	8.50	12.13	17.83	15.48
15	Straw yield /plant (g)	19.52	24.88	22.55	9.91	12.84	10.99	19.44	24.98	22.12

Table 18(a). Morphological characteristics of parents and BC_2F_{4s} selected for background selection

Sl. No.	Plant	Plant height (cm)	Days to flowering	Leaf width (cm)	Leaf blade length (cm)	Productive tillers/ plant	Panicle length (cm)	Spikelets/ panicle	Grains/panicle
1	Jyothi	67.69	85.52	1.58	37.95	7.84	20.99	123.12	100.48
2	ISM	47.29	116.44	0.95	35.50	7.92	22.89	96.64	80.44
3	Plant No. 5	67.38	87.00	1.66	29.83	9.00	36.66	130.38	109.17
4	Plant No. 9	50.33	105.00	1.03	35.32	7.00	26.88	108.23	82.76
5	Plant No. 21	53.05	104.00	1.06	35.91	7.00	27.63	113.37	81.48
6	Plant No. 25	64.50	92.00	1.34	34.46	8.00	35.43	140.32	97.54
7	Plant No. 27	67.23	87.00	1.96	36.67	8.00	38.60	130.23	110.47

Table 18(b). Grain characteristics of parents and BC_2F_{4s} selected for background selection

Sl. No.	Plant	1000 grain weight (g)	Grain length (mm)	Grain width (mm)	Decorticated grain length (mm)	Decorticated grain width (mm)	Grain yield/ plant (g)	Straw yield /plant (g)
1	Jyothi	26.41	7.49	1.75	5.87	1.55	15.97	22.52
2	ISM	10.53	6.47	1.29	4.63	0.84	8.5	10.98
3	Plant No. 5	21.43	8.46	1.73	5.88	0.98	15.34	21.24
4	Plant No. 9	18.25	6.38	1.67	5.87	1.03	12.52	20.72
5	Plant No. 21	19.42	6.14	1.74	5.91	1.09	12.13	19.44
6	Plant No. 25	23.4	6.39	1.58	5.23	0.88	15.54	22.69
7	Plant No. 27	20.12	6.98	1.63	4.82	0.86	17.81	20.46

4.2.5. Productive tillers/ plants

The average number of productive tillers per plant in the donor parent ISM (7.92) was higher than in the recurrent parent Jyothi (7.84). Among the 51 BC_2F_4s, it ranged between 6.00 and 9.00. The average number of productive tillers per plant in BC_2F_4s was 8.04. Number of productive tillers of BC_2F_4 Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 was 9.00, 7.00, 7.0, 8.00 and 8.00 respectively.

4.2.6. Panicle length

The average length of panicle in the BC_2F_4s was 36.82 cm and it varied from 26.88 cm to 39.68cm. The recurrent parent Jyothi recorded a panicle length of 20.99 whereas the average panicle length in donor parent ISM was 22.89 cm long. Panicle length of ranged from 36.66cm, 26.88cm, 27.63cm, and 35.43cm to 38.60cm in BC_2F_4 Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 respectively.

4.2.7. Spikelets/ panicle

The average number of spikelets per panicle of BC_2F_4s was 131.01 and it was found to range from 108.23 to 140.32. The average number of spikelet'/ panicle in the recurrent parent Jyothi was 123.12 while it was 106.00 in the donor parent ISM. Spikelets / panicle was 130.38, 108.23, 113.37, 140.32 and 130.23 in BC_2F_4 Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 respectively.

4.2.8. Grains/ panicle

The average number of grains per panicle recorded in parents Jyothi and ISM was 100.48 and 80.44 respectively. The number of grains per panicle in the BC_2F_4s ranged from 80.34 to 134.14. Grains / panicle in BC_2F_4 Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 was 109.17, 82.76, 81.48, 97.54 and 110.47 respectively.

4.2.9. 1000 grain weight

The BC_2F_4s registered an average 1000 grain weight of 22.13g. Thousand grain weight ranged between 18.25g to 24.95g in BC_2F_4s. The recurrent parent Jyothi registered an average 1000 grain weight of 26.41g while it was found to be 10.54g in the donor parent ISM. Thousand

grain weight was 21.43g, 18.25g, 19.42g, 23.40g and 20.12g in BC_2F_4 Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 respectively.

4.2.10. Grain length

Average length of grain in the recurrent parent Jyothi was 7.49 mm and that of the donor parent ISM 6.48 mm. The BC_2F_4s recorded an average grain length of 7.05 mm ranging between 6.11 mm and 8.46 mm. Grain length of BC_2F_4 Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 was 8.46mm, 6.38mm, 6.14mm, 6.39mm and 6.98mm respectively.

4.2.11. Grain width

An average grain width of 1.75 mm was recorded in the recurrent parent Jyothi while it was 1.29 mm in donor parent ISM. Average grain width observed in the BC_2F_4s was 1.62 mm and it ranged from 1.14mm to 1.96 mm. Grain width was 1.73mm, 1.67mm, 1.74mm, 1.58mm and 1.63mm in BC_2F_4 Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 respectively.

4.2.12. Decorticated grain length

The average grain length of the susceptible parent Jyothi after decortication was 5.87 mm and that of the resistant parent ISM was 4.63 mm. Decorticated grain length of the BC_2F_4s ranged between 4.70 mm to 5.91 mm with an average value of 5.36mm. Decorticated grain length of BC_2F_4 Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 was 5.88mm, 5.81mm, 5.91mm, 5.23mm and 4.82mm respectively.

4.2.13. Decorticated grain width

The average decorticated grain width of the BC_2F_4s was 0.93mm and ranged between 0.84mm to 1.09mm. Average grain width after decortication in the recurrent parent was 1.56 mm while it was 0.85 mm in the donor parent. Decorticated grain width was 0.98mm, 1.03mm, 1.09mm, 0.88mm and 0.86mm in BC_2F_4 Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 respectively

4.2.14. Grain yield/ plant

Average grain yield/ plant in Jyothi was 15.97g and 8.50 g in ISM. The average grain yield of the BC_2F_4s was 15.48 g and ranged between 12.13g to 17.83g. Grain yield/ plant of

the selected $BC_2F_{4}s$ *i.e.,* Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 was 15.34g, 12.52g, 12.13g, 15.54g and 17.81g respectively

4.2.15. Straw yield / plant

The BC_2F_4s recorded an average straw yield per plant of 22.12 g. It ranged between 19.44g to 24.98g. An average straw yield 22.52g was recorded in the recurrent parent Jyothi, while it was 10.99 g in donor parent ISM. Straw yield / plant of BC_2F_4 Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 was 21.24g, 21.72g, 19.44g, 22.69g and 20.46g respectively

`4.3. Clustering of parents and BC_2F_4s selected for background selection based on morphological characters

Clustering of the five BC_2F_4s and parents based on morphological characters grouped the recurrent parent Jyothi and BC_2F_4 Plant No. 5, 27 and 25 in one cluster I and donor ISM and BC_2F_4 Plant No. 9 and 21 in cluster II at 75 per cent similarity (Figure 2). Plant No. 5 and Plant No. 27 were more similar to Jyothi than Plant No. 25.

4.4. Production of BC_3F_1s

The selected BC_2F_4s individuals *i.e.,* Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 were backcrossed individually to both recurrent parent Jyothi and donor ISM to obtain BC_3F_1s and BC_1F_1s respectively (Table19).

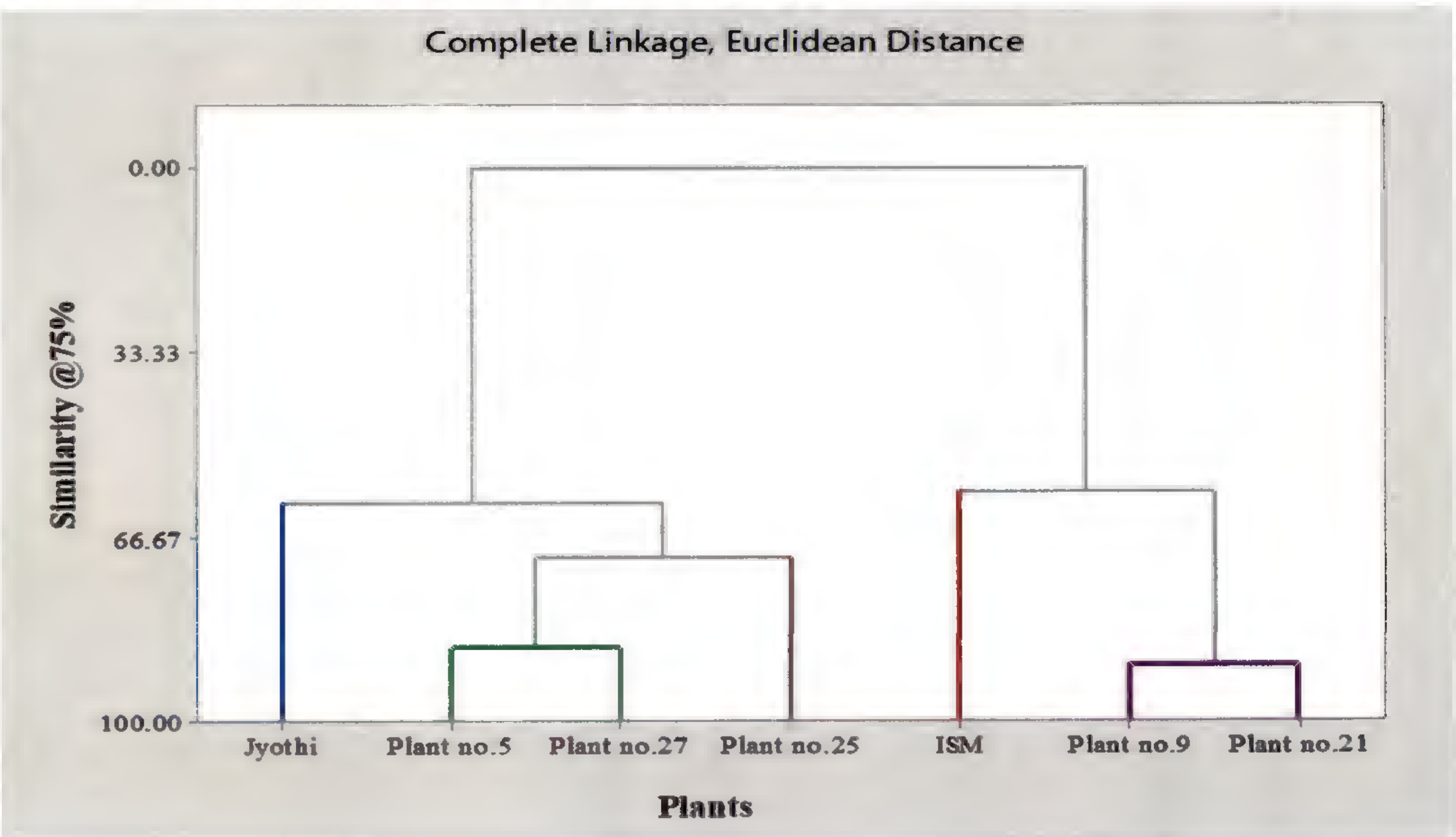
4.5. Production of BC_2F_5s

Selfing was also done in BC_2F_4 Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25 and Plant No. 27 which resulted in the production of BC_2F_5 (Table19).

Table 19. BC_3F_1s and BC_2F_5s produced from BC_2F_4s selected for background selection

Sl. No.	BC_2F_4 source plant	BC_3F_1 (Nos.)	BC_1F_1 (Nos.)	BC_2F_5 (Nos.)
		(BC_2F_4 / Jyothi)	(BC_2F_4 / ISM)	
1	Plant No. 5	9	7	142
2	Plant No. 9	13	22	165
3	Plant No. 21	19	5	47
4	Plant No. 25	8	8	174
5	Plant No. 27	14	14	140
	Total	**63**	**56**	**668**

Figure 2. Clustering of parents and selected BC_2F_4s based on morphological characters



Discussion

Plate 7. Preparations for transplanting of BC_2F_4s

Plate 8. Transplanting of BC_2F_4s

Plate 9. BC_2F_4 plants one-month after sowing

Plate 10. Donor parent – ISM (RP Bio-226) and recurrent parent- Jyothi (PTB 39)

Plate 11. Staggered sowing of parents Jyothi and ISM

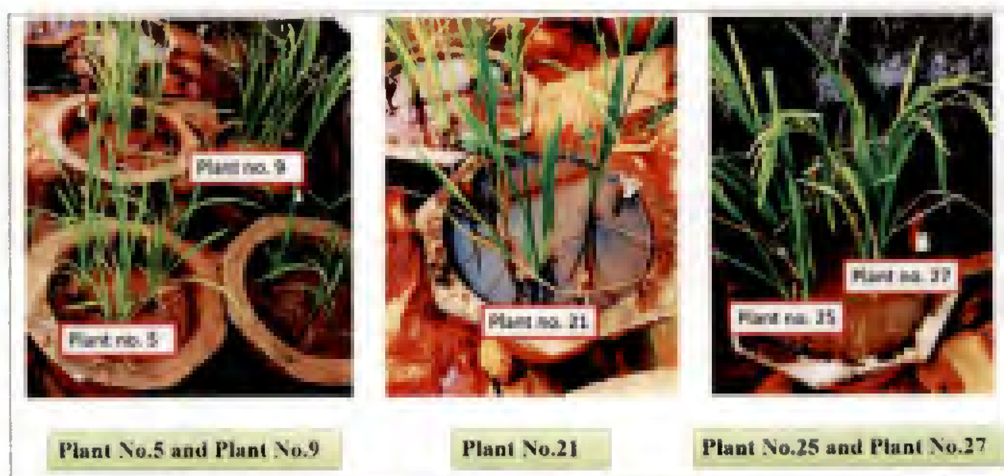


Plate 12. Selected BC_2F_4 plants used for background selection

becomes activated and infest the rice plants usually through the roots. According to Tagami *et al*. (1964), rice plants also get infected with *Xoo* pathogen from the inoculum surviving in the seeds, tillers or roots that are abandoned at harvest, and additionally from alternative weed hosts.

On infecting the plants, the pathogen reproduces inside the xylem to cause widespread infection and ooze out, on to the leaf exteriors as droplets. Wind, rain-water and irrigation water assists in spreading of the pathogen inoculum in the ooze to non-infected parts or plants. It is observed that cutting off the seedling tips while transplanting also encourages disease spread (Ou, 1985).

Epidemics of bacterial blight are common during the rice cropping seasons in Kerala which usually coincides with the monsoons. Resorting to recommended chemical or biological means to control the disease like spraying of antibiotics *e.g.* Streptocycline or cow dung extract (KAU, 2011), usually becomes ineffective due to washing off of the spray fluid. Moreover, neither antibiotics nor cow dung extract provide complete insulation of the crop from BB pathogen. According to Liu *et al*. (2004), the amount and pattern of rainfall in rainy season fundamentally decides the disease spread. The high rate of rainfall and relative humidity (90% for 12-14 hours per day) support quick disease development. In addition, temperatures between 23^0C - 31^0C experienced during the rice cropping seasons is also said to favour the disease occurrence during the wet season.

Further, owing to high variability among the *Xoo* populance, the chemical control strategy of BB pathogen is not economic and effective. Additionally, the evolution and presence of drug-resistant strains impairs the effectiveness of the adopted disease management technique (Gnanamanickam *et al*., 1999). Hence, in spite of resorting to disease forecasting, biological, chemical, cultural strategies for the management of BB pathogen, a total check of the pathogen on rice crop is not realised. According to Chen *et al*. (2011), although prophylactic measures and chemical control of the disease are recommended, durable resistance of varieties is a much better option to combat this biotic stress. Peng *et al*. (2015) stressed that, in addition to the ecological and food safety issues that the chemical pesticides and biocontrol agents (plant extracts and antagonistic organisms) may produce, their effectiveness and efficiency considerably decreases on continuous application. Thus, in the long run, exploiting durable host plant resistance governed by R-genes would prove to be more reliable and beneficial.

Singh *et al.* (2015), recommended resorting to host plant resistance coupled with good crop management practices as the most effective, economical, and environmentally safe measure to combat the *Xoo* pathogen. Till date, about 42 resistant genes to combat *Xoo* have been identified (Khan *et al.*, 2014; Zhang *et al.*, 2014; Busungu *et al.*, 2016). 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. Studies conducted at Regional Agricultural Research Station, Kerala Agricultural University, Pattambi further revealed that the R-gene combinations *Xa4* + *xa13* + *Xa21, xa5* + *xa13* + *Xa21 and Xa4* + *xa5* + *xa13* + *Xa21* confers broad spectrum resistance to Kerala isolate of *Xoo* (DRR, 2015). However, breakdown of resistance of cultivars with *Xa4* has been reported earlier by Mew *et al.* (1992).

Conventional breeding tools have proved to be inefficient for gene pyramiding, particularly in the case of recessively inherited resistance genes such as *xa5* and *xa13* (Singh *et al.* 2015). Several reports on the advantage of molecular markers in breeding for improved agronomic traits such as resistance to pathogens, insects and nematodes, tolerance to abiotic stresses, quality parameters and quantitative traits that are otherwise difficult to tag are available. The use of DNA markers, permits complete gene identification of the progeny in each generation and increases the speed of pyramiding process (Edwards and Johnson., 1994; Openshaw *et al.*, 1994; Hospital and Charcosset, 1997; Joshi and Nayak, 2010).

5.1.1. Foreground selection

Results confirmed that the genomic DNA extracted from the parents and the BC_2F_4 plants were of good quality (A_{260}/A_{280} of DNA extract: 1.7 to 1.9). Sufficient quantity of good quality total genomic DNA extracted from 51 BC_2F_4 plants, were subjected to foreground selection along with recurrent parent Jyothi and the donor parent ISM (Table 20a) using rice microsatellites.

STS marker RG 556 is reported to be tightly linked to R-gene *xa5* at a distance of 0.1cM. The PCR analysis of the genomic DNA of the 51 BC_2F_4 and the two parents with the marker did not produce any polymorphism even after restriction digestion of the PCR product with *Dra*I restriction enzyme. In all the 51 BC_2F_4 and the parents, alleles of size 238 bp and 438 bp were present, indicating that all the BC_2F_4 individuals and the parents carried the R-

96

Table 20 (a). Segregation of molecular markers in selected BC_2F_4s and parents (Foreground selection)

Sl. No.	Markers	Nature of amplification	Number of amplicon	Size of amplicon (bp)		
				Polymorphic		
				Recurrent parent (Jyothi)	R-gene introgressed BC_2F_4 plants	Donor parent (ISM)
Markers employed in the foreground selection						
1	*xa5*SR	Monomorphic	1	196		
2	RG 556	Monomorphic	2	238, 438		
3	*xa13* promoter	Polymorphic	2	280		428
4	RG 136	Polymorphic	2	945		635
5	pTA 248	Polymorphic	2	678	992	992

gene *xa5* (Table 20a). On resolving the DNA of BC_2F_4s and parents on agarose gels, the functional marker *xa5*SR proved to be monomorphic as amplicon of size 190 bp was present in all the BC_2F_4 individuals and parents Jyothi and ISM. This further confirmed the presence of *xa5* gene in both the parents as well as in all the BC_2F_4s studied.

During a polymorphism survey, Singh *et al.* (2015) observed that out of thirty four rice cultivars, no amplicons specific to *Xa21* and *xa13* allele were detected, indicating the absence of these two genes in all the cultivars evaluated. However, in twenty cultivars a 219 bp size fragment indicating the presence of *xa5* was observed. Similar to the present study, Tuyen and Lang (2004) had also found monomorphic banding pattern on using the xa5 SR marker for BB resistance in several local rice cultivars.

According to Sundaram *et al.* (2008), R-gene *xa5* provides only moderate resistance against the *Xoo* pathogen. Studies by Bharathkumar *et al.* (2008) revealed that resistance in rice cultivars with single BB resistance gene breaks down in the field whereas a R-gene pyramid is more durable. This substantiates the susceptibility reaction of parent Jyothi in spite of occurrence of the R-gene *xa5*. Higher level of resistance to the *Xoo* pathogen, than would be expected from the sum of the parental levels has been reported in multiple BB resistance gene pyramided lines compared to those with single resistance gene (Yoshimura *et al.*, 1996, Huang *et al.*, 1997 and Sundaram *et al.*, 2008).

Restriction digestion of PCR products of STS marker RG 136 with *Hinf*I enzyme had produced homozygous alleles for the gene *xa13* in all BC_2F_4 individuals as found in the susceptible parent. Hence, it was evident that none of the BC_2F_4 plants possessed the R-gene *xa13*.

Similarly, the 280bp amplicon associated with the resistant allele found in donor parent ISM was absent in the BC_2F_4 individuals when amplified with functional marker *xa13* promoter. This further confirmed that the 51 BC_2F_4 individuals screened were not introgressed with the R-gene *xa13*. The results thus pointed that all the 51 BC_2F_4s possessed only a single R-gene (*xa5*).

The STS marker pTA 248 reported to be tightly linked to dominant R-gene *Xa21* is located at a distance of 0.2cM from *Xa21* (Dokku *et al.*, 2013). Hence, it has been widely used for precise and early detection of genotypes carrying the R-gene *Xa21*. Out of the 51 BC_2F_4

individuals scored with the STS marker pTA 248, two individuals *i.e.,* Plant No. 9 and Plant No. 21 were found to possess alleles (855 bp) similar to the donor parent ISM. These were also found to be homozygous with the donor parent allele.

Swamy *et al.*, (2006) reported that stacking of multiple BB resistance genes by MAS into the susceptible variety Pusa Basmati 1 background was most effective in imparting durable resistance. The pyramids that carried two or more BB resistant genes were reported to show a higher degree of resistance over lines containing single BB resistant genes. The single *Xa* gene containing lines IRBB 4 (*Xa4*), IRBB21 (*Xa21*), MH2R (*xa5*) were susceptible or only moderately resistant to the *Xoo* isolates. Marker aided introgression of *xa13* and *Xa21* into MH2R showed clear resistance against the same *Xoo* isolates. The result indicated that combinations of R-gene provided a broader spectrum of resistance to the disease.

To summarise, the foreground selection of the 51 BC_2F_4 individuals revealed that two BC_2F_4 plants (Plant No. 9 and Plant No. 21 in Plate No. 12) were introgressed with the two R-genes *xa5* and *Xa21.* Absence or no recovery of 3 R-gene pyramids, recovery of 2 R-gene pyramids with various combinations of R-genes and occurrence of single R-gene introgressions, are a common occurrence in a backcross programme owing to gene segregation and independent assortment. Arunakumari *et al.* (2016) in their attempt to pyramid two major bacterial blight (BB) resistance genes (*Xa21* and *xa13*) and a major gene for blast resistance (*Pi54*) into an Indian rice variety MTU1010 through marker-assisted backcross breeding found that, out of a total of 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*.

Pinta *et al.* (2013) resorted to MAS to identify individual plants introgressed with resistance alleles. Although $BC_2F_{2:3}$ introgression lines were selected on markers, they suspected occurrence of crossing over owing to the genetic distance between flanking markers. Consequently, none of the $BC_2F_{2:3}$ lines had the same resistance alleles as donor. Despite the pyramiding of lines containing four QTLs for blast and one gene for BLB, they failed to recover any genotype that was resistant to all isolates of the two disease pathogens. This indicated that further backcrossing and pyramiding are required to broaden the spectrum of resistance. According to Kumar and Nayak, (2010), the success of gene pyramiding depends upon factors like the number of genes to be introgressed, the distance between the target genes and flanking markers, the number of genotype selected in each breeding generation, the nature of germplasm

etc. However, innovative tools molecular markers play a significant role in gene pyramiding for crop plant improvement, and the prospect and challenges in integrating MAS based gene pyramiding with conventional plant breeding programmes.

The study also pointed out the presence of R-gene *xa5* in both parents (Jyothi and ISM) as well as in the BC_2F_{4s}. The alleles of the R-genes in each of the two 2 R-gene pyramids (*xa5* + *Xa21*) thus obtained were also found to be in the homozygous state as in the donor parent and therefore expected to show no segregation in further selfed generation. It is also expected to exhibit a higher degree of resistance to the BB pathogen than the remaining single gene introgressions.

Single *Xa* gene containing lines IRBB 4 (*Xa4*), IRBB21 (*Xa21*), MH2R (*xa5*) were found to be susceptible or only moderately resistant to the *Xoo* isolates. Marker aided introgression of *xa13* and *Xa21* into MH2R showed clear resistance against the same *Xoo* isolates (Singh *et al*., 2001). However, Gnanamanickam *et al.* (1999) had observed a sub-population of *Xoo* virulent to rice line IRBB21 from a pathogen isolate from Kerala. According to Priyadarsini and Gnanamanickam (1999), rice line NH56 carrying four R-genes, (*Xa4* + *xa5* + *xa13* + *Xa21*) was found to be resistant to Kerala isolates of the *Xoo* pathogen. The three gene combination (*xa5*+*xa13*+*Xa21*) of pyramiding was also reported to be highly effective in providing resistance to BB pathogen by several earlier workers (Sanchez *et al*., 2000; Singh *et al*., 2001; Nguyen *et al*., 2004; Sundaram *et al*., 2008; Shanti *et al*., 2010; Bharani *et al*., 2010; Salgotra *et al*., 2011; Pradhan *et al*., 2015b).

Sundaram *et al.* (2008), suggested that a quantitative complementation through which the multiple genes have an additive effect on the overall resistance may be the reason for higher resistance offered by the multiple gene pyramided lines. Therefore, introgression of the three R-genes (*xa5*, *xa13* and *Xa21*) into a rice genotype would be most ideal to impart durable resistance against the BB pathogen.

5.2. Background selection

According to Joseph *et al.* (2004), background selection in the backcross generation would greatly enhance the efficiency of marker assisted backcross breeding and help release a

cultivar with increased BB resistance. The background profiling of the two 2 R-gene introgressed BC_2F_4 plants (Plant No. 9 and Plant No. 21) was done along with the donor parent ISM and the recurrent parent Jyothi. In addition, Plant No. 5, Plant No. 25 and Plant No. 27 that were near similar to recurrent parent with respect to plant height and days to flowering were also included for background screening (Plate 12). These plants had produced higher number of productive tillers/ plant, panicle length and spikelets/panicle. Fifty rice microsatellite markers that were earlier reported to be polymorphic between the two parents; Jyothi and ISM were used for the background selection.

Results (Table 20b) revealed that the banding pattern in all the BC_2F_4s individuals (Plant No. 5, Plant No. 9, Plant No. 21, Plant No. 25, and Plant No. 27) were similar to recurrent parent on analysis with sixteen RM markers (RM1, RM10871, RM11069, RM11342, RM11390, RM15583, RM16, RM19255, RM19514, RM19793, RM20416, RM214, RM248, RM26868, RM307 and RM439). This indicated that the BC_2F_4s possessed the same allele as in recurrent parent Jyothi at these marker loci.

However, there was no consistency in banding pattern among the selected BC_2F_4s on genotyping with the remaining 34 RM markers. BC_2F_4 Plant No. 9 followed by Plant No. 21 possessed higher number of donor alleles than the other three BC_2F_4 individuals screened. In addition, these plants along with Plant No. 5 also registered higher number (4 nos.) of heterozygous loci. Such variations may be attributed to the segregation and independent assortment of alleles in the early backcross generations. These plants could be expected to segregate for the alleles in subsequent generation.

Predominance of donor alleles in the BC_2F_4s is due to linkage drag from the donor parent. Higher linkage drag from the donor parent responsible for the reduced background recovery has been earlier reported by Rajpurohit *et al.* (2010). Repeated backcrossing followed by rigorous background selection through MAS is preferable to enhance the recurrent parent genome at a faster pace. The contribution of the recurrent parent increases by one-half with each generation of backcrossing while the undesirable donor allele contribution reduces considerably (Singh, 2001). Higher recovery of recurrent parent genome have been reported in the later generations of backcrossing *viz.*, BC_2 and further generations (Sundaram *et al.*, 2008; Salgotra *et al.*, 2011; Suh *et al.*, 2013).

Table 20 (b). Segregation of molecular markers in selected BC_2F_4s and parents (Background selection)

Sl. No.	Markers	Nature of amplification	Number of amplicon	Size of amplicon (bp)						
				Recurrent parent (Jyothi)	Plant No. 5	Plant No. 9	Plant No. 21	Plant No. 25	Plant No. 27	Donor parent (ISM)
1	RM1	Polymorphic	2	107						132
2	RM6340	Polymorphic	2	154						148
3	RM10871	Polymorphic	2	190						232
4	RM11069	Polymorphic	2	210						280
5	RM11313	Polymorphic	2	350						303
6	RM11342	Polymorphic	2	130						125
7	RM12038	Polymorphic	2	310						321
8	RM482	Polymorphic	2	487						469
9	RM3340	Polymorphic	2	150						146
10	RM12941	Polymorphic	2	180						167
11	RM11390	Polymorphic	2	210						180
12	RM16	Polymorphic	2	187						230
13	RM49	Polymorphic	2	179						192
14	RM85	Polymorphic	2	98						130
15	RM251	Polymorphic	2	147						123
16	RM15583	Polymorphic	2	140						122
17	RM307	Polymorphic	2	198						220
18	RM5586	Polymorphic	2	110						105
19	RM6089	Polymorphic	2	174						154
20	RM6679	Polymorphic	2	110						106
21	RM17377	Polymorphic	2	290						285
22	RM18225	Polymorphic	2	409						429
Key:	Recurrent parent allele			Heterozygous allele			Donor parent allele			

23	RM18919	Polymorphic	2	485						460
24	RM19218	Polymorphic	2	172						160
25	RM217	Polymorphic	2	187						170
26	RM214	Polymorphic	2	132						121
27	RM248	Polymorphic	2	360						309
28	RM295	Polymorphic	2	210						200
29	RM20833	Polymorphic	2	198						172
30	RM21345	Polymorphic	2	218						215
31	RM72	Polymorphic	2	179						164
32	RM5545	Polymorphic	2	130						125
33	RM5556	Polymorphic	2	146						103
34	RM23087	Polymorphic	2	410						392
35	RM107	Polymorphic	2	129						98
36	RM205	Polymorphic	2	127						110
37	RM524	Polymorphic	2	190						170
38	RM23998	Polymorphic	2	279						260
39	RM304	Polymorphic	2	120						125
40	RM7545	Polymorphic	2	234						203
41	RM202	Polymorphic	2	141						165
42	RM224	Polymorphic	2	200						179
43	RM26213	Polymorphic	2	309						299
44	RM26868	Polymorphic	2	170						175
45	RM17	Polymorphic	2	459						472
46	RM19	Polymorphic	2	208						206
47	RM247	Polymorphic	2	198						175
48	RM260	Polymorphic	2	94						82
49	RM28277	Polymorphic	2	240						209
50	RM308	Polymorphic	2	149						141

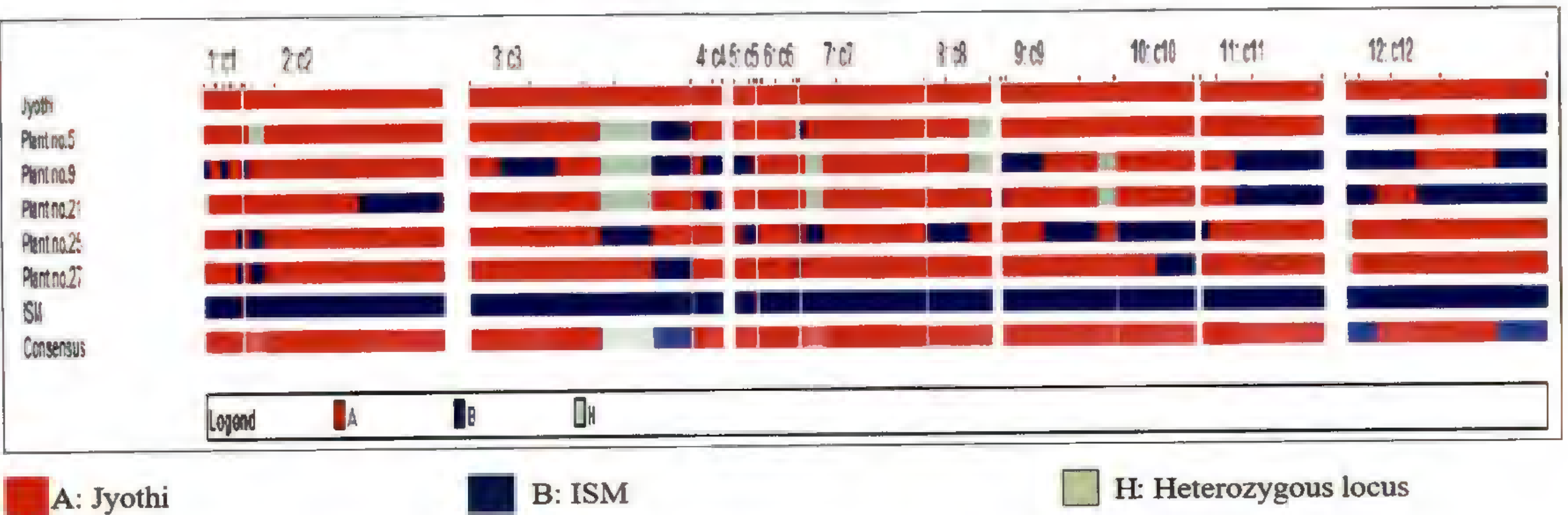
Key: **Recurrent parent allele** | **Heterozygous allele** | **Donor parent allele**

Rice microsatellites have been successfully used to estimate the recovery of recurrent parent genome during background selection. The expected recovery of background of recurrent parent in BC_2F_4 generation is 87.50 per cent (Meksem *et al.*, 2009). In the present study, based on the segregation of the 50 markers, the recurrent parent genome contribution among the selected the BC_2F_4 plants were estimated. Among the five BC_2F_4 individuals, the recovery of recurrent parent genome was found to be highest in Plant No. 5 (92.20 %) followed by Plant No. 27 (91.60%) while it was lower than the expected recovery in Plant No. 25 (76.40%), Plant No. 21 (64.40%) and Plant No. 9 (58.80%). The graphical representation of the results of genotyping of the BC_2F_4 plants done using the GGT software (Figures 3 to 12) also confirmed the above findings.

Rajpurohit *et al.* (2010) had also observed variable background recovery in BC_2F_2 progenies (81.57 per cent to 92.10 per cent). Sundaram *et al.* (2008) had also obtained similar low contribution of the recurrent parent to the genome of the backcross progeny in early background generations of the cross between Samba Mahsuri and SS1113. The recurrent parent recovery was less than the expected (87.50%). However, the group were able to pyramid three BB resistance genes *xa5, xa13* and *Xa21* and recover nearly 97 per cent background of recurrent parent by BC_4F_1 generation through foreground and background selection during each backcross generations. However, the same group had also reported low background recovery of recurrent parent during the transfer of the three resistance gene *xa5, xa13* and *Xa21* from SS1113 into a rice cultivar, Triguna. They attributed the low recovery of recurrent parent genome in the latter case to a 'pull', through still unknown mechanism which results in the inheritance of additional undesirable loci from the donor parent genome. Pradhan *et al.* (2015a), Cuc *et al.* (2012), Mackill *et al.* (2012) and Dash *et al.* (2016) have also reported high recurrent parent genome contribution in advanced backcross generations.

The rice genome of 400-450 Mbp size has around 50,000 gene loci. The low per cent recovery of recurrent parent genome may also be due to the extremely lower number of rice microsatellite markers used for background screening. In the present study, only a small fraction of the marker loci has been covered (50 RM markers) and this might be the reason for the low recovery obtained. Repeated backcrossing of the pyramided lines to the recurrent parent has been found essential as in conventional breeding to obtain pyramided genotypes with maximum background recovery of recurrent parent. Pradhan *et al.* (2015a), suggested that higher recovery of the recurrent parent genome in the later backcross generation may be

Figure 3. Recovery of recurrent parent genome in selected BC_2F_4s



A: Jyothi

B: ISM

H: Heterozygous locus

c: Chromosome; c1: Chromosome no. 1 to c12: Chromosome no. 12

Figure 4. Graphical genotyping of recurrent parent Jyothi

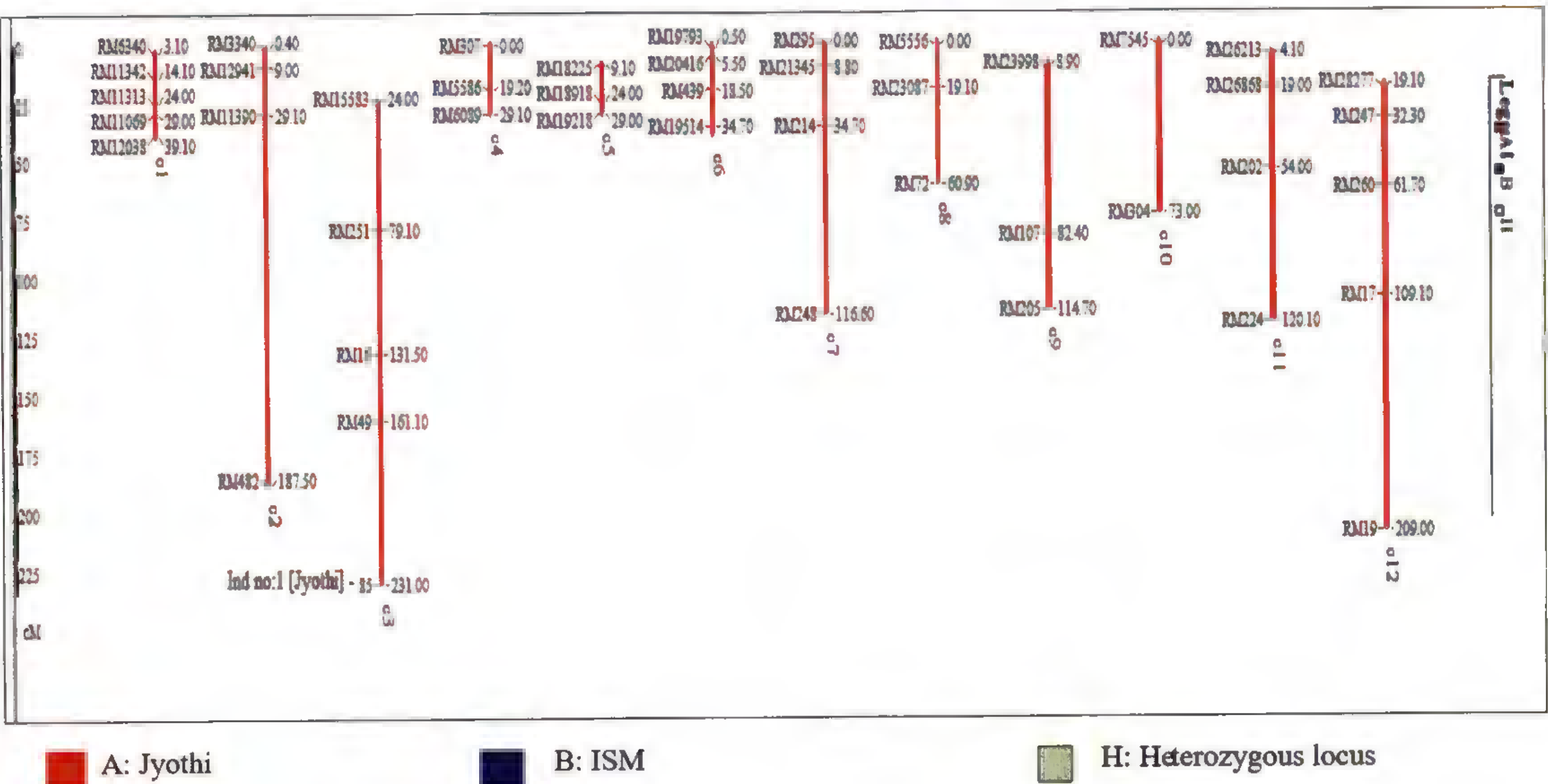
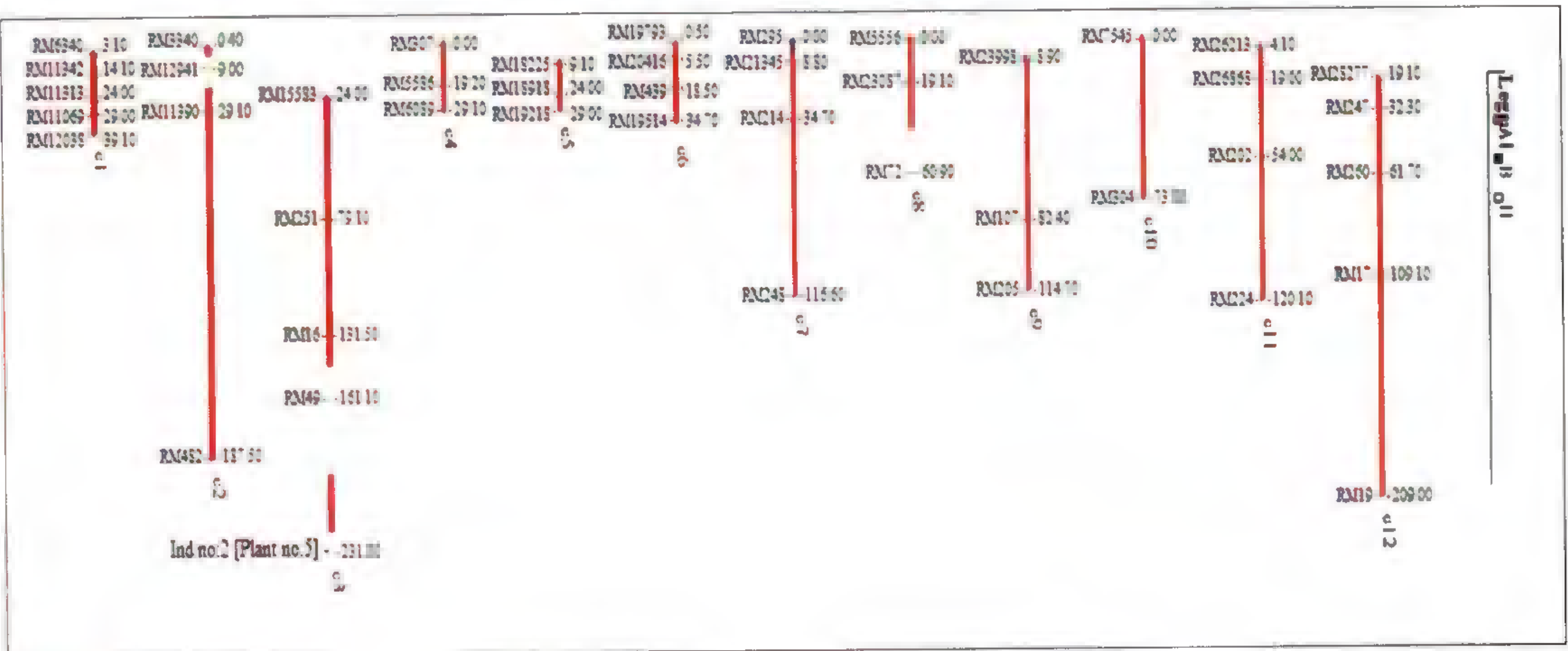


Figure 5. Graphical genotyping of BC_2F_4 Plant No. 5

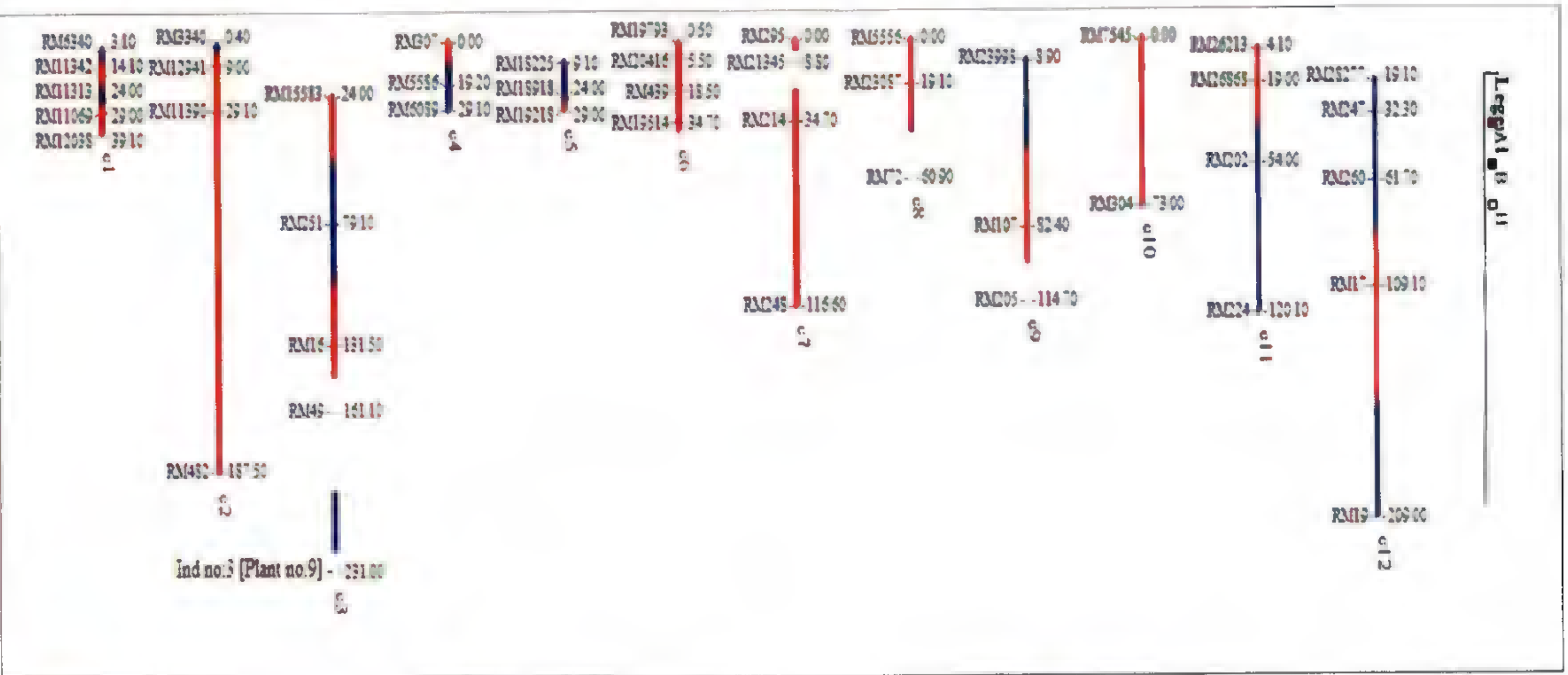


A: Jyothi

B: ISM

H: Heterozygous locus

Figure 6. Graphical genotyping of BC_2F_4 Plant No. 9



A: Jyothi

B: ISM

H: Heterozygous locus

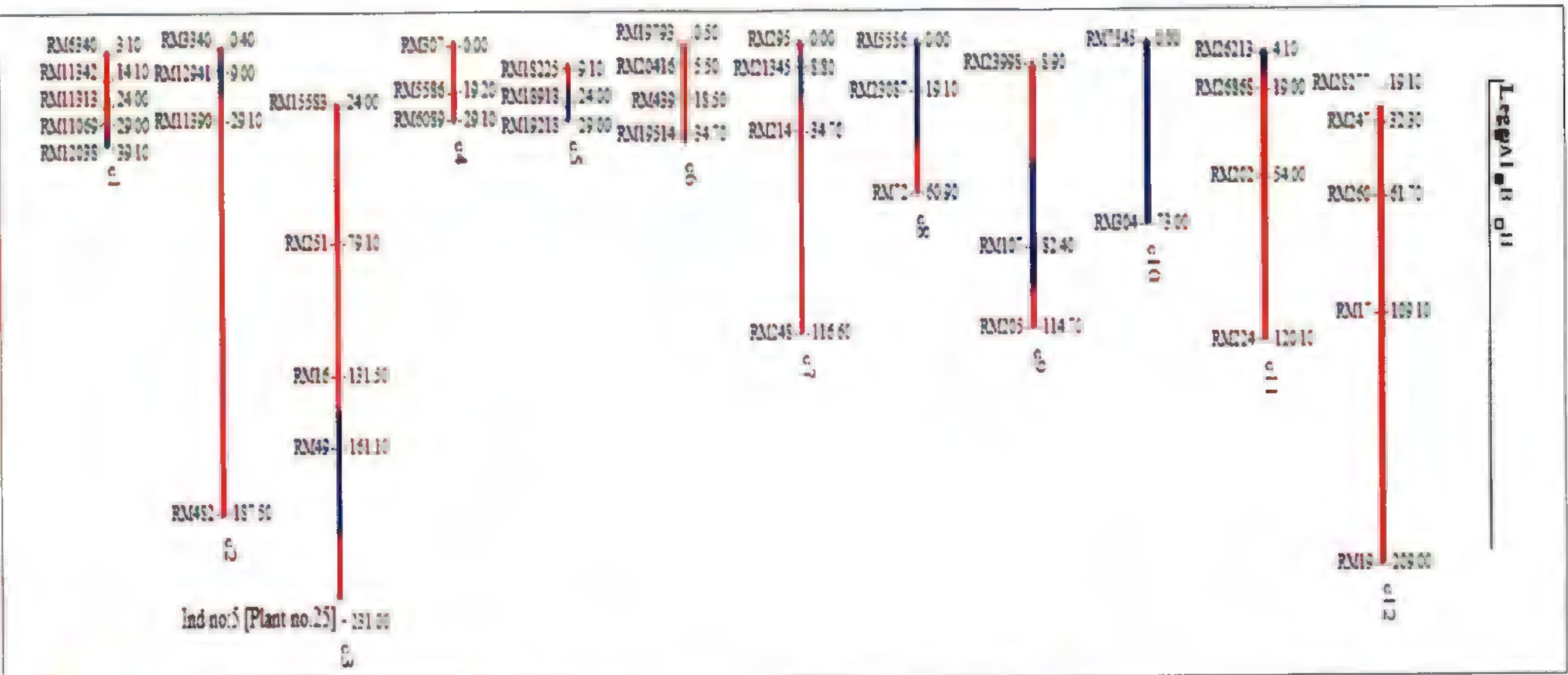
Figure 7. Graphical genotyping of BC_2F_4 Plant No. 21

A: Jyothi

B: ISM

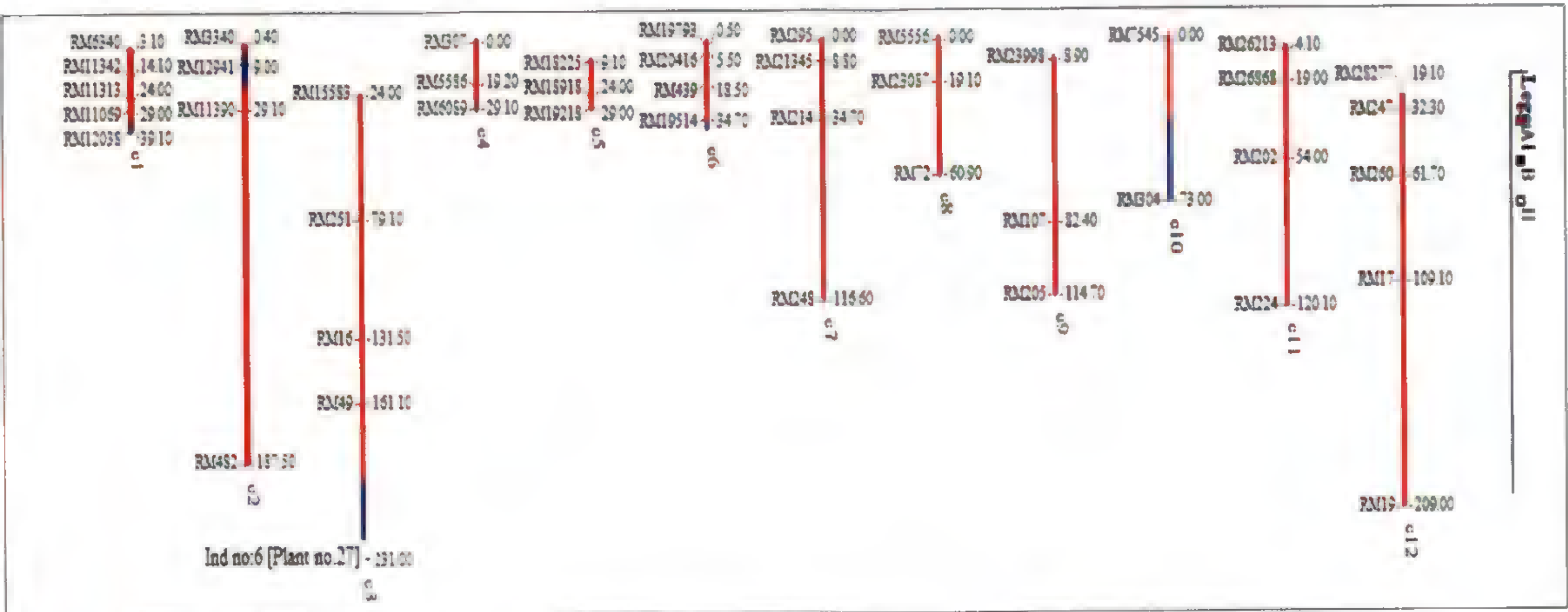
H: Heterozygous locus

Figure 8. Graphical genotyping of BC_2F_4 Plant No. 25



A: Jyothi   B: ISM   H: Heterozygous locus

Figure 9. Graphical genotyping of BC_2F_4 Plant No. 27

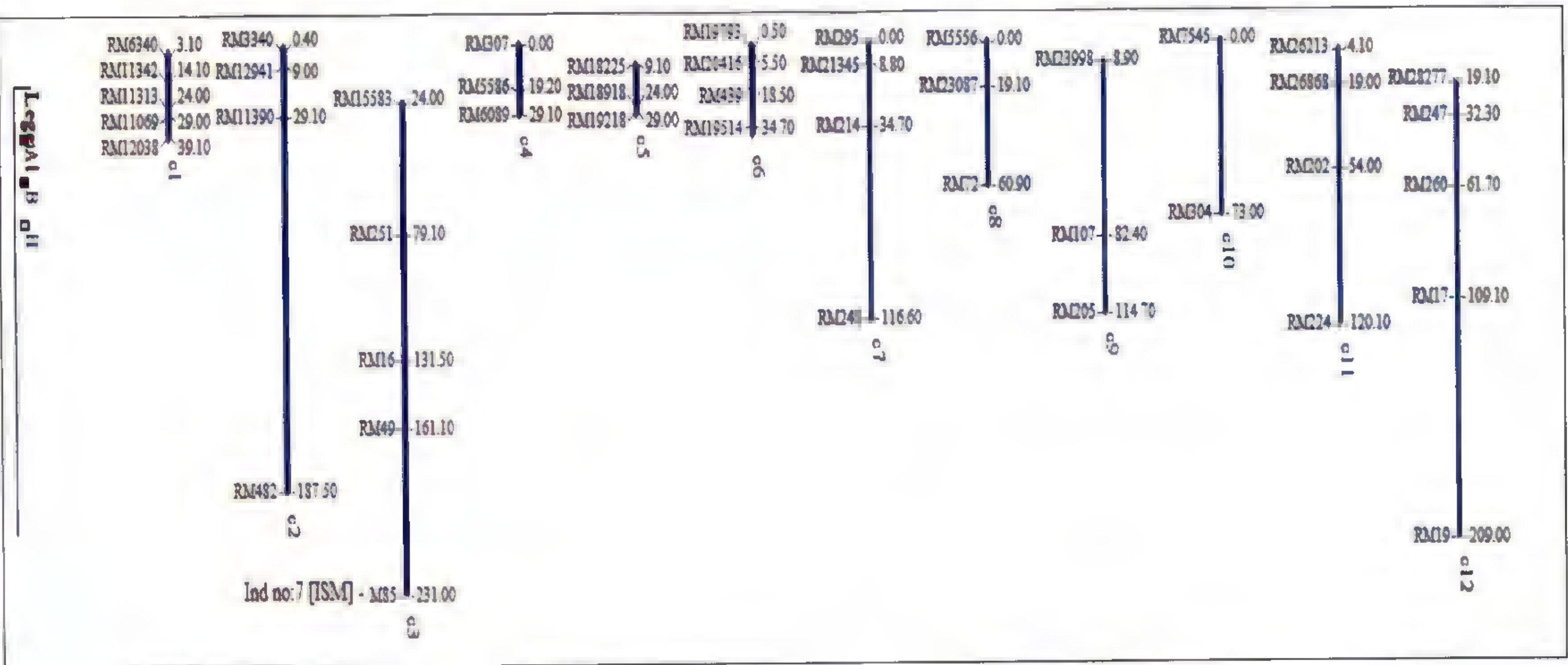


A: Jyothi

B: ISM

H: Heterozygous locus

Figure 11. Graphical genotyping of donor parent Improved Samba Mahsuri (ISM)



attributed to a reduced linkage drag in the fragments flanking the three resistance genes (*xa5*, *xa13* and *Xa21*) and the use of more number of polymorphic microsatellite markers. Similar results were obtained earlier by Suh *et al.* (2013) and Dokku *et al.* (2013).

It is advisable to grow a large back cross population to recover multi-gene pyramids. According to Rajpurohit *et al.* (2011), Ye, (2010) 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.

The dendrogram generated out of the marker data resulted in two clusters, one with the five selected BC_2F_4 individuals along with the recurrent parent Jyothi. However, Plant No. 9 and Plant No. 21 were clustered into a sub- cluster of the cluster I containing Jyothi, indicating that they were less similar to the recurrent parent when compared to the other three BC_2F_4 individuals studied. This indicated that, the R-gene introgressed plants were genetically more similar to the donor parent ISM owing to larger linkage drag with the introgressed R-genes. Similar to the present study, the dendrogram generated by Dokku *et al.* (2008) grouped the 10 pyramided lines analysed and two parents into two clusters. They found that the donor IRBB60 and four pyramided lines were clustered into a single group while cluster II had all the remaining lines and the recurrent parent Tapaswini. Rajpurohit *et al.* (2010) had also reported clustering of R-gene introgressed lines with the recurrent parents in later backcross generations rather than with the donor parent

According to Shanti *et al.* (2001), two gene combinations confer a less degree of resistance than the three gene combinations and so use of three gene combinations was the most feasible option. Multiple genes have an additive effect on overall levels of resistance. According to Shanti and Shenoy (2005), durable resistance conferred is due to the complementary action of the resistance genes. This corroborates to the earlier reports showing that multiple genes have higher levels of resistance as compared to those with single genes (Yoshimura *et al.*, 1996; Huang *et al.*, 1997 and Sundaram *et al.*, 2008; Devi *et al.*, 2013).

To ensure the release of a cultivar with durable resistance to the *Xoo* pathogen further backcrossing and selfing of the R-genes introgressed lines have been advocated (Suh *et al.*,

2013; Dokku *et al.*, 2013). Frisch and Melchinger (2005) and Deshmukh *et al.* (2017) concluded that the effectiveness of marker assisted breeding depends on the availability of closely linked markers and/or flanking markers for the target locus, the size of the population as well as the number of backcrosses, and the position and number of background markers. Considering this, backcrossing of the identified two 2-R-gene pyramided BC_2F_4s *i.e.*, Plant No. 9 and Plant No. 21 to recurrent parent Jyothi was done. Foreground and background profiling of these BC_3F_1s need to done to identify and recover the 2-R-gene pyramided lines with maximum recovery of recurrent parent (Jyothi).

Simultaneously, backcrossing of the selected BC_2F_4s to donor parent ISM was also done in order to introgress *xa13* into the two 2-R-gene pyramids (BC_2F_4 Plant No.9 followed by Plant No. 21), resulting in production of BC_3F_1s. This was found necessary as stacking of genes was found to offer better broad spectrum resistance to the Kerala isolate of BB pathogen (Priyadarsini and Gnanamanickam, 1999). These may further be subjected to foreground and background profiling to identify the stable 3-R-gene pyramided lines and incorporate them in further breeding programmes.

Selfing would help achieve homozygosity at the various loci in an individual. Joseph *et al.* (2004) were able to recover 80.40 per cent to 86.70 per cent recurrent parent (Pusa Basmati 1) background in BC_1F_3 generation with two BB resistant genes, *xa13* and *Xa21* by a combination of phenotypic and molecular marker aided selection. Hence, selfing of the selected BC_2F_4s were also attempted. These may further be subjected to foreground and background profiling to identify the stable 2-R-gene pyramided lines and initiate their performance evaluation.

5.4. Morphological characterization of BC_2F_4 individuals

Backcross breeding programme aims to integrate one or few desirable traits into an otherwise desirable cultivar. In the present study, transfer of resistance genes to BB pathogen was envisaged into elite highly acceptable susceptible cultivar Uma. To assess the similarity in morphological traits, each backcross individuals (BC_2F_4s) along with the parents (donor parent ISM and recurrent parent Jyothi) were characterized (Tables 17, 18a, 18b, Appendices 3 to 8).

Results indicated the presence of wide variability for the morphological characters across the BC_2F_4 population. Suh *et al.* (2013) found that even though the R-gene introgressions

showed highly recovered chromosome segments, they could not exhibit a similar phenotype with the recurrent parent since the insertion of small chromosome segments also affected phenotype.

The agronomic evaluation of the BC_2F_4 population for number of productive tillers, panicle length, spikeltes/ panicle, grains/panicle, indicated the presence of segregants that were better than the parental genotypes while in a few genotypes the magnitude for the traits studied was found to be lower than that of the parental genotypes.

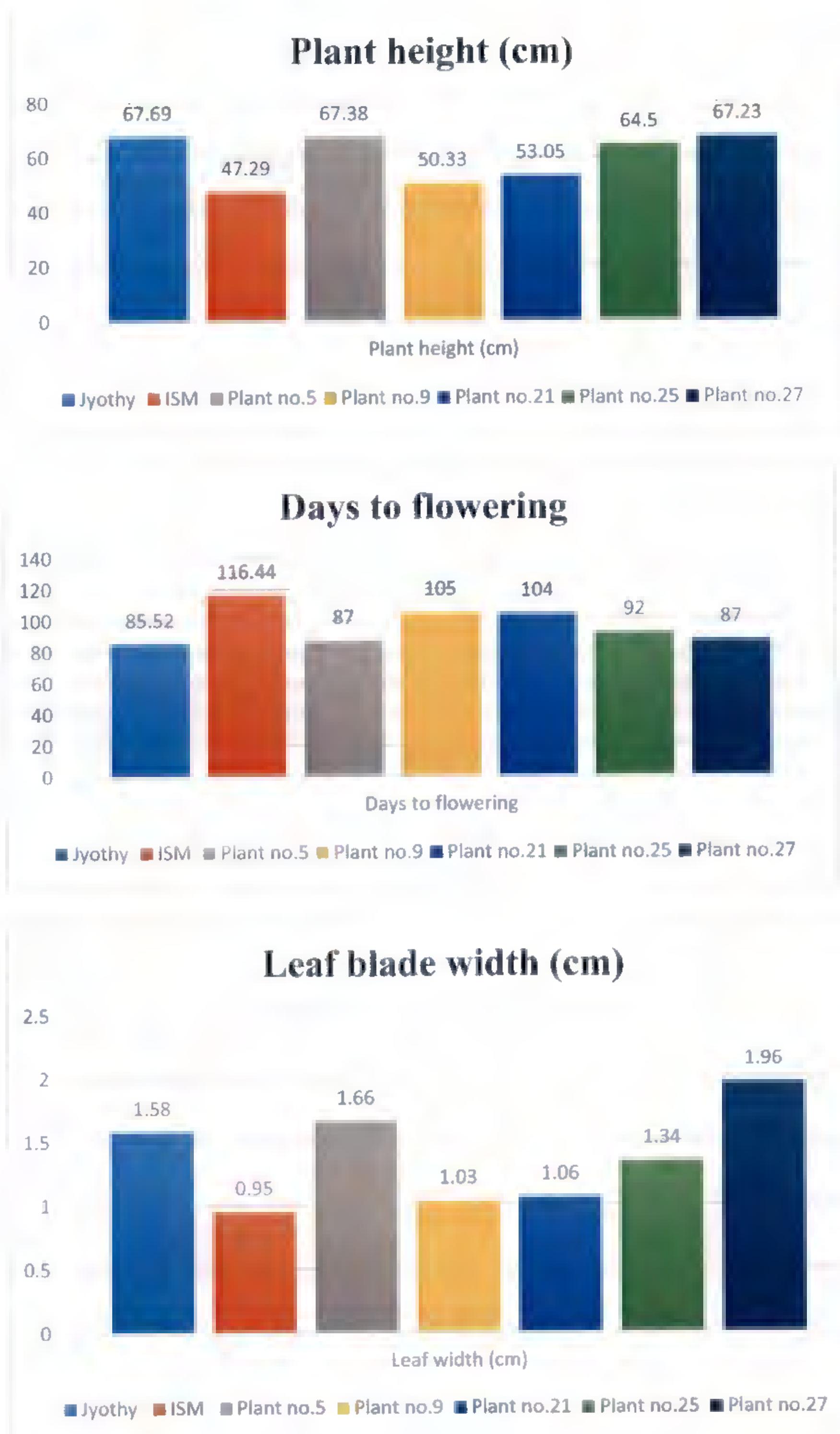
Similar to the findings in the present study, Arunakumari *et al*. (2016), also observed occurrence of R-gene introgressed individuals that exhibited significant superiority over recurrent parent MTU1010 with respect to number of filled grains, panicle length and grain yield per plant, with plant height and duration being similar to the recurrent parent. They attributed this to occurrence of transgressive segregation for yield and yield-related traits.

As opined by Sundaram *et al*. (2008), the complete recovery of yield and other morphological characters of recurrent parent must be ensured in the gene introgressed lines, as pyramiding the R-genes imparting resistance to the BB pathogen without recovery of yield and other characters would be futile if the developed lines is not be acceptable to farmers. It is therefore essential to ensure the recovery of the agro-morphological characters in the gene introgressed lines. The selected BC_2F_4s found to be intermediate between the two parents for characters like plant height, days to flowering, leaf blade length, 1000 grain weight, grain width (Plate No. 13), decorticated grain length and width, and straw yield/ plant (Figure 13). In addition, the selected BC_2F_4s were red kernelled as recurrent parent Jyothi (Plate No. 14).

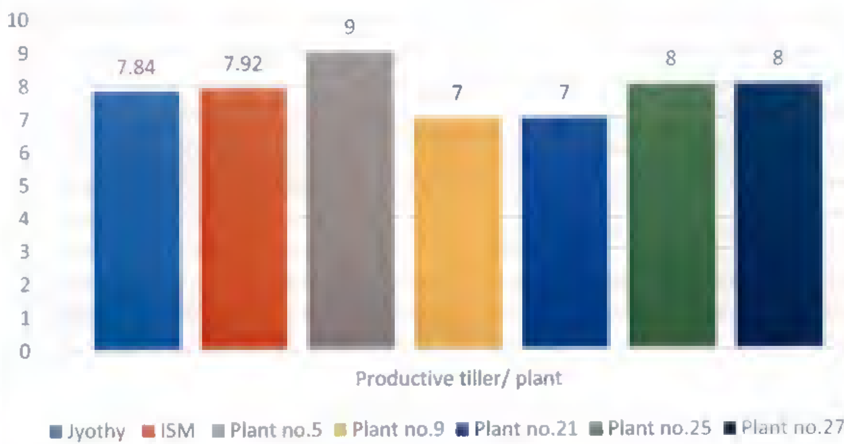
However, the BC_2F_4 Plant No. 9 and Plant No. 21 were near similar to the donor parent ISM with respect to plant height, leaf blade length and width, productive tillers/ plant, panicle length, spikelets and grains per panicle, grain length and decorticated grain width.

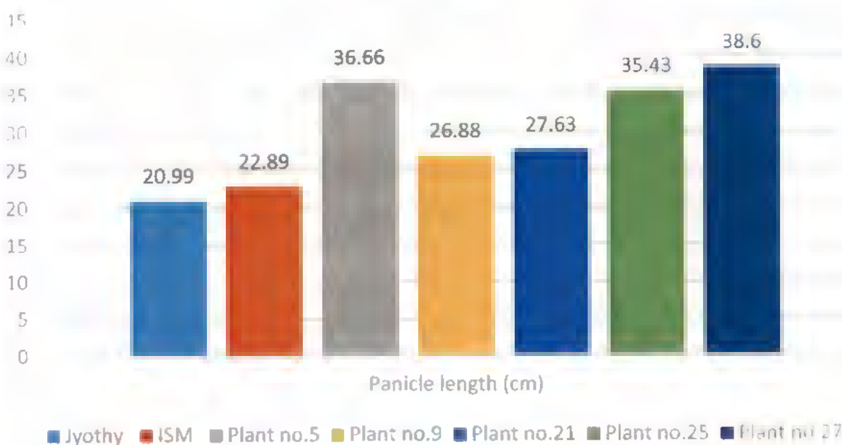
The leaf blade width, number of productive tillers/ plant, spikelets/ panicle and grain length in Plant No. 5 was found to be higher than in both the parents, while plant number 27 recorded higher leaf blade width, grain yield per plant than both the parents. Yield being a quantitative trait, the performance *per se* of Plant No. 5 and Plant No. 27 need to be further confirmed through evaluation trials in order to assess their superiority to both the parents. Joseph *et al*, 2004 had also recovered a few segregants with favourable characteristics of Pusa Basmati 1 with two BB resistant genes through MAS just in BC_1 due to stringent phenotypic selection. Vasal *et al*. (1993) and Babu *et al*. (2005) had however reported low frequency of

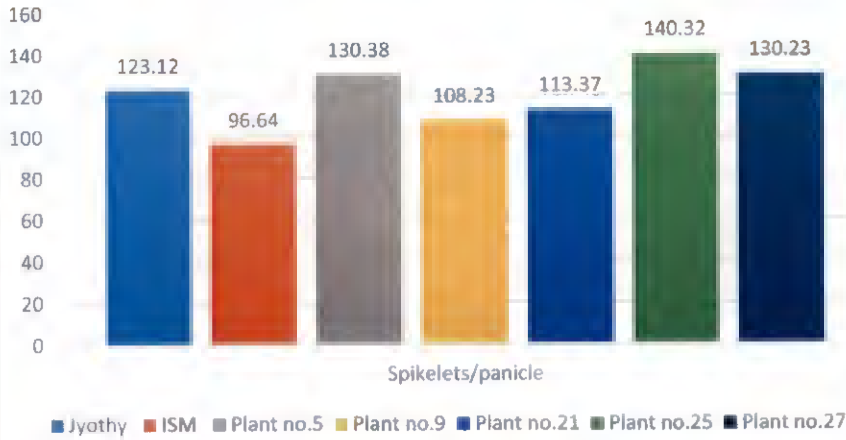
Figure 11. Morphological characteristics of selected BC_2F_4s and parents

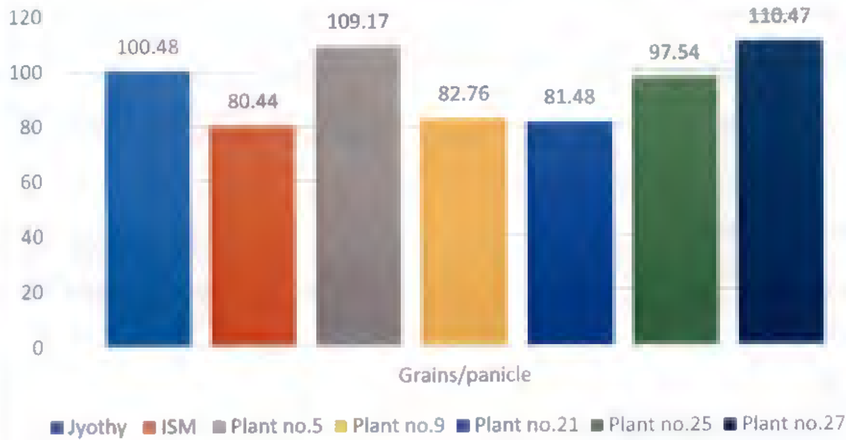


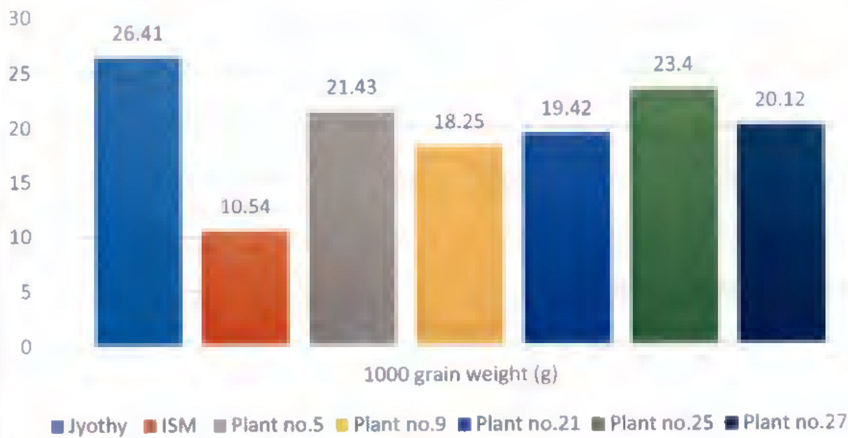
Leaf blade length (cm)
40
35
30
25
20
15
10
5
0
37.95
35.5
29.83
35.32
35.91
34.46
36.67
Leaf blade length (cm)
Jyothy
ISM
Plant no.5
Plant no.9
Plant no.21
Plant no.25
Plant no.27

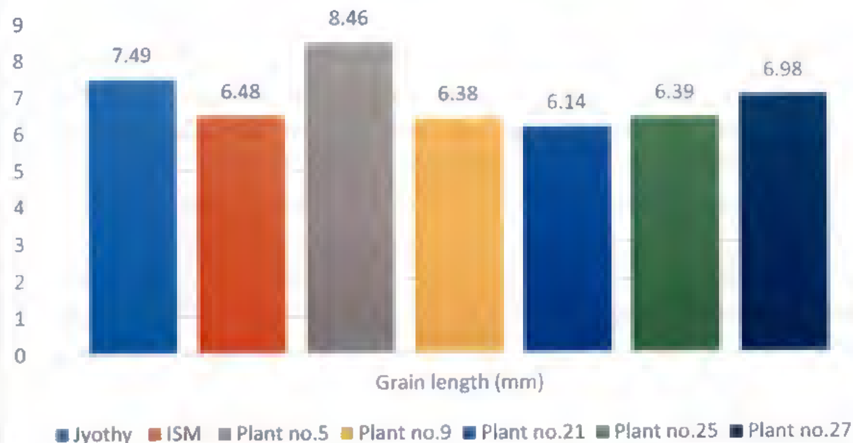

Productive tillers/ plant
10
9
8
7
6
5
4
3
2
1
0
7.84
7.92
9
7
7
8
8
Productive tiller/ plant
Jyothy
ISM
Plant no.5
Plant no.9
Plant no.21
Plant no.25
Plant no.27

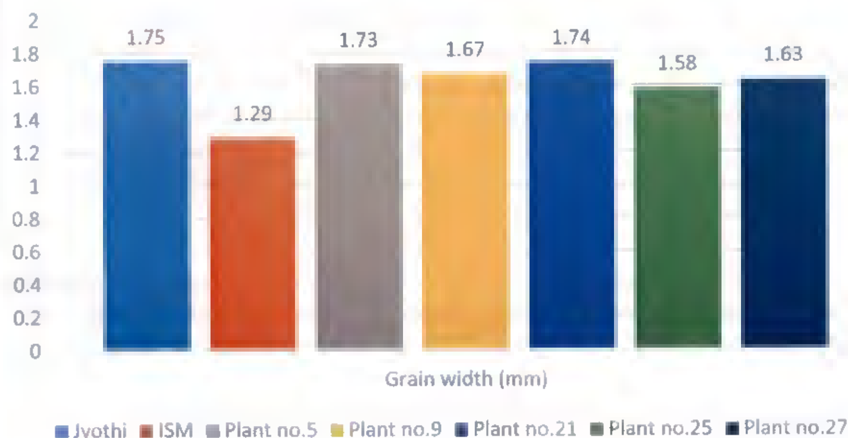

Panicle length (cm)
40
35
30
25
20
15
10
5
0
20.99
22.89
36.66
26.88
27.63
35.43
38.6
Panicle length (cm)
Jyothy
ISM
Plant no.5
Plant no.9
Plant no.21
Plant no.25

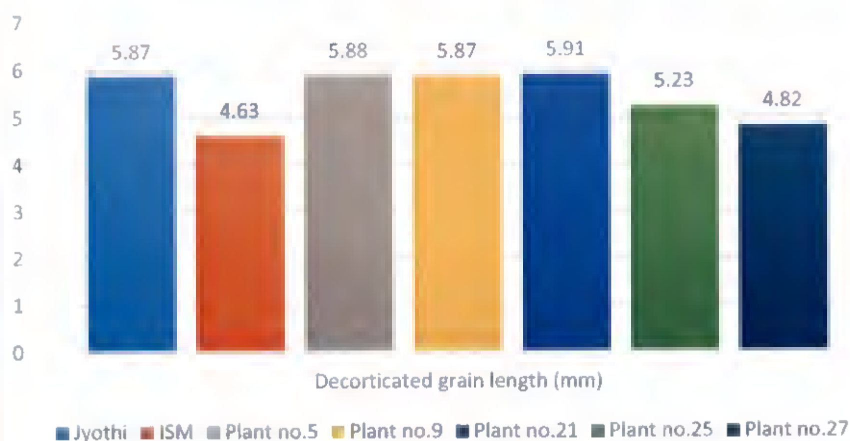

Spikelets/panicle
160
140
120
100
80
60
40
20
0
123.12
96.64
130.38
108.23
113.37
140.32
130.23
Spikelets/panicle
Jyothy
ISM
Plant no.5
Plant no.9
Plant no.21
Plant no.25
Plant no.27


Grains/panicle
120
100
80
60
40
20
0
100.48
80.44
109.17
82.76
81.48
97.54
110.47
Grains/panicle
Jyothy
ISM
Plant no.5
Plant no.9
Plant no.21
Plant no.25
Plant no.27

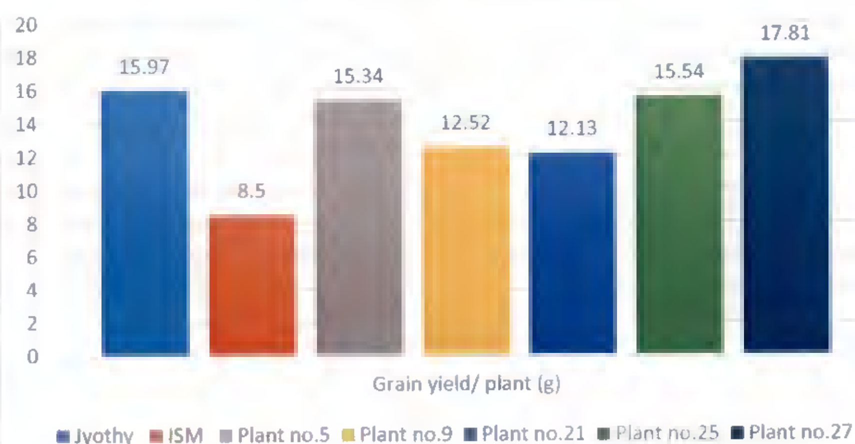

Thousand grain weight (g)
30
25
20
15
10
5
0
26.41
10.54
21.43
18.25
19.42
23.4
20.12
1000 grain weight (g)
Jyothy
ISM
Plant no.5
Plant no.9
Plant no.21
Plant no.25
Plant no.27


Grain length (mm)
9
8
7
6
5
4
3
2
1
0
7.49
6.48
8.46
6.38
6.14
6.39
6.98
Grain length (mm)
Jyothy
ISM
Plant no.5
Plant no.9
Plant no.21
Plant no.25
Plant no.27


Grain width (mm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1.75
1.29
1.73
1.67
1.74
1.58
1.63
Grain width (mm)
Jyothi
ISM
Plant no.5
Plant no.9
Plant no.21
Plant no.25
Plant no.27


Decorticated grain length (mm)
7
6
5
4
3
2
1
0
5.87
4.63
5.88
5.87
5.91
5.23
4.82
Decorticated grain length (mm)
Jyothi
ISM
Plant no.5
Plant no.9
Plant no.21
Plant no.25
Plant no.27

Decorticated grain width (mm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1.55
0.84
0.98
1.03
1.09
0.88
0.86
Decorticated grain width (mm)
Jyothi
ISM
Plant no.5
Plant no.9
Plant no.21
Plant no.25
Plant no.27


Grain yield/ plant (g)
20
18
16
14
12
10
8
6
4
2
0
15.97
8.5
15.34
12.52
12.13
15.54
17.81
Grain yield/ plant (g)
Jyothy
ISM
Plant no.5
Plant no.9
Plant no.21
Plant no.25
Plant no.27

Straw yield /plant (g)
25
20
15
10
5
0
22.52
10.99
21.24
20.72
19.44
22.69
20.46
Straw yield /plant (g)
Jyothy
ISM
Plant no.5
Plant no.9
Plant no.21
Plant no.25
Plant no.27

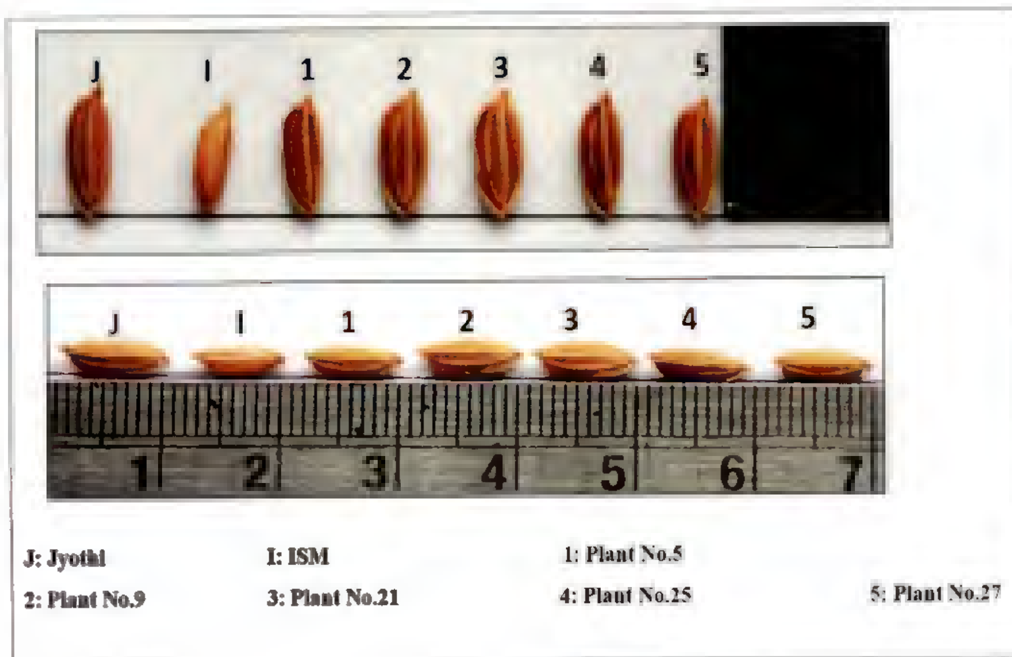


Plate 13. Grains of parents and selected BC_2F_4s

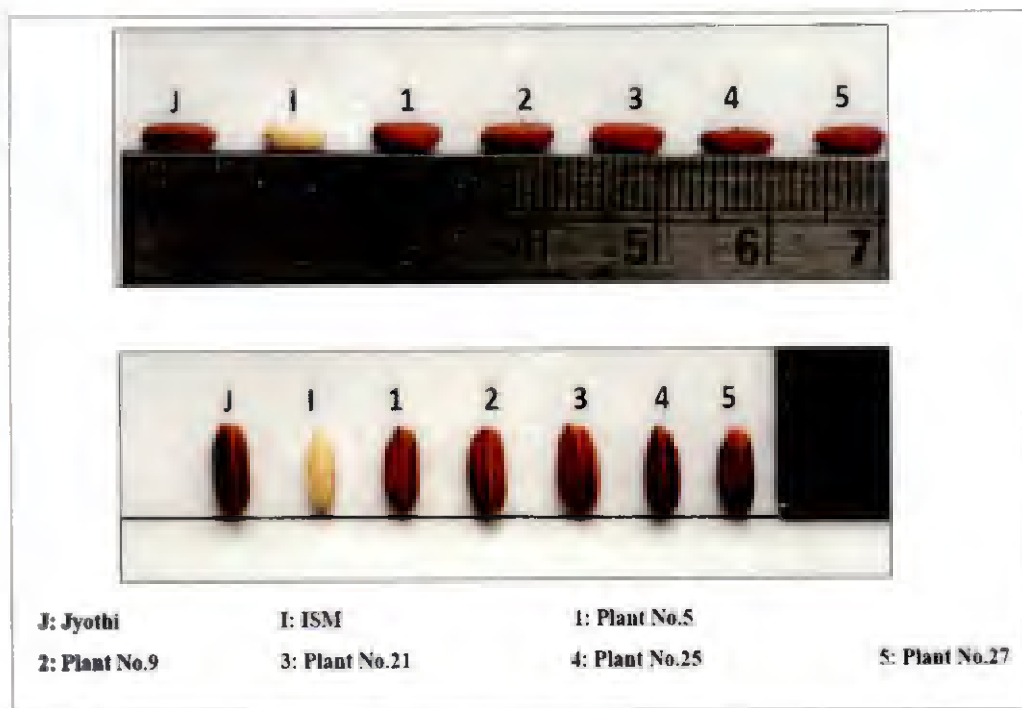


Plate 14. Decorticated grains of parents and selected BC_2F_4s

100 per cent opaque hard kernelled quality protein maize segregants in the BC_2F_2 families. Sundaram *et al.* (2008) had obtained promising three R-gene pyramided lines in the BC_4F_6 generation of the cross between Samba Mahsuri and donor line SS1113 yielding EDV, Improved Samba Mahsuri.

The dendrogram generated out of the morphological characters (Figure 2) grouped the two 2-R-genes pyramids, Plant No. 9 and Plant No. 21 into a single cluster along with donor parent ISM, while, the BC_2F_4 Plant No. 5, Plant No. 27 and Plant No. 25 clustered separately with recurrent parent Jyothi. This indicated less similarity between two 2-R-genes pyramids, Plant No. 9 and Plant No. 21 and recurrent parent Jyothi owing to lower recovery of recurrent parent genome. There was near total correlation between the clustering of the selected BC_2F_4 plant based on molecular (Figure 1) and morphological data (Figure 2).

Hence, it can be concluded that rapid line conversion strategy requires application of a combination of both marker aided and phenotypic selection approaches in handling segregating generation to fix target locus, reduction of linkage drag and recovery of maximum amount of recurrent parent genome. Higher success would be achieved through this approach by advancing the backcross population. Considering this, the identified 2 R-gene pyramids (BC_2F_4s *i.e.,* Plant No. 9 and Plant No. 21) were backcrossed to both recurrent parent Jyothi as well as donor parent ISM, resulting in production of BC_3F_1s (63 Nos.) and BC_1F_1s (56 Nos.) respectively. Simultaneously, selfing of these individuals resulted in production of 668 BC_2F_5 seeds.

To summarise, the use of molecular markers closely linked to BB resistance along with phenotype based-selection in the present study had resulted in identification of two 2 R-gene pyramided BC_2F_4s (Plant No. 9 and Plant No. 21) from among a population of 51 BC_2F_4s. This has greatly reduced the number of BC_2F_4s individuals that are required to be backcrossed to the recurrent parent Jyothi to accelerate the recovery of recurrent parent genome.

According to Dwivedi *et al.* (2007), efficient and precise identification of desired multigene pyramided genotypes is possible using the molecular marker assisted selection compared to the conventional breeding method. Development of advanced lines with resistance gene combinations have practical breeding value by providing a wider spectrum of resistance against most of the existing isolates of BB in the region and will have a high impact on yield stability and sustainability of rice crop in the region (Singh *et al.,* 2001). The two R-genes introgressed BC_2F_4s plants are expected to show moderate resistance to the *Xoo* pathotypes

prevalent in Kerala. Further, backcrossing and selfing of the identified backcrossed individuals as executed in the programme in combination with marker assisted evaluation of these lines for BB resistance will result in the production of advanced breeding lines of Jyothi. Moreover, this will enable pathogen inoculation and disease scoring studies in order to assess the extent of resistance offered by the pyramided individuals to various strains of *Xoo* pathogen.

The 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. According to Ragimekula *et al.* (2013), although conventional breeding has had a significant impact on improving resistance of cultivars, the time-consuming process of making crosses and backcrosses, and the selection of the desired resistant progeny takes ten or more years. By this time, in some instances, the pathogen has already evolved and the variant is not recognized by the improved genotype leading to susceptibility. New technologies such as DNA markers serve as a new tool to detect the presence of allelic variations in the genes underlying economic traits. DNA markers have enormous potential to improve the efficiency and precision of conventional plant breeding via marker-assisted selection by reducing reliance on laborious and fallible screening procedures.

Summary

VI. Summary

The present study 'Pyramiding of bacterial leaf blight resistance genes in rice variety Jyothi (PTB 39) through Marker Assisted Selection,' was conducted during 2015-2017, at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University. Jyothi (Ptb 39), one of the most widely cultivated high yielding rice variety in Kerala is highly susceptible to bacterial leaf blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Fifty-one BC_2F_4s generated in an earlier backcross breeding programme involving Jyothi as the recurrent parent and Improved Samba Mahsuri (ISM) as the donor parent comprised the experimental population. The study aimed to identify the R.-gene pyramids (*xa5*, *xa13* and *Xa21*) from among the BC_2F_4 population and generate further backcross progenies. Marker assisted selection (MAS) using functional markers and Sequence Tagged Sites (STS) tightly linked to the genes conferring resistance (R-genes) to BB pathogen was employed for this purpose. The research programme was categorized under four major experiments *viz*., I) Genotyping of BC_2F_4 population, II) Morphological characterization of pyramided lines (BC_2F_4 population) and parents, III) Production of BC_3F_1 population and IV) Production of BC_2F_5 population. The salient findings of the study are summarized below.

Foreground selection of BC_2F_4s

1. Sufficient quantity of good quality total genomic DNA was extracted from 51 BC_2F_4s, the donor parent Improved Samba Mahsuri (ISM) and recurrent parent Jyothi.
2. The PCR analysis with the STS marker RG 556 that is reported to be tightly linked to R gene *xa5* at a distance of 0.1cM, did not produce any polymorphism in the BC_2F_4s (51 Nos.) and parents.
3. On restriction digestion of the PCR product with *Dra*I restriction enzyme, alleles of size 238 bp and 438 bp were found in the BC_2F_4s and the parents, indicating that the BC_2F_4 individuals and both the parents carried the R-gene *xa5*.
4. Resolving the DNA of BC_2F_4s and parents on agarose gels using the functional marker *xa5*SR, produced monomorphic banding pattern. Amplicon of size 190 bp was resolved in the BC_2F_4 individuals and parents Jyothi and ISM, reconfirming the presence of *xa5* gene in both the parents as well as in all the BC_2F_4s studied.
5. Restriction digestion of PCR products of STS marker RG 136 with *Hinf*I enzyme produced homozygous alleles for the R-gene *xa13* in all the BC_2F_4 individuals

similar to that found in the susceptible parent. Hence, it was evident that none of the BC_2F_4 plants possessed the R-gene *xa13*.

6. A 280bp amplicon associated with the resistant allele of R-gene *xa13* that is found in donor parent ISM was not found in the BC_2F_4 individuals when amplified with functional marker *xa13* promoter. This further confirmed that the 51 BC_2F_4 individuals screened were not introgressed with the R-gene *xa13*.
7. Out of the 51 BC_2F_4s scored with the STS marker pTA 248 tightly linked to dominant R-gene *Xa21*, two plants (Plant No. 9 and Plant No. 21) were found to possess the resistance alleles (855 bp) similar to that found in the donor parent ISM. These were also found to be homozygous with the donor parent allele.
8. The results of foreground selection thus pointed out that, among the 51 BC_2F_4s studied, two individuals (Plant No. 9 and Plant No. 21) were the only 2-R-gene pyramids. All the other individuals possessed only a single R-gene (*xa5*).

Background selection of selected BC_2F_4s

1. Background profiling of the two 2-R gene introgressed BC_2F_4 plants (Plant No. 9 and Plant No. 21) and BC_2F_4 Plant No. 5, Plant No. 25 and Plant No. 27 along with parents revealed the presence of alleles similar to that found in the recurrent parent in case of 16 rice microsatellite markers out of the total 50 used.
2. No consistency in banding pattern among the selected BC_2F_4s was observed on genotyping the BC_2F_{4s} with the remaining 34 RM markers.
3. BC_2F_4 Plant No. 9 followed by Plant No. 21 possessed higher number of donor alleles (17 RM markers) than the other three BC_2F_4 individuals screened. In addition, these plants along with Plant No. 5 also registered higher number (4 nos.) of heterozygous loci.
4. Among the five BC_2F_4 individuals, the recovery of recurrent parent genome was found to be highest in Plant No. 5 (92.20 %) followed by Plant No. 27 (91.60%) while it was lower than the expected recovery in Plant No. 25 (76.40%), Plant No. 21 (64.40%) and Plant No. 9 (58.80%). The graphical representation of the results of genotyping of the BC_2F_4 plants done using the GGT software also reaffirmed the above.
5. The dendrogram generated using the marker data resulted in two clusters, one with the five selected BC_2F_4 individuals and the recurrent parent Jyothi. The donor

parent ISM was the lone member of the second cluster. The dissimilarity between these two clusters was 58 per cent.

6. The R-gene pyramids (Plant no. 9 and Plant No. 21) grouped into a separate sub-cluster farthest from recurrent parent Jyothi indicating that they were less similar to the recurrent parent when compared to the other three BC_2F_4 individuals studied. Conversely, this indicated that, the R-gene introgressed plants were genetically more similar to the donor parent ISM
7. BC_2F_4 Plant No. 5 exhibited maximum similarity with recurrent parent Jyothi.

Morphological characterization of BC_2F_4s

1. Presence of wide variability in morphological characteristics was observed among the BC_2F_4 population.

2. The agronomic evaluation of the BC_2F_4 population for number of productive tillers, panicle length, spikelets/ panicle, grains/panicle, indicated the presence of segregants that were better than the parental genotypes. However, in a few genotypes the magnitude for the traits studied was found to be lower than that of the parental genotypes.

3. The selected BC_2F_4s were found to be intermediate between the two parents for characters like plant height, days to flowering, leaf blade length, 1000 grain weight, grain width, decorticated grain length and width and straw yield/ plant.

4. The BC_2F_4 Plant No. 9 and Plant No. 21 were near similar to donor parent ISM with respect to traits plant height, leaf blade length and width, productive tillers/ plant, panicle length, spikelets and grains per panicle, grain length and decorticated grain width.

5. The number of productive tillers/ plant and grain length in BC_2F_4 Plant No. 5 was found to be higher than in both the parents, while plant number 27 recorded higher grain yield per plant than both the parents. Both these plants also registered a higher value than both the parents for leaf width, panicle length, spikelets and grains per panicle.

6. These R-genes introgressed plants also possessed red, medium sized kernels as observed in the recurrent parent Jyothi.
7. There was near total correlation between the clustering of the selected BC_2F_4 plant based on molecular and morphological data. The dendrogram generated based on the morphological characters grouped the two R-gene pyramids, Plant No. 9 and Plant No. 21 into a single cluster along with donor parent ISM, while, the BC_2F_4 Plant No. 5, Plant No. 27 and Plant No. 25 clustered separately with recurrent parent Jyothi. This indicated less similarity between two 2-R-genes pyramids, Plant No. 9 and Plant No. 21 and recurrent parent Jyothy owing to lower recovery of recurrent parent genome.

Production of BC_3F_1s and BC_2F_5s

1. The identified two R-gene pyramids (BC_2F_4s *i.e.*, Plant No. 9 and Plant No. 21) were backcrossed to both recurrent parent Jyothi and donor parent ISM resulting in production of BC_3F_1s (63 Nos.) and BC_1F_1s (56 Nos.) respectively.

2. Simultaneously, selfing of the above individuals were also undertaken resulting in production of 668 BC_2F_5 seeds.

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Appendices

Appendix 1. Quantity and quality of genomic DNA of BC_2F_4s

Sl. No.	Genotype	Quantity (ng/ml)	Quality (A_{260}/A_{280})	Sl. No.	Genotype	Quantity (ng/ml)	Quality (A_{260}/A_{280})
1	1	1084	1.8	27	27	1189	1.8
2	2	937	1.8	28	28	1208	1.9
3	3	948	1.9	29	29	1290	1.8
4	4	1237	1.8	30	30	1098	1.8
5	5	1193	1.8	31	31	1056	1.8
6	6	1200	1.9	32	32	1034	1.9
7	7	1400	1.8	33	33	997	1.9
8	8	1034	1.8	34	34	928	1.8
9	9	1102	1.9	35	35	903	1.7
10	10	894	1.8	36	36	909	1.9
11	11	898	1.9	37	37	988	1.9
12	12	986	1.8	38	38	1067	1.8
13	13	1039	1.8	39	39	1039	1.9
14	14	980	1.8	40	40	667	1.8
15	15	935	1.9	41	41	738	1.9
16	16	1293	1.9	42	42	1098	1.8
17	17	1403	1.8	43	43	883	1.8
18	18	798	1.8	44	44	893	1.9
19	19	1204	1.9	45	45	983	1.8
20	20	1119	1.7	46	46	1037	1.9
21	21	1008	1.9	47	47	1190	1.9
22	22	934	1.8	48	48	1320	1.8
23	23	927	1.8	49	49	1040	1.8
24	24	959	1.8	50	50	1200	1.8
25	25	789	1.8	51	51	680	1.8
26	26	1056	1.8				

Appendix 2. Quantity and quality of genomic DNA of parents

Parent	Plant No.	Quantity (ng/ml)	Quality (A_{260}/A_{280})
Jyothi (Recurrent parent)	1	938.00	1.80
	2	1029.00	1.90
	3	896.00	1.80
	4	979.00	1.80
	5	987.00	1.90
	6	1005.00	1.80
	7	1020.00	1.80
	8	1102.00	1.80
	9	703.00	1.80
	10	934.00	1.80
	Mean	959.30	1.82
ISM (Donor parent)	1	1025.00	1.80
	2	969.00	1.80
	3	946.00	1.90
	4	1026.00	1.80
	5	968.00	1.80
	6	1123.00	1.80
	7	984.00	1.90
	8	987.00	1.80
	9	915.00	1.90
	10	1003.00	1.80
	Mean	994.60	1.83

Appendix 3. Morphological characteristics of recurrent parent Jyothi

Plant No.	Plant height (cm)	Days to flowering	Leaf width (cm)	Leaf blade length (cm)	Productive tillers/plant	Panicle length (cm)	Spikelets/ panicle	Grains/panicle
1	68.25	80.00	1.73	38.00	7.00	21.00	130.00	114.00
2	66.59	81.00	1.58	37.90	7.00	20.77	117.00	103.00
3	29.29	80.00	1.16	38.25	8.00	21.12	115.00	102.00
4	66.35	82.00	1.90	37.36	7.00	20.98	124.00	98.00
5	67.48	84.00	1.87	38.72	7.00	21.00	128.00	101.00
6	69.72	85.00	1.10	37.53	10.00	20.78	112.00	95.00
7	68.33	82.00	1.73	38.03	9.00	20.96	117.00	97.00
8	67.48	86.00	1.80	38.09	9.00	21.17	130.00	107.00
9	68.59	90.00	1.05	38.12	9.00	21.05	124.00	95.00
10	70.88	91.00	1.73	38.17	9.00	21.25	126.00	98.00
11	72.91	86.00	1.52	38.27	7.00	21.12	112.00	92.00
12	72.98	87.00	1.83	37.61	7.00	21.19	117.00	95.00
13	68.61	80.00	1.95	38.54	7.00	20.96	128.00	110.00
14	69.22	81.00	1.10	37.44	8.00	20.97	130.00	108.00
15	69.31	84.00	1.58	37.83	8.00	20.97	112.00	92.00
16	69.38	86.00	1.46	37.26	8.00	20.05	115.00	90.00
17	70.41	90.00	1.36	37.22	8.00	20.89	118.00	95.00
18	67.10	84.00	1.10	38.16	9.00	20.98	128.00	99.00
19	69.29	85.00	1.58	38.23	7.00	21.17	130.00	102.00
20	70.74	86.00	1.73	38.64	7.00	21.12	126.00	101.00
21	71.21	86.00	1.68	38.31	9.00	21.08	124.00	90.00
22	72.49	90.00	1.79	38.52	7.00	21.09	130.00	100.00
23	69.12	91.00	1.80	37.53	8.00	21.00	128.00	112.00
24	68.31	90.00	1.68	37.00	7.00	21.05	130.00	110.00
25	68.16	91.00	1.60	38.06	7.00	21.11	127.00	106.00
Mean	**67.69**	**85.52**	**1.58**	**37.95**	**7.84**	**20.99**	**123.12**	**100.48**

Appendix 4. Grain characteristics of recurrent parent Jyothi

Plant No.	1000 grain weight (g)	Grain length (mm)	Grain width (mm)	Decorticated grain length (mm)	Decorticated grain width (mm)	Grain yield/plant (g)	Straw yield/plant (g)
1	26.82	7.60	1.80	6.02	1.53	14.23	20.03
2	26.10	7.01	1.59	5.98	1.36	14.78	22.85
3	25.82	7.28	1.67	5.87	1.45	14.77	23.34
4	26.31	7.01	1.72	5.83	1.51	15.21	22.96
5	27.02	7.13	1.92	5.78	1.72	14.23	21.34
6	26.45	7.20	1.76	6.06	1.56	15.41	19.52
7	26.86	7.54	1.68	5.92	1.46	15.54	22.12
8	26.45	7.68	1.79	5.90	1.54	15.59	24.35
9	24.67	7.76	1.85	5.82	1.65	16.62	23.38
10	24.41	7.14	1.68	5.81	1.49	16.70	22.18
11	27.90	7.09	1.69	5.79	1.48	16.26	23.19
12	27.81	7.18	1.76	5.93	1.56	17.40	24.29
13	25.04	7.40	1.59	5.72	1.39	17.21	22.30
14	25.64	7.64	1.64	5.70	1.48	16.22	21.82
15	26.84	7.49	1.78	5.64	1.51	17.43	22.01
16	26.07	7.68	1.76	6.05	1.53	17.11	20.50
17	27.47	7.70	1.73	6.11	1.50	17.13	24.44
18	26.07	7.74	1.84	6.14	1.67	16.82	22.36
19	26.48	7.78	1.93	6.13	1.74	14.94	22.88
20	26.46	7.84	1.91	5.87	1.75	15.46	21.46
21	26.28	7.64	1.64	5.72	1.76	15.43	23.57
22	26.48	7.82	1.79	5.68	1.54	15.38	24.88
23	26.96	7.84	1.68	5.64	1.49	15.51	23.30
24	26.48	7.69	1.94	5.65	1.74	16.80	22.10
25	27.46	7.40	1.71	6.01	1.57	17.13	21.86
Mean	**26.41**	7.49	**1.75**	**5.87**	**1.56**	**15.97**	**22.52**

Appendix 5. Morphological characteristics of donor parent ISM

Plant No.	Plant height	Days to flowering	Leaf width (cm)	Leaf blade length (cm)	Productive tillers/plant	Panicle length (cm)	Spiklets/ panicle	Grains / panicle
1	42.27	110.00	1.01	37.62	7.00	22.37	94.00	82.00
2	44.21	112.00	0.88	41.37	8.00	21.38	101.00	86.00
3	43.26	114.00	0.94	43.22	9.00	22.38	106.00	91.00
4	44.78	117.00	0.85	36.38	8.00	23.49	103.00	89.00
5	48.15	121.00	0.99	32.35	6.00	29.03	98.00	85.00
6	47.39	113.00	0.96	31.67	9.00	22.89	98.00	79.00
7	46.81	112.00	0.89	34.58	7.00	21.39	96.00	81.00
8	49.67	114.00	0.94	30.21	7.00	22.10	97.00	80.00
9	50.71	121.00	1.08	29.27	9.00	22.84	105.00	88.00
10	53.55	120.00	1.10	29.21	9.00	22.82	105.00	89.00
11	47.61	119.00	1.02	30.81	9.00	23.79	106.00	84.00
12	40.81	122.00	0.96	42.31	9.00	23.39	92.00	79.00
13	50.30	114.00	0.92	40.38	7.00	23.64	93.00	78.00
14	50.76	114.00	0.93	36.65	9.00	23.69	81.00	74.00
15	53.08	118.00	0.88	35.44	8.00	21.37	97.00	77.00
16	51.28	117.00	1.06	38.21	8.00	22.88	98.00	79.00
17	45.39	118.00	1.03	33.03	8.00	23.68	101.00	90.00
18	46.52	112.00	1.09	38.74	8.00	22.93	100.00	91.00
19	53.17	114.00	0.09	37.69	7.00	22.11	89.00	72.00
20	50.11	117.00	0.99	32.64	7.00	21.98	88.00	73.00
21	42.17	115.00	1.04	31.17	9.00	21.92	90.00	71.00
22	46.78	119.00	1.10	36.71	7.00	22.01	92.00	74.00
23	41.61	122.00	1.09	40.09	7.00	22.19	99.00	76.00
24	43.27	114.00	0.98	33.02	8.00	23.09	92.00	75.00
25	48.68	122.00	0.88	34.73	8.00	22.85	95.00	68.00
Mean	**47.29**	**116.44**	**0.95**	**35.50**	**7.92**	**22.89**	**96.64**	**80.44**

Appendix 6. Grain characteristics of donor parent ISM

Plant No.	1000 grain weight (g)	Grain length (mm)	Grain width (mm)	Decorticated grain length (mm)	Decorticated grain width (mm)	Grain yield/ plant (g)	Straw yield /plant (g)
1	10.12	6.72	1.36	5.07	0.96	7.84	11.10
2	10.81	6.38	1.28	4.38	0.68	8.80	9.91
3	11.78	6.15	1.23	4.68	0.58	7.27	10.09
4	10.97	6.11	1.34	5.03	0.76	7.59	10.09
5	10.91	6.09	1.38	5.08	0.92	8.46	10.68
6	10.37	6.91	1.40	5.12	0.74	8.52	10.76
7	11.22	6.28	1.22	4.52	0.68	8.76	10.84
8	11.03	6.31	1.14	4.27	0.99	8.34	11.04
9	10.04	6.36	1.28	4.68	0.92	9.20	11.76
10	10.08	6.38	1.36	4.74	0.84	9.28	12.06
11	9.82	6.12	1.38	5.14	0.68	7.79	12.84
12	10.31	6.29	1.24	4.42	0.78	9.09	9.96
13	10.28	6.52	1.26	4.44	0.77	7.95	10.90
14	9.63	6.58	1.30	4.39	0.91	7.93	10.91
15	9.34	6.87	1.32	4.48	0.84	7.94	10.87
16	9.45	6.91	1.34	4.53	0.82	9.34	10.95
17	10.21	6.84	1.38	4.34	0.92	9.46	9.98
18	10.28	6.80	1.31	4.44	1.01	9.23	10.76
19	11.12	6.75	1.28	4.16	0.90	7.65	10.88
20	10.78	6.81	1.20	4.20	0.88	8.83	10.84
21	11.19	6.33	1.18	4.22	0.82	8.41	11.36
22	11.15	6.23	1.10	4.14	0.99	8.71	11.92
23	11.21	6.20	1.24	5.12	0.92	9.09	11.79
24	10.66	6.21	1.39	5.08	0.94	9.13	11.69
25	10.73	6.73	1.36	5.08	0.93	7.89	10.72
Mean	**10.54**	**6.48**	**1.29**	**4.63**	**0.85**	**8.50**	**10.99**

Appendix 7. Morphological characteristics of BC_2F_4s

Plant No.	Plant height (cm)	Days to flowering	Leaf width (cm)	Leaf blade length (cm)	Productive tiller/ plant	Panicle length (cm)	Spikelets/ panicle	Grains/ panicle
1	61.03	87.00	1.70	36.78	6.00	38.17	138.87	110.50
2	64.27	90.00	1.58	34.09	8.00	30.16	137.90	110.39
3	63.63	90.00	1.58	30.19	9.00	39.03	135.91	114.03
4	65.34	87.00	1.88	33.43	9.00	37.85	134.34	103.13
5	67.38	87.00	1.66	29.83	9.00	36.66	130.38	109.17
6	64.28	92.00	1.73	30.29	9.00	37.37	130.29	109.37
7	60.17	90.00	1.38	29.71	8.00	36.83	128.17	98.28
8	62.23	94.00	1.16	36.49	9.00	37.61	117.94	94.29
9	50.33	105.00	1.03	35.32	7.00	26.88	108.23	82.76
10	55.34	92.00	1.19	37.33	8.00	28.91	139.33	99.89
11	59.38	99.00	1.82	36.43	8.00	38.28	124.34	97.29
12	60.40	96.00	1.63	34.83	8.00	38.36	127.38	98.78
13	68.62	97.00	1.54	36.04	8.00	36.45	138.40	99.41
14	70.72	99.00	1.24	34.26	7.00	36.42	130.62	110.49
15	72.68	100.00	1.60	33.27	7.00	37.06	134.72	112.34
16	64.90	102.00	1.60	29.68	8.00	36.06	127.66	114.81
17	67.60	98.00	1.34	34.96	7.00	31.41	134.07	117.24
18	69.73	96.00	1.60	28.06	8.00	37.06	134.13	100.27
19	62.10	95.00	1.94	36.37	8.00	37.44	130.91	103.53
20	69.19	104.00	1.34	38.31	8.00	36.43	132.13	103.54
21	53.05	104.00	1.06	35.91	7.00	27.63	113.37	81.48
22	55.99	92.00	1.76	38.50	9.00	39.67	117.05	110.12
23	60.33	94.00	1.48	38.29	9.00	38.84	139.14	80.34
24	63.40	94.00	1.64	38.36	9.00	36.46	137.46	96.47
25	64.50	92.00	1.34	34.46	8.00	35.43	140.32	97.54
26	66.64	86.00	1.68	36.42	8.00	36.86	139.32	96.13

27	67.23	87.00	1.96	36.67	8.00	38.60	130.23	110.47
28	58.11	86.00	1.06	38.85	8.00	38.94	134.46	134.14
29	59.49	87.00	1.34	37.58	9.00	37.85	132.06	116.24
30	60.58	93.00	1.86	38.29	7.00	38.05	128.43	111.24
31	63.50	92.00	1.70	38.39	7.00	38.46	130.40	92.24
32	65.64	94.00	1.74	38.36	7.00	36.64	137.44	90.16
33	64.46	95.00	1.64	36.46	9.00	39.68	133.44	93.24
34	68.86	90.00	1.54	34.05	8.00	38.69	130.85	94.91
35	69.96	87.00	1.05	32.28	9.00	37.63	134.37	110.18
36	63.36	89.00	1.24	34.39	8.00	38.24	136.68	102.14
37	64.46	91.00	1.36	39.40	7.00	38.38	130.62	103.53
38	64.42	96.00	1.17	38.78	7.00	37.43	128.32	104.18
39	68.83	98.00	1.19	37.98	7.00	36.93	139.19	105.29
40	64.34	99.00	1.34	36.34	8.00	36.04	128.40	112.45
41	69.34	100.00	1.46	37.41	9.00	38.14	130.57	114.41
42	70.40	102.00	1.47	36.06	8.00	36.04	134.73	119.20
43	72.41	88.00	1.51	36.33	8.00	36.94	138.39	110.88
44	63.49	89.00	1.62	38.41	8.00	38.85	138.19	101.78
45	60.58	90.00	1.58	38.42	9.00	37.62	118.40	97.34
46	58.26	92.00	1.72	32.39	7.00	38.54	130.26	98.09
47	56.45	94.00	1.34	29.10	8.00	37.83	128.51	99.34
48	55.38	96.00	1.70	38.16	9.00	38.41	128.88	100.26
49	62.14	97.00	1.16	37.36	8.00	38.24	127.20	102.41
50	61.52	96.00	1.98	36.43	9.00	38.20	124.17	101.45
51	66.73	92.00	1.92	34.46	9.00	38.37	124.97	98.38
Mean	**63.59**	**93.76**	**1.51**	**35.49**	**8.04**	**36.82**	**131.01**	**103.44**

Appendix 8. Grain characteristics of BC_2F_4s

Plant No.	1000 grain weight (g)	Grain length (mm)	Grain width (mm)	Decorticated grain length (mm)	Decorticated grain width (mm)	Grain yield/ plant (g)	Straw yield/ plant (g)
1	21.63	6.40	1.82	5.37	0.94	14.36	20.52
2	21.31	7.69	1.73	5.81	0.96	15.13	22.46
3	21.96	6.84	1.64	5.25	0.92	16.69	21.74
4	22.46	7.82	1.60	5.11	0.92	14.64	22.43
5	21.43	8.46	1.73	5.88	0.98	15.34	21.24
6	23.27	7.84	1.74	4.92	0.88	15.72	24.14
7	23.42	7.78	1.28	5.07	0.87	14.24	22.60
8	20.40	7.68	1.14	4.86	0.92	15.24	23.50
9	18.25	6.38	1.67	5.81	1.03	12.52	20.72
10	21.08	7.46	1.36	5.09	0.92	15.80	22.62
11	21.45	7.52	1.48	5.25	0.93	15.54	20.44
12	20.84	7.34	1.82	4.91	0.95	15.78	21.04
13	21.30	7.77	1.96	5.02	0.96	16.68	22.24
14	22.10	7.17	1.72	5.17	0.98	17.48	23.42
15	21.12	7.13	1.49	5.34	0.91	15.03	24.24
16	21.39	6.48	1.63	5.83	0.90	15.01	22.12
17	23.25	6.45	1.64	5.89	0.92	16.21	23.21
18	23.46	7.24	1.74	5.87	0.89	16.03	22.68
19	21.47	6.72	1.72	5.90	0.90	16.21	21.17
20	21.34	7.14	1.69	5.29	0.98	16.93	20.03
21	19.42	6.14	1.74	5.91	1.09	12.13	19.44
22	22.14	7.20	1.42	5.62	0.92	17.48	24.98
23	22.60	7.32	1.39	4.96	0.94	14.03	22.06
24	22.27	7.31	1.47	5.22	0.96	14.14	23.04
25	23.40	6.39	1.58	5.23	0.88	15.54	22.69
26	21.21	7.41	1.64	5.29	0.92	16.92	21.21

27	20.12	6.98	1.63	4.82	0.86	17.81	20.46
28	20.17	7.22	1.47	5.62	0.84	13.76	21.38
29	21.31	6.29	1.84	4.70	0.85	14.42	20.24
30	20.48	7.27	1.34	5.34	0.90	15.73	22.19
31	21.67	6.11	1.27	5.11	0.94	17.83	21.17
32	23.26	7.22	1.24	5.25	0.95	16.19	20.79
33	23.92	6.48	1.44	4.86	0.96	16.57	22.81
34	23.13	7.49	1.61	5.37	0.92	16.12	23.75
35	21.09	6.50	1.72	5.12	0.91	16.15	22.72
36	22.40	6.58	1.60	5.10	0.90	15.19	21.83
37	23.52	6.68	1.75	5.09	0.92	16.24	22.32
38	22.57	6.78	1.74	5.39	0.99	16.43	22.80
39	23.48	7.82	1.82	5.17	0.98	15.16	23.85
40	22.52	6.36	1.84	5.82	0.98	14.17	22.54
41	24.42	6.92	1.84	5.84	0.97	14.23	21.24
42	22.43	6.91	1.76	5.51	0.96	14.12	20.18
43	24.81	6.94	1.70	5.17	0.90	14.24	20.68
44	24.64	6.95	1.64	5.79	0.89	15.18	21.24
45	24.95	6.94	1.82	5.17	0.90	15.34	22.75
46	24.20	6.82	1.86	5.17	0.91	14.51	23.24
47	22.14	7.14	1.44	5.87	0.97	16.15	22.34
48	22.01	6.90	1.90	5.23	0.92	16.34	21.31
49	22.02	7.14	1.91	5.82	0.98	16.16	22.33
50	21.33	7.17	1.24	5.85	0.97	16.17	24.96
51	22.20	7.08	1.30	5.14	0.96	14.27	22.87
Mean	**21.92**	**7.06**	**1.61**	**5.33**	**0.93**	**15.50**	**22.05**

PYRAMIDING OF BACTERIAL LEAF BLIGHT RESISTANCE GENES IN RICE VARIETY JYOTHI (PTB 39) THROUGH MARKER ASSISTED SELECTION

By

KABADE PRAMOD GORAKHANATH

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ABSTRACT OF THE THESIS

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CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR – 680656

KERALA, INDIA

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ABSTRACT

As in other rice growing locales around the world, in Kerala too, various climatic, edaphic, biological, physical, physiological and socio-economic variables impact the area, production, and productivity of the rice. Bacterial blight (BB), an important biotic stress caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) assumes a huge role in deciding rice profitability in Kerala.

PTB 39 (Jyothi) and Mo 16 (Uma) are both elite rice varieties of Kerala, but extremely susceptible to the 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) in contrast to the use of hazardous plant protection chemicals. Breeders have attempted to introgress disease resistance genes (R-genes) into rice cultivars to impart BB resistance. Marker-assisted selection (MAS) enables pyramiding multiple R-genes along with rapid background recovery of the recurrent parent, while maintaining the exquisite quality characteristics of rice.

Considering the impact of the disease on food security and sustainability, efforts to introgress three R-genes (*xa5, xa13* and *Xa21*) into the variety Jyothi from donor parent Improved Samba Mahsuri (ISM) through Marker Assisted Selection (MAS) were made. Further, backcrossing to Jyothi (recurrent parent) and advancing the resultant BC_1F_1s have resulted in production of BC_2F_4 generation (51 plants). The present study aimed to identify BC_2F_4 lines pyramided with genes (*xa5, xa13* and *Xa21*) conferring resistance to bacterial blight using functional marker as well as R-gene linked Sequence tagged site (STS) markers. In addition, advancing the R gene introgressed BC_2F_4s to BC_2F_5 generation was also envisaged.

Foreground selection of the BC_2F_4 individuals was done using the *xa5* gene linked STS marker RG556. Restriction digestion of the PCR product of the STS marker with *Dra*1 restriction enzyme, resulted in production of alleles of size 238bp and 438bp in all the BC_2F_4 individuals including the parents, indicating the presence of R-gene *xa5*. Amplification of DNA of the individuals with the functional marker *xa5*SR further confirmed the presence of R-gene *xa5* in the parents as well as in the 51 BC_2F_4 individuals.

Restriction digestion of the PCR product of STS marker RG 136 linked to R-gene *xa13*, with *Hinf* 1, produced alleles similar to that of the recurrent parent Jyothi in all the BC_2F_4

individuals. This indicated the absence of R-gene *xa13*. The absence of gene *xa13* in the BC_2F_4s was further confirmed by using the functional marker *xa13* promoter. The analysis has resulted in the production of 280bp allele associated with the susceptible allele of R-gene *xa13*, indicating the absence of R-gene *xa13* in all the BC_2F_4 plants.

Foreground selection with STS marker pTA 248 to detect the presence of *Xa21* gene revealed that in BC_2F_4 Plant No. 9 and Plant No. 21 amplicon of size 992 bp, as found in the donor parent ISM was present. This confirmed that the BC_2F_4s Plant No. 9 and Plant No. 21 possessed resistant allele of R-gene *Xa21*. Results obtained thus revealed that, of the 51 BC_2F_4s subjected to foreground selection, BC_2F_4 Plant No. 9 and Plant No. 21 were the only R- gene introgressed pyramids (*xa5*+ *Xa21*).

In addition, the R-gene pyramided individuals, BC_2F_4 Plant No. 5, Plant No. 25 and Plant No. 27 were also selected for background screening as they were near similar to recurrent parent Jyothi with respect to plant height and days to flowering. Background profiling of the selected five BC_2F_4s using 50 rice microsatellite (RM) markers, revealed that the banding pattern in all the BC_2F_4s individuals was similar to recurrent parent in case of sixteen RM markers. This indicated that the BC_2F_4s possessed the same allele as in recurrent parent Jyothi at these marker loci. However, there was no consistency in banding pattern among the selected BC_2F_4s on genotyping with the remaining 34 RM markers. BC_2F_4 Plant No. 9 followed by Plant No. 21 possessed higher number of donor alleles than the other three BC_2F_4 individuals screened. In addition, these plants along with Plant No. 5 also registered higher number (4 nos.) of heterozygous loci. Such variations may be attributed to the segregation and independent assortment of alleles in the early backcross generations. These plants could be expected to segregate for the alleles in subsequent generation.

Considering the segregation of the 50 markers, the magnitude of recovery of recurrent parent genome was found to be highest in Plant No. 5 (92.20%), followed by Plant No. 27 (91.60%), Plant No. 25 (76.40%) and Plant No. 21 (64.40%). It was found to be the least in Plant No. 9 (58.80%). This was also confirmed by graphical genotyping. The dendrogram based on molecular data grouped the individuals into two major clusters. Cluster one was monogenic with only the donor parent ISM, while cluster 2 comprised of the recurrent parent Jyothi and all the five BC_2F_4 individuals. The highest similarity was observed between the R gene pyramids Plant No. 9 and Plant No. 21. These plants grouped into a separate sub-cluster

2 farthest from recurrent parent Jyothi under cluster 2, while Plant No. 5 showed maximum similarity with recurrent parent Jyothi.

Wide variability was observed among the BC_2F_4 individuals for various morphological traits. The BC_2F_4 plant No. 9 and Plant No. 21 were near similar to donor parent ISM with respect to plant height, leaf blade length and width, productive tillers/ plant, panicle length, spikelets and grains/ panicle, grain length and decorticated grain width. The number of productive tillers/ plant and grain length in Plant No. 5 was found to be higher than both the parents, while Plant No. 27 recorded higher grain yield per plant than both the parents. Both these plants also registered a higher value than both the parents for leaf width, panicle length, spikelet's and grains per panicle.

A near total correlation was observed between the clustering of the selected BC_2F_4 plant based on molecular and morphological data. The dendrogram generated out of the morphological characters indicated less similarity between the two 2-R-gene pyramids (Plant No. 9 and Plant No. 21) and recurrent parent Jyothi.

The identified two 2-R-gene pyramids (BC_2F_4s *i.e.*, Plant No. 9 and Plant No. 21) were backcrossed to both recurrent parent Jyothi and donor parent ISM resulting in production of BC_3F_1s (63 Nos.) and BC_1F_1s (56 Nos.) respectively. Simultaneously, selfing of these individuals resulted in production of 668 BC_2F_5 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 these generations can ensure precise identification of R-gene introgressed genotypes that resemble the recurrent parent Jyothi.

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