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Research Article

Marker assisted pyramiding of major brown plant hopper resistance genes in an elite culture CBMAS14065

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Abstract
Rice is an important source of energy and nutrition for half of the world's population. Various biotic stress factors severely affect rice production. Among the biotic stresses, brown plant hopper is an important destructive pest affecting rice yield around the world. Host Plant Resistance which involves the 'R' gene factors is considered the most effective strategy for controlling brown plant hopper infestation. Introgression of single 'R' genes doesn't confer complete protection against BPH damage due to continuous evolution of new BPH biotypes and hence multiple 'R' gene pyramiding can be done for developing durable resistance. In our study, BPH resistance was established in a pre-release high yield culture CBMAS14065 through QTL/Gene pyramiding strategy. Advanced backcross inbred progenies (BC_1F_3) were developed in the background of CBMAS14065 by crossing with the donor IR71033-121-15B harbouring Bph20 and Bph21 genes. Foreground selection was carried out using SSR markers RM261 and RM3331 closely linked to the respective Bph20 and Bph21 resistant genes. The BILs of CBMAS14065 harboring*Bph20* and *Bph21* were phenotypically screened for brown plant hopper resistance under greenhouse conditions. The developed BILs *viz*., 48-5-3 and 2-3-2 exhibited enhanced resistance against BPH infestation with decreased yield loss, in comparison with the susceptible parent CBMAS14065. The BILs developed through gene pyramiding in this study will serve as a novel donor source for developing durable BPH-resistance in rice cultivars.

Keywords: Rice, Gene pyramiding, CBMAS14065, BPH resistance genes

INTRODUCTION

Rice is an important cereal food crop for more than half of world's population. Annual rice production was severely affected by the incidence of numerous pests and diseases (Rakshana *et al.*, 2019; Hu *et al.*, 2011). Nearly 52 percent of the overall rice production is impacted by several biotic stress factors, among which 21% is caused by damages due to pest attack (Brookes and Barfoot, 2003). Brown plant hopper (*Nilaparvata lugens*) is considered as an important insect pest affecting rice cultivation with an annual yield loss up to 80% which is $300 million in Asia (Satturu *et al*., 2020). BPH is a sucking pest that severely damages the rice plants by feeding on its xylem and phloem saps, and itproduces the characteristic'hopper burn' symptoms in the rice fields. Sometimes BPH also serves as a carrier pest/vector for spreading viral diseases such as 'ragged stunt virus' and 'grassy stunt virus', severely affecting rice production (Lakshmi *et al.,* 2021; Wei *et al.,*2018). There are four major biotypes of BPH prevalent in South Asian countries, and particularly biotype 4 is considered as

the deadliest BPH in the Indian subcontinent. BPH in the rice ecosystem is usually controlled by adopting different chemical and biological management measures (Normile, 2008). The "Green Revolution" got glorified in Indian agriculture through the late 1960s, and the rice production was sustained and considerably improved by the application of chemical fertilisers / pesticides. Continuous high-dose application of these chemical pesticides has eventually developed pesticide-tolerance in the BPH biotypes through evolution and this severed the incidence/frequency of BPH injury in rice plants (Rashid *et al.,* 2022). The most efficient and cost-effective method for combating BPH, however, has been through the development of host plant resistance through introgression of BPH resistance genes, as opposed to conventional chemical control measures. Several 'R' genes from BPH resistant genetic stocks have been introduced into our elite rice cultivars for developing resistance against BPH injury (Alam and Cohen, 1998). A better and safer alternative for BPH control is cultivation of resistant cultivars in contrast to the use of chemical pesticides (Hong-Xing *et al.,* 2017). Several effective 'R' genes is being used in rice breeding for developing cultivars with durable resistance against BPH damage (Suh *et al*., 2011; Tenguri *et al.,* 2023).However, because of rapid adaptability and emergence of novel BPH biotypes, resistance is easily broken in several resistant cultivars harbouring single R gene in a brief period (Jing *et al.,* 2014). Sustainable and environmentally beneficial approach "gene pyramiding" is being used to develop resilient resistant cultivars against BPH through introgression of multiple BPH resistant 'R' genes using marker breeding (Verma *et al.,* 2023).

Nearly 39 BPH, resistant genes or polygenes have been studied from several indica wild species (Sani *et al.,* 2020; Wang *et al.,* 2018; Hu *et al.,* 2018). Rice cultivars IR70, IR72 and IR74 harbouring Bph3 resistance gene have durable resistance against BPH and hence it is being cultivated in Philippines for more than 30 years (Penalver Cruz *et al.,* 2011).Myint *et al.* (2012) reported that, NILs harbouring the resistance genes Bph25 and Bph26 exhibited significantly higher levels of BPH resistance in comparison to their susceptible parents. Three strong BPH resistant genes *viz*., Bph14, Bph15, and Bph18, were pyramided into elite indica rice variety 93-11, and an additive impact was seen in the pyramided lines compared totheir single gene and double gene introgressions (Muduli *et al.,* 2023; Hu *et al.,* 2013).

Durable BPH 'R' genes *viz*., *Bph20* (chromosome 4) and *Bph21*(chromosome 12)*, discovered* from *O. minuta,* are commonly used as resistant sources for brown plant hopper. An elite line IR71033-121-15B (obtained from the cross *O. minuta* x *O. sativa*) harbors two BPH 'R' genes *viz*., *Bph20* and *Bph21* and it shows higher expression of resistance against BPH damage. Therefore, our research was focused towards developing advanced generation BILs (BC_1F_3) harbouring two R genes (*Bph20 and Bph21*) in the background of pre-release culture CBMAS14065 for durable BPH resistance.

MATERIALS AND METHODS

CBMAS14065, a pre-release culture is a medium duration, semi-dwarf, high yielding, and fine grain type rice line developed from a cross between (Improved White Ponni x Apo). Apo was developed from a traditional variety; 'Benong' from Indonesia and it harbours three major drought tolerant QTLs *viz*., *qDTY1.1, qDTY2.1* and *qDTY3.1*. CBMAS14065 harbours two drought tolerant QTLs (qDTY1.1 and qDTY2.1) from the Apo rice genotype. The Brown plant hopper donor parent IR71033-121-15B harbouring two BPH resistance genes (Bph20 and Bph21) was crossed with the recurrent parent CBMAS14065.

Marker Assisted gene pyramiding approach was used to develop two BPH resistance genes (*Bph20* and *Bph21*) in the background of CBMAS14065 (**Fig.1**). Parental polymorphism survey between CBMAS14065 and IR71033-121-15B parents identified two polymorphic SSR markers, RM261 and RM3331, closely linked to respective BPH genes , *Bph20* and *Bph21*. DNA was isolated from all the developed progenies and parents using modified CTAB protocol (Richards *et al.,* 1994). DNA quality was checked using agarose gel electrophoresis, and quantified by Nanodrop using spectrophotometer (Thermo Fisher Scientific, USA). PCR reaction was carried out in a total reaction volume of 10µl containing 100ng of template DNA, 6 µl of master mix (10x Taq Buffer, dNTPs and Taq polymerase), Each 0.5 µl of both forward primer and reverse primers at 10µM concetration. All PCR reactions were amplified following the temperature profiles of initial denaturation at 94˚C for 4 min after that 37 cycles of 94˚C for 1 min, annealing at 55– 60˚C for 30s, extension at 72˚C for 1 min, subsequently a final extension at 72˚C for 10 min using a thermal cycler (C1000 TOUCH PCR, BIO-RAD Inc., USA). PCR products were ruined in a 3.5% agarose gel stained by ethidium bromide and the gel documented by documentation system (BIO-RAD, USA).

Selected BC_1F_3 progenies of CBMAS14065 along with respective parents were raised in the nursery bed in individual rows during the *Kharif* season (July, 2021). The 21 days old seedlings were transplanted to well puddle main field with 30 x 30 cm spacing at Paddy Breeding Station (PBS), Coimbatore. Data collected on different Agro-morphological traits *viz*., Days to Flowering (DF), Days to 50% flowering (DFF), Plant Height (PH), Number of productive tillers (NPT), Flag leaf Width (FLW), Flag leaf Length (FLL), Panicle length (PL), number of filled grains per panicle (NFGP), Single Plant yield (SPY) and 100 grain weight (GW) were recorded under field conditions.

The homozygous BC_1F_3 progenies of CBMAS14065 (harbouring both *Bph20*and*Bph21* genes) were used for the bioassay study on BPH resistance traits along with the recurrent parent (CBMAS14065), donor parent (IR71033-

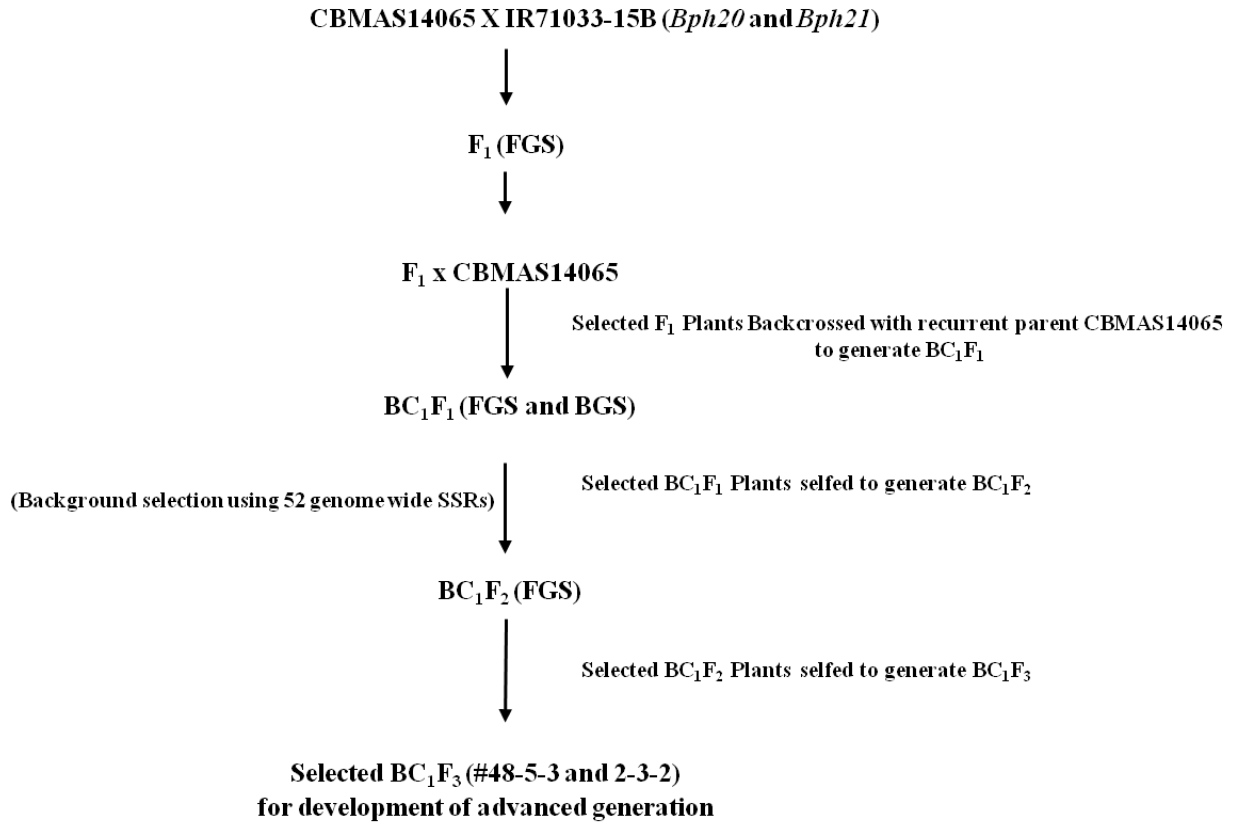

Fig. 1. Marker-assisted backcrossing scheme for the development of Brown Plant Hopper Resistant rice in the background CBMAS14065

121-15B), resistant check (PTB33), and susceptible check (Taichung Native1) in the Entomology glasshouse at the Department of Rice, TNAU, Coimbatore. Standard seed box screening method recommended at IRRI was used to conduct the bioassay trial at relative humidity levels of 75–80% and temperature level of 28–30˚C (Heinrichs, 1985). Respective parents and developed progenies seeds were pre-soaked in water for a day, and the next day all pre-soaked seeds were sown in the seed box of size 60x45x10 cm with 10-15 seedlings maintained in each row. BPH, second and third instar stage nymphs were released to the seven-day-old seedling for our bioassay studies. The characteristic "hopper burn" symptoms were observed on rice seedlings in a week after releasing the BPH insects. BPH damage was scored in all progenies using the International Rice Research Institute's grading system (**Table 1**) at the stage whenmore than 90% of the TN1(susceptible check) showed signs of drying and wilting (IRRI, 1996).

RESULTS AND DISCUSSION

Generation and evaluation of back cross progenies of CBMAS14065 harbouring *Bph* resistant genes: CBMAS14065, a pre-release culture is a medium duration, semi-dwarf, high yielding, and fine grain type rice line. CBMAS14065 harbours two droughts tolerant QTLs (qDTY1.1 and qDTY2.1) of Apo but it is susceptible to Brown Plant Hopper infestation. Developing BPH resistant backcross progenies in the background of CBMAS14065, attempt were made to crossing the CBMAS14065 (recurrent parent) with the IR71033-121-15B (donor parent).

Developing BC_1F_3 progenies of CBMAS14065 harboringBph20 and *Bph21*:BPH resistant rice cultivars harbouring single resistant gene is broken simply through continuous development of new BPH biotypes. Durable resistance can be achieved by introgressing multiple resistant genes against this devastating insect pest (Qiu *et al.,* 2012: Liu *et al.,* 2016). Till date, 32 BPH 'R' genes are identified, in that nearly 7 genes were cloned and characterized (Ren *et al.,* 2016). The *Bph20* gene performed better than *Bph21* gene with high survival rate against BPH infestation (Jena *et al.,* 2017). Both *Bph20* and *Bph21* genes when combined showed improved level of resistance against BPH due to additive effects (Rahman *et al.,* 2009). In our study, efforts were made to pyramid two BPH resistant genes namely *Bph20* and *Bph21* from the donor IR71033-121-15Binto CBMAS14065 through MABC. True F_1s were identified

Table 1. IRRI Standard evaluation system for BPH resistance

Scale	Damage	Resistance level
0	No Damage	Immune
1	Very slight damage	Highly resistant
3	Most of the plants First and second leaves Partially started yellowing	Resistant
5	About 10 to 25% of the plants severely stunted/dying	Moderately resistant
7	More than 50% of the plants dead	Moderately susceptible
9	All plants dead	Susceptible

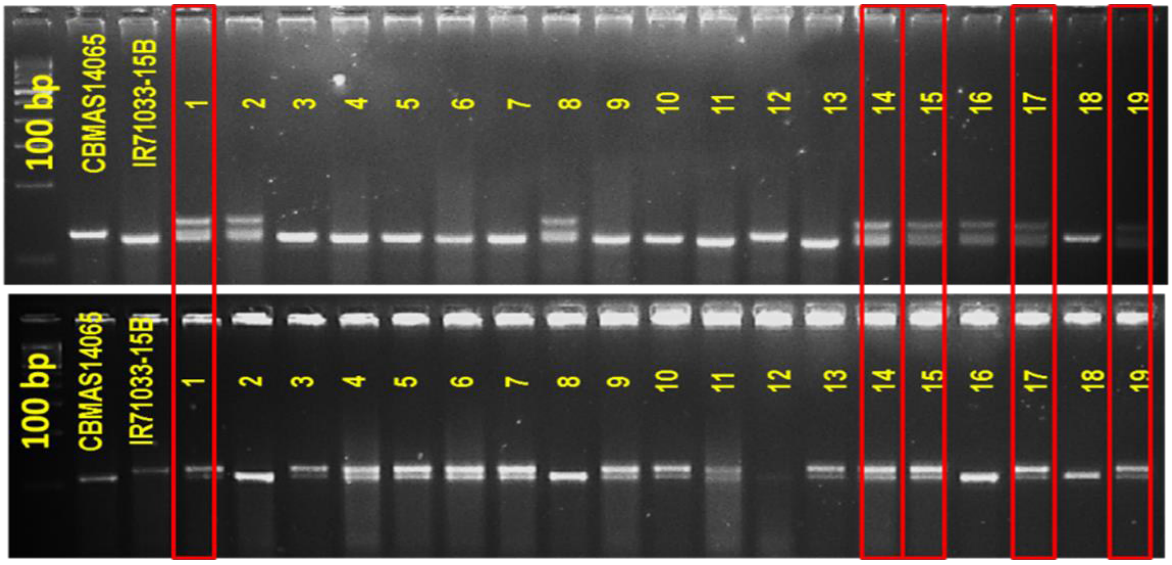

Fig. 2. Evaluation of BC$_1$F$_1$ progenies of CBMAS 14065 harbouring *Bph20* and *Bph21*

using closely linked SSR markers RM261 (*Bph20*) and RM3331 (*Bph21*) and then backcrossed with the recurrent parent CBMAS14065. Out of nineteen BC_1F_1 progenies developed, five progenies (#1, #14, #15, #17 and #19) were found to harbour both *Bph20* and *Bph21* genes (**Fig.2**). These five plants were selected for generation advancement through selfing. A total of 53 BC_1F_2 plants were genotyped using SSR markers and two plants # 2-3-2 and # 48-5-3 were resulted, homozygous for both the genes. These two plants were forwarded to BC_1F_3 generation and genotyped to confirm the presence of both the target genes under homozygous conditions (**Fig.3**). Recurrent parent genome recovery was estimated to be around 80-84.3% in our BC_1F_3 using 52 genome-wide SSR markers. Adoptions of MABC enabled development of back cross progenies of CBMAS14065 harboring two major BPH resistant genes of *O.minuta* namely *Bph20* and *Bph21*.

Evaluation of BC_1F_3 progenies under field condition: Developed progenies of CBMAS14065, #48-5-3 and #2-3-2 were evaluated under field condition for their phenotypic performance along with CBMAS14065 (recurrent parent) andIR710033-121-15B (Donor parent) during *Kharif* 2020. The agronomic data recordedunder field conditions were described in **Table 2**. Days to flowering and Plant height werereduced in progenies compared to the recurrent parent (Days to flowering in progenies were around 106 days while the recurrent parent was 125 days; Plant height in the progenies were around 100cm while the recurrent parent was 116cm. NPT were increased in the progenies to 27-30 Nos when compared to its recurrent parent which was only 24 Nos. Flag leaf length, Panicle length, single plant yield, Number of filled grains and 100 grain weight were increased in progenies compared to the recurrent parent (PL of progenies was 25-27cm and the recurrent parent was 21cm; NFGP of progenies was 235 -261 and the recurrent parent was 226; SPY and GW of progenies were 65-69g &1.525-1.795g while the recurrent parent were 56g and 1.245g respectively). The developed progenies were performing well in the agronomic performance compare to the recurrent parent *viz*., 10-15 days early flowering, 5-7 numbers of increased productive tillers, 6cm of increased panicle length, 30 numbers of increased grain number and 11-14g of increased single plant yield.

Evaluation of BILs for their responses against *Bph*

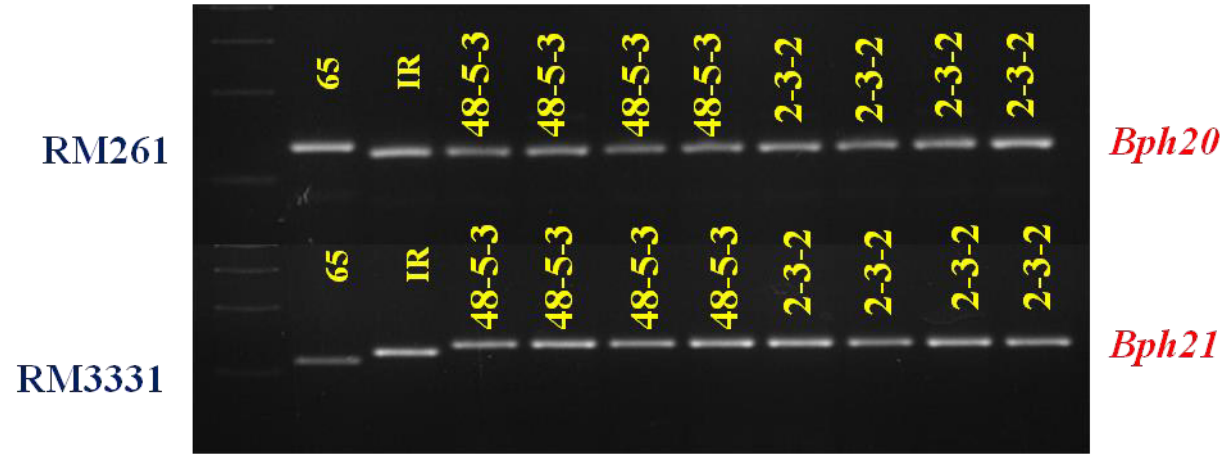

Fig. 3. Foreground selection of BC F progenies of CBMAS 14065
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Table 2. Agronomic Performance of BC_1F_3Progenies of CBMAS14065 pyramided with BPH Resistant genes under field condition

Plant No	Days to Flowering (First)	Days to Flowering (50%)	Plant Height (cm)	Number of Tillers	Flag Leaf Length (cm)	Flag Leaf Width (cm)	Panicle Length (cm)	Number of Filled Grains	Number of Chaffy Grains	100 Grain Weight (gm)	Grain Yield/ Plant (gm)
CBMAS14065	125	128	116	24	23.5	1.2	21	226	62	1.245	56
IR71033-121-15B	100	103	100	20	29	1.2	21	125	21	2.185	50
48-5-3	106	109	100	27	26	1.5	25	235	40	1.525	65
2-3-2	107	110	103	30	28	1.7	27	261	30	1.795	69

through Standard Seed box Screening Technique (SSST): An advance in gene pyramiding has enabled us to introgress two or more BPH resistance 'R' genes to develop resistant rice varieties. BILs of CBMAS14065 with different combinations of *Bph* resistant genes were evaluated for their resistance against brown plant hopper (BPH) along with CBMAS14065 (recipient parent), IR71033-15B (donor parent), PTB33 (resistant check) and susceptible check (TN1). Well-established BPH resistant rice cultivars have shown various reactions against the Coimbatore (India) biotype of BPH (Thamarai and Soundararajan, 2017). Donor parent IR71033-121-15B shows moderate resistance against *Nilaparvatalugens* population of AP (Andhra Pradesh) (Bhanu *et al.,* 2014). In our study, the donor parent IR71033-121-15B and fewBILs, notably # 48-5-3 and 2-3-2 (harbouring*Bph20* and *Bph21* under homozygous conditions) were found to be moderately resistant (MS) (SES score = 5) against BPH infestation. Resistant check (PTB33) was found to exhibit highly resistant (HR) with an SES score of 3 and the susceptible check (TN 1) was highly susceptible (HS) with an SES score of 9 (**Fig.4 and Table 3**). Other BILs possessing either *Bph20* or *Bph21* genes (homozygous or heterozygous alleles) exhibited increased susceptibility against BPH with SES score in range of 5-7. Hence, introgression of two BPH 'R' genes has provided high resistance / higher effect on phenotype compared to lines with single BPH genes.

The durable gene (*Bph6* and *Bph12*) pyramided lines have shown additive effect against BPH, compared to the single gene (*Bph6* /*Bph12)* introgressed lines (Qiu *et al.,* 2012). Similarly, *Bph14* and *Bph15* resistance genes have shown increased resistance than the single introgression lines harbouring either *Bph14/Bph15* resistance gene (Kim *et al.,* 2022; Hu *et al.,* 2012). Our results clearly demonstrated that pyramiding two or more BPH resistant genes is an efficient strategy to develop higher and durable resistance against BPH in rice (Kaur *et al.,* 2022; Tu *et al.,* 2000; Hittalmani *et al.,* 2000). The BILs with two 'R' genes (*Bph20* and *Bph21*) developed in this study might be used as genetic resource in rice breeding programs for achieving durable host-plant resistance against BPH.

Table 3. Responses of rice genotypes including BILs against BPH

S. No.	Genotypes	Score	IRRI Scale
1	TN1 (Susceptible check)	9	S
2	PTB33 (Resistant check)	3	R
3	CBMAS14065	7	MS
4	IR71033-15B	5	MR
5	Genotype no 48-5-3 (homozygous resistant allele of *Bph20* and heterozygous for *Bph21*)	5	MR
6	Genotype no 2-3-2 (heterozygous for *Bph20* and homozygous susceptible allele of *Bph21*)	5	MR

MR: Moderately Resistant;R: Resistant; MS: Moderately Susceptible andS: Susceptible

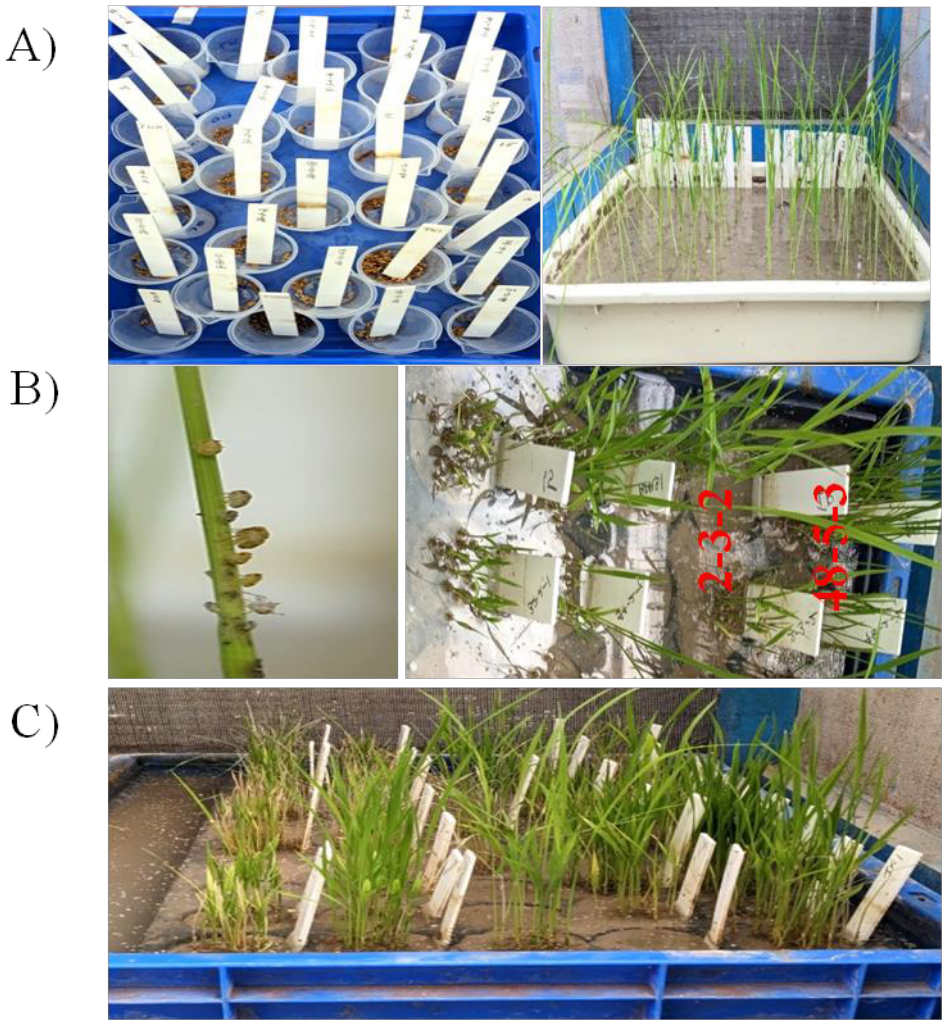

Fig. 4. Bph Bioassay Study:
A) Sowing of pre germinated seeds; B) Establishment of test lines;
C) Release of second and third instar nymphs in 7 days old seedling

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