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Marker assisted screening of recombinant inbred lines for bacterial blight and phosphorus starvation tolerance in rice (*Oryza sativa* L.)

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Abstract
Bacterial blight (BB) and phosphorus starvation tolerance are key factors that limit the rice production. Resistant cultivars developments are an excellent approach for managing biotic and abiotic stress challenges at a low cost. Morphological and genotypic screenings are commonly used method in the marker assisted crop improvement programme. Improved recombinant lines were screened by using functional markers and resistance reaction was confirmed through phenotypic screening. An active virulent local strain of *Xanthomonas oryzae* Pv. Oryaze (*Xoo*), IS (PNT-4) was employed to identify the resistant genotypes from the homozygous lines developed from popular rice varieties (ADT43, ASD16 and Co51), and donor lines (IRBB60, ACM18220, ISM pup1) combinations. Pyramided *Pup1* lines were phenotypically evaluated under hydroponic condition. The improved lines were confirmed by using functional markers *PTA248, xa13, xa5, K29-3* and *K46-1* to identify BB (*Xa21, xa13, xa5*,) and Phosphorus starvation tolerance (Os*PSTOL1*) genes. Among the genotypically confirmed twenty seven RILs, (AD (Bio) 13056, AD(Bio)13060, ACM18089,ACM18091, ACM18097, ACM18068, and ACM20015 were found to be promising for agronomic traits. These pyramided lines will be useful to breeders to develop newer variety/donor line in stress breeding programme.

Keywords: Assisted, screening, recombinant, phosphorus, *Oryza sativa* L.

1. Introduction
Rice bacterial blight caused by *Xanthomonas oryzae* pv. *Oryzae* (Xoo.) is one of the major biotic stress causing substantial yield losses. The disease infestation was observed almost in every rice field in south Asiatic rice growing regions. It can reduce yield by 20-50%.Similarly in abiotic stresses, Pi deficiency can reduces the crop yield upto 30 −40% in arable rice growing crop conditions. The worldwide Pi deficiency is a predominant nutrient factor which decides the crop productivity (Aluwihare *et al.,* 2016) [1]. The P shortage results in decreased plant growth, curled leaves, hairy-lateral roots, purple pigmentation in leaves, and impaired tillering, resulting in substantial yield losses. A major quantitative trait locus (QTLs) has been identified the Phosphorus uptake (Pup1) in a traditional *aus* type rice line (Kasalath) which located on chromosome 12 of longer arm (Wissuwa *et al.,* 2002) [16]. The QTL introgression was highly benefitted to promote primary, secondary root growth and high N update efficiency. In bacterial blight, the susceptible cultivars were severely holding the yield loss (Mew *et al.,* 2004) [7]. The cultural practices, chemical control, biological control, disease forecasting is useful but use of resistant cultivar is the best and cost-effective approach for controlling this disease in plant gene compositional levels. (Pinta *et al.,* 2013) [8]. Furthermore, lines bearing four major BB genes (*Xa21, xa13 and xa5*) four bacterial blight resistance genes exhibited a wider range of resistance to compared with a single gene resistance. In cultivated rice and its wild relatives, more than 40 BB resistance genes (R genes) have been found. Kim *et al*.,(2015) [6]. Phosphorus starvation tolerance responsive PHT1 family genes have been found in the cereal crops which express in different regions of roots in P starvation tolerance attributes. Thirteen number of PHT1 genes were encoded high-affinity P transporters have been found to be expressed in rice, with some of these functionally identified genes, such *as OsPHT1;1, OsPHT1;2, OsPHT1;3, OsPHT1;6, and OsPHT1;8.*The *OsPSTOL1*gene promotes the extensive root development in rice, the enhanced uptake of 'P' from the soil (Wissuwa *et al.,* 2001) [16] (Roy *et al.,* 1997) [12].

In this present study, Six functional SSR primers (*PTA248, xa13, xa5, RM224, RM336, K29-3, and K46-1*) have been used for DNA amplification to identify the BB and P starvation tolerance in rice resistance. The objective of this study is to assess the performance of recombinant inbred lines pyradmided with major P starvation tolerance gene, *OsPSTOL1* and *Xa21, xa13 and xa5* genes. These lines were developed in a marker assisted breeding programme using popular cultivar Co51 as recipient parent. The identified progenies to comprehensive phenotyping to test traits related to P uptake efficiency and BB disease resistance.

2. Materials and Methods

2.1. Experimental Site

The plant materials were laid out at the experimental plots in AC&RI, Madurai. The area is located at a 9.9252° N, 78.1198° E. The bacterial blight and phosphorus starvation tolerance screening was done at both open field and artificial screening facility. The molecular analyses were carried out at Department of Biotechnology, AC&RI, Madurai.

2.2. Plant materials

The experimental material consisted of Recombinant Inbred Lines (RILs) developed from crossing of popular high yielding varieties, ADT 43, Co51 and ASD 16 and donor lines ACM18220, IRBB60 and ISM pup1. The donor line, IRBB60 carrying *Xa21, xa13 and xa5,* ACM18220 and ISM pup1 harbouring both bacterial blight (*Xa21, xa13 and xa5*) and and phosphorus starvation tolerance (Os*PSTOL1*) genes were used in this study. The details of improved lines were presented in Table 2.

2.3. DNA extraction and genotypic confirmation by SSR functional markers

The young leaves from 21 days old seedling were collected. Total genomic DNA was extracted using modified CTAB (Cetyl trimethyl ammonium bromide) method as described by Doyle and Doyle (1987) [3]. Isolated DNA was diluted with double distilled water and stored at -40ºC for subsequent marker analysis. The diluted 50 ng/µl DNA template was used for the PCR amplifications. The amplified products were resolved by electrophoresis on a 1.5% agarose gel in 0.5x TBE buffer to determine whether PCR amplification was successful. When the combinations of said primers (Table.1) were used to amplify the DNA of the lines and parents method was earlier discussed by Williams *et al.,* (1999) [15] used DNA markers for confirmation BB (Bacterial blight) resistant and P starvation tolerant QTL with gene specific markers for BB and *OsPTOL1* resistant genes in each generation (Table 1). All the RILs with parents were evaluated from each generation and selected plants were forwarded to phenotypic and yield performance screening.

2.4. Preparation of bacterial inoculums for bacterial blight screening

The different isolates were collected from various rice growing regions IS(PNT)-Poonjuthi, IS(ARL)-Ariyalur, IS(MLR)-Melur and IS(ADT Aduthurai, Among the isolates IS(PNT)-4 isolate showed maximum virulence. The highest virulence was confirmed through the microscopical and disease incidence level. The isolate was maintained in slants with peptone sucrose agar medium (Khan *et al.,* 2009) [5]. After growing of *Xoo* the inoculum was produced by slanting 10 ml sterile distilled water with the cultured bacteria for 72 hours at 30 °C. Using sterile distilled water, the bacterial culture was adjusted to a concentration of 108 CFU/ml for inoculation (Chithrameenal *et al.,* 2018) [2].

2.5. Screening for phosphorus starvation tolerance in improved RILs

Hydroponic experiment was conducted with Yoshida nutrient solution (Roumen *et al.,* 1997) [11]. Yoshida solution with 100% (High P) and 50% (Low P) of phosphorus concentrations were used to screen the genotypes under artificial hydro environmental condition**.** (Pic.2). Seeds RILs with *OsPTOL1* gene along with parental genotypes were sprouted in moist paper towels. After ten days, the seedlings were transferred to trays containing hydroponic solution. Under a completely randomized complete block design, replications for each treatment were maintained. The pH level was kept at 5.0, and the solution was replaced every two weeks. Full plants were collected separately after 50 days after transplanting, and root length (cm) and root biomass (g) were measured. The plant roots and shoots were sampled to measure phosphorus content (Piper *et al*., 1997) [9].

3. Result and Discussion

3.1. Confirmation of bacterial blight resistance in RILs

The twenty seven Recombinant Inbred Lines (RILs) from ADT43, ASD16 and Co51 crosses with IRBB60, ISM *pup1* and ACM18220 were evaluated with predominantly available six BB isolates, Among the six isolates IS (PNT)-4 Poonjuthi was recorded highest lession length (>15cm) than other five isolates. Hence, bacterial blight pathogen culture (IS (PNT)-4) was used to evaluate the resistance in artificial disease screening A representative picture is given as (Pic.1.) The mean lesion lengths of plants with three resistance genes in RILs and parental population were evaluated (Table.2). As expected, all the selected RILs AD (Bio)13056, AD(Bio)13060, AD(Bio)13049, ACM18040, ACM18071, ACM18068, ACM20012, ACM20015 were showed higher resistance with a mean lesion length of less than 3.0 cm. The recipient parent Co51 showed highly susceptible character and it showed more than 14 cm of lesion length and categorized as highly susceptible to bacterial blight pathogen. (Table.2) The donor parent, IRBB 60 and ACM18220 showed high level of resistance compared with recipient parents. that the resistance gene combination, *Xa21, xa13, xa5* showed more effective resistance to compare other gene combinations (Ramalingam *et al*., 2017) [10].

3.2. Evaluation of phosphorus starvation tolerance in selected RIL population with parents

To evaluate phosphorus starvation tolerance under hydroponic condition with +P and -P treatments. (Fig.1). The twelve improved RILs showed high root length, observed in Low P condition, ACM 18089, ACM 18091, ACM18097, ACM20012 and ACM20013. ACM 18079, ACM18064, ACM20011 and ACM20015 genotypes obtained that moderate root growth were noted to compared with resistant check (ISM *pup*1). The similar results in arabidopsis, root growth and shoot biomass production recorded by (Fang *et al.,* 2009) [4]. The poor biomass and root production indicated in low P condition is Co51, ADT43 and ASD16. (Pic.2) Based on the hydroponic (Yoshida) media experiment were conducted in two different concentrations (50% and 100%).

The popular rice cultivar of ADT43, ASD16 and Co51 susceptible showed lower biomass and root development to compare with improved RILs and the plants produced detectable expression and P-deficiency tolerance capacity was clearly demonstrated by (Sarkar *et al.* 2011) [13].

3.2. Marker assisted screening by functional SSR markers in RILs

The functional SSR markers were used to inculcate four important BB genes *xa5, xa13, Xa21* and (Os*PSTOL1*) into targeted recipient varieties. During the marker assisted breeding procedure, SSR functional marker (Table.1) selection was practiced from parental lines to improved RILs (Fig.1). Each stage, the plants having resistance alleles of all the targeted genes/QTLs of both stresses were identified. Based on the genotypic evaluation twenty seven plants are selected and these plants contained different BB and phosphorus starvation tolerance. The improved RILs was also possessed good agronomic attributes. Vidya *et al.*, 2018 [14] stated that in BC_1F_1 progenies from the background of CB14002 and out of twenty four BC_1F_1 plants, eight plants showed heterozygous for RM209 and Chithrameena *et al.*, 2018 [2] also was evaluated (BC_2F_2) lines with pyramided the major P starvation tolerance gene *OsPSTOL1* into these NILs, ADT43 (CB14002) and ASD16 (CB14004) which already harbor major disease resistance genes for blast and BB.

Table 1: Details of molecular markers used for foreground selection

Target Trait	Gene/QTL	Marker	Primer sequence (5'-3')	AT	Chr	Reference
Bacterial Blight resistance	*xa5*	xa5 F xa5 R	F-CGGATAGCAGCATTTCCAAGAG R-GATTCCTTTAGCAAGGTGTG	56	5	Iyer-Pascuzzi and Mccouch (2007)
	xa13	xa13 F xa13 R	F-GAGCTCCAGCTCTCCAAATG R-GGCCATGGCTCAGTGTTTAT	59	8	Chu *et al.* (2006)
	Xa21	pTA248	F-ATAGCTAGTTCATAGAGG R-ACATCCGTCACTCTGCCA	65	11	Ronald *et al.* (1992)
Phosphorus Tolerance	*OsPSTOL 1*	K29-3F, 3R	F: TTCGTCCAGATGCTGCTATG R: TCTTCGGTGTAATTGGCACA	58	12	Chin *et al.* (2010)
	OsPSTOL 1	K 46-1	F: TGAGATAGCCGTCAAGATGCT R: AAGGACCACCATTCCATAGC	58	12	Chin *et al.* (2010)

Table 2: The gene composition in improved lines with resistance characters

S. No	Line	Cross combination	Gene composition	Resistance
1	AD(Bio) 13083	ADT43 x IRBB60	*Xa21+xa13+xa5*	BB
2	AD(Bio) 13060	ADT43 x IRBB60	*Xa21+xa13+xa5*	BB
3	AD(Bio) 13056	ADT43 x IRBB60	*Xa21+xa13+xa5*	BB
4	AD(Bio) 13082	ADT43 x IRBB60	*Xa21+xa13+xa5*	BB
5	AD(Bio) 13049	ADT43 x IRBB60	*Xa21+xa13+xa5*	BB
6	AD(Bio) 13064	ADT43 x IRBB60	*Xa21+xa13+xa5*	BB
7	AD(Bio) 13090	ADT43 x IRBB60	*Xa21+xa13+xa5*	BB
8	AD(Bio) 13068	ADT43 x IRBB60	*Xa21+xa13+xa5*	BB
9	ACM 18089	ADT43 x ISM pup	*Xa21+xa13+xa5+OsPSTOL1*	BB, *PSTOL*
10	ACM 18091	ADT43 x ISM pup	*Xa21+xa13+xa5+OsPSTOL1*	BB, *PSTOL*
11	ACM 18079	ADT43 x ISM pup	*Xa21+xa13+xa5+OsPSTOL1*	BB, *PSTOL*
12	ACM 18042	ADT43 x IRBB60	*Xa21+xa13+xa5*	BB
13	ACM 18040	ADT43 x IRBB60	*Xa21+xa13+xa5*	BB
14	ACM18243	ASD16 x IRBB60	*Xa21+xa13+xa5*	BB
15	ACM18097	ASD 16 x ISM pup	*Xa21+xa13+xa5+OsPSTOL1*	BB, *PSTOL*
16	ACM18072	ASD 16 x ISM	*Xa21+xa13+xa5*	BB
17	ACM18074	ASD 16 x ISM	*Xa21+xa13+xa5*	BB
18	ACM18071	ASD 16 x ISM pup	*Xa21+xa13+xa5+OsPSTOL1*	BB, *PSTOL*
19	ACM18068	ASD 16 x ISM pup	*Xa21+xa13+xa5+OsPSTOL1*	BB, *PSTOL*
20	ACM18064	ASD 16 x ISM pup	*Xa21+xa13+xa5+OsPSTOL1*	BB, *PSTOL*
21	ACM 18042	ADT43 x IRBB60	*Xa21+xa13+xa5*	BB
22	ACM18040	ADT43 x IRBB60	*Xa21+xa13+xa5*	BB
23	ACM 20011	Co51 X ACM18220	*Xa21+xa13+xa5+OsPSTOL1*	BB, *PSTOL*
24	ACM 20012	Co51 X ACM18220	*Xa21+xa13+xa5+OsPSTOL1*	BB, *PSTOL*
25	ACM 20013	Co51 X ACM18220	*Xa21+xa13+xa5+OsPSTOL1*	BB, *PSTOL*
26	ACM 20014	Co51 X ACM18220	*Xa21+xa13+xa5+OsPSTOL1*	BB, *PSTOL*
27	ACM 20015	Co51 X ACM18220	*Xa21+xa13+xa5+OsPSTOL1*	BB, *PSTOL*
28	ADT43	IR (50) x White ponni	-	NA
29	ASD 16	ADT 31 x Co 39	-	NA
30	Co51	ADT 43 x RR 272-1745	-	NA

Table 3: Phenotypic disease score of Recombinant Improved Lines (RILs) against bacterial blight

Pl. No	Lines	Lesion length (cm)	Scale	Resistance
1	AD(Bio) 13083	2.85	1	R
2	AD(Bio) 13060	4.56	1	R
3	AD(Bio) 13056	2.00	1	R

4	AD(Bio) 13082	0.85	1	R
5	AD(Bio) 13049	0.90	1	R
6	AD(Bio) 13064	5.11	3	MR
7	AD(Bio) 13090	1.84	1	R
8	AD(Bio) 13068	1.77	1	MR
9	ACM 18089	5.00	3	R
10	ACM 18091	1.10	1	R
11	ACM 18079	6.64	3	=R
12	ACM 18042	0.99	1	R
13	ACM 18040	1.78	1	R
14	ACM18243	1.11	1	R
15	ACM18097	2.45	1	R
16	ACM18072	5.11	3	MR
17	ACM18074	2.94	1	R
18	ACM18071	0.95	1	R
19	ACM18068	2.34	1	R
20	ACM18064	0.99	1	R
21	ACM 18042	2.95	1	R
22	ACM18040	2.22	1	R
23	ACM 20011	1.0	1	R
24	ACM 20012	2.85	3	R
25	ACM 20013	2.05	3	R
26	ACM 20014	2.0	1	R
27	ACM 20015	0.9	3	R
28	IRBB60	0.5	0	I
29	ADT 43	21.5	9	S
30	ASD16	18.45	9	S
31	CO51	16.5	9	S
32	IL7	0.5	1	R

BB culture-Local virulent isolate IS (PNT)-4 Poonjuthi; Replication-5 leaves/Hill; BB- Lesion length >5cm = Resistant (R)-1, 5-10cm = moderately resistant (MR)-3; 10-15cm = moderately susceptible (MS)-5; >15 cm = Susceptible (S).-9.

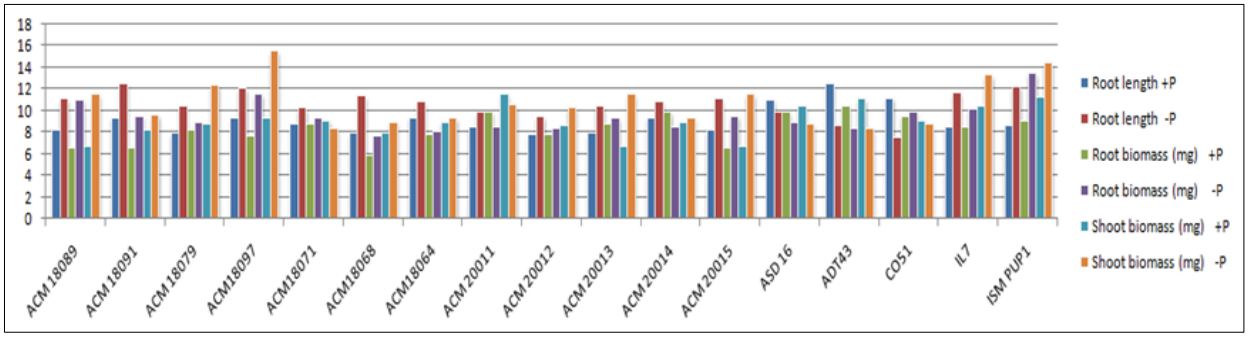
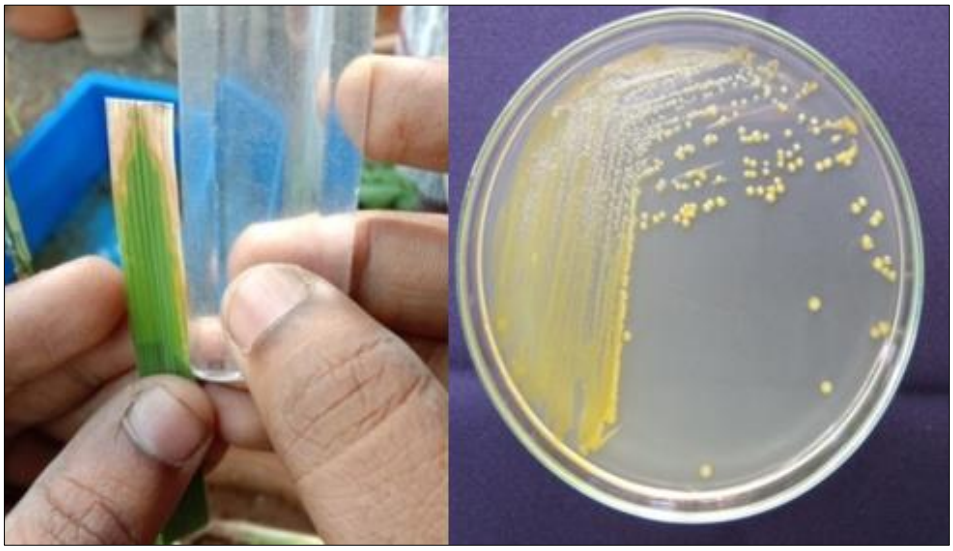
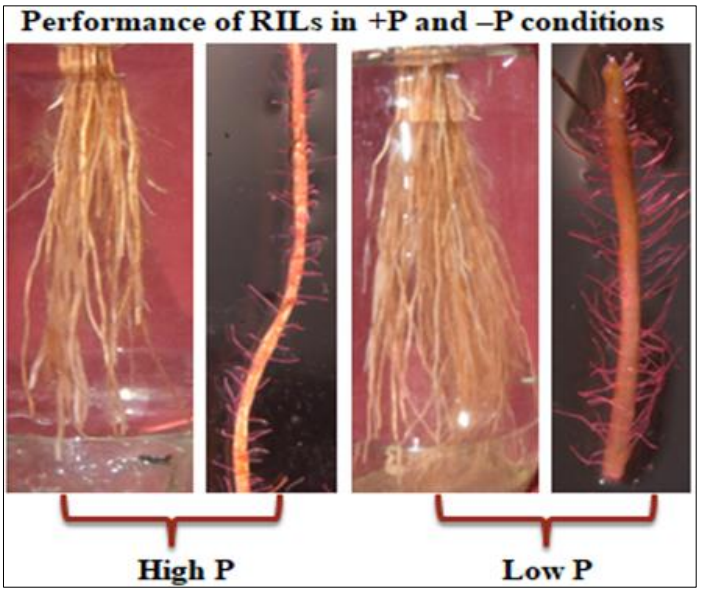


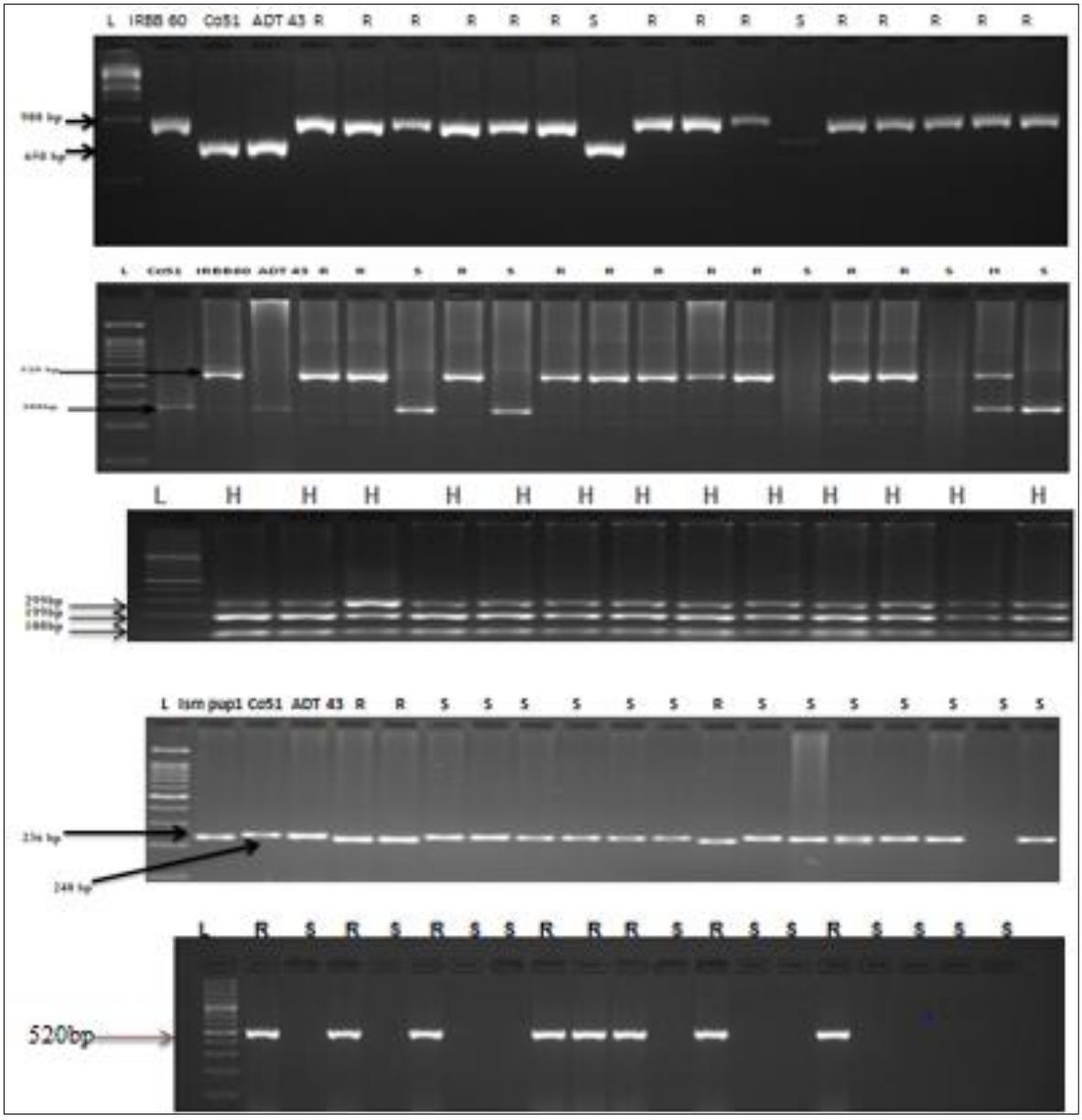
Fig 1: Observation of root parameters in RILs with parental lines under high & low P condition RILs and parents



Pic 1: Artificial BB screening of RILs against IS (PNT)-4 isolate of bacterial blight (BB). Parents with five RILs harboring *xa5*, *xa13*, and *Xa21* genes.



Pic 2: Phosphorus starvation tolerance study of RIL in Yoshida hydroponic solution with Low P and High P conditions



Pic 3: Agarose gel electrophoresis pictures depicting the presence and absence of (A) *Xa21*, (B*) xa13*, (C) *xa5*, (D), (E) *OsPSTOL 1*alleles. M – 100-bp ladder, R, resistant; H, heterozygote; S, susceptible In RILs and parents.

4. Conclusion

Marker assisted gene pyramided lines with bacterial blight (BB) genes (*Xa21, xa13, xa5*) and phosphorus starvation tolerant gene (*OsPSTOL1*) were developed and evaluated. These improved homozygous resistant lines were confirmed for the presence of resistance genes through functional markers. AD(Bio)13056, AD(Bio)13060, ACM18089, ACM18091, ACM18097, ACM18068, ACM20015 were found to be promising for bacterial blight resistance and phosphorus starvation tolerance with good agronomic traits. Marker assisted screening along with artificial screening and stringent phenotypic selection thus help us to identify promising lines for future use. In addition to offering the potential for release as new cultivars, the pyramided lines will serve as useful donor for various resistances breeding programmes in rice.

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