



Unveiling Genetic Potential: Marker-Assisted Selection for Phosphorus Deficiency Tolerant Rice Genotypes

Rishiraj Raghuvanshi^{1*}, Miranda Kongbrailatpam¹, Datta P Kakade¹, Jyoti Singh¹, Vinay Bachkaiya², S B Verulkar¹ and Shubha Banerjee¹

¹Department of Plant Molecular Biology and Biotechnology, Indira Gandhi Krishi Vishwavidyalaya, India

²Department of Soil Science and Agricultural chemistry, Indira Gandhi Krishi Vishwavidyalaya, India

*Corresponding author: Rishiraj Raghuvanshi, Department of Plant Molecular Biology and Biotechnology, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh.492012, India

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Abstract

Improving phosphorus (P) use efficiency of indica rice is a major concern of rice breeders. Promising rice genotypes and potential genes contributing phosphorus use efficiency (PUE) in indica rice were identified using marker assisted selection and bulk expression profiling. Segregating population (F3) derived from Sahbhagi Dhan and RRF-78 was evaluated for root traits and yield attributing factors under P-supplemented and P-deficient conditions. The marker RM242 and RM212 were found to be associated to number of tillers/plant and RM212 with plant height in P-deficient condition. Bulk expression profiling of Pup1 QTL underlying genes showed differential expression. Pup1 specific gene OsPupK04-1 showed 2 amplicons of length ~800bp and ~250bp in tolerant and susceptible bulk and in contrasting genotypes, indicating alternate splicing under P-deficiency. The longer fragment was found to be associated with higher Phosphate use efficient rice genotypes. Six recombinant lines SDxRRF-78(63), SDxRRF-78 (83), SDxRRF-78(124), SDxRRF-78(99), SDxRRF-78(26) and SDxRRF-78(81) were identified as P-deficiency tolerant rice genotypes.

Keywords: Phosphorus deficiency tolerance, MAS, Bulk expression in rice

Introduction

Phosphorus (P) is the most essential nutrient after nitrogen. P-deficiency stress is one of the major issues in rice cultivation especially under low input non irrigated farming in India. District wise soil mapping of arable land have shown that about 51% of the soils are having low P content while 40% were medium in P [1]. Moreover, the chemically P fertilizer are made from rock phosphate source which are nonrenewable and are estimated to exhaust very soon [2]. Thus, the identification and development of P-efficient rice cultivar is required with improved root traits, enabling them to better access phosphorus in the soil, or they can have mechanisms to utilize phosphorus more efficiently. Few of the DNA markers-based association studies have also been conducted

in last two decades [3-9]. Pup1 QTL is mapped on chromosome 12 of Kasalath that contributes significant P-deficiency tolerance to this landrace. Wissuwa et al. [4] studied P uptake and use efficiency and identified a major quantitative trait locus (QTL) named Pup1 on chromosome 12 of (Kasalath) rice. Further introgression of Pup1 have shown that Nippon bare near-isogenic genotypes having Pup1 could increase phosphate uptake in a severely affected P-deficient plot, relative to the recurrent parent Nippon bare [10]. Fine mapping, sequencing and gene annotation of Pup1 QTL have led to identification of underlying candidate genes (PSTOL1) [4, 11-12]. QTL, Qyd-12 was also identified by Jewel et al. [13] in P-deficient plot which is co-localized with Pup1 QTL. Combining the QTLs validation and transcriptome profiling approaches have been found more effective in study of genes and genomic regions

governing complex traits like P-deficiency tolerance & drought [14]. In this view, in the present study the combination of marker assisted selection (MAS) and expression profiling approaches was used for identification of P-efficient rice genotypes having root trait suitable for higher P-uptake and better P- utilization efficiency depicted by higher yield.

Materials and methods

The experiment was conducted at Department of Plant Molecular Biology & Biotechnology and research cum instructional farm, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.), India. (210 16' N and 810 36'E at altitude of 289.6 meter above sea level).

Population development

Sahbhagi Dhan and RRF-78 were crossed in order to develop phosphorus efficient rice genotype. The genotype RRF-78 has been reported to have P-deficiency responsive modification in root traits (increased root volume and surface area) and relatively lower yield penalty. While Sahbhagi Dhan (IR743I71-70-1-1) is the drought stress tolerant rice variety with high yield released for cultivation under unirrigated conditions in India by IRRI.

Field screening

The 148 F3 segregating lines were evaluated in P-supplemented and P-deficient plots derived from Sahbhagi Dhan and RRF-78. Seedlings were transplanted in the P-supplemented and P-depleted sick plot (P205 level 7.00 Kg per hectare having 6.8 pH). The observation of phenotypic parameters viz. plant height 45 days after transplanting (DAT), total number of tillers per plant (45 DAT), number of panicles per plant (60 DAT), flag leaf length & width, biological yield, grain yield was recorded in five replications in both conditions.

Genotyping with SSR and Pup1 sequence specific marker

DNA was extracted from fresh leaf with of Mini Prep method [15]. PCR was done in a total volume of 20µl and the reaction contained (10 X PCR Assay buffer, 1 mM dNTP mix, 5 pM forward and reverse primers, 50ng of template DNA and 1 unit Taq polymerase in Applied Biosystems thermal cycler) After an initial, denaturation step at 95 C for 5 min, the amplification was done for 35 cycles comprising of 1 min each of 94C, 55C and 72C. The final elongation step was extended to 7 min at 72C followed by 4C. After the PCR amplification was completed, the PCR products were run at 5 % PAGE in a mini-vertical electrophoresis unit. DNA fragments were then stained with ethidium bromide and visualized with a UV trans-illuminator, Bio-Rad XLR+ make. A total 60 SSR and 14 Pup1 QTL specific markers were screened, out of which 17 polymorphic SSR markers were used for genotyping. The bands observed were designated as A, B, and H, where A represents RRF-78 like allele, B represent Sahbhagi Dhan like allele, H represents heterozygous. The phenotypic and genotypic data were used for Single marker analysis for identification of markers associated to P-deficiency tolerant traits.

Bulk expression analysis

Based on field evaluation ten best and ten poor performing RI (Recombinant inbred) lines were selected for expression analysis. These RI lines were grown in hydroponics using modified Yoshida nutrient solution [16]. under P-supplemented and P-deficient conditions. The pH of the nutrient solution was maintained at 4.8 to 5.2. Total RNA was extracted from 45 days old plant from root tissue and quantified using nanodrop spectrophotometer. The RNA from the 10 best performing RI lines were pooled in equimolar concentration to form tolerant bulk (1 micro gram). Similarly, process was followed for susceptible bulk. The cDNA synthesis was done from susceptible and tolerant bulk along with 3 best genotypes (SDxRRF-78(83) SDxRRF-78(81), SDxRRF-78(99), 2 poor performing lines (SDxRRF-78(127) SDxRRF-78(138) and a check (MTU1010) along with Parents. For expression analysis Semi-quantitative PCR was done using five Pup1 QTL co-localized candidate genes [11]. The presence of amplicons and their respective intensity were recorded for characterization of relative gene expression levels.

Statistical analysis

Descriptive statistics and Percent change $\{(1 - P\text{-stress}/P\text{-control}) * 100\}$ was calculated for each trait in both the conditions. Single-marker analysis was done using winQTL Cartographer 1.13 [17] for identification of significant markers. It can be conducted using a variety of statistical analyses including t-tests, ANOVA, regression, maximum likelihood & log likelihood ratios.

Results and Discussion

P-deficiency tolerance is a complex phenomenon which is manifested at various levels as changes in whole rice plant [18-19]. Since, most of these traits are also affected by other environmental factors such as water availability, soil type, heat etc. apart from the inherent genetic difference, therefore it has been difficult to characterize the response of a genotype in field level or controlled condition screening alone [20]. Thus, in present study, a combination of field level screening for yield and attributing traits, characterization of root morphological features in root rhizotron experiments and bulk expression profiling for understanding the critical molecular components was used to identify the promising rice genotypes.

Field evaluation under P-deficiency stress

The F3 population developed from cross between Sahbhagi Dhan and RRF-78. Sahbhagi Dhan is the released variety for direct seeded un-irrigated condition and RRF-78, advanced breeding line (ABL) developed for drought tolerance. Although both the parents, Sahbhagi Dhan and RRF-78 are developed for direct seeded low water input cultivation but the genotypes have reported to show differential response under P-deficiency stress. Particularly delay in flowering, reduced yield have been observed in Sahbhagi Dhan while the ABL RRF-78 showed P-deficiency responsive enhancement in root volume and surface area (Figure

1). P-deficiency stress is known to be intensified under water stress therefore population was developed with variety, Sahbhagi Dhan, that has better yield under unirrigated condition. The field evaluation of the F3 population (148 RILs) was done in the wet season, in the P-deficient sick plot with ~7.00 kg P2O5. Phenotypic traits that are known to be affected by abiotic stresses were recorded under both the conditions (Table 1). Among all traits studied, highest reduction of 24.04% was observed in biomass followed by plant height (19.15%) and number of panicle/plant (17.3%) indicating significant effect of P-deficiency stress on these three traits in rice (Figure 2). These 3 traits were thus considered more informative for field screening and selection of P-deficiency tolerant plants [18-19]. Hung [21] have also reported that tillering

ability is the suitable morphological marker to screen phosphorus deficient tolerant rice genotype. However, in our study we observed non-significant decrease in average number of tillers per plant (NOT) under P-supplemented (8.28) and P-stress (7.25) conditions (Table 1). Thus, an average reduction of 7.63 % was observed. Compared to NOT a higher reduction in number of effective tillers (reproductive tillers bearing panicles) was recorded i.e., 17.3% which again suggested importance of phosphorus for reproductive growth phase in plants. Delay in flowering (Figure 3) in almost all plants in the population was observed under P-deficiency stress which ranged from 2-5 days. Therefore, reduced plant, height, delay in flowering and reduced number of reproductive tillers were found to be important for screening out of the susceptible rice genotypes.

Table 1: Descriptive statistics for morphological traits under P+ and P- conditions.

	PHT		NOT		NOP		FLL		FLW		BY		GY		HI	
	P+	P-	P+	P-	P+	P-	P+	P-	P+	P-	P+	P-	P+	P-	P+	P-
Mean	108.75	87.72	8.28	7.53	5.74	4.97	29.62	29.84	1.23	1.25	295.44	223.12	79.36	72.97	0.27	0.33
P-1 (SD)	106.6	84.14	5.8	6.2	4.6	5.1	32.75	24.2	1.3	0.96	357.75	102.8	113.25	50	0.32	0.49
P-2 (R)	87.6	74.04	8.4	9.8	5.1	6.2	33.18	26.4	1.3	1.09	340	122.8	148.5	56.8	0.44	0.46
SE	1.85	1.48	0.17	0.15	0.1	0.23	0.4	0.42	0.01	0.01	6.66	4.69	2.58	1.98	0.01	0.01
SD	22.34	17.9	2.09	1.85	1.15	2.8	4.88	5.11	0.17	0.13	80.5	56.62	31.14	23.95	0.08	0.09
Range	205.34	77.5	8.6	13	6.6	33.98	24.88	22.4	1.14	0.8	394	292	153	126	0.42	0.11
Min	69.5	54.42	4.4	2.4	3.6	3	17.65	19.42	0.6	1	98	119	24	25	0.12	0.78
Max	274.84	131.92	13	15.4	10.2	36.98	42.53	41.82	1.74	1.8	492	411	177	151	0.54	0.01

PHT: plant height; NOT: No. of tillers; NOP: No. of panicle; FLL: Flag leaf length; FLW: Flag Leaf Width; BY: Biological Yield; GY: Grain Yield; HI: Harvest Index; P+, P-supplemented; P-, P-stress; SE, P-1(SD), Sahbhagi Dhan; P-2(R), RRF-78 standard error, SD; standard deviation, Min; minimum, Max, maximum.

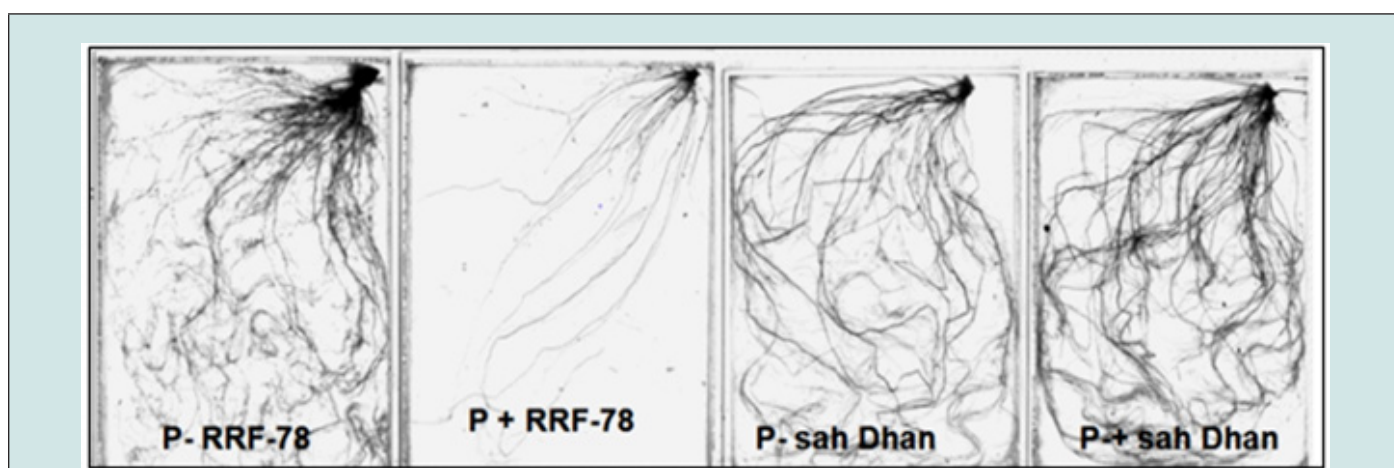


Figure 1: Root scanning of parents, RRF-78 and Sahbhagi Dhan under P-supplemented (P+) and P-deficient (P-) conditions. RRF-78 is an advanced breeding line (ABL) developed for drought tolerance, showing increased root growth and higher root volume in P-deficient condition, under P deficiency stress condition than Sahbhagi Dhan.

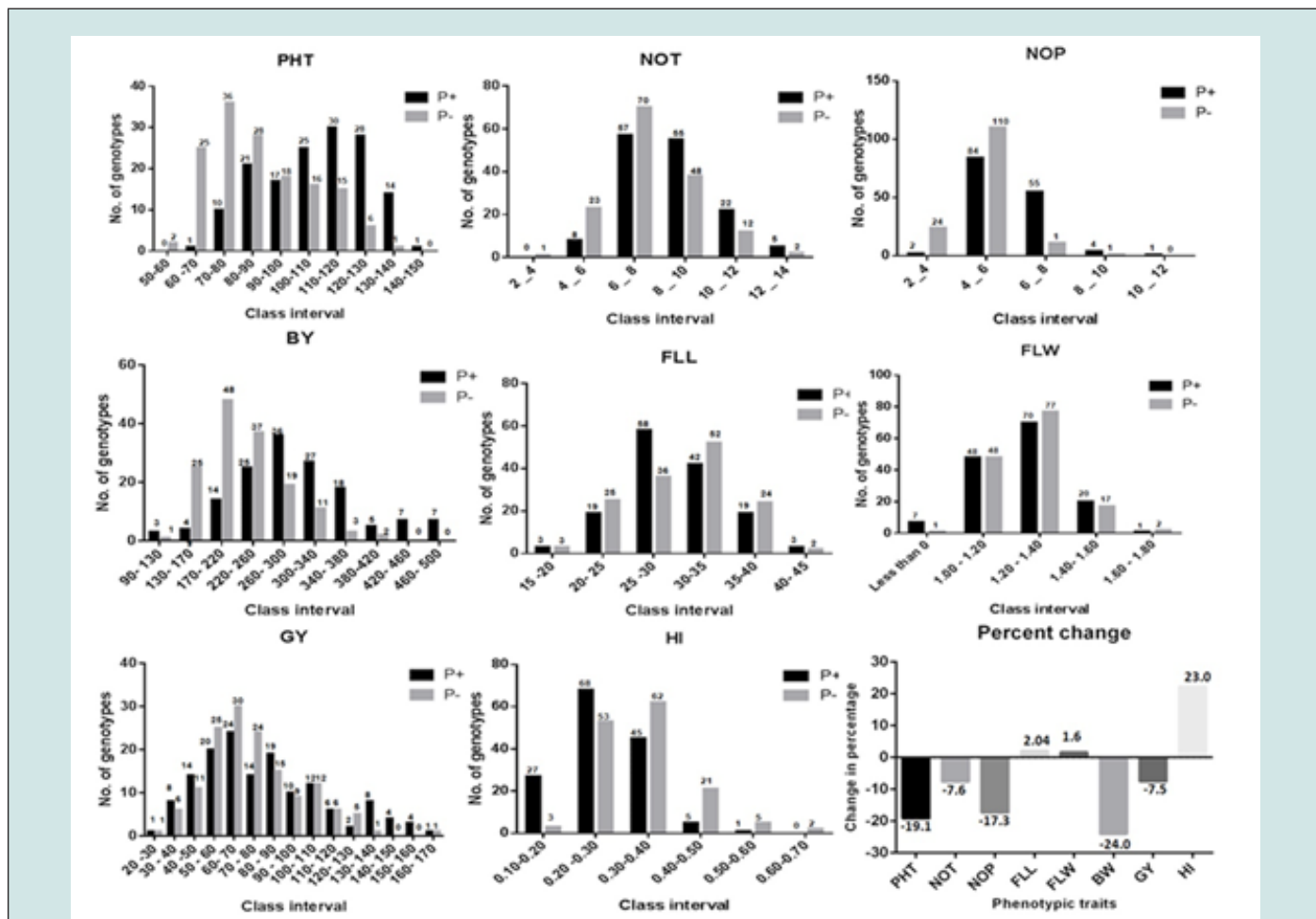


Figure 2: Frequency distribution and percent change for different morphological under P-supplemented and P-deficient field conditions. PHT, plant height; NOT, No. of tillers; NOP, No. of panicle; FLL, Flag leaf length; FLW, flag leaf width; B.Y., biological yield; G.Y, grain yield; HI, harvest index; P+, P-supplemented; P-, P-stress.



Figure 3: Differential response in flowering percentage under P-supplemented and P-deficient field conditions. Field photograph were recorded at days to 50 % flowering stage in P-supplemented and P-deficient plots. Arrow indicates higher no of flowered plants in P- Supplemented plot than P-Deficiency plot due to P-deficiency stress.

Similarly, relative number of tillers (ratio of tillers in stress and control conditions) under P-deficient condition has been reported as an important character to quantify P-deficient tolerance genotype in rice [22-23] but as per our observations the relative values of relative biomass and number of panicle (NOP) provide a better index for screening and identifying rice plant tolerant to P-deficiency stress. To assess the effect of P-deficiency stress on flag leaf observation were recorded at 60 days after transplanting (DAT). Our study indicated non-significant change in the flag leaf length (FLL) and width (FLW) with -0.02 & +0.01 percent change respectively indicating that FLL and FLW are not affected by P-deficiency stress. Reduction in the biomass under P-deficiency stress has been reported [24-26] and biological yield is thus considered as important trait for screening of P-deficiency tolerance genotype. An average reduction of 24.04 % was observed. The RILs having lesser reduction in biomass in comparison to supplemented P-condition were thus considered tolerant to P-deficiency stress.

P is an essential macro nutrient for plants that has a major role in influencing yield when deficient [27-28]. Our observations for grain yield in the segregating population showed an average of 79.36 gm under P supplemented condition which was reduced to 72.9 gm in P-deficient condition. Although the reduction of 7.5 % was significant but is lesser than biological yield indicating that the rice plants tend to maintain grain yield under stress. Apart from population mean values the individual F3 lines showed significant effect of P-deficiency stress as depicted by change in the grain yield per plant as shown in graphs of grain yield (Figure 2). It was observed that several RI lines belonging to the higher grain yield classes (120 gm/plant and above) were shifted to lower grain yield classes (120gm and lower). The grain yield data thus showed that the grain yield penalty was more in higher yielding genotypes as compared to moderate or poor yielding genotypes. The high yielding lines that either showed no significant change in grain yield or very less reduction was thus classified as tolerant

genotypes. The harvest index (HI) was found to be relatively higher in P-deficiency stress than the supplemented condition with -0.22 percent reduction. Grasses tend to shift to flowering phase as an escape response to abiotic stress thus early flowering is observed in many rice varieties native to problem soils. This earliness or escape is usually accompanied with the yield penalty and is not the true reflection of better P use efficiency. An increase in HI is expected due to reduction in biomass as observed in most of the genotypes. Therefore, the RI lines that were able to maintain their biomass, number of tillers and panicles per plant along with non-significant reduction in grain yield were selected.

Marker identification and validation

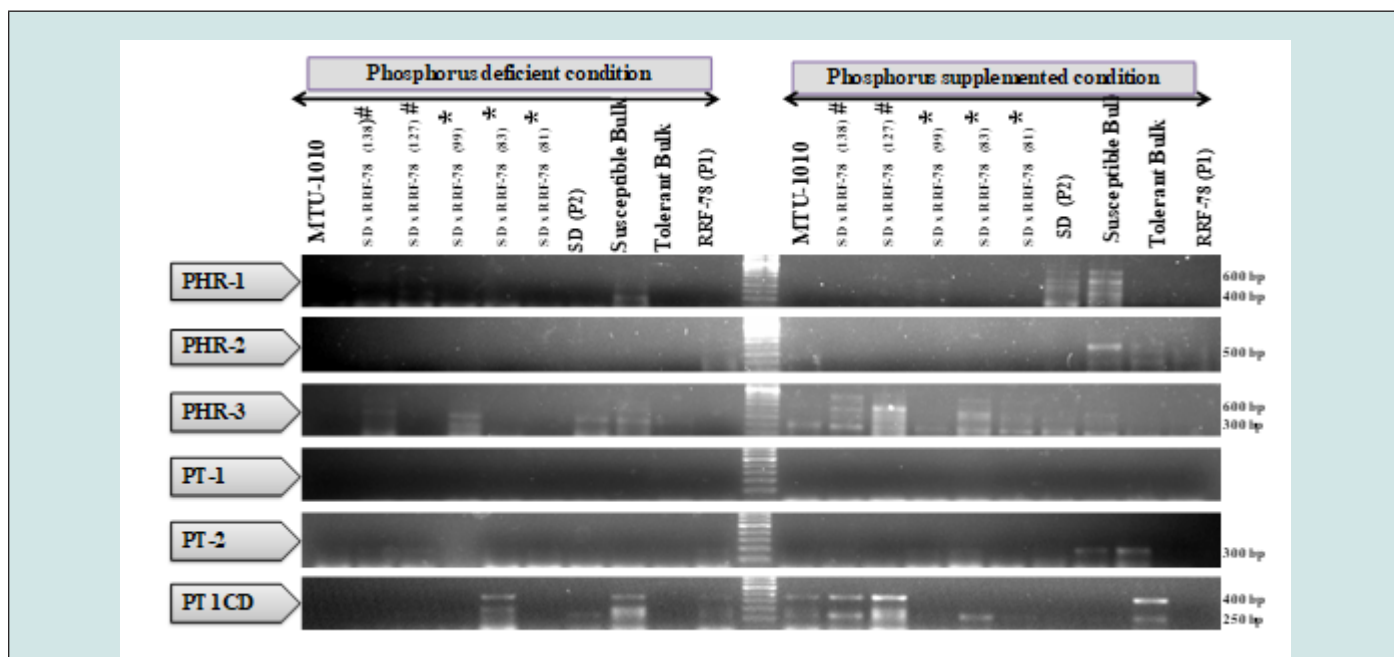
60 QTL linked RM markers were used for genotyping of the F3 population (Supplementary file1) along with the 14 markers developed from Pup1 QTL region. Both the parent derived from japonica and indica genetic base thus both the genotypes were found to be Pup1 haplotypes showing Pup1 alleles in all 14 markers but their response to P-deficiency stress varied significantly. P-deficiency stress responsive stimulation in root growth leading to higher root volume and surface area was observed in RRF-78, while non-significant effect was observed in Sahbhagi Dhan (Supplementary Figure 1). The observations indicated the interaction of genetic background on the phenotype of Pup1 QTL and also suggested that different mechanism may operate under P-deficiency which influences the performance of the genotype. Previous studies conducted on these parents have also indicated that both the parents (Sahbhagi Dhan and RRF-78) are pup1 haplotypes however they showed different performance under P-deficiency stress [29-30]. Out of 60 RM markers, 17 RM markers exhibited parental polymorphism and were subsequently used to generate the genotypic data whereas all 14 Pup1 specific marker were found to be monomorphic hence they were not used for genotyping. Single Marker Analysis was done for 17 polymorphic markers and identified associated markers under both the conditions (Table 2).

Table 2: Result obtained from Single Marker Analysis (SMA) under P-supplemented and P-deficient.

P+					P-				
Traits	Ch.	Marker	pr(F)	R2	Traits	Ch.	Marker	pr(F)	R2
PHT	1	RM212	0.02369738*	0.0127	PHT	2	RM237	0.015381059*	0.04
	8	RM447	0.033672974*	0.0016		1	RM212	0.00050316***	0.018
NOT	NF	NF	NF	NF	NOT	8	RM152	0.035381449*	0.041
	NF	NF	NF	NF		9	RM242	0.013830203*	0.001
FLL	5	RM413	0.01744997*	0.0032	FLL	1	RM212	0.012618059*	0.014
	8	RM408	0.033058663*	0.0158		8	RM447	0.010370108*	0.004
	9	RM242	0.034257367*	0.0077					
FLW	2	RM154	0.017649998*	0.0385	FLW	8	RM152	0.00837748**	0.027
	3	RM55	0.020262456*	0.0351		9	RM242	0.043887955*	0.011

BY	2	RM71	0.0006032***	0.076
	5	RM413	0.00716222**	0.0146
GY	8	RM408	0.024449193*	0.0064
	8	RM152	0.01043456*	0.0106
	2	RM71	0.044956022*	0.03
	8	RM152	0.011166134*	0.0057

PHT: plant height; NOT: No. of tillers; NOP: No. of panicle; FLL: Flag leaf length; FLW: flag leaf width; BY: Biological Yield; GY: Grain Yield; P+, P-supplemented ; P-, P-deficient conditions; Ch, chromosome no.; pr(F) P value, *, **, *** and**** = level of significance at 5%, 1%, 0.1% and 0.01% respectively; R², coefficient of determination



Supplementary files:

Figure 1: Expression Patterns of different Phosphorus signaling and transporter genes. Phosphorus signaling genes OsPHR1, OsPHR2 and OsPHR3 and phosphorus transporter genes (PT1, PT2 and PT1CD) were analyzed in root tissue of selected best performer, poor performer lines and their tolerant and susceptible bulk with one check (MTU1010).

Markers RM212 on Ch#01 and RM447 on Ch#8 were found to be significantly associated with plant height under P-supplemented condition whereas RM237 on Ch#2 and RM212 on Ch#01 were found to be associated with plant height under P-deficient condition. Gomez et al. [31]. also reported for QTL for plant height on chromosome no. 1, 4, 5 by using recombinant inbred (RI) lines of Bala × Azucena. Similarly, several minor QTLs for plant height have been identified on chromosome no 1, 2, 4, 7, 8, and 9 under control and water stress conditions in cross derived from CT9993-5-10-1-M/ IR62266-42-6-2 [32]. Minor QTLs related to P-deficiency stress tolerance have been identified on chromosome 1, 6 and 9 under [12]. In our study also, the marker RM152 on Ch#8 and RM242 on Ch#9 were found to be significantly associated to number of tillers in P-deficiency stress. For P-deficiency stress the marker RM242 has been found associated with number of panicles per plant and plant height under P-deficiency stress [33]. The same

marker has been reported as a linked marker for root trait under drought stress [34]. The number of tillers per plants is an important trait for characterizing drought tolerance also, as indicated by QTLs identified on chromosome 1, 3 for the trait under drought stress [31]. Tillering ability reflects significant GXE interaction and is influenced by most of the abiotic stresses like water stress, nutrient deficiency, heat etc. Therefore, the F3 genotypes showing higher number of tillers under P-deficiency stress or lesser reduction in number of tillers in relation to P-control are the promising candidate genotypes for un-irrigated cultivation conditions.

The F3 RI lines SDxRRF-78(63), SDxRRF-78(83), SDxRRF-78(124), SDxRRF-78(99), SDxRRF-78(26) and SDxRRF-78(81) were thus identified (Table 3). The number of panicles is also an important trait contributing to yield potential of a genotype. In present study RM71 on Ch# 2 and RM408 on

Ch# 8 were found to be significantly associated with number of panicles per plant under P-supplemented condition but there were no markers found to be associated to the No. of panicles under P-deficiency. Analysis of the FLL and FLW in the F3 RI lines under P-deficiency stress led to identification of markers, RM 413, RM 408, RM242 on Ch# 5,8, 9 respectively, as significantly associated with FLL under P-supplemented condition. Whereas RM212 and RM 447 located on Ch# 1 and 8, were found to be associated to

FLL in P-deficiency stress. Gomez et al. [31] reported that RM212 is linked to biological yield under drought stress. The significant association of the linked marker RM212 in our population under P-deficiency stress validates the presence of QTL. Similarly, the markers RM154 on Ch#1 and RM 55 on Ch#8 were found to be significantly associated with FLW under P-supplemented condition and the markers RM152 on Ch#8 and RM242 on Ch#9 in P-deficiency stress (Supplementary Tables 1 & 2).

Table 3: Selected genotypes and their performance under P-supplemented and P-deficient condition.

S.N.	line No.	PHT		NOT		NOP		FLL		FLW		BY		GY	
		P-	P+	P-	P+	P-	P+	P-	P+	P-	P+	P-	P+	P-	P+
1	SDXR 63	104.36	134	6.4	7.6	5.2	6	39.8	38.2	1.32	1.18	288	435	125	151
2	SDXR 83	86.38	114	7.6	9.2	5.6	7.8	36.9	24.3	1.36	1.18	250	319	124	143
3	SDXR 124	101.7	106	7.2	8.2	5.4	7.2	30.6	34.2	1.3	1.18	322	451	123	142
4	SDXR 99	78.18	78.9	7.2	10.4	5.4	6.6	31.5	29.8	1.12	1.18	268	271	122	150
5	SDXR 26	80.42	101	11.4	14	8	9.2	26.8	29.7	1.48	1.28	411	449	120	159
6	SDXR81	96.34	132	9.6	11	4.8	5.8	33.8	27.5	1.16	1.1	315	283	118	147
7	P1(SD)	84.14	107	6.2	5.8	5.1	4.6	24.2	32.8	0.96	1.3	253	340	116	149
8	P2(R)	74.04	87.6	9.8	8.4	6.2	5.1	26.4	33.1	1.09	1.3	226	280	93.9	117

PHT: plant height; NOT: No. of tillers; NOP: No. of panicle; FLL: Flag leaf length; FLW: flag leaf width; BY: Biological Yield; GY: Grain Yield; P+, P-supplemented condition; P-, P- deficient condition; SDxR, Sahbhagi Dhan (P1) x RRF-78 (P2).

Supplementary Table 1: List of SSR and Pup 1 sequence specific primers were used for parental polymorphism.

SN	Marker	Forward Primer	Reverse Primer
1	RM495	AATCCAAGGTGCAGAGATGG	CAACGATGACGAACACAACC
2	RM283	GTCTACATGTACCCTTGTGGG	CGGCATGAGAGTCTGTGATG
3	RM237	CAAATCCCGACTGTGTCC	TGGGAAGAGAGCACTACAGC
4	RM431	TCCTGCGAAGTGAAGAGTTG	AGAGCAAAACCTGGTTTCC
5	RM154	ACCCTCTCCGCTCGCCTCCTC	CTCCTCTCTGCGACCGCTCC
6	RM452	CTGATCGAGAGCGTTAAGGG	GGGATCAAACCACGTTTCTG
7	OSR13	CATTTGTGCGTCACGGAGTA	AGCCACAGCGCCATCTCTC
8	RM338	CACAGGAGCAGGAGAAGAGC	GGCAAACCGATCACTCAGTC
9	RM514	AGATTGATCTCCATTTCCC	CACGAGCATATTACTAGTGG
10	RM124	ATCGTCTGCGTTGCGGCTGCTG	CATGGATCACCGAGCTCCCCC
11	RM507	CTTAAGTCCAGCCGAAATG	CTCACCTCATCATCGCC
12	RM413	GGCGATTCTTGGATGAAGAG	TCCCCACCAATCTTGTCTTC
13	RM161	TGCAGATGAGAAGCGGCGCTC	TGTGTCATCAGACGGCGCTCCG
14	RM133	TTGGATTGTTTGTGGCTCGC	GGAACACGGGTCGGAAGCGAC
15	RM162	GCCAGCAAAACCAGGGATCCGG	CAAGGTCTTGTGCGGCTTGGCG
16	RM125	ATCAGCAGCCATGGCAGCGACC	AGGGGATCATGTGCCGAAGGCC
17	RM455	AACAACCACCACCTGTCTC	AGAAGGAAAAGGGCTCGATC
18	RM118	CCAATCGGAGCCACCGGAGAGC	CACATCTCCAGCGACGCGGAG
19	RM408	CAACGAGCTAATTCCGTCC	ACTGCTACTTGGGTAGCTGACC
20	RM152	GAAACCACCACCTCACCG	CCGTAGACCTTCTTGAAGTAG
21	RM44	ACGGGCAATCCGAACAACC	TCGGGAAAACCTACCCTACC
22	RM284	ATCTCTGATACTCCATCCATCC	CCTGTACGTTGATCCGAAGC

23	RM433	TGGGCTGAACTAAACACAGC	AGACAAACCTGGCCATTAC
24	RM447	CCCTTGTGCTGTCTCTCTC	ACGGGCTTCTTCTCTCTC
25	RM316	CTAGTTGGGCATACGATGGC	ACGCTTATATGTTACGTCAAC
26	RM215	CAAAATGGAGCAGCAAGAGC	TGAGCACCTCCTTCTCTGTAG
27	RM271	TCAGATCTACAATTCATCC	TCGGTGAGACCTAGAGAGCC
28	RM484	TCTCCCTCCTACCATTGTC	TGCTGCCCTCTCTCTCTC
29	RM536	TCTCTCTCTTGTGGGCTC	ACACACCAACACGACCACAC
30	RM277	CGGTCAAATCATCACCTGAC	CAAGGCTTGAAGGGAAG
31	RM1	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC
32	RM259	TGGAGTTTGAGAGGAGGG	CTTGTTCGATGGTGCCATGT
33	RM312	GTATGCATATTGATAAGAG	AAGTCACCGAGTTACCTTC
34	RM5	TGCAACTTCTAGCTGCTCGA	GCATCCGATCTTGATGGG
35	RM489	ACTTGAGACGATCGGACACC	TCACCCATGGATGTTGTCAG
36	RM55	CCGTCGCGTAGTAGAGAAG	TCCCGGTTATTTAAGGCG
37	RM307	GTACTACCGACCTACCGTTCAC	CTGCTATGCATGAACTGCTC
38	RM178	TCGCGTGAAGATAAGCGGCGC	GATCACCGTTCCTCCGCTGC
39	RM334	GTTTCAGTGTTCAGTCCACC	GACTTTGATCTTTGGTGGACG
30	RM510	AACCGGATTAGTTTCTCGCC	TGAGGACGACGAGCAGATTC
41	RM454	CTCAAGCTTAGCTGCTGCTG	GTGATCAGTGCACCATAGCG
42	RM 11	TCTCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG
43	RM25	GGAAGAATGATCTTTTCATGG	CTACCATCAAAACCAATGTTTC
44	RM105	GTCGTCGACCCATCGGAGCCAC	TGGTCGAGGTGGGATCGGGTC
45	RM474	AAGATGTACGGGTGGCATTTC	TATGAGCTGGTGAGCAATGG
46	RM171	AACGCGAGGACACGTACTTAC	ACGAGATACGTACGCCCTTTG
47	RM552	CGCAGTTGTGGATTTCAGTG	TGCTCAACGTTTACTGTCC
48	RM287	TTCCCTGTAAAGAGAGAAATC	GTGTATTTGGTGAAAGCAAC
49	RM144	TGCCTGGCGCAAATTTGATCC	GCTAGAGGAGATCAGATGGTAGTGCATG
50	RM19	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA
51	RM235	AGAAGCTAGGGCTAACGAAC	TCACCTGGTCAGCCTCTTTC
52	RM302	TCATGTCATCTACCATCACAC	ATGGAGAAGATGGAATACTTGC
53	RM71	CTAGAGGCGAAAACGAGATG	GGGTGGCGAGGTAATAATG
54	RM242	GGCCAACGTGTGTATGTCTC	TATATGCCAAGACGGATGGG
55	RM231	CCAGATTATTTCTGAGGTC	CACTTGATAGTTCTGCATTG
56	RM288	CCGGTCAGTTCAAGCTCTG	ACGTACGGACGTGACGAC
57	RM49	TTCGGAAGTTGGTACTGATCA	TTGGAGCGGATTCGGAGG
58	RM26	GAGTCGACGAGCGGCAGA	CTGCGAGCGACGGTAACA
59	RM245	ATGCCGCCAGTGAATAGC	CTGAGAATCCAATTATCTGGGG
60	RM212	CCACTTTCAGCTACTACCAG	CACCCATTTGTCTCTCATTATG
Pup 1 sequence specific primers			
1	K1	AGTCTGGATGGACAACCTGCCTG	TGCTAGCTCATTGCCGTTACGTCG
2	K5	ATTCAGACATCGACGGCGAC	CCTCGTAAACATGGCTTGC
3	K29-1	TATGGCCAACGGGGTAGAG	GTCCAGGTAACCACGAGGAA
4	K29-2	CCCGTCTGCGTTCTACCTTA	CTCCCGTCAAGCACAATCT
5	K29-3	TTCGTCCAGATGCTGTATG	TCTTCGGTGTAAATTGGCACA
6	K41	TGATGAATCCATAGGACAGCGT	TCAGGTGGTCTCGTTGGTA
7	K42	CCCGAGAGTTCATCAGAAGGA	AGTGAGTGGCGTTTGGCAT
8	K43	AGGAGGATGAGCCTGAAGAGA	TCGCACTAACAGCAGCAGATT

9	K45	GCGGAAGAAGAGGATAACGA	TCTAGGCTTCGTTGGCAAG
10	K46-1	TGAGATAGCCGTC AAGATGCT	AAGGACCACCATTCCATAGC
11	K46-2	AGGAAGATGGTTGTCGTTGG	TTCACACCAAACAGTGTGTC
12	K48	CAGCATT CAGCAAGACAACAG	ATCCGTGTGGAGCAACTCATC
13	K52	ACCGT TCCCAACAGATTCAT	CCCCTAATAGCAACAACCCAA
14	K59	GGACACGGATTCAAGGAGGA	TGCTTCCATTTGCGGCTC

Supplementary Table 2: Primer sequences used for expression analysis in root tissue.

S.N.	Primers	Sequence
1	OsPupK04-1	F-GGGATATCAAGCTTGTGGTG
		R- GAATGCTGTTTCGCTTATGG
2	OsPupK20-2	F- CTGGACTTGACCCCAATGTA
		R- TCTGATGGAGTGTTCGGAGT
3	OsPupK29-1	F- CCAATGCATCCAATTCTTGT
		R-ATGAGCCAGATTACGAATG
4	OsPupK46-2	F-AGGAAGATGGTTGTCGTTGG
		R-TTCACACCAAACAGTGTGTC
5	OsPupK671	F-CACGAGCTATTTCTGTGGG
		R- GGTAGGAGAACTTGCCGAAC

The marker RM 71, RM 413, RM 408, RM 152 on Ch# 2, 5, 8, 8 respectively were found to be significantly associated with biological yield under P-supplemented condition whereas no marker was found under P-deficient condition. The marker RM152 was found to be significantly associated with harvest index and grain yield under P-deficiency stress. Grain yield is the ultimate objective in any crop improvement program. Yield reduction by the P-deficiency stress has been reported to be as high as 47%, even without any drought

symptoms [35-36]. In this study the markers RM 71, RM152 on Ch# 2,8 respectively were found to be significantly associated with grain yield under P-supplemented condition whereas no marker was found to be significantly associated to grain yield in P-deficient condition. The markers for relative grain yield and biomass under P-deficiency stress will be of substantial importance for the screening of P-deficiency tolerance ability in rice.

Bulk expression analysis of Pup1 QTL co-localized candidate genes

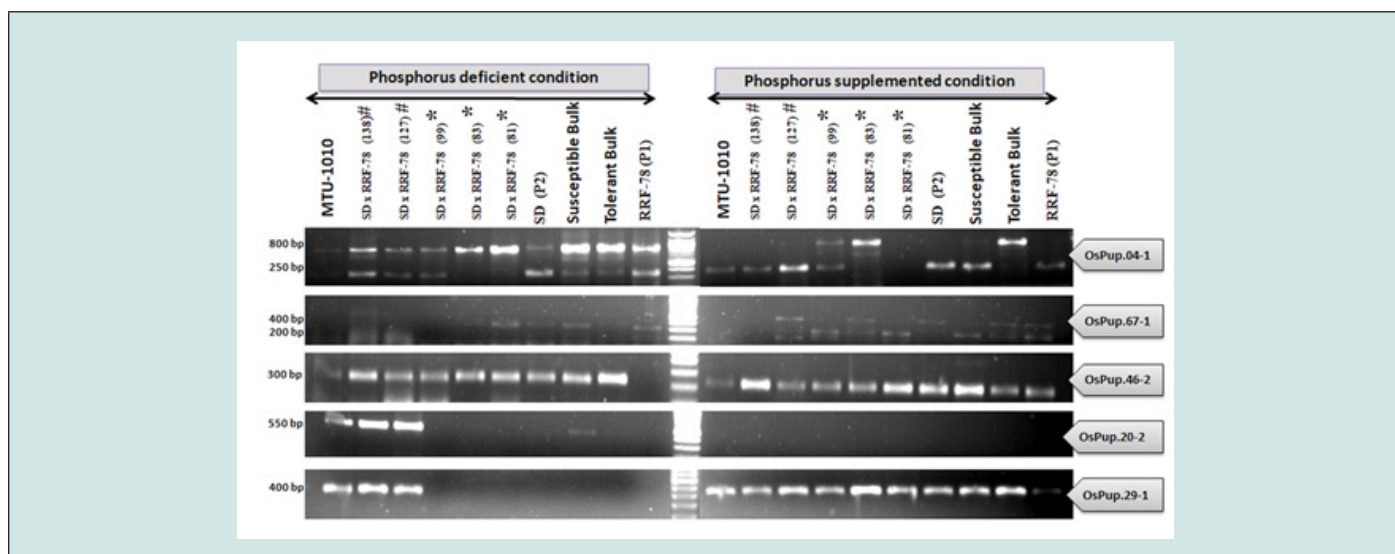


Figure 4: Expression patterns of Pup1 QTL co-localized candidate genes in root tissue. Pup1 QTL underlined genes (Os-PupK04-1, OsPupK67-1, OsPupK46-2, OsPupK20-2, and OsPupK29-1) were analyzed in root tissue of selected best performer (*) and poor performer (#) and their tolerant and susceptible bulk with one check (MTU1010) under P-supplemented and P-deficiency conditions.

Five genes were used in this study for characterization of expression in rice root tissue under P-supplemented and deficiency stress conditions (Figure 4). The genes OsPupK04-1 and OsPupK05-1 are known to be ubiquitously expressing genes with higher level of expression of OsPupK05-1 under P deficiency [11,26]. The expression pattern of OsPupK04-1 showed differential expression. In our experiment the OsPupK04-1 gene expressed with two different lengths of mRNA under P-supplemented condition in tolerant bulk, SDxRRF-78(83), SDxRRF-78(99) (800bp) and susceptible bulk (250bp), parents (250bp) and check genotype MTU1010 (250bp) (Figure 4). With onset of phosphorus deficiency stress the 800 bp mRNA transcript was observed in both tolerant and susceptible bulk, best and poor performing genotypes, SDxRRF-78(83), SDxRRF-78(81), check genotype MTU1010 and both the parents, with different level of expressions. This indicated that the gene might be alternatively spliced for regulation of P-uptake in rice roots. Still the higher intensity of 800 bp band in tolerant bulk, better root trait parent RRF-78, and better performing genotypes SDxRRF-78(83) and SDxRRF-78(81) while very low level of expression in Sahbhagi Dhan (water stress tolerant variety) suggested differential regulation of the gene is correlated to the growth of root in response to P-deficiency stress in rice. The results indicated that either two copies of genes are present in tolerant and susceptible lines or there is post transcriptional modification occurring more efficiently in tolerant genotype under P deficient condition.

The Pup1 gene model described by Chin et al. [11] also suggested that OsPupK05 may be independent gene or it may represent a spliced variant of OsPupK04-1. The presence of two amplicons for the same primer set obtained in our study indicated that OsPupK05 gene model proposed is actually a spliced variant of OsPupK04-1 and not an independent gene. The functional marker (K5) located within OsPupK05 is therefore a potential target for marker assisted selection strategy. In a parallel study carried in our lab with 177 diverse rice genotypes of different genetic background, it was found that the K5 marker has been found significantly associated to number of tillers per plant and grain yield under P-deficient condition [33]. Thus, the K5 marker and the OsPupK04-1 gene may be further analyzed for development for functional markers capable of screening and identification of indica rice genotypes having higher P uptake efficiency in deficient soils. The OsPupK20-2 is known supplemented to encode divergent protein, putative, expressed (LOC_Os12g26380) [11], but in our study no expression of this gene was observed in all ten samples under P-supplemented condition. The gene however expressed at significant high level in 3 P-deficiency susceptible genotypes, check genotype MTU1010 and susceptible bulk (faint expression) suggesting role of OsPupK20-2 in P-starvation sensing or signaling mechanism. Up-regulation of OsPupK20-2 in genotypes susceptible to P-deficiency stress showed its association with susceptibility. The OsPupK29, that codes for a hypothetical protein and having high but partial similarity to two expressed protein genes (Os12g26390 and 12g26410) in the Nippon bare reference genome [10], showed

higher expression in all the genotypes and both tolerant and susceptible bulk in P-supplemented condition.

Similar to the OsPupK20-2 the expression of OsPupK29 was found to be down-regulated in all the tolerant genotypes and tolerant bulk but high level of expression was observed in P-deficiency susceptible genotypes, check MTU1010 and susceptible bulk with very low level of expression. The results thus suggested that the gene OsPupK29-1 expression is also associated to susceptibility in terms of yield under P-stress response. OsPupK46-2 gene expressed at equal level in all the genotypes as well as tolerant and susceptible bulk and parent under both conditions. Although it was down regulated in RRF-78 under P-deficiency stress but non-significant effect was observed in the tolerant and susceptible bulk. The results indicated that non-significance correlation between the level of expression of the gene and response of genotypes to P-deficiency stress. OsPupK67-1 is reported to be similar to aspartic proteinase precursor nepethesin-1 precursor LOC_Os12g262470) [11]. Data showed that there is slight up-regulation of OsPupK67-1 genes except RRF-78 and one best performance line (SDxRRF-78(81) under P-supplemented condition but onset of P-deficiency down regulation was observed except RRF-78 and one best performance lines SDxRRF-78(81) the data showed that there were two tolerant genotypes showing slight up-regulation under P-deficiency stress only which suggested that there was positively correlation between genotypes and P-deficiency stress, however the intensity of expression was low in both the conditions.

Conclusion

The combined approach led to identification of six RILs, SDxRRF-78(63), SDxRRF-78(83), SDxRRF-78(124), SDxRRF-78(99), SDxRRF-78(26) and SDxRRF-78(81) as the promising tolerant genotypes that showed highest number of tillers per plant and least reduction in number of effective tillers (panicles) per plant as well as highest yielder among 148 lines under P-deficiency condition. The Pup1 colocalized gene, OsPupK04-1, showed two distinct amplicons (800 and 250 bp) in tolerant and susceptible bulk indicating alternatively splicing of this gene under P-deficiency stress. The sequencing of the amplicons is in process to understand the gene regulation in these genotypes and its functional significance. The marker RM242 and RM212 were found to be associated to number of tillers/plant and number of tillers as well as plant height in P-deficient condition thus validating the presence of the corresponding QTLs in our population.

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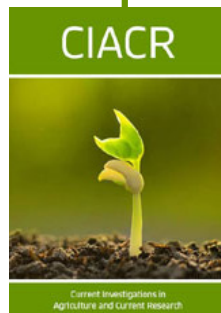
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