Polymorphic survey of KMR-3R and Kasalath(*Oryza sativa* L.) by using SSR markers and morphological traits.

Abstract: Low phosphorus stress has become more important abiotic factor affecting rice yield under present changing climate conditions. KMR-3R is one of the popular restorer line used in commercial rice hybrids production in India. Most of the rice hybrids released for the diverse ecosystem conditions using three-line system for normal phosphorus conditions. Consequently, these derived hybrids suffer drastic yield decline under low phosphorus conditions. To facilitate the efficient use of complex polygenic traits in hybrid rice molecular breeding research, we developed the KMR-3R BILs with *Pup1* QTL by introgression of major QTL *Pup1* controlling grain yield under low phosphorus stress into KMR-3R.To begin marker assisted back cross breeding for introgression of a desirable trait into a varietyfrom the donor genotype, study of parental polymorphism is a pre-requisite. Unless the parents are polymorphic for the traitsof interest, the further selection of plants carrying the traits of interest is not possible in the progenies. We screened all reported SSR primers linked to low phosphorus tolerance and observed K-46-1 and K-46-2 linked to *Pup1* QTL was polymorphic between Kasalath and KMR-3R. Simultaneously we screened 300 full-genotype SSR markers covering entire genome and observed 86 SSR primers as polymorphic between Kasalath and KMR-3R. After polymorphic survey, we evaluated Kasalath and KMR-3R.under low phosphorus stress and normal phosphorus conditions and recorded significant differences among the parents. Kasalath performed well when compared to KMR-3R for all the characters studied under low phosphorus stress conditions.Construction of a Linkage Mapcould be ensued procuring the generated genotyping data which could further avail QTL analysis and identification ofmarkers linked to low phosphorus stress.

Keywords: Rice, KMR-3R, low phosphorus stress, Yield, BILs and Marker assisted backcross breeding.

INTRODUCTION

Rice is one of the most important food crops for more than half of the world's population. Over 90% of global rice production is in Asia, and it contributes 20% of total caloric intake on a

daily basis. The green revolution has revolutionized the world's rice production in the last six decades, and the production of rice is equivalent to the population growth in India. With an average annual population growth rate (1.5%) and an average daily rice consumption of 250g per person, the demand for rice is expected to reach at least 140 million tonnes (Mt) by 2050.By 2050, India's population is going to need production of rice to rise by 2.5 M T annually (*http://www.fao.org/rice2004/en/pdf/khush.pdf*). The global problem of climate change is currently affecting rice cultivation worldwide by influencing resources like land, water, and nutrients to become scarcer. The development of rice cultivars resistant to climate change is urgently needed. As ofnow, as many as 8000 QTLs governing various traits have been identified and flanking/tightly linked/functional markers for these traits can be used to transfer into elite varieties through MAS. However, identification of background markers for each pair of donor and recipient parental varieties is very laborious and time consuming also.

Hence, the current study was carriedout to identify ready to use SSR markers forbackground selection of two widely used donor and recipient varieties. One of the readily available and economicallyviable technological options for meeting theprojected global demand of rice is exploitationof heterosis through large-scale cultivation ofrice hybrids. To increase the nation's rice yield, hybrid rice technology is essential.According to the AICRIP progress report 2021, 127 rice hybrids have been certified for commercial cultivation in India as of now. In *Kharif* 2017, 3.5 million ~~tonnes~~tons of hybrid rice were produced on 3 million hectares of land. Majority of the rice hybrids were developed for irrigated conditions. Currently, three-lineCMS system was widely used for exploitation of hybrid ~~vigour~~vigor in rice.Phosphorus (P), one of the most important macronutrients necessary for healthy rice plant growth and development, is one of the most deficient elements in rice soils, and its low availability in the soil is one of the major yield-limiting factor in rice production (Wissuwa*et al.,* 1999).Global demand for P fertilizer is steadily rising, despite the fact that commercial phosphate supplies are expected to run out in a few decades. In India, soils are either low (49.3% of soils) or medium (48.8% of soils) in terms of available Pnecessitating the country's importation of phosphorus-based fertilizers on a massive scale. As a result, it is critical to be prepared with alternate solutions to this problem, such as improved crop residue management, adoption of integrated nutrient management, and creation of low soil P tolerant rice cultivars to handle low soil P levels.Genetic enhancement of rice tolerance to P-limiting soils should be one of the focus areas of rice research and development in

order to reduce the use of phosphatic fertilizers, which is required not only to increase farmers' income by lowering fertilizercosts, but also to maintain rice production. Therefore, creating hybrids resistant to low phosphorus stress will aid in preventing losses in quantum yield.Though conventional plant breeding made significant impact on increasing the yield coupled with resistance to various biotic and abiotic stresses for target environments, these efforts are slow, need expertise ~~labour~~labour and resource intensive.

Before initiating marker assisted selection or marker assisted backcross breeding for transferring a desired trait into a variety from the donor parent, an analysis of parental polymorphism is required. Further selectionof plants possessing the traits of interest isnot achievable in the progenies unless theparents are polymorphic for the traits of interest.Polymorphism is an index of geneticvariation that shifts with different sets of parents.According to the survey of parentalpolymorphism, there is demonstrable variationbetween parents. Due to their many advantageous features, microsatellites (SSRs) have been used more than any other kind of molecular marker. These include having a large genomic distribution, being co-dominant and highly polymorphic, and being amplified by PCR. Through their ability to facilitate precise transfer of specific genomic regions, these markers have also demonstrated significant promise in enhancing breeding efficiency.

SSRs have been widely utilized by most crop species for which sequence information is available, including rice. Many genetics-related domains, such as gene tagging, genetic diversity research, population genotyping mapping, linkage map creation, tracking marker-trait associations, single marker analysis, and quantitative trait loci (QTL) mapping, can benefit from these. Finding the relevant polymorphic SSR markers for the donor and recipient parents was the goal of the current investigation. After that, these markers were included to a backcross breeding experiment using marker support, which was used to insert low phosphorus deficiency tolerance QTLs from the donor parent Kasalath into the background of the extensively used restorer line of hybrid rice *i.e.*, KMR-3R.

MATERIALS AND METHODS

The parents used for the study were KMR-3R and Kasalathand their characteristics werementioned below.

1. **KASALATH**: Kasalath is a low P tolerant indica genotype harboring major effect QTL *Pup1* controlling grain yield under phosphorus stress.
2. **KMR-3R**: KMR-3R is the restorer line of popular hybrid KRH2**.**

Leaf sample collection and DNA isolation

Total genomic DNA was isolated from the parental linesusing the method of Zheng e (1991) with modifications.Healthy leaf pieces (2-3 cm) from the youngest leaves of a 20-25 day old rice seedlings were taken into the well of a spot testplate (M/s Thomas Scientific, USA). 400 ml of extractionbuffer (50 mM Tris HCL, pH 8.0, 25 mM EDTA, 300 mM NaCland 1 % SLS) was added to the well. The leaf tissues weremacerated for about 15-20 seconds till it was completely homogenized. Another 400 µl of extraction buffer was addedto the well containing the leaf sample. Using a micropipette of1 ml capacity, the entire content from the well was transferredto a 1.5 ml capacity micro centrifuge tube. About 400-500 µlPhenol (pH 8.0) ~~÷~~, Chloroform ÷ andIsoamyl alcohol (25:24:1) wasthen added to the micro centrifuge tube. The contents weremixed well by inversion for about 10 minutes and centrifugedat 12000 rpm for about 10 minutes at room temperature. Afterthe centrifugation, the supernatant was aliquoted from themicro-centrifuge tube without disturbing the intermediatelayer into a fresh 1.5 ml micro-centrifuge tube. To the clearsupernatant, 5-10 µl of RNAase (10 mg per ml) was addedand incubated for 45-60 minutes at room temperature (~ 37C). After completion of the incubation period, about 500 µl ofChloroform and ~~÷~~Isoamyl alcohol (24:1) was added to the micro-centrifuge tube and mixed well by inversion for 10 minutes.The content was centrifuged at 12000 rpm for 10 minutes andthe supernatant was collected into another fresh 1.5 ml micro-centrifuge tube without disturbing the intermediate layer. Tothe clear supernatant equal volume (~500-600 ml) of chilledisopropanol was added. The contents were mixed gently andcentrifuged at 12000 rpm for 10 minutes at room temperature.The supernatant was drained gently without disturbing theDNA pellet. About 150-200 µl of 70 % ethanol was added tothe pellet at the bottom of the micro-centrifuge tube. It wasensured that the pellet was completely immersed in 70%alcohol. Then the content was centrifuged at 5000 rpm for 5minutes at room temperature. After

centrifugation, thesupernatant was drained and 70% alcohol wash wasrepeated once again. Finally, after centrifugation and drainingout the supernatant, the pellet was left overnight air drying atroom temperature. After complete drying of the pellet,depending upon the size of the pellet, about 50-100 µl ofsterile distilled water was added for dissolving the pellet.About 3 µl of the DNA sample was taken and loaded in 0.8 %ethidium bromide stained agarose-TBE (concentration ofTBE) gel, electrophoresed for about an hour and the bands ofgenomic DNA were visualized and documented in a UV geldocumentation system (M/s Alpha Innotech Corporation,USA).

Polymerase Chain Reaction (PCR) using SSR markers

The genomic DNA of parental lines subjected to PCRamplification as per the procedure described by Chen (1997). PCR amplification was carried out in a thermocycler usingthe temperature profile, followed by rapid cooling to 4°C prior to analysis. For electrophoretic studies, The SSR-PCR products were resolved in a 3%Agarose gel prepared in 0.5 X TBE buffer stained with Ethidium Bromide. The gel was run ata constant voltage of 90 V for 1 h and visualized under UV light and documented using gel documentation system. The temperature specifications for the denaturation of DNA strands, Annealing of primers and extension steps are as following:

S.No.	Steps	Temperature	Time
1	Initial denaturation	94^0C	5 min
2	Denaturation	94^0C	30 sec
3	Annealing	55^0C	1 min
4	Extension	72^0C	1 min
5	Final extension	72^0C	7 min
6	Cooling	4^0C	A

Agarose gel electrophoresis and image visualization

PCR products were analyzed by electrophoresis using a 3.0% agarose gel. About 9.0 g of agarose was weighed and applied to a conical flask and 400 ml of 1X TAE buffer was added and mixed well. The ingredients were boiled slowly in the microwave by stirring occasionally. Complete melting of the agarose and a clear solution were maintained during the

process. To clean the gel cast plate, it was soaked in water and then wiped with ethanol. After cooling the agarose to room temperature, two milliliters of ethidium bromide (10 mg/ml) was added to the melt and the mixture was then poured onto a gel casting tray with the necessary gel combs and allowed to settle at 20°Cfor 30 minutes.The gel was transferred to an electrophoresis apparatus containing 1X TAE buffer. Before loading, PCR-amplified products were mixed with 1/6 volumes of gel loading dye (ethidium bromide) and loaded into wells. A 100 bp DNA ladder was added to one well to determine the size of the amplified fragments. Ethidium bromide-stained gels were documented in a UV gel documentation system (Syngene) and the subsequent banding pattern was evaluated.

Statistical Analysis

The percentage of polymorphism was calculated by using the formula (reference):

Formatted: Highlight

$$\text{Polymorphism (\%)} = \frac{\text{Number of molecular markers showing polymorphism}}{\text{Total number of molecular markers run}} \text{ X } 100$$

Graphical representation of the genome

Graphical representation of molecular marker data was performed using Graphical Genotype (GGT2.0) software.

Phenotypic evaluation of IR58025B and Apo in field under control and moisture stress conditions

In normal phosphorus experiment, regular irrigation and flooded conditions were maintained until maturity of the crop and soil was maintained with P > 18.3 ppm. In low phosphorus experiment,regular irrigation and flooded conditions were maintained until maturity of the crop and soil was maintained with P < 2 ppm. The crop was harvested when the grains reached physiological maturity.Low ‘P’ level in the field was maintained without external application of P fertilizers. The levels of phosphorus in the field were estimated from the collected soil sample in each season through Olsen P method before taking up of planting of

experimental breeding material in the field plot.The differences between the parents on yield and drought tolerated traits were tested using paired t-test.

Results and Discussion

Genotypic polymorphism by molecular markers

The presence of two or more discontinuousgenotypes or alleles on a specific locus in agiven population is referred to as geneticpolymorphism. In the present study, clear polymorphism was observed between the parents both for full genome coverage markers and drought tolerant markers. A total of 300full genome coverage SSRmarkers (forward and reverse) and 10 phosphorus uptake yield QTL markers reported by Chin *et al.* (2010) were analyzed for molecular polymorphism.Polymerase Chain Reaction was done to check eachsample for parental polymorphism using specific Rice Microsatellite (RM) primers. Out of 300full genome coverage primers used the parents showed polymorphic only to 86 RM primers listed in Table 1 were polymorphic whereas K-46-1 and K-46-2 markers linked to *Pup1* QTL listed in Table 2 were polymorphic between the parents for low phosphorus deficiency tolerant markers. These are used in foreground selection. This type of parental polymorphic survey was done by previous workers *i.e.,*Collard *et al.* (2006), Chin*et al.* (2010), Sarkar*et al.* (2011), Tyagi*et al.* (2012), Aluwihare*et al.* (2014), Goncharova and Kharitonov(2016), Neelam *et al.* (2017), Zhang *et al.* (2018) and Lapitan and Mercado (2023), before starting marker assisted backcross breeding for introgression of desired yield controlling *qDTYs* under moisture stresses in rice. Chromosome wise distribution of polymorphic markers used in this study was presented in the Table 3. Percentage of polymorphism was highest on 1 chromosomeand least on 8 chromosomes. The average per centpolymorphism on all the chromosomes was 28.74.The lack ofdetectable polymorphism between two parents could be due to the fact that both parents, Kasalath and KMR-3Rareindica lines. Lack of molecular marker polymorphismbetween indica genotypes has been earlier noticed in studiesby Xu *et al.*(2002) and Biradar *et al.*(2004). In the present study dinucleotide repeats in the microsatellite region showed more polymorphism than tri and tetra nucleotide repeats. It may be due to the abudance of dinucleotide repeats distributed throughout the genome of rice. The prevalence of dinucleotide repeats in parental polymorphism was reported by previous workers. The usage of molecular marker technology in breedingprograms has greatly increased its efficiency and fastens the transfer of desirable genes among varieties and to introgressnovel genes from related wild species. Polygenic markerswhich were previously

difficult to analyze using traditional breeding methods, would now be easily tagged using molecular markers. The screening of markers for parental polymorphism among the rice cultivars forms the basis for tagging of the desired gene, fine mapping of the gene in the rice chromosome and in the subsequent Marker Assisted Selection (MAS) programmes.

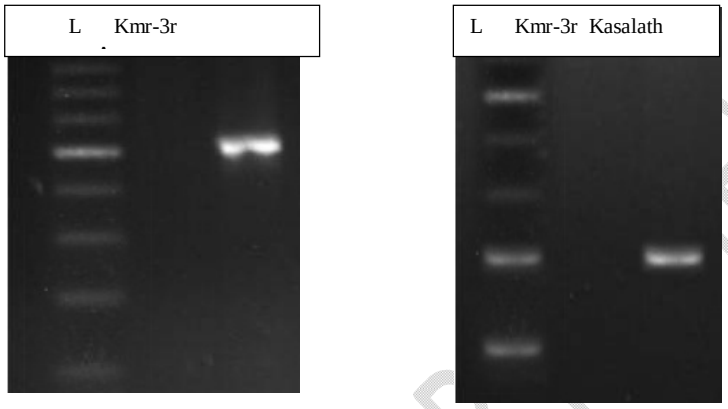

Fig 1 : Parental polymorphism shown by K-46-1 and K-46-2 for *Pup1 QTL*

Phenotypic polymorphism by morphological traits under control and moisture stress conditions

KMR-3R and Kasalath were assessed for grain yield and its attributing traits under low phosphorus stress and normal phosphorus conditions (Table 4). KMR-3R recorded significant differences for all traits studied between normal phosphorus and low phosphorus stress conditions. Similarly, Kasalath recorded significant difference for all the traits studied except for grain yield per plant between normal phosphorus and low phosphorus stress conditions. These findings are used for development of low P tolerant backcross inbred lines of KMR-3R by marker assisted backcross breeding.

CONCLUSION

Phosphorus is one of the major plant nutrients and plays a vital role in productivity of rice. It is one of the least abundant of all essential nutrients in soil. The present situation in India in both high and low input farming systems is unsustainable because P is a finite resource. It is

difficult to recognize the deficiency of mild to moderate P in the field. As a result, phosphorus deficiency in rice is known as hidden hunger. Application of phosphorus fertilizer can solve this problem. But it is difficult for resource poor farmers in India to apply phosphorus fertilizer every year. Therefore selection of rice cultivars capable of extracting phosphorus from phosphorus limiting soils with higher phosphorus use efficiency is considered as a significant cost-effective solution.

Fortunately, significant genetic variation has been reported in rice for tolerance to soil phosphorus deficiency (Fageria and Baligar, 1997). A large effect QTL known as *Pup1* controlling grain yield under phosphorusdeficiency has been mapped on chromosome12 of Kasalath rice cultivar (Wissuwa*et al.,* 1998). Marker Assisted Selection (MAS) programmes rely on the identification of parental polymorphism in rice cultivars through the screening of markers. This allows for the subsequent tagging and fine mapping of the target gene within the rice chromosome. Polymorphic markers discovered in this work were used to genotype the entire mapping population and facilitated the introgression of *Pup1* QTL involved in grain production under low phosphorus stress into KMR-3R. This study helped in the development of backcross inbred lines of KMR-3R by introgression of *Pup1* from Kasalath into KMR-3R by marker assisted backcross breeding. These BILs were utilized in development of hybrids tolerant to low phosphorus stress.

References

1. Aluwihare, Y.C., Lelwala, R.V., Ishan, M., Sirisena, D.N., Samarasinghe, W.L.G and Sooriyapathirana, S.D.S.S. 2014. DNA marker genotyping for *pup1* locus in rice to identify tolerant varieties under low phosphorus soil conditions in srilanka. *Proceedings of post graduate institute of science research congress,* Srilanka.
2. Biradar S K, Sundaram R M, Thirumurugan T, Bentur J S, Amrudhan S, Shenoy V V, Mishra B, Bennett J and Sharma N P. 2004. Identification of flanking SSR markers for a major rice gall midge resistance gene and their validation. Theoretical and applied genetics 109:1468-1473.
3. Chin, J.H., Lu, X., Haefele, Gamuyao, R., Ismail, A., Wissuwa, M and Heuer, S. 2010. Development and application of gene-based markers for the major rice QTL Phosphorus uptake 1. *Theoretical and Applied Genetics.* 120: 1073-1086.

4. Collard,B.C.Y., Thomson, M., Penarubiai, M., Lu, X., Heuer, S., Wissuwa, M., Mackill, D.J and Ismail, A.M. 2006. SSR analysis of rice near-isogenic lines (NILs) for P-deficiency tolerance. *SABRAO Journal of Breeding and Genetics*. 38(2): 131-138.

5. Goncharova, J.K and Kharitonov, E.M. 2016. Genetic control of traits associated with phosphorus uptake in rice (*Oryza sativa* L.) varieties. *Russian Journal of Genetics and Applied Research*. 6(3): 270-278.

6. http://www.fao.org/rice2004/en/pdf/khush.pdf.

7. Lapitan, V.C and Mercado, M.J.T. 2018. Genetic improvement of upland rice through marker assisted selection (MAS) for tolerance to phosphorus deficiency. http://www.uplb.edu.ph

8. Murray, H.G and Thompson, W.F. 1980. Rapid isolation of high molecular weight DNA. *Nucleic Acids Research.* 8: 4321-4325.

9. Neelam, K., Thakur, S., Neha, I., Yadav, S., Kumar, K., Dhaliwal, S.S and Singh, K. 2017. Novel alleles of phosphorus-starvation tolerance 1 gene (*PSTOL1*) from *Oryzarufipogon* confers high phosphorus uptake efficiency. DOI: 10.3389/fpls.2017.00509.

10. Sarkar, S., Yelne, R., Chatterjee, M., Das, P., Debanth, S., Chakraborthy, A., Mandal, N., Bhattacharya, K and Bhattacharya, S. 2011. Screening for phosphorus(P) tolerance and validation of *Pup-1* linked markers in indica rice. *Indian Journal of Genetics.* 71(3): 209-213.

11. Tyagi, W., Rai, M and Dohling,A. 2012. Haplotype analysis for *pup1* locus in rice genotypes of north eastern and eastern india to identify suitable donors tolerant to low phosphorus. *SABRAO Journal of Breeding and Genetics*. 44 (2): 398-405.

12. XuW, Virmani S S, Hernanadez J E, Sebastian L S, Redona E D and Li Z. 2002. Genetic diversity in the parental lines and heterosis of the tropical rice hybrids. Euphytica. 127: 139- 148.

13. Zhang, Y., Anis, G.B., Wang, R., Wu, W., Yu, N., Shen, X., Zhan, X., Cheng, L and Cao, L. 2018. Genetic dissection of QTL against phosphate deficiency in the hybrid rice 'Xieyou9308'. *Plant Growth Regulators*. 84: 123-133.

Table 1 Microsatellite markers exhibiting polymorphismbetween KMR-3R and Kasalath

1	RM 10694	21	RM 250	41	RM 3558	61	RM 408	81	RM 7391
2	RM 8094	22	RM 29	42	RM17486	62	RM 331	82	RM 209
3	RM 3412	23	RM 3316	43	RM6909	63	RM 256	83	RM 457
4	RM 140	24	RM 262	44	RM2275	64	RM 447	84	RM 2136
5	RM 10794	25	RM 530	45	RM127	65	RM 126	85	RM 28048
6	RM 10825	26	RM 424	46	RM5270	66	RM 342	86	RM 571
7	RM 562	27	RM 569	47	RM 413	67	RM 23679	87	RM 28199
8	RM 10927	28	RM 3766	48	RM 169	68	RM 23911	88	RM 27100
9	RM 543	29	RM 15087	49	RM 5140	69	RM 24173		
10	RM 486	30	RM 231	50	RM 3638	70	RM 24179		
11	RM 315	31	RM 468	51	RM 3321	71	RM 24390		
12	RM 472	32	RM 16232	52	RM 204	72	RM 201		
13	RM 12023	33	RM 16236	53	RM 6836	73	RM 105		
14	RM 165	34	RM 523	54	RM 3827	74	RM 26076		
15	RM 8051	35	RM 15914	55	RM20546	75	RM 286		
16	RM 312	36	RM 15087	56	RM 20801	76	RM596		
17	RM 12611	37	RM 545	57	RM 21330	77	RM 6132		
18	RM 145	38	RM 551	58	RM 21427	78	RM 206		
19	RM 6374	39	RM 16616	59	RM 5481	79	RM 590		
20	RM 3275	40	RM 5757	60	RM 248	80	RM 286		

Table 2 Details of polymorphic primer used for foreground selection of *Pup1* QTL

QTL	Chromosome	Position	Primer
PUP1	12	523 bp	K-46-1
PUP1	12	227 bp	K-46-2

Table 3 Chromosome wise polymorphic primers between KMR-3R and Kasalath

Chromosome	No. of SSR markers used	No. of polymorphic markers on each chromosome	No. of monomorphic markers on each chromosome	Percentage of primers showing polymorphism on each chromosome
1	35	14	21	40.00
2	30	6	24	20.00
3	28	9	19	32.14
4	25	5	20	20.00
5	25	7	18	28.00
6	28	8	20	28.57
7	25	7	18	28.00

8	27	5	22	18.52
9	20	8	12	40.00
10	19	5	14	26.32
11	20	6	14	30.00
12	18	6	12	33.33

Table 4 Comparison of KMR-3R and Kasalath for grain yield and its attributes under normal P and low P stress conditions

S.No.	Trait	KMR-3R			Kasalath			S.E. of difference
		Normal P (N.P.) (n=5)	Low P stress (L.P.) (n=5)	Mean difference (N.P-L.P)	Normal P (N.P.) (n=5)	Low P stress (L.P.) (n=5)	Mean difference (N.P-L.P)	
1	Plant height (cm)	115.12	105.12	10.00**	94.24	83.19	11.05**	1.16
2	Days to 50% flowering	103.00	110.00	-7.00**	85.00	90.00	-5.00**	0.64
3	Days to maturity	128.00	130.00	-2.00**	115.00	120.00	-5.00**	0.58
4	No. of tillers per plant	15.00	8.00	7.00**	16.00	10.00	6.00**	0.37
5	No. of panicles per plant	12.00	7.00	5.00**	14.00	8.00	6.00**	0.35
6	Panicle length (cm)	25.10	19.92	5.18**	23.62	21.24	2.38**	0.64
7	1000 grain weight (g)	24.12	20.21	3.91**	30.56	21.90	8.66**	0.55
8	No. of filled grains per panicle	150.00	74.00	76.00**	135.00	98.00	37.00**	1.24
9	No. of unfilled grains per panicle	8.00	58.00	-50.00**	35.00	48.00	-13.00**	0.96
10	Spikelet fertility (%)	94.93	56.06	38.87**	79.41	65.77	13.64**	0.60
11	Grain yield per plant (g)	24.24	12.41	11.83**	19.12	18.04	1.08	0.71
12	Biological yield per plant (g)	60.14	50.14	10.00**	70.21	65.21	5.00**	0.98
13	Harvest index (%)	40.30	24.75	15.55**	27.23	27.66	-0.43**	1.25
14	Leaf phosphorus content (%)	0.50	0.30	0.20**	0.90	0.80	0.10**	0.03
15	Seed phosphorus content (%)	1.20	1.00	0.20**	1.60	1.50	0.10**	0.03

* Significant at 5% level; ** Significant at 1 % level