Oryza Vol. 56 No. 2, 2019 (185-192)
DOI 10.35709/ory.2019.56.2.2

Understanding the physiological responses to low nitrogen and molecular screening of selected rice genotypes for *TOND1* gene

Sujata SB[1], Nirakar SNP[1], Bishal B Batta[2], Ranjit K Nagireddy[1], S Sabarinathan[1], HN Subudhi[1] , J Meher[1] , JN Reddy[1] and Anandan A[1]*

[1]ICAR-National Rice Research Institute, Cuttack, Odisha, India

[2]Orissa University of Agriculture and Technology, Bhubaneswar, Odisha, India

Corresponding author e-mail: anandanau@yahoo.com

Received : 23 May 2019 Accepted: 28 June 2019 Published : 30 June 2019

ABSTRACT

Nitrogen (N) plays a major role in the growth and development of a plant. Extensive application of N fertilizers results in low N use efficiency (NUE) generated by N loss due to denitrification byammonia volatilization, surface runoff, and leaching in the soil-flood water system. Therefore, there is an urgent requirement for the development of rice varieties with high NUE, which may improve the yield and decrease the N application which is harmful to the environment. In the present study, variability and correlation of morpho-physiological traits among the rice genotypes under low N in hydroponic solution was carried out for further genotyping with Tolerance of Nitrogen Deficiency 1 TOND1 gene markers. The root parameters and traits associated with shoot growth observed from 30 days old rice seedlings under low N condition suggested that shoot length was positively associated with leaf and root number followed by root length. The genetic diversity was estimated among the 36 selected genotypes with TOND1 gene primers. A total of 14 alleles were identified with an average number of alleles of 2.33 per locus. Allele frequency ranged from 0.62 to 0.86 with an average of 0.76. Genetic Diversity index ranged from 0.23 to 0.46 with an average of 0.35. The observed heterozygosity ranged from 0.00 to 0.1429 with an average of 0.056. The PIC values ranged between 0.61 and 0.77 with an average of 0.69. The unweighted neighbour-joining dendrogram grouped the 36 genotypes into 3clusters, wherein the local land race IC517708 clustered with known N deficiency tolerant Tequing. Therefore, the identified N deficiency tolerant genotype may be used as donor in developing N use efficient cultivar.

Key words: *Nitrogen use efficiency, nitrogen deficiency tolerance, TOND1, genetic diversity*

INTRODUCTION

Rice is the most important cereal food crop and the staple food feeding over half of the world's population. With the expanding growth of world's population and gradually deteriorating environment, food security has become a major challenge around the world. Increasing rice yield has become the most important goal of rice production with limited land and resources. Rice yield is affected by several biotic and abiotic stresses. Nitrogen (N) is one of the most significant minerals required by plants for their growth and development. N plays an extensive role during vegetative growth of the plant, it also aids in branching of root, carbon allocation and increased grain yield (Ali et al., 2018, Anandan et al., 2018). Earlier investigations disclosed that the proper use of fertilisers may considerably enhance the yield and the quality of rice (Mahender et al., 2016; 2017), but the excessive application of fertilisers not only decrease the N use efficiency (NUE) but may also cause several negative effects on the environment and human life. Partial or incomplete transformation of N fertilisers may lead to global warming by emitting nitrous oxide (Bouwman et al., 2002), polluting groundwater through nitrate leaching (Davies and Sylvester-Bradley 1995; Ferguson et al., 2002; Hashimoto et al., 2007; Anandan et al., 2018) and ammonia emission (Misselbrook et al.,2000). In

China, field trials conducted by various organisations reported that about 34% of the chemical fertilisers used was lost by denitrification, 11.5% by ammonium volatilisation, 2% via leaching and 5% via surface erosion (Zhu, 2003) creating a threat to the ecosystems (Nosengo, 2003; Giles, 2005). Therefore, there is an urgent requirement for the development of rice varieties with high NUE apart from preventing N losses with improved agronomic practices.

Plant's capacity to manage normal growth and yield in low N content in the soil is called N tolerance (NT), which is related to NUE. Till date several QTLs were identified for NT (Lian et al., 2005; Shan et al., 2005; Tong et al., 2006; Wang et al., 2009; Feng et al., 2010; Wei et al., 2012; Zhao et al., 2014). Zhang et al. (2015) cloned a gene, *TOND1* (Tolerance to Nitrogen Deficiency) on chromosome 12, associated with NT and NUE, which regulates the expression of its interacting proteins to change plant's response to nitrogen under low N condition. This gene encodes a thaumatin protein containing an N-terminal signal peptide enhances the tolerance of N deficiency. The *TOND1* overexpressing transgenic plants exhibited an increase in dry weight, N concentration, total N amount per plant, SPAD, plant height and root length with increase in grain yield of 20.2% under N-deficient condition (Zhang et al., 2015).

In the present experiment, we have used hydroponic system to screen the population under limited N in controlled condition. Plant growth solution described by Yoshida (1976) serves as a good source for the plants in hydroponic conditions in which growth and its response to the environment can be exactly measured. Therefore, genetic diversity for abiotic stress tolerance (salinity, nutrient deficiency etc), within and among the species, can be determined using hydroponic system (Shavrukov et al., 2012; Anandan et al., 2016). Hence, the present study aimed to estimate genetic diversity among the set of land races and improved rice cultivars to identify suitable local source of N tolerant donor from short duration group of *indica* sub species.

MATERIALS AND METHODS

Plant material

A total of 173 rice genotypeswere selected for the study and their morpho-physiological traits were studied under low Nin net house condition using nutrient solution described by Yoshida (1976).

Physiological responses of rice genotypes to low N

Pre-germinated rice plantlets of each genotype after attaining a height of 4 - 5 cm were treated with standard Yoshida solution for 15 days by renewing the solution after 7 days and pH of the solution was adjusted to 4.50 - 4.55 on each alternative day (Fig. 4). After 15 days, the plants were transferred to an N deficient solution with reduced concentration of ammonium nitrate (the sole source of N in the Yoshida solution) 1/4x (0.358mM) against the standard concentration of 1.43 mM, while all other macronutrient and micronutrient solutions of Yoshida solution was maintained as same. The plants were maintained in N deficient solution for

Table 1. Descriptive statistics of rice seedlings grown under low N

Parameters	Leaf number	R/S ratio	Root number	Root length (cm)	SPAD	Shoot length (cm)	Tiller number
No. of genotypes	173	173	173	173	173	173	173
Median	5.88	0.29	18.00	13.59	21.36	48.62	1.13
Minimum	3.00	0.16	7.00	4.50	13.99	19.50	1.00
Maximum	7.13	0.46	37.14	21.79	27.34	63.10	2.25
Lower quartile	5.63	0.26	15.62	11.95	19.57	42.34	1.00
Upper quartile	6.13	0.31	20.50	14.95	22.84	53.37	1.38
Mean	5.84	0.29	18.58	13.36	21.17	47.61	1.24
SD	0.56	0.05	4.67	2.39	2.59	7.08	0.31
SE	0.04	0.00	0.36	0.18	0.20	0.54	0.02
CV %	9.64	16.40	25.15	17.85	12.24	14.88	24.50
Skewness	-1.13	0.41	1.23	-0.18	-0.23	-0.58	1.13
Kurtosis	3.87	0.66	2.69	1.01	-0.32	0.44	0.23

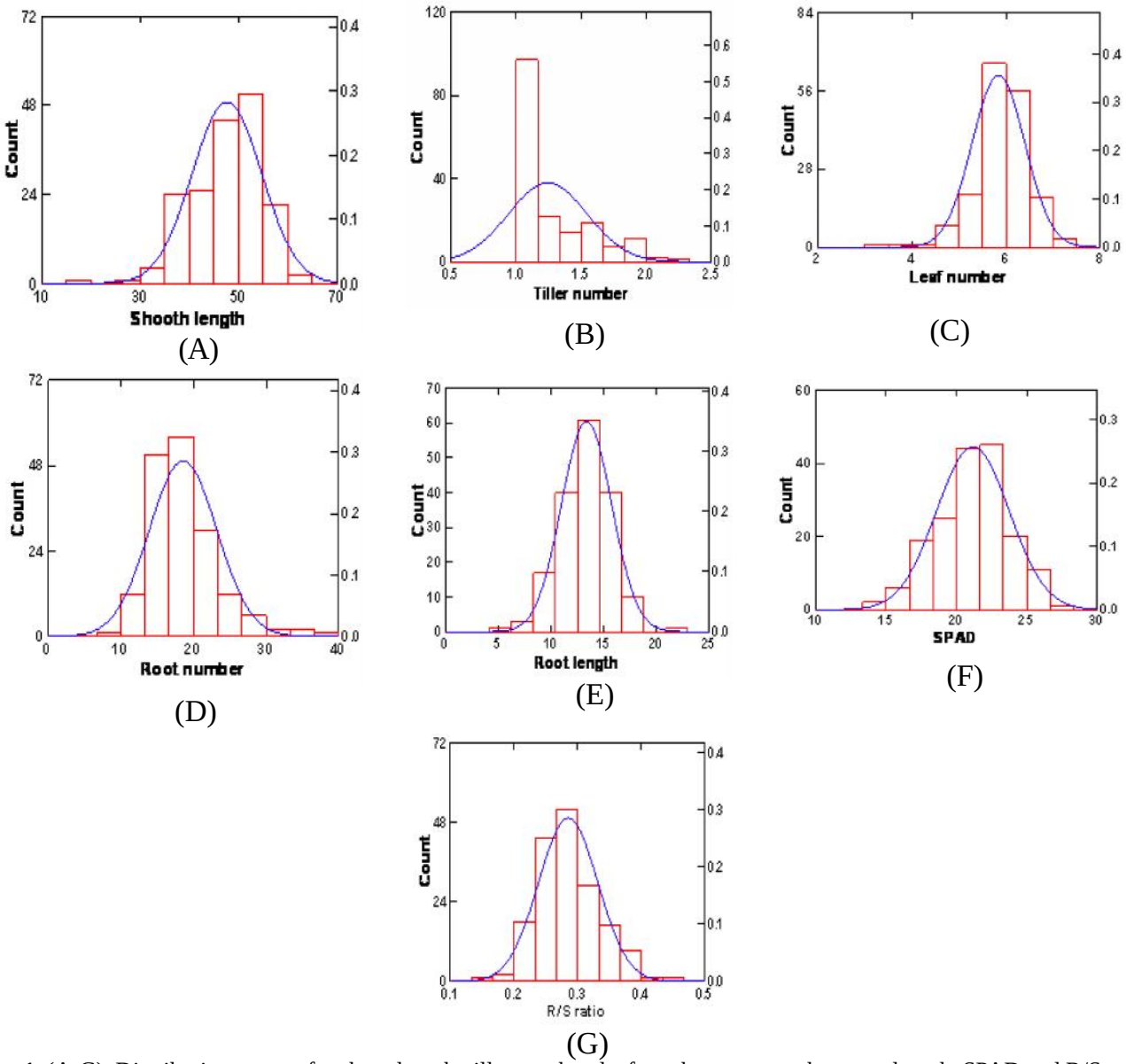

Fig. 1. (A-G). Distribution pattern for shoot length, tiller number, leaf number, root number, root length, SPAD and R/S ratio under low N condition during seedling stage among 173 rice genotypes.

15days by renewing the deficient solution after 7days by adjusting the pH to 4.50 - 4.55 every alternate day. After 15 days of growth in N deficient solution, various morphological traits (leaf number, root number, root length, shoot length, root shoot ratio, and tiller number) and physiological traits (SPAD) were observed (Fig. 5).

Molecular screening of selected rice genotypes for *TOND1* gene

Based on morphological and physiological data collected, 28 genotypes from 173 genotypes (6 genotypes which were negatively skewed from the normal distribution were also included) and 8 genotypes tolerant to N deficiency (reported earlier) were selected for genotyping with *TOND1* gene (Zhang et al., 2015).

DNA isolation and molecular screening with *TOND1* gene

DNA from 36 selected rice genotypes was isolated by

Table 2. Correlation coefficients among morhpo-physiological traits of 173 rice genotypes under low N.

Characters	Shoot length	Tiller number	Leaf number	Root number	Root length	SPAD
Shoot length	1.000					
Tiller number	-0.026	1.000				
Leaf number	0.649**	0.077	1.000			
Root number	0.656**	0.039	0.593**	1.000		
Root length	0.513**	0.093	0.456**	0.270**	1.000	
SPAD	0.289**	0.323**	0.452**	0.362**	0.423**	1.000
R/S ratio	-0.348**	0.135	-0.069	-0.262**	0.608**	0.209**

following modified CTAB method (Murray and Thompson, 1980). Eleven internal markers of the *TOND1* gene were amplified with the isolated 36 DNA samples. The isolated DNA samples were amplified using T100 thermal cycler (BIO-RAD, U.S.A.) with a total of 10 µl reaction consisting of 2 µl of 50 ng DNA, 1 µl of Tris-HCl 10 mM (pH 8.3), 0.5µl of 1.5 mM $MgCl_2$, 0.1 µl 0.5 unit of TaqPolymerase (New England Biolabs, U.K.), 1 µl of 50 µM dNTP mixture and 0.2 µl each of forward and reverse primers (5 pico molar).

Conditions for carrying out DNA amplification were as follows: initial denaturation at 94 ºC for 5 min, followed by 35 repeated cycles of denaturation at 94 ºC for 45 s, annealing for 45 s (temperature specific to primer) and extension for 60 s at 72 ºC followed by a final extension for 8 min at 72 ºC. Bromophenol blue was added to the samples and the molecular weight of the amplified DNA was estimated in 3.5% agarose gel with the 50-bp ladder (New England Biolabs, U.K.) as standard in 1X Tris-Boric acid-EDTA (TBE) buffer. The resolved PCR bands were documented using Molecular Imager Gel Doc XR System(Bio-Rad).

Table 3. Principal component analysis of morpho-physiological traits of rice under low N.

Parameters	PC1	PC2
Percentage variation	75.91	12.54
Characters	Latent vectors (loadings)	
Leaf numbers	0.0488	0.0169
Root shoot ratio	-0.0017	-0.00016
Root number	0.4650	0.8452
Root length	0.1570	-0.1703
SPAD	0.1222	0.2172
Shoot length	0.8612	-0.4571
Tiller number	0.0003	0.0110

Data analysis

Only clear and intense bands were recorded. The molecular size of the amplified fragments were determined by image lab software (Bio-rad) using 50-bp DNA ladders as standard. Amplification of DNA samples with the primers were recorded as '1' for the amplified and '0' for unamplified regions according to the molecular size of the marker. A data matrix with '0' or '1' against the molecular size was prepared for further analysis. Genetic diversity parameters *viz*., number of alleles, allele frequency, genetic diversity index and heterozygosity were calculated by Power Marker software.

Polymorphism information content (PIC) for every polymorphic marker was computed using the formula: PIC = 1 - ∑Pi2 - ∑ ∑Pi2 Pj2; where 'i' is the sum of alleles detected, 'Pi' is the frequency of the i^{th} allele and j = i+1. A neighbour-joining tree with bootstrap values (1000) was constructed using unweighted pair group method with arithmetic averages (UPGMA) algorithm with the help of DARwin version 6.0.

RESULTS AND DISCUSSION

Variability in morpho-physiological traits of rice genotypes under low N

The distribution pattern of 173 rice genotypes for seven traits at low Ncondition of the 30-day old seedling is presented in Fig. 1 (A-G). The genotypes studied in the present experiment have a wide diversity of morphological and physiological traits. Shoot length, root length, root shoot ratio and SPAD under low N were found to be normally distributed with skewness ranged between -1 to +1 (Table 1). Leaf number was found to deviate significantly from the normal distribution with negative skewness (S < 1), while the root number/plant

Table 4. Showing the genetic diversity parameters and PIC values of the 6 polymorphic primers.

S. no.	Primer	Allele no.	Major allele frequency	Genetic diversity	Heterozygosity	PIC
1	M1	2.0000	0.7778	0.3457	0.0000	0.6728
2	M3	3.0000	0.7500	0.3966	0.0833	0.7795
3	RM171	3.0000	0.8056	0.3306	0.0278	0.7749
4	RM235	2.0000	0.8611	0.2392	0.0000	0.6195
5	RM1227	2.0000	0.6250	0.4688	0.0833	0.6909
6	RM6411	2.0000	0.7857	0.3367	0.1429	0.6141
	Mean	2.3333	0.7675	0.3529	0.0562	0.6942

and tiller number/plant were positively skewed (S > 1). Among the traits studied in the seedling phase, shoot length under low N varied from 19.50 cm to 63.10 cm as measured in IRGC 29108-1 and IRGC 11482-1 respectively with mean of 47.61 cm and IRGC 11482-1 exhibited 1.32 times of the mean value. Genotypes in low N at the seedling phase exhibited maximum differences for root number per plant (7.00-37.14) followed by root length (4.50 - 21.79 cm) and minimum differences were observed for SPAD (13.99 - 27.34) followed by tiller number (1- 2.25) and leaf number per plant (3-7.13). Coefficients of variation (Cv) indicating variability among the genotypes were >25% for tiller number and root number per plant. On the other hand, leaf number per plant, SPAD and shoot length exhibited Cv of <15%.

Correlation among morpho-physiological traits of 173 genotypes under low N

The relationship among morpho-physiological traits was analysed by Pearson correlation analyses (Table 2). Three root parameters and four traits associated with shoot growth were observed from 30 days old rice seedlings under low N condition are subjected to Pearson correlation (Table 2). The study brings out that shoot length was found to be positively correlated with leaf number and root number (>0.6) followed by root length (0.513) (Fig. 2), while significantly associated with root shoot ratio in negative direction. Leaf number was found to be positively associated with root parameters like number and length. The leaf colour or green pigment was estimated using SPAD meter. The SPAD value was found to be associated with leaf number and root length. This implies the estimating of chlorophyll (SPAD) is highly required parameter to understand the performance of genotypes under low N condition. Conversely, root shoot ratio was found to be negatively associated with shoot length (-0.348) and root number (-0.262).

Principal component analysis of rice genotypes under low N condition

Table 3 shows the two principal components (PCs) and their corresponding variances. The first two PCs

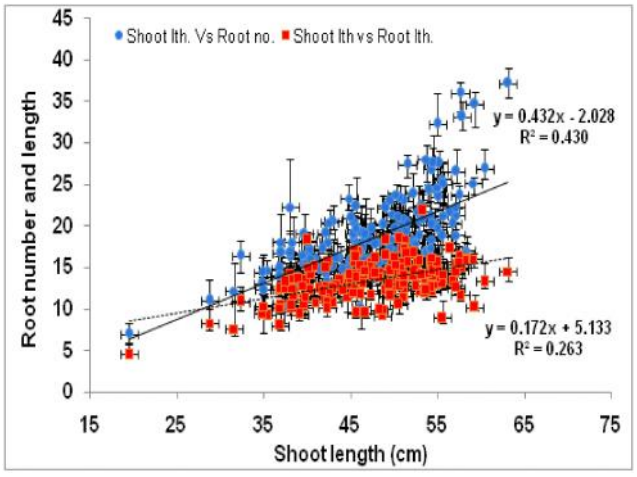

Fig. 2. Relationship between shoot length and root number and length under low N condition

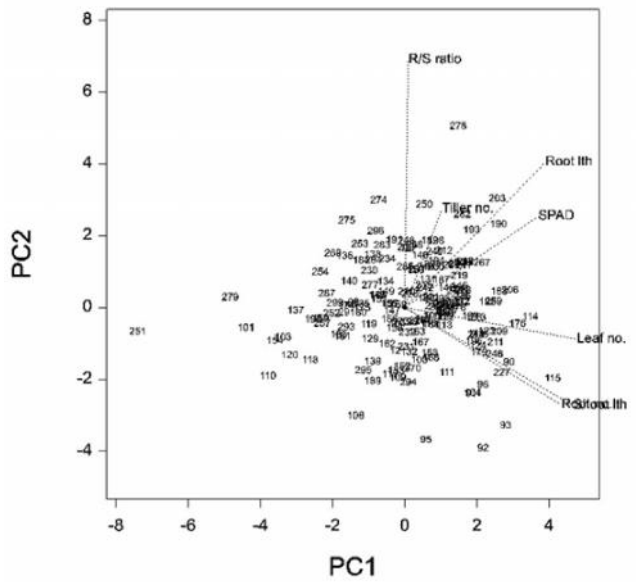

Fig. 3. Principal component analysis of morpho-physiological traits under low N condition

Fig. 4. Rice seedlings screened under low N condition.

Fig. 5. Some selected genotypes based on level of their growth under low N.

accounted for about 88.45% of the total variability, with > 1.0 eigenvalue. Naturally, the result showed that, seven variables could be condensed and represented by the 1st 2 PCs. The 75.91% variation in the 1st PC was mainly due to the variation in shoot length and root number, for which the eigenvectors were 0.8612 and 0.1570, respectively. Thus, 1st PC was represented by shoot length. The 2nd PC explained 12.54% of the total variance. The variation was again explained by root number and shoot length with eigenvectors of 0.8452 and -0.4571, respectively.

Biplot (Fig. 3) analysis has grouped the genotypes into two groups *viz*., tolerant and susceptible. The right side of the biplot Figure 3 has categories the tolerant lines having necessary shoot and root growth under low N condition. Conversely, genotypes on left of the graph showed poor performance under low N condition. Further, the right side of plot could be separated into two quadrants. The quadrant 1 (top right) has grouped the genotypes having lengthy roots, more tiller numbers, more of root shoot ratio and high SPAD. Genotype on quadrant 3 (bottom left), opposite to the quadrant 1 exhibited poor root system with low SPAD value. On the other hand, genotypes clustered in between quadrant 1 and 3, exhibited greater shoot length, leaf number and root number under low N status. Opposite to the quadrant 2, quadrant 4 grouped the poor performers having limited shoot and root growth.

PCR amplification and visualization of *TOND1* gene

Eleven internal primers of the *TOND1* gene were amplified with 36 genotypes. Six of the 11 primers were found to be polymorphic in the set of 36 genotypes.

Genetic diversity analysis

Six out of eleven primers were found to be polymorphic

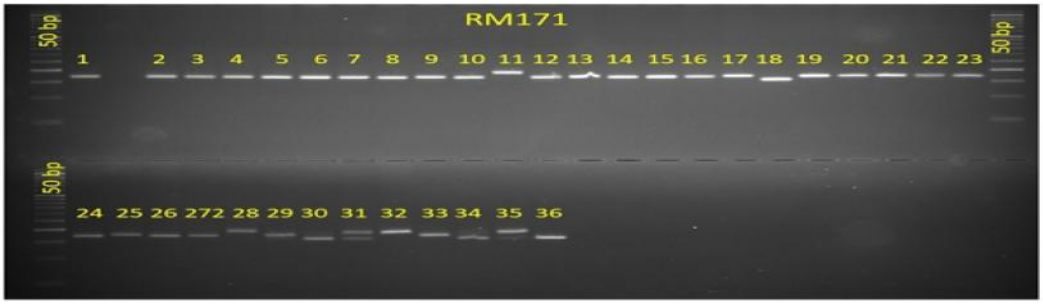

Fig. 6. PCR profile of 36 genotypes with primer RM171

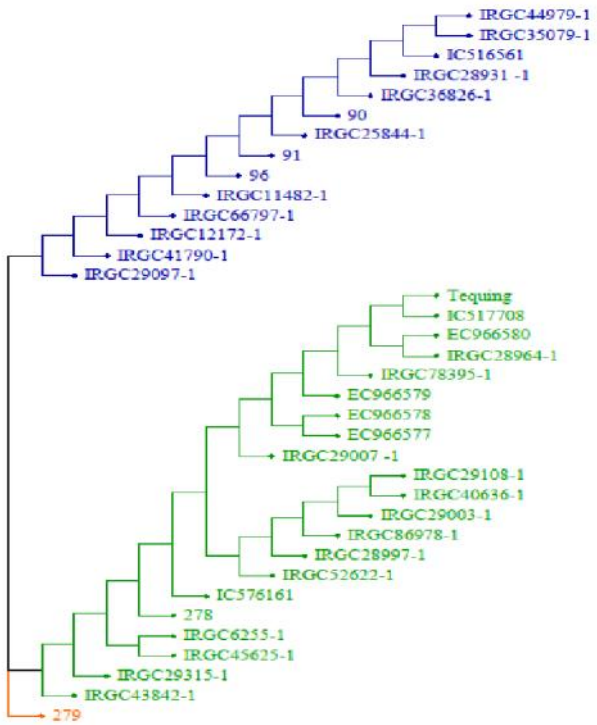

Fig. 7. Dendrogram showing the genetic similarity among the 36 genotypes.

(54.54%). A total of 14 alleles were identified by amplification of the 6 polymorphic primers with an average number of alleles of 2.33 per locus, 2 loci were with 3 alleles and 4 loci were with 2 alleles (Fig. 6). Genetic diversity parameters were calculated using Power Marker. The allele frequency ranged between 0.62 (RM1227) and 0.86 (RM235) with an average of 0.76. Genetic diversity index ranged from 0.23 (RM235) to 0.46 (RM1227) with an average of 0.35. The observed heterozygosity ranged from 0.00 (M1 and RM235) to 0.1429 (M3 and RM1227) with an average of 0.056 (Table 4).

The unweighted neighbour-joining (UNJ) dendrogram constructed using DARwin 6.0 (Fig. 7) with 1000 bootstraps grouped all the 36 genotypes into three major clusters. Genotype 279 formed a separate cluster (cluster C) suggesting that it is highly diverse genotype from all the other genotypes used in the study. Cluster A consisted of 14 genotypes, genotypic pairs IRGC44979-1 and IRGC35079-1 were more similar. Cluster B consisted of 21 genotypes. Genotypic pairs Tequing - IC517708 and EC966580 - IRGC28964-1 clustered separately making them more similar. Genotype IC517708 belongs to mid-early group (120 days) of indica. As the genotype IC517708 was clustered along with Tequing, it may be used as a donor for N tolerance as that of Tequing used in several studies earlier.

The present study provides a detailed insight into the genetic diversity for N deficiency tolerance in rice. The results obtained from the phenotype and genotype with known tolerant markers revealed a great genetic diversity and identified N deficiency tolerant donor from the indigenous collection. Correlation studies identified shoot length has a positive association with leaf number and root number. The SPAD value was found to be associated with most of the traits studied, suggests an important parameter to understand the performance of genotypes under low N condition. We strongly believe that the results of this study will share its contribution to rice breeding for NUE.

REFERENCES

Ali J, Jewel Z, Mahender A, Anandan A, Hernandez J and Li Z (2018). Molecular genetics and breeding for nutrient use efficiency in rice. International Journal of Molecular Sciences 19(6): 1762

Anandan A, Anumalla M, Pradhan SK and Ali J (2016). Population structure, diversity and trait association analysis in rice (*Oryza sativa* L.) germplasm for early seedling vigor (ESV) using trait linked SSR markers. PLoS One 11(3), p.e0152406

Anandan A, Meher J, Sah RP, Samantaray S, Parameswaran C, Panneerselvam P, Dash SK, Swain P, Annamalai M, Prabhukarthikeyan SR and Kumar G (2018). Enhancing input use efficiency in direct-seeded rice with classical and molecular breeding. In: rice research for enhancing productivity, profitability and climate resilience, H. Pathak, A.K. nayak, Mayabini Jena, O.N. Singh, P. Samal and S.G. Sharma. (eds), ICAR- national Rice Research Institute, Cuttack, Odisha, India pp. 73-89

Bouwman AF, Boumans LJM and Batjes NH (2002). Emissions of N2O and NO from fertilized fields: Summary of available measurement data. Global Biogeochemical Cycles 16(4): 6-1

Davies DB and Sylvester Bradley R (1995). The contribution of fertiliser nitrogen to leachable nitrogen in the UK: a review. Journal of the Science of Food and Agriculture 68(4): 399-406

Feng Y, Cao LY, Wu WM, Shen XH, Zhan XD, Zhai RR, Wang RC, Chen DB and Cheng SH (2010). Mapping QTLs for nitrogen deficiency tolerance at seedling stage in rice (*Oryza sativa* L.). Plant Breeding 129(6): 652-656

Ferguson B and Zhang XC (2002). Materials for terahertz science and technology. Nature Materials 1(1): 26

Giles J (2005). Millennium group nails down the financial value of ecosystems. Nature pp. 434-547

Good AG, Johnson SJ, De Pauw M, Carroll RT, Savidov N, Vidmar J, Lu Z, Taylor G and Stroeher V (2007). Engineering nitrogen use efficiency with alanine aminotransferase. Botany 85(3): 252-262

Han-Hua TONG, Han-Wei MEI, Xin-Qiao YU, Xiao-Yan XU, Ming-Shou LI, ZHANG SQ and Li-Jun LUO (2006). Identification of related QTLs at late developmental stage in rice (*Oryza sativa* L.) under two nitrogen levels. Acta. Genetica Sinica 33(5): 458-467

Hirel B, Le Gouis J, Ney B and Gallais A (2007). The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. Journal of Experimental Botany, 58(9): 2369-2387

Lian X, Xing Y, Yan H, Xu C, Li X and Zhang Q (2005). QTLs for low nitrogen tolerance at seedling stage identified using a recombinant inbred line population derived from an elite rice hybrid. Theoretical and Applied Genetics 112(1): 85-96

Mahender A, Anandan A, Pradhan SK and Pandit E (2016). Rice grain nutritional traits and their enhancement using relevant genes and QTLs through advanced approaches. Springerplus 5(1): 2086

Mahender A, Anandan A, Pradhan SK and Singh ON (2018). Traits-related QTLs and genes and their potential applications in rice improvement under low phosphorus condition. Archives of Agronomy and Soil Science 64(4): 449-464

Maruyama T, Hashimoto I, Takimoto H and Nakade D (2009). Assessment for nitrogen pollution loads from farmland, Japan, by objective yield and standard fertilizer usage. Paddy and Water Environment 7(2): 151-159

Misselbrook TH, Van Der Weerden TJ, Pain BF, Jarvis SC, Chambers BJ, Smith KA, Phillips VR and Demmers TGM (2000). Ammonia emission factors for UK agriculture. Atmospheric Environment 34(6): 871-880

Murray MG and Thompson WF (1980). Rapid isolation of high molecular weight plant DNA. Nucleic acids Research 8(19): 4321-4326

Nicolaisen K, Moslavac S, Samborski A, Valdebenito M, Hantke K, Maldener I, Muro-Pastor AM, Flores E and Schleiff E (2008). Alr0397 is an outer membrane transporter for the siderophoreschizokinen in Anabaena sp. strain PCC 7120. Journal of Bacteriology 190(22): 7500-7507

Nosengo N (2003). Fertilized to death. Nature 425: 894-895

Pathak H, Singh R, Bhatia A and Jain N (2006). Recycling of rice straw to improve wheat yield and soil fertility and reduce atmospheric pollution. Paddy and Water Environment 4(2): 111

Shan YH, Wang YL and Pan XB (2005). Mapping of QTLs for nitrogen use efficiency and related traits in rice (Oryzasativa L.). Agricultural Sciences in China, 4(10): 721-727

Shavrukov Y (2012). Salt stress or salt shock: which genes are we studying. Journal of Experimental Botany 64(1): 119-127

Wei D, Cui K, Ye G, Pan J, Xiang J, Huang J and Nie L (2012). QTL mapping for nitrogen-use efficiency and nitrogen-deficiency tolerance traits in rice. Plant and Soil 359(1-2): 281-295

Yoshida S (1976). Routine procedure for growing rice plants in culture solution. Laboratory manual for physiological studies of rice pp. 61-66

Yu WANG, Yong-Jian SUN, Deng-Yin CHEN and Si-Bin YU (2009). Analysis of quantitative trait loci in response to nitrogen and phosphorus deficiency in rice using chromosomal segment substitution lines. Acta Agronomica Sinica 35(4): 580-587

Zhang Y, Tan L, Zhu Z, Yuan L, Xie D and Sun C (2015). *TOND1* confers tolerance to nitrogen deficiency in rice. The Plant Journal 81(3): 367-376

Zhao CF, Zhou LH, Zhang YD, Zhu Z, Chen T, Zhao QY, Yao S, Yu X and Wang CL (2014). QTL mapping for seedling traits associated with low nitrogen tolerance using a set of advanced backcross introgression lines of rice. Plant Breeding 133(2): 189-195

Zhu JK (2003). Regulation of ion homeostasis under salt stress. Current opinion in plant biology 6(5): 441-445