



Breeding rice for nitrogen use efficiency

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Abstract

Development of nitrogen use efficient (NUE) rice varieties is inevitable for sustainability of environmental friendly and economical agricultural practices. Several management practices are being studied for increasing efficiency of spatial and temporal inputs of N under National Innovations for Climate Resilient Agriculture (NICRA). Attempts are being made to develop NUE rice varieties with multidisciplinary approach and conventional selection along with mapping and next generation sequencing strategies. Around 800 rice genotypes were characterized under low and recommended nitrogen for two consecutive seasons and the promising donors further evaluated to identify consistent NUE rice genotypes. Several mapping populations were developed using the NUE donors and popular rice varieties. QTL/genomic regions were identified for yield under low N using biparental and association mapping. Using minimum marker set of 50 rice SSR markers, 12 genomic regions were identified for yield and yield associated traits under low nitrogen. Several promising recombinants of yield and NUE were identified and these breeding lines were evaluated under multi-locations and stable performers were identified under AICRIP Trial - Evaluation of Radiation and Nitrogen use efficient promising rice genotypes -Plant Physiology during *Kharif* 2016, 2017 and 2018. As nitrogen is the building block of biomass, an optimum N is required for realizing the yield. The strategy should be to maximise uptake and improve utilization, so that remobilization of N to yield is achieved under low N.

Key words: Rice, low nitrogen, donors, genomic regions, breeding

Introduction

India has the world's largest area under rice with ~44 million ha and world's second largest production of 110.15 million tonnes during 2017-18 (<http://agricoop.nic.in/>). Within the country, rice occupies

35.4% of the total cropped area, contributes about 43% of total food grain production and continues to play a key role in the national food and livelihood security system. With the estimated Indian population of 1.63 billion by 2050 and a per capita demand of ~250 g/day, the productivity needs to be enhanced from the present 2.05 t ha⁻¹ to 3.3-4.05t ha⁻¹ to meet the requirement of ~150 million tonnes of rice (DRR Vision, 2050 <http://www.icar-iirr.org/>). Irrigated rice ecology accounts for 24.5 million ha with highest productivity followed by rainfed shallow lowlands are considered as favourable ecologies for rice production in India with a response to intensive resource inputs *viz.*, fertilizers and pesticides. Before the introduction of semi-dwarf high yielding rice varieties (HYVs) during green revolution period of 1960's, rice was mostly cultivated under native soil fertility conditions without any major inputs. With its direct response as high grain yield to artificial fertilizers, a large amount of N fertilizer is applied to fields to maximize rice yields for HYVs. In India, around ~220 kg of urea is applied for irrigated rice in general as three split doses *viz.*, basal, vegetative and panicle initiation stage. Through fertilizer management strategies, considerable progress has been achieved to reduce N losses by new application methods and modified N sources in the past decades (De Datta and Buresh 1989). However, farmers generally apply more N fertilizer than the recommended dose required for maximum crop growth. In irrigated rice ecology, N losses are rapid because of ammonia volatilization, denitrification, surface runoff and leaching in soil-floodwater system (De Datta and Buresh 1989). Leaching of nitrogen to water table causes water contamination and eutrophication of water bodies (Hirel et al. 2007). Over 40 years, the amount of mineral N fertilizers applied to agricultural crops increased by

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7.4 fold, whereas the overall yield increase was only 2.4 fold (Tilman et al. 2002), thus increasing the production costs of rice. Recent awareness about the climate change and the role of greenhouse gases viz., methane and nitrous oxide emitting from rice fields has emphasized the rational use of nitrogen fertilization. Nitrous oxide is 310 times more potent greenhouse gas than CO₂ and 21 times potent than methane on a 100 year time scale, though atmospheric loading of nitrous dioxide is low (IPCC, 1995 <https://www.ipcc.ch/reports/>).

In India, the current average nitrogen use efficiency (NUE) in the field is approximately 33% and a substantial proportion of the remaining 67% is lost into the environment, especially in the intensively cropped areas (Abrol and Raghuram 2007). Therefore, it is important to aim at efficient use of nitrogen through management techniques and also by developing varieties with high nitrogen use efficiency. Though efficient fertilizer management practices improve NUE, unless the cultivar is responsive, there is a limited scope of adopting those costly or labour intensive practices by the farmers. Thus, development of nitrogen use efficient rice varieties is inevitable for sustainability of environmental friendly and economical agricultural practices. While several management practices are being studied for increasing efficiency of spatial and temporal inputs of N, at IIRR under NICRA, efforts being made to develop NUE rice varieties with multidisciplinary approach are summarized here.

Identification of useful germplasm

Genetic variation in NUE in rice has been reported (Broadbent et al. 1987; DeDatta and Broadbent 1988; Tirol-Padre et al. 1996; Zhang et al. 2009). Rice landraces adapted to the local environments and selected by the farmers for better yields under low or zero inputs form an interesting genetic material for identification of donors and genomic regions for NUE. Landraces, given their past evolutionary history and adaptation to stress environments, often out-yield modern cultivars under low-input production systems (Dwivedi et al. 2016). Many candidate genes have been identified for stress tolerance and yield in landraces of rice viz., SUB1 in 'FR13A' for submergence, PUP1 in 'Kasalath' for phosphorus uptake, NAL1 in 'Daringan' (Xu et al. 2006; Gamuyao et al. 2012; Fujita et al. 2013) proving the potential of rice landraces as a source of genes of agronomic interest.

Around 800 rice genotypes under low and recommended nitrogen were characterized using agro morphological, yield and N related traits/parameters in IIRR farm for two consecutive seasons. Wide variation was observed for all the traits/parameters comprising morphological, yield components, nitrogen content and NUE indicators under low and recommended N. A sub set of genotypes analysed for panicle topology also showed a wide genetic variability but for grain filling % of secondary branches (Vijayalakshmi et al 2015; Rao et al 2018).

Under low N, for four leaf traits (length, width, thickness and area), the reduction ranged from 18 to 40% (wet) and 6.9 to 17% (dry) at vegetative stage and 14 to 43% (wet) and 10 to 27% at reproductive stage (Table 1). The increase of leaf related parameters with the increase of N has always been reported for rice (Anzoua et al. 2010; Zhang et al. 2013; Mahajan et al. 2012; Metwally et al. 2010). The increased translocation of N to the leaves increases the leaf length, width, area and thickness along with chlorophyll content, thus main effect of N is to increase the rate of leaf expansion, leading to increased interception of daily solar radiation by the canopy and so increased dry matter production (Ohnishi et al. 1999). Increase of 35% increase of leaf N content resulted in 15% increase in carboxylation activity of Rubisco and photosynthetic rate in rice seedlings. The decrease of N content affects the leaf parameters drastically (Nguyen et al. 2014). Low N conditions were reported to impair chloroplast size, composition and function; decrease the content of chloroplastidic pigments and synthesis of several enzymes involved in the Calvin cycle, particularly Rubisco; affect the diversion of proportion of soluble proteins relevant to the Calvin cycle (Evans 1989). When the genotypes were classified as high and poor yielders under low N, SPAD has shown positive association in our study. Leaf area index was also shown to be positively correlated with total N at plant maturity with high NUE of elite rice varieties under low N (Wu et al. 2016). Overall, leaf parameters appears to be playing a critical role for N content and there by total biological yield under low N. However, the possibility of using the leaf traits for selection of efficient genotypes under low N appears to be discouraging because we found the leaf traits of the efficient genotypes to be promising, but not all the genotypes with promising leaf traits were efficient under low N.

Under low N, the general trend of reduction of plant height, number of tillers and productive tillers

Table 1. Mean and range of leaf traits of ~ 800 germplasm grown under low and recommended (Rec.) N across two seasons

Trait	Kh 11		Rb 12		Kh 11		Rb 12	
	Range		Range		Mean		Mean	
	Low N	Rec. N	Low N	Rec. N	Low N	Rec. N	Low N	Rec. N
Vegetative stage								
Leaf length	11-56.52	14-66.01	12.47-40.56	12.5-44.04	32.65	39.69	23.88	26.59
Leaf width	0.18-1.4	0.29-2.01	0.37-1.54	0.37-1.95	0.76	1.02	0.81	0.87
Leaf area	4.09-53.63	5.58-69.71	3.6-34.59	4.41-63.72	18.58	30.72	14.62	17.65
Leaf thickness	0.02-0.56	0.02-0.53	0.05-0.24	0.05-1.16	0.14	0.13	0.13	0.14
SPAD	12.8-46.03	18.38-58.6	20.07-51.52	27.57-55.73	31.74	34.52	35.17	38.4
Reproductive stage								
Leaf length	12.97-42.26	14.59-66.21	7.57-40.52	7.8-71.73	25.38	33.72	18.05	21.78
Leaf width	0.48 - 1.92	0.89 - 2.41	0.47-2.02	0.47-2.06	1.13	1.5	0.97	1.08
Leaf area	8.42-57.28	12.68-106.16	3.7-35.3	4.19- 61.33	21.9	38.15	13.26	18.2
Leaf thickness	0.03-0.26	0.03-1.14	0.06-0.29	0.04-0.27	0.12	0.14	0.16	0.14
SPAD	10.10-38.95	10.08-45.91	14.80-44.47	13.89-49.59	27.44	30.72	32.2	30.18

was observed in our studies owing to the limitation of N for the increase of biomass. The reduction was by 30.8% (wet) and 17.7% (dry) for plant height; by 17% (wet) and 35.4% (dry) for tillers m^{-2} and 16% (wet) and 32% (dry) for productive tillers under low N. Nitrogen fertilization promotes tillering in rice with the significant different number of tillers between the basal and additional N fertilization as well as the top dressing (Lee et al. 2010). An optimum concentration of N >35g/kg reported to activate tillering and concentrations < 25 N/kg could reduce tillering with further lower concentration leading to the death of tillers (Evans, 1975). The wet season appears to be more vulnerable for low N conditions for leaf related parameters and plant height, whereas, the tillers, productive tillers and panicle number were relatively reduced more in dry season under low N.

Grain yield of rice is the final product of yield components viz., the number of panicles per unit area, the number of spikelets per panicle, the percentage of filled spikelets and the grain weight (Yoshida 1983). From the studies on nutrient management practices influencing the yield and yield components reported that the panicle or spikelet number per unit area is the most variable among the yield components (Fageria et al. 1997). The panicle number was reduced by ~20%, panicle weight by ~36% and panicles m^{-2} were reduced by 18% across wet and dry seasons. Under low N, grain yield (kg/ha), total grain weight, straw

weight and total dry matter were reduced by 30 to 50% across seasons. The grain yield increments under recommended N were reported to be possible mainly through increasing the number of spikelets of the panicle and then the number of the tillers (Anzoua et al. 2010).

Grain yield mostly depends on the total number of fertile and sterile spikelets among the panicle parameters (Matsushima 1970). Analysis of panicle topology under low N in wet season has shown the decrease is more for spikelets and grains on secondary branches (~27%) of lower portion of the panicle (~23%). However the grain filling percentage has increasing trend across the panicle which could be explained by the decrease of the number of spikelets under low N situation. The spikelet number per panicle was determined by the number of differentiated spikelets and the number of degenerated spikelets (Hoshikawa 1989). Nitrogen is needed essential initially for the development of spikelets and later for the reduction of the spikelet degeneration. Significant increase of spikelets per panicle with the increase of N application was earlier reported (Mae et al. 2006; Singh et al. 1998; Sui et al. 2013; Yoshinaga et al. 2013). The significant observation from the panicle topology studies for NUE was the overall reduction of number of spikelets and grains across the panicle and spikelets on secondary branches under low N (Rao et al. 2018). The yield increase in high yielding varieties of rice under green

revolution was attributed to the increase of the grain and spikelets number on secondary branches is dependent on the nitrogen fertilization (DRR 2008; Yamagishi et al. 2002).

N content in grain, straw and total biomass was reduced by 33 to 56% in wet season and ~46% in dry season. Genotypic differences cause differences in N use because of their inherent and internal N requirements for tissue expansion and differentiation (Singh and Buresh 1994; Yoshida 1983; Cassman et al. 1996). The nitrogen content in grain and straw and their association to biomass or yield appears to highly variable across the locations, genotypes, duration of the genotypes, stage of application and sampling as per reported studies. In our studies, we've found several genotypes with similar total N uptake, but with differential and efficient remobilization of N into yield.

In rice, NUE can simply be defined as the ratio of grain yield to N supplied. In literature, NUE has been calculated taking the output, biological yield viz., total above ground plant dry matter or total plant N or economical yield viz., grain. The N supply can be from soil, fertilizer (organic or inorganic), or soil plus fertilizer (Ladha et al. 2005). Basically NUE is the uptake efficiency (whether from indigenous soil N or fertilizer N) and the utilization efficiency of internal N to the grain yield of the plant. In addition, recovery efficiency indicating the ratio of plant N to N supplied was also often reported. The nomenclature of NUE indicators sounds similar viz., agronomic nitrogen use efficiency, physiological use efficiency, internal N use efficiency, however, the definitions and parameters used in the various reports are variable (Fagerial and Baligar 2003; Ladha et al 2005; Mahajan et al. 2012; Mae et al. 2006; Metwally et al. 2010; Sui et al. 2013; Tirol-Padre et al. 1996). NUE indicators have been calculated for low and recommended N individually viz., physiological NUE (total dry weight (g/plant)/total N absorbed (g/plant), NGPE (grain yield/total N accumulation), internal N use efficiency (INUE N grain/N plant), and NAE (plant N accumulation amount/soil available N), NGPE (grain yield/total N accumulation) (Anzoua et al. 2010). And also using the data of both low and recommended N viz., agronomic efficiency, physiological efficiency, agro physiological efficiency, apparent recovery efficiency, utilization efficiency also were calculated (Fagerial and Baligar 2003). Because of the wide genetic variability of the genotypes of our study, the range was also wide for all NUE indicators suggesting the varied mechanisms involved for N uptake and utilization in various genotypes. The useful

indicators for the identification of efficient genotypes are calculated based on mostly on grain yield.

From our studies, we observed that there is no ready and easy selection criterion from agromorphological, yield or N content traits/parameters. Based on yield under low N, we could identify several promising genotypes from the landraces as well the varieties (Table 2). The selection criterion for NUE is

Table 2. Top promising genotypes (> 10 g single plant yield) under low N

Genotypes	Single plant yield (g) Year I	Single plant yield (g) Year II
IC466475	15.4	14.6
IC576984	14.9	16.8
IC462271	12.5	14.5
IR88634:3-B-1	17.7	10.8
GQ25	14.8	15.4
Rasi	10.5	9.8
Varadhan	12.2	11.8

different in landraces and HYVs viz., the landraces were identified for their promising traits under low N to be used in breeding programs, whereas HYVs were selected based on the lesser reduction in yield with buffering capacity under low N. The short listed genotypes were further evaluated for three years and consistent NUE rice genotypes were identified, which have been used as source for developing breeding lines with NUE.

Identification of genomic regions/genes for yield under low N

NUE including N uptake, translocation, assimilation, and remobilization is inherently complex, and is governed by several quantitative trait loci (QTL) and environmental factors (Xu et al. 2012). Through molecular mapping, major QTL/genomic regions associated with NUE components in rice can be detected and can be deployed in the development of rice varieties suitable for low N situation (Vinod and Heuer 2012). Since 2001, around 200 QTL for various traits associated N metabolism and NUE have been identified using many biparental populations in rice. Association mapping is a high-resolution method for the dissection of complex genetic traits with the power of simultaneous detection of multiple loci with multiple alleles on a locus. Linkage disequilibrium (LD),

defined as the non-random association of alleles at separate loci, is a prerequisite for association mapping (Flint-Garcia et al. 2003). Using rice SSR markers, several agronomically important traits including NUE-related traits in rice have been investigated through association mapping approach (Liu et al. 2016). Through association mapping, using a marker set of 50 rice SSR markers and 472 rice genotypes, 12 genomic regions were identified for yield and yield associated traits under low nitrogen in our studies. Out of four subpopulations identified in the set of 472 genotypes, no common traits across the seasons or no common marker across all the four subpopulations indicating the complexity of NUE trait. Several positive alleles and a few negative alleles were identified for the traits across subpopulations for low and recommended N. Within alleles of same locus, some alleles showed negative effect, some showed positive effect and some showed negative effect for some traits and positive some traits in the same population e.g. RM 271. Four associated genomic regions on chromosomes 5, 7 and 10 were fine mapped and QTL for yield under low N were identified from the marker delimited regions. Three candidate genes viz., 2-oxoglutarate/malate translocator (Os05g0208000), alanine aminotransferase (Os07g0617800) and pyridoxal phosphate-dependent transferase (Os10g0189600) from QTL regions showed enhanced expression in the genotypes with promising yield under low N (Rao et al. 2018). With rapid development of sequencing technologies and computational methods, Genome-Wide Association Studies (GWAS) is becoming a powerful tool for detecting natural variation underlying complex traits in crops, especially in rice using SNPs (Wang et al. 2017).

Using the donors identified for NUE, several populations were developed for mapping. A set of 291 recombinant inbred lines between PTB1 (NUE variety) and BPT5204 (popular variety) were screened under low and recommended nitrogen for two seasons. Polymorphism survey and selective genotyping showed five genomic regions associated with yield under low nitrogen. With the saturation of the identified genomic regions with more RM markers, several markers trait associations under low N were identified using single marker analysis (Data not shown). A subset of 96 RIL population derived from Rasi (NUE variety) and Vibhava (high yielding variety) was screened under low and recommended N and the promising RIL were identified (Table 3). Validation of SSR markers from the association mapping study (Rao

Table 3. Mean and range of agro-morphological traits of 96 RILs of Rasi and Vibhava grown under low and recommended N

	Low N			Recommended N		
	Min.	Max.	Mean	Min.	Max.	Mean
Leaf length (cm)	13.0	45.0	26.7	17.2	50.3	32.1
Leaf width (cm)	0.1	1.1	0.7	0.8	1.9	1.1
Plant height(cm)	47.0	95.3	72.8	76.3	131.0	96.2
Total number of tillers/plant	2.3	6.7	3.8	4.3	11.7	7.1
Total no. of productive tillers/plant	2.3	6.7	3.6	4.3	11.3	7.1
Total no. of panicles/plant	2.6	7.0	4.0	2.6	10.4	6.6
Weight of total panicles (g)/plant	0.9	14.9	5.6	4.0	19.0	10.5
Total grain weight(g)/plant	0.6	14.2	5.2	3.9	18.0	9.8
Total dry matter (g)/plant	3.0	14.3	7.8	6.9	31.3	17.6

Min. = Minimum; Max. = Maximum

et al. 2018) showed seven traits under low N with eight RM markers and 24 traits with 13 RM markers under recommended N found to be associated in the RIL population (Table 4).

Table 4. Association between RM markers and phenotypic traits ($P < 0.05$) of 96 RIL population of Rasi and Vibhava using GLM and MLM analysed by TASSEL 3.0

Marker	Trait
RM534	Total grain weight(g)/plant
RM11	Total dry matter(g)/plant
RM495	Total number of tillers/plant
RM495	Total number of productive tillers/plant
RM1381, RM190	Plant height(cm)
RM452	Leaf width (cm)
RM495	Leaf length (cm)

Transcriptome sequencing of two efficient (Basmati 370 and Thurur Bhog) and two poor genotypes (Kola Joha and Suraksha) at recommended and low nitrogen at panicle developmental stage using the 454 Roche and IlluminaHiSeq 2000 platforms. In the efficient genotypes, the number of down-regulated transcripts were higher in number than up-regulated

and vice-versa was recorded in poor genotypes (Data not shown).

The candidate genes of N metabolism in rice are well characterized and differential expression of several candidate genes directly involved in N metabolism and transcription factors in relation to NUE has been earlier reported in rice under differential N applications (Li et al. 2017). Expression analysis of *OsSPL14* (LOC_Os08g39890) gene reported to be associated with increased panicle branching and higher grain yield through real time PCR in leaf and three stages of panicle has shown differential temporal expression and its association with yield and yield related components across the genotypes. The expression of *OsSPL14* at panicle stage 3, has shown correlation ($P < 0.05$) with N% in grain. Since *OsSPL14* is a functional transcription activator, its association of expression in leaf and three panicle stages with yield components as observed in the present study suggests the role of nitrogen metabolism related genes in plant growth and development and its conversion into yield components in rice (Srikanth et al. 2015).

Several recombinants of yield and NUE were identified from evaluation under low N. These breeding lines were evaluated under multi-locations and stable performers were identified under All India Coordinated Rice Improvement Project (AICRIP) Trial - Evaluation of Radiation and Nitrogen use efficient promising rice genotypes -Plant Physiology during Kharif 2016, 2017 and 2018 (<http://www.icar-iirr.org/>).

Conclusion

As nitrogen is the building block of biomass, an optimum N is required for realizing the yield. So, the strategy should be maximum uptake, maximum utilization and maximum remobilization of the optimum N inputs to give maximum possible yield. From the observations of the studies conducted at IIRR and reported from other studies across the world, the genotypes do exist in rice with differential ability for maximum uptake, utilization and remobilization under low N. However, all the three traits are not observed in a single genotype. Thus, these genotypes can be donors for some of the critical traits for yield under low N e.g. grains on secondary branches. Remobilization or conversion of the available N into yield appears to be most important mechanism for NUE in rice. With advent of rice genome sequencing and next generation sequencing, the identification of allelic variation for NUE appears to be a promising

strategy. With the resources of information of candidate genes associated with N metabolism from rice genome sequencing, their expression pattern using transcriptomics and germplasm identified for NUE at IIRR, the genomic regions and the alleles of candidate genes associated with NUE are being identified at IIRR and other research institutes. But the generated information is still to be deployed in the development of rice breeding lines with NUE through conventional breeding approach. Meanwhile, the selection based on yield under low N found to be reasonably successful for breeding rice lines with relative higher yield under low N and the evaluation is under progress through AICRIP trials.

Declaration

The authors declare no conflict of interest.

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