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Biochemical and physiological characterization for nitrogen use efficiency in aromatic rice genotypes

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ABSTRACT

In a set of 78 aromatic rice genotypes, cluster analysis was performed for yield and its related traits in field under two nitrogen (N) conditions viz., application of N fertilizer (N100) and without application of N fertilizer (N0) during wet season, 2011 and dry season, 2012. Basmati370 and Ranbir basmati were selected as high nitrogen use efficiency (NUE) genotypes and Kolajoha-3 and Ratnasundari as low NUE genotypes for characterization in terms of biochemical, physiological and agronomical aspects of NUE. A total of 32 biochemical, physiological and agronomical characters were measured in the selected four genotypes, growing in field under two N levels i.e., N0 and N100 during wet season 2012. Five efficiency parameters were also studied to determine their NUE. GS activity increased under low N and the increase was more in two high NUE genotypes (41.3%) than that of two low NUE genotypes (5.43%). NR activity increased with application of N fertilizer and low NUE genotypes expressed higher NR activity (8.8% and 2.02% more in N0 and N100 respectively). Chlorophyll content recorded maximum (3.6 mg g^{-1}) in low NUE genotypes under N100 condition, where as the chlorophyll content was minimum (0.43 mg g^{-1}) in high NUE genotypes under N0 condition. Electron transport rate (ETR), quantum yield (Φ_{PSII}) and F_v/F_m were not affected by N levels but there were significant variations in non-photochemical quenching (q_N) (15% more in N0) and photochemical quenching (q_P) (25% more in N0). Grain yield, total dry matter and N uptake by grain and straw were higher in high NUE genotypes. Higher GS activity, maintenance of sufficient chlorophyll fluorescence and chlorophyll content in case of high NUE genotypes support their higher grain yield and total dry matter content under low N conditions by efficient N uptake, and utilization of nitrogen.

1. Introduction

Nitrogen (N) is a very important nutrient for plant growth and development. India stands third and second in N fertilizer consumption and production respectively (FAI, 2008). Nitrogen is one of the most expensive though but an essential nutrient to plant as the commercial N fertilizers represent the major cost in plant production (Singh, 2005). Though, application of N fertilizers increases crop yields, increased use of N fertilizers effects global N cycle, depletion of ozone layer and also causes nitrate leaching problems in soil (Hakeem et al., 2012). Moreover, crop plants are able to utilize only 30–40% of applied nitrogen for food production and the remaining N is left over into environment leading to hazardous environmental pollution by N contamination (Raun and Johnson, 1999). Genetic variations in N uptake and/or grain yield per unit of N applied has also been studied in different crops such as wheat, rice, maize, sorghum, and barley (Ortizmonasterio et al., 1997; Muchow, 1998; Le Gouis et al., 2000; Presterl et al., 2003; Anbessa et al., 2009; Namai et al., 2009). Nitrogen use efficiency is relatively low in rice as major part of N applied to rice is released as gaseous N, effecting environment and reducing economic efficiency of applied N (Hakeem et al., 2012). Nitrogen use efficient (NUE) genotypes/species can be defined as the plants which can

Abbreviations: GS, glutamine synthetase; NR, nitrate reductase; NUE, nitrogen use efficiency; P_N, rate of photosynthesis; g_s, stomatal conductance; C_i, intercellular CO_2 concentration; *E*, *transpiration* rate; TE (P_N/T), transpiration efficiency; WUEi (P_N/g_s), intrinsic water use efficiency; P_N/C_i ratio, carboxylation efficiency; F_v/F_m, maximum photochemical efficiency of PSII; ETR, electron transport rate; Φ_{PSII}, in vivo quantum yield of PSII photochemistry; q_P, coefficient of photochemical quenching; q_N, coefficient of non photochemical quenching; chl, chlorophyll; car, carotenoids; FM, fresh mass.

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absorb and accumulate higher N content and grow well and yield better under low N conditions (Mi et al., 2007). Genetic variation in nitrogen use efficiency in rice was earlier reported (Broadbent et al., 1987; DeDatta and Broadbent, 1988; Tirol-Padre et al., 1996; Inthapanya et al., 2000; Zhang et al., 2008). It is very important to identify or develop high NUE genotypes in rice for its production under low cost crop management practices and also to protect environment (Lea and Azevedo, 2006; Hirel et al., 2007).

A comprehensive knowledge on physiological, biochemical and molecular aspects of NUE particularly in low N environment is crucial for developing NUE varieties. Plants absorb nitrogen in the form of nitrate (NO_3^-) and ammonia (NH_3) from the soil through root transporter systems and it is assimilated by a series of nitrate assimilatory enzymes, *viz*., nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS) and glutamate synthetase (GOGAT). Glutamine synthetase (GS) is a key enzyme in ammonia assimilation, converts ammonium to glutamine. Ammonium is also produced via few internal metabolic reactions such as photorespiration, nitrate/nitrite reduction, storage molecules, and amino acid conversion (Ireland and Lea, 1999; Vijayalakshmi et al., 2013). GS exists in two forms viz., cytosolic GS (GS1) and chloroplastic GS (GS2). GS1 is critical for normal growth and grain filling where as GS2 is used to recycle assimilated ammonia, derived from photorespiration and also involved in photosynthesis. In C_3 plants such as tobacco, photo respiratory ammonium was produced from oxidative decarboxylation of glycine. The amount of ammonia released during photorespiration is up to 10-fold greater than the amount of primary nitrogen taken up by the plant (Keys et al., 1978; Maheswari et al., 1993). The earlier studies revealed that the cytosolic GS played a key role in nitrogen remobilization for grain filling in wheat, rice and maize (Tabuchi et al., 2007; Martin et al., 2006). Nitrate reductase (NR), present in cytosol, is one of the foremost metabolic enzymes in plants involved in the reduction of nitrate to nitrite (Chandna and Hakeem, 2013). This enzyme shows great variability in its activity at different levels of N (Hakeem et al., 2012) and there are also genotypic differences in NR activity in rice (Chandna et al., 2010).

Total chlorophyll content increased with increasing nitrogen levels, resulting in higher photosynthesis rates leading to more sugar formation (Pramanik and Bera, 2013; Dikshit and Paliwal, 1989). Achieving higher yield with reduced nitrogen fertilization, without considerable effects on the normal physiological processes of functional leaves, has become an important challenge in rice (Long et al., 2007). The relation between slow or controlled release of N fertilizers, supporting more N absorption and related physiological mechanisms have been well studied (Liu et al., 2002; Li et al., 2004; Luo et al., 2007; Long et al., 2013). But there are only a few studies on the effect of nitrogen fertilizer on rice leaf absorption, transmission and distribution of light energy, dissipation of excess excitation energy and related mechanisms. Chlorophyll fluorescence is a quick tool to study the plant photosynthetic capacity using rapid light curve with less impact on leaf (Ralph et al., 1998; Kühl et al., 2005; Gitelson et al., 1999). Electron transport rate (ETR) of the flag leaf was affected mostly by the photosynthetically active radiation and by nitrogen levels during the initial heading stage to some extent. Chlorophyll fluorescence studies suggested a nitrogen rate of 135–180 kg/h for super hybrid rice for improving the photosynthetic electron transport rate, the effective quantum yield and the PS reaction centre openness (Long et al., 2013). The light-saturated photosynthetic rate is correlated with nitrogen content of leaf and Rubisco content. Higher photosynthetic rate at higher N levels was attributed to higher chloroplast CO_2 content, chloroplast size, Rubisco activity and carboxylation capacity (Yong et al., 2009).

Agronomic practices and environmental factors related to N use, grain yield and N accumulation can be measured by determining NUE in cereal based agro ecosystems (Hugins and Pan, 2003). Nitrogen use efficiency in rice can be divided into different efficiencies namely agronomic efficiency (AE), physiological efficiency (PE), agro physiological efficiency (APE), apparent recovery efficiency (ARE) and utilization efficiency (UE). Agronomic efficiency is defined as the response of crop/genotype to applied fertilizer or profitable production obtained per unit of nitrogen applied (Aynehband et al., 2012; Fageria et al., 2010). Physiological efficiency is defined as biological yield per unit nutrient uptake or represents grain yield or plant biomass relative to nitrogen uptake (De Datta, 1986; Peng et al., 2002). Apparent recovery efficiency, as the financial production of grain yield obtained per unit of nutrient uptake (Fageria et al., 2010) and UE as the capacity of the plant to assimilate N and remobilize the N taken up from the soil, producing into amino acids resulting in final grain yield (Moll et al., 1982; Good et al., 2004; Moose and Below, 2009). Genotypic variation in the above NUE indices exists in rice. Classifying these genotypes by using cluster analysis to disperse genotypes into qualitative groups based on response similarities was shown earlier (Yau, 1991; Rincon et al., 1996; Sezener et al., 2006). This method is based on Euclidean distances among group means and produces a dendrogram showing successive combination of individuals. Cluster analysis in the present study was undertaken for classifying 78 aromatic rice genotypes as high NUE and low NUE genotypes based on yield and its related traits performance in field under two N conditions viz., with recommended application of N fertilizer; @ 100 kg of urea (N100) and without application of N fertilizer (N0) during wet season 2011 and dry season 2012. The present work was also planned to investigate biochemical viz., GS and NR activities, photosynthetic viz., photosynthetic pigments content, leaf area and thickness, chlorophyll fluorescence, gaseous exchange parameters and NUE related parameters viz., % N content and N uptake in grain and straw and NUE indices in two genotypes with high NUE and low NUE under two N conditions, N0 and N100 during wet season 2012.

2. Materials and methods

2.1. Experimental conditions and genotypes screening

A total 78 genotypes, belong to aromatic group were evaluated for their agronomic performance under low nitrogen level (N0) and recommended nitrogen level (N100) with application of 100 kg urea/ha in field conditions during two consecutive seasons of wet (kharif) 2011 and dry (rabi) 2012 at Directorate of Rice Research (DRR), Hyderabad, India. For screening the genotypes under field conditions with two different N levels, a nitrogen deficient plot (N0) of dimensions 19.0 m length and 24.5 m width was developed and maintaining at DRR since wet season, 2010 along with a plot supplied with recommended dose of nitrogen (N100). Soil samples were collected from these plots before start of the experiment in wet season 2011and dry season 2012 to determine the initial soil properties. Soil samples were collected from 4 different areas in plot and the mixed composite was used to determine N content by using semi micro Kjeldahl method (Kjeldahl, 1883). The details of soil properties of both N0 and N100 plots were given in Table 1. The soil pH and EC are normal and organic carbon content is medium.

Hierarchical cluster analysis was carried out using Euclidean distance metric and UPGMA method (un-weighted paired group method and arithmetic averages) (web: http://darwin.cirad.fr/darwin) and data of five traits viz., panicle weight, filled grain weight, total grain weight (filled grain and unfilled grain), dry straw weight, total dry matter (dry panicle and stover) from each season and each environment. Cluster analysis was performed using the data in the following nine different pooled data sets of season and

Table 1
Soil properties of two N level fields before beginning of experiment.

Season	Soil property	Without application of N (N0)	With application of N (N100)
Wet season 2011	pH	7.2	7.29
	Electronic conductivity (dS/M)	0.22	0.24
	Organic carbon (%)	0.58	0.69
	Available N (kg/ha)	202	220
	Available P_2O_5 (kg/ha)	56	60
	Available K_2O (kg/ha)	628	682
Dry season 2012	pH	7.42	7.29
	Electronic conductivity (dS/M)	0.26	0.28
	Organic carbon (%)	0.66	0.72
	Available N (kg/ha)	243	255
	Available P_2O_5 (kg/ha)	52	63
	Available K_2O (kg/ha)	692	705
Wet season 2012	pH	7.46	7.42
	Electronic conductivity (dS/M)	0.29	0.28
	Organic carbon (%)	0.66	0.72
	Available N (kg/ha)	233	252
	Available P_2O_5 (kg/ha)	50	61
	Available K_2O (kg/ha)	721	789

treatment: (1) wet season N0; (2) wet season N100; (3) wet season both N0 and N100; (4) dry season N0; (5) dry season N100; (6) dry season both N0 and N100; (7) N0 data of both wet and dry seasons; (8) N100 data of both wet and dry seasons; (9) all four combination of N0 and N100 data of wet and dry seasons. Dendrogram trees were constructed for all these pooled data sets.

Based on above cluster analysis results, genotypes viz., Basmati 370 and Ranbir basmati were selected as high NUE whereas Kolajoha-3 and Ratnasundari were chosen as low NUE genotypes for further biochemical, physiological and NUE studies. Total 32 parameters related to biochemical, physiological and agronomic traits were characterized in the selected four genotypes, growing in field under two different N levels i.e., N0 and N100 during wet season 2012 at DRR. Activity of Nitrogen assimilatory enzymes viz., glutamine synthetase (GS) and nitrate reductase (NR), chlorophyll content (chl a and chl b), carotenoids content (car), chlorophyll fluorescence and photosynthetic parameters were measured in flag leaves using following methods. Grain weight, total dry matter, % N content and N uptake in both grain and straw were also measured and various efficiencies related to NUE were calculated using these observations. All the above observations were taken in three replications.

2.2. Estimation of GS enzyme activity

The enzymatic activity was determined using the methodology proposed by Rowe et al. (1970). 500 mg of leaf tissue was extracted with 3 ml of Tris-buffer (pH 7.5) containing 0.765 g of Tris–HCl, 0.11 g of cysteine hydrochloride, 0.246 g of $MgSO_4{\cdot}7H_2O$ and 0.02 g EDTA was dissolved in 100 ml of water. The pH of the extraction medium is adjusted to 7.5 and final volume is made up to 250 ml. Centrifuge the homogenate for 45 min at 10,000 rpm at 4 °C. The supernatant was used as enzyme extract. This crude extract was assayed for enzymatic activity. Reaction mixture containing Tris–HCl buffer (pH 8.0), ATP, sodium glutamate, $MgSO_4$, L-cysteine, and hydroxylamine was added to enzyme extract. Reaction was started by the addition of hydroxylamine. The mixture was incubated at 30 °C for 30 min. Reaction was stopped by the addition $FeCl_3$ mixture containing 10% $FeCl_3$, 20% TCA, 50% HCL (1:1:1) ratio. Glutamyl hydroxymate (GH) was measured at the absorbance of 540 nm by using the (Spectrascan 2600, Chemito). The GS activity was calculated by using the standard curve of γ-glutamyl hydroxymate and was expressed as $\mu mol/g^{-1}\ h^{-1}$.

2.3. Estimation of NR enzyme activity

The NR activity was estimated by using the method described by (Hageman and Hucklesby, 1971). 500 mg of freshly harvested flag leaf tissue was cut into small pieces and transferred into test tubes containing 2.5 ml of 25 mmol potassium phosphate buffer (pH 7.5) containing 5 mmol cysteine hydrochloride, 1 mmol EDTA and 1 mmol DTT Centrifuge the homogenate for 45 min at 10,000 rpm at 4 °C. The 0.3 ml of supernatant was used immediately for enzyme assay. Enzyme extract and reaction mixture containing 0.1 mol potassium phosphate buffer (pH 7.5), 0.1 mol potassium nitrate, and 14 mg/10 ml of NADH was incubated at 33 °C for 30 min. After incubation 1 ml of sulphanilamide and 1 ml of NEDD reaction was stopped by the addition of zinc acetate. Nitrite, produced was estimated at 540 nm using a (Spectrascan UV2600, Toshniwal Instruments (India) Pvt. Ltd.). The enzyme activity was expressed as $\mu M/(g^{-1}\ h^{-1})$.

2.4. Leaf pigment extraction and quantification

One gram of the fresh leaf tissue was cut into small pieces and placed into a volumetric flask containing 25 ml of 80% acetone (Porra et al., 1989) and stored in the dark for 1–2 days. The absorbance of the extracted solution was measured at 663.2, 646.8 and 470 nm using a UV–vis double beam spectrophotometer (Spectrascan 2600, Chemito) to estimate chlorophyll a, chlorophyll b and carotenoids content using formulas reported by Lichtenthaler and Wellburn (1983).

2.5. Measurement of chlorophyll fluorescence

Chlorophyll fluorescence was measured with a portable PAM-210 fluorometer (Walz, Effeltrich, Germany) connected to a desktop computer. The fluorescence parameters were calculated: maximum efficiency of open PS II reaction centres in the light, $F_V'/F_M' = (F_M' - F_O')/F_M'$; quantum yield of PS II photochemistry, $FPS\ II = (F_M' - F_S)/F_M'$ (Genty et al., 1989); non-photochemical quenching, $q_N = 1 - [(F_M' - F_O')/(F_M - F_O)]$; and photochemical quenching $q_P = (F_M' - F_S)/(F_M' - F_O')$ (Schreiber et al., 1986); where F_O is the minimum fluorescence signal of dark adapted leaves, F_M is the maximum fluorescence signal of dark adapted leaves after a pulse of saturating light, F_S is the minimum fluorescence signal of an illuminated leaf, F_O' is the minimal fluorescence signal of light adapted leaves and F_M' is the maximum

fluorescence signal of an illuminated leaf after a pulse of saturating light. For the determination of F_M and F_O, leaves were dark adapted for one hour before measurements. For the measurements of F_O', light adapted leaves were rapidly covered with a black cloth after the saturation pulse and 50 mmol m^{-2} s^{-1} of far-red light were applied for 5 s.

2.6. Specific leaf mass, specific leaf area and leaf thickness

Leaf area of flag leaf was measured using electronic leaf area metre, LI-3100C (LI-COR Environmental, USA) and its fresh and dry weights were measured using digital weighing balance. Leaf thickness (in mm) was measured with the help of digital callipers (06-664-16, Fisher Scientifics, USA). Specific leaf area (SLA) and specific leaf weight (SLW) were calculated by using the formulas reported by Radford (1967).

2.7. Leaf photosynthetic characters

Leaf photosynthetic characteristics were measured between at 10 and 13 h on 50th day of transplantation on flag leaves using LI6400XT portable photosynthesis measuring system (LI-COR Environmental, USA) connected to Leaf Chamber Florometer (6400-40, LI-COR, USA) which was used as light source (Vishnukiran et al., 2013). During photosynthesis measurements, leaf temperature was maintained at ambient conditions of temperature (35 °C), PAR (1000 μM m^{-2} s^{-1}) and CO_2 levels (387 μM mol^{-1}). By using this equipment eight leaf photosynthetic traits were measured viz., photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (T), internal CO_2 concentration (C_i), Internal CO_2 concentration/ambient CO_2 ratio (Ci/Ca), intrinsic water use efficiency, WUEi, (P_N/g_s), transpiration efficiency TE (P_N/T) and carboxylation efficiency CE (P_N/C_i).

2.8. N estimation and efficiencies

At physiological maturity, panicles from each plant were harvested, sun dried, threshed, cleaned and weight of grains was recorded and expressed in g/plant. Similarly, straw weight was measured after drying each plant without panicles, in oven at 70 °C. Dried leaves and grains were powdered and the N content was determined by using the semi-micro Kjeldahl procedure (Kjeldahl, 1883). N uptake by grain and straw, agronomic efficiency (AE), physiological efficiency (PE), agro physiological efficiency (APE), apparent recovery efficiency (ARE) and utilization efficiency (UE) were determined by using formula provided by Fageria et al. (2010).

2.9. Statistical analysis

For each trait, mean values for both high NUE and low NUE genotypes were calculated. Differences among treatment, genotypes and genotype verses treatment means were determined using the least significant differences (LSD) comparison method in Statistica 8.1 System (SAS, 1989).

3. Results

3.1. Cluster analysis

All nine dendrograms constructed by cluster analysis with different data sets as described in materials and methods showed mainly 3–4 clusters, classifying genotypes into high, medium and low performance genotypes. In case of wet N0 and dry N0 treatments, the selected genotypes for low NUE viz., Kolajoha-3 and Ratnasundari were found in the same where as high NUE viz., Basmati 370 and Ranbir Basmati were found to be in two different clusters (Figs. 1 and 2). The dendrogram resulted from pooled data of wet N0 and dry N0 also showed Basmati 370 as high, Ranbir basmati as medium and the other two genotypes (Kolajoha-3 and Ratnasundari) as low efficient genotypes (Fig. 3). As per wet season, N100 dendrogram, Ranbir Basmati and Ratnasundari were in the same cluster and Basmati 370 and Kolajoha-3, in two different groups (Supplementary Figure 1). The same results were also shown by tree constructed using pooled data of all four environments (Supplementary Figure 2). Dry season N100 dendrogram showed that all four selected genotypes except Basmati 370 were in the same main cluster though belonged to different sub clusters (Supplementary Figure 3). The average dissimilarity between two high NUE genotypes viz., Basmati 370 and Ranbir Basmati was 10.8 where as between two low NUE genotypes viz., Kolajoha-3 and Ratnasundari was 6.6. The average dissimilarity between selected high NUE and low NUE genotypes was 58. These four genotypes were selected for further study of biochemical, photosynthetic and NUE studies and the results were shown in Table 2.

3.2. GS activity

GS activity was 41% and 5% more in leaves of N0 treatment than that of in N100 in high NUE and low NUE genotypes respectively. Significant difference between treatments was exhibited only in case of high NUE. Substantial difference between GS activity of high NUE and low NUE genotypes was observed only in N0 levels but not in N100. The GS activity in high NUE genotypes was 1.76 and 1.09 folds that of low NUE in case of N0 and N100 treatments respectively.

3.3. NR activity

Leaves from N0 treatment showed significantly lower NR activity than that of from N100 treatment in case of both high and low NUE genotypes. The NR activity in N100 was increased by 77% and 79% in high and low NUE genotypes respectively when compared to that of in N0. With regard to genotypes, NR activity was more in low NUE genotypes than in high NUE and the difference was significant in N100 environment only but not in N0 unlike GS activity.

3.4. Leaf pigment content

Significant differences were observed between genotypes for chl a and chl b contents in both N0 and N100 levels. These pigment levels were more in low NUE genotypes when compared with pigment levels in high NUE genotypes in case of both N treatments. Total chlorophyll content in low NUE genotypes was five and two times that of high NUE genotypes in N0 and N100 treatments respectively. Chl a/b ratio was significantly different between treatments in case of high NUE genotypes (2.4) only but not in low NUE genotypes (0.7). There was no considerable difference for carotenoids content and total chl/car ratio either between treatments or genotypes.

3.5. Leaf thickness, SLA, SLM and leaf area

Application of N fertilizer increased leaf area, leaf thickness and SLW by 4%, 18% and 11% respectively but decreased SLA by 8% in case of high NUE genotypes. However these changes were not significant except for leaf thickness. With regard to genotypic variances, high NUE genotypes showed higher values for all these traits except for leaf thickness and SLW when compared with those of low NUE genotypes. Specific leaf area in high NUE genotypes was 1.24 times that of low NUE genotypes in N100 treatment where as in N0, it was only 1.05 times. Specific leaf width was significantly more in low NUE genotypes (2 times more) when compared with that of high NUE.

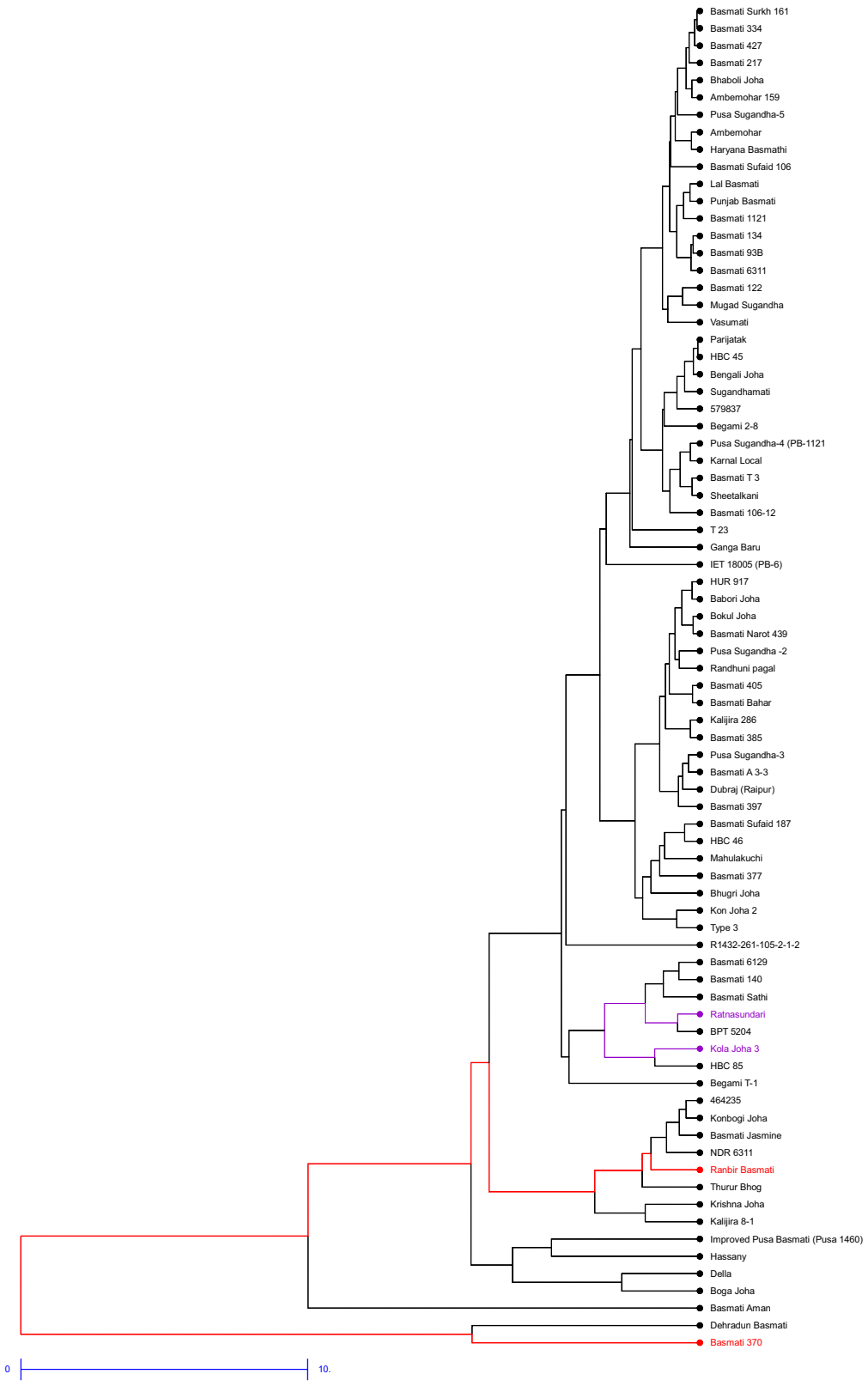

Fig. 1. Dedrogram of 78 aromatic rice genotypes by UPGMA method based on yield and its related traits in N0 treatment during wet season 2011.

3.6. *Chlorophyll fluorescence parameters*

There were no major changes in values of F_v/F_m and ETR between two N treatments but in case of Φ_{PSII}, q_N and q_P, there was considerable decrease in its values in N100. The decrease was 17, 18 and 34 percent for Φ_{PSII}, q_N and q_P respectively in high NUE genotypes. In case of F_v/F_m, significant variations were not observed between high NUE and low NUE genotypes. High NUE genotypes exhibited significantly higher (21–10% more) Φ_{PSI} and q_P than that of low NUE genotypes in N0 levels. ETR in high NUE

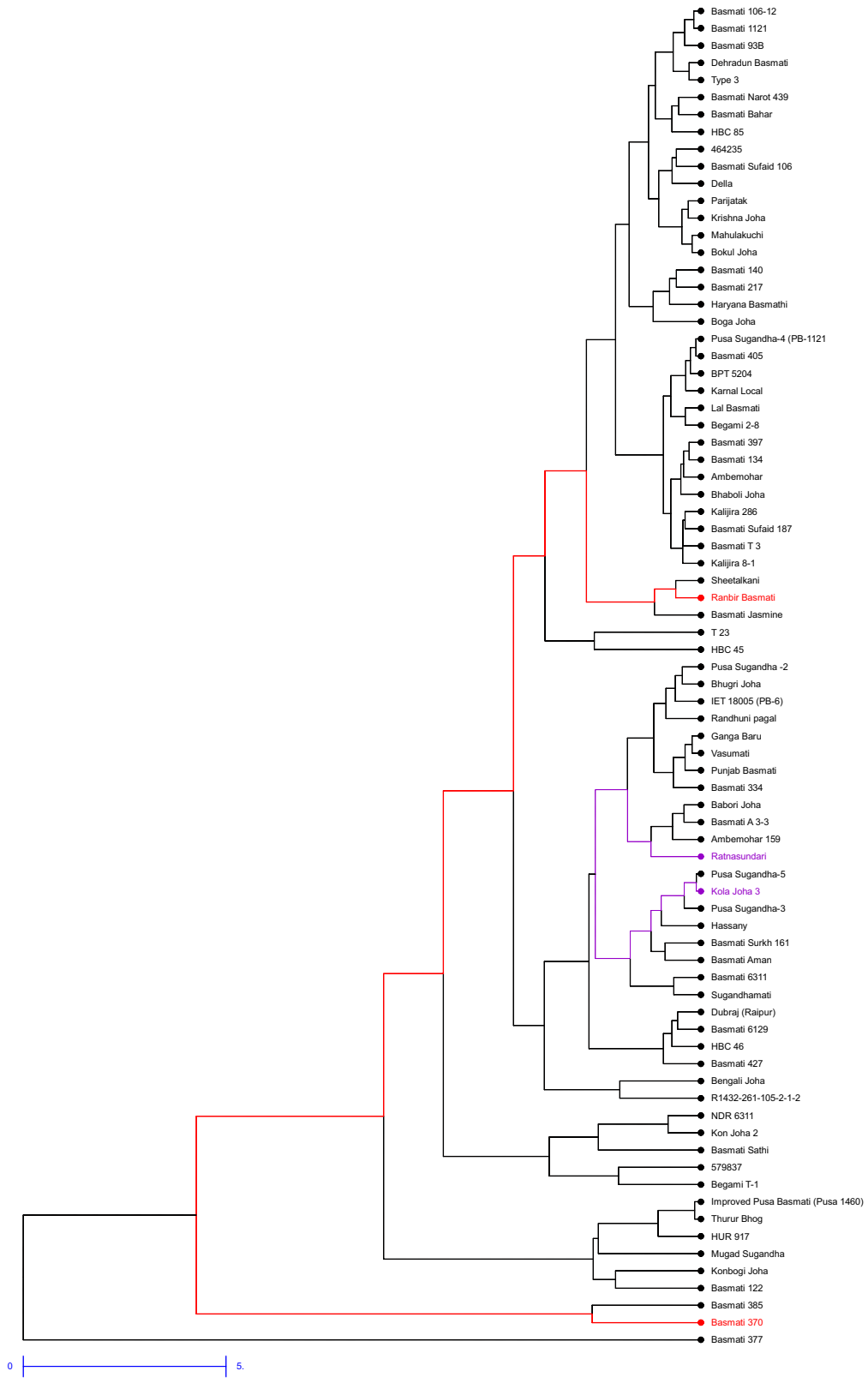

Fig. 2. Dedrogram of 78 aromatic rice genotypes by UPGMA method based on yield and its related traits in N0 treatment during dry season 2012.

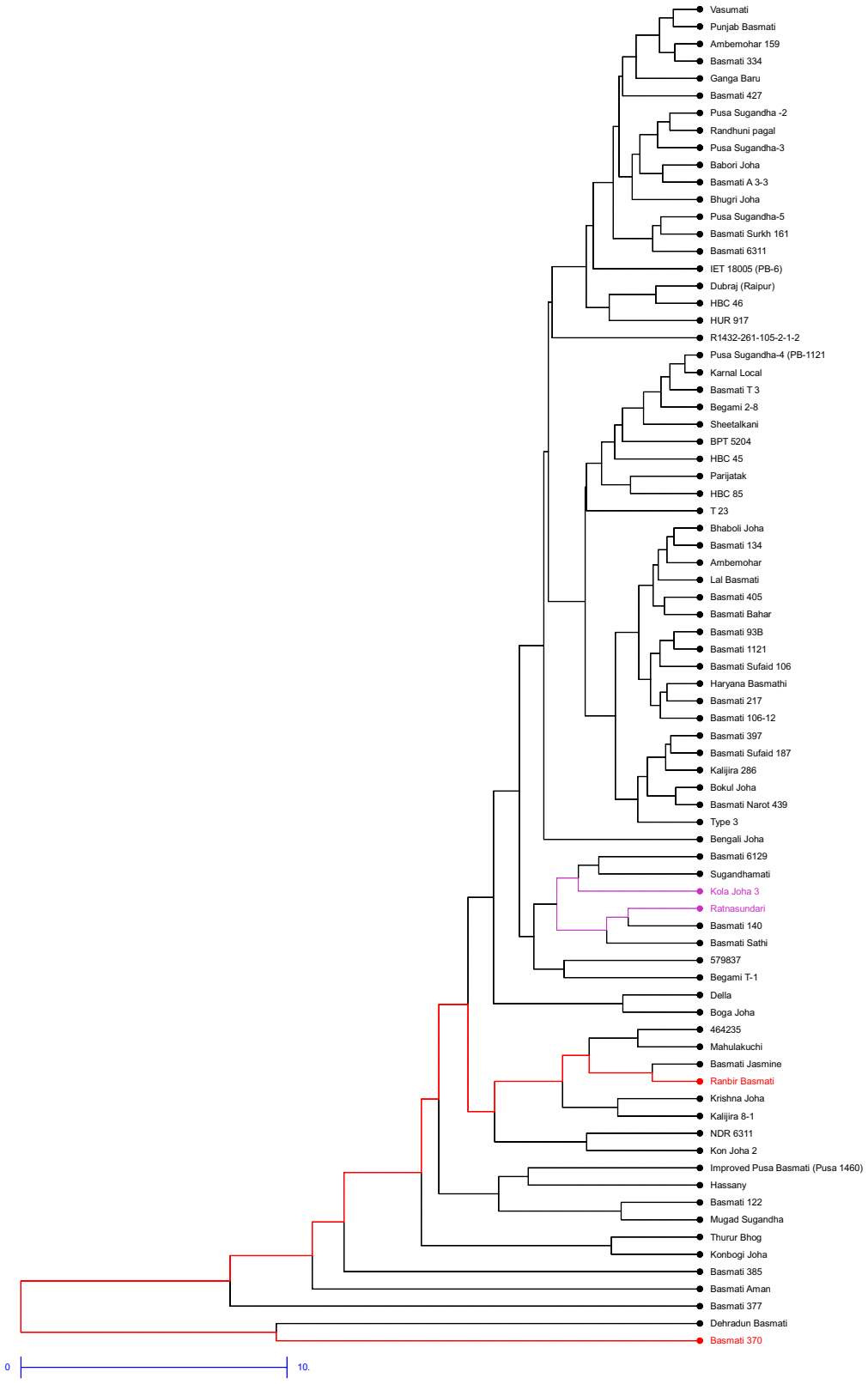

Fig. 3. Dedrogram of 78 aromatic rice genotypes by UPGMA method based pooled data of yield and its related traits in N0 treatments of both wet 2011 and dry season 2012.

Table 2
Mean performance of high NUE and low NUE genotypes and least significant differences (LSD) for 32 traits in two N treatments.

Source	Mean				LSD between		
Treatment	Without application of N (N0)		With application of N (N100)		Treatment	Genotype	Treatment * genotype
Trait/Genotype	High NUE	Low NUE	High NUE	Low NUE			
Glutamine synthetase (μmol g^{-1} h^{-1})	16.2^{a}	9.2^{b}	9.5^{b}	8.7^{b}	1.320	1.320	1.870
Nitrate reductase (μmol g^{-1} h^{-1})	11.3^{c}	12.4^{c}	49.4^{b}	59.3^{a}	2.300	2.300	3.200
Chlorophyll a (mg g^{-1})	0.29^{c}	1.44^{b}	1.74^{b}	2.86^{a}	0.685	0.685	0.960
Chlorophyll b (mg g^{-1})	0.14^{c}	0.61^{ab}	0.45^{b}	0.82^{a}	0.190	0.190	0.270
Chlorophyll (a + b) (mg g^{-1})	0.43^{c}	2.0^{b}	2.19^{b}	3.6^{a}	0.750	0.750	1.060
Carotenoids (mg g^{-1})	0.048^{a}	0.29^{a}	0.69^{a}	0.71^{a}	0.470	0.470	0.667
Chlorophyll a/b ratio	2.1^{b}	2.8^{b}	3.8^{a}	3.5^{ab}	1.160	1.160	1.600
Chlorophyll/carotenoid ratio	14.2^{a}	12.6^{a}	11.0^{a}	12.6^{a}	14.000	14.000	19.800
Leaf area (mm)	13.9^{a}	11.1^{a}	14.6^{a}	14^{a}	3.200	3.200	4.600
Specific leaf area (SLA) (mg cm^{-2})	198.3^{a}	188.4^{a}	183.7^{a}	148.1^{a}	55.900	55.900	79.000
Specific leaf weight (SLW) (mg cm^{-2})	0.53^{a}	0.54^{a}	0.6^{a}	1.6^{a}	1.190	1.190	1.600
Leaf thickness (mm)	0.09^{b}	0.11^{ab}	0.11^{a}	0.13^{a}	0.014	0.014	0.020
Maximum photochemical efficiency of PSII (F_v/F_m)	0.76^{b}	0.74^{b}	0.79^{a}	0.78^{a}	0.013	0.013	0.019
Electron transport rate (ETR)	21.2^{a}	17^{ab}	21.2^{a}	17.1^{b}	2.600	2.600	3.700
Quantum yield of PSII photochemistry (Φ_{PSI})	0.33^{a}	0.26^{b}	0.28^{b}	0.25^{b}	0.030	0.030	0.040
Coefficient non-photochemical quenching (q_N)	0.72^{a}	0.67^{ab}	0.61^{bc}	0.54^{c}	0.050	0.050	0.070
Coefficient of photochemical quenching (q_P)	0.59^{a}	0.47^{b}	0.44^{bc}	0.38^{c}	0.050	0.050	0.080
Net photosynthetic rate (P_N) [μmol (CO_2) m^{-2} s^{-1}]	9.05^{b}	14.175^{a}	13.9^{a}	15.7^{a}	1.541	1.541	2.180
Stomatal conductance (g_s) [mol (H_2O) m^{-2} s^{-1}]	0.15^{c}	0.25^{b}	0.25^{b}	0.35^{a}	0.046	0.046	0.065
Internal CO_2 concentration (C_i) [μmol mol^{-1}]	245.6^{b}	244.4^{b}	283.0^{a}	286.8^{a}	26.171	26.171	37.011
Transpiration rate (T) [mmol (H_2O) m^{-2} s^{-1}]	4.08^{c}	5.8^{b}	7.8^{a}	9.3^{a}	1.215	1.215	1.718
Internal CO_2 concentration/ambient CO_2 ratio (C_i/Ca)	0.7^{a}	0.7^{a}	0.725^{a}	0.75^{a}	0.097	0.097	0.137
Transpiration efficiency (TE) (P_N/T)	2.4^{ab}	2.45^{a}	1.825^{bc}	1.675^{c}	0.439	0.439	0.620
Intrinsic water use efficiency, WUEi, (P_N/g_s)	64.407^{a}	62.902^{a}	50.267^{a}	46.123^{a}	16.424	16.424	23.227
Carboxylation efficiency, CE (P_N/C_i)	0.0375^{b}	0.0575^{a}	0.05^{a}	0.0575^{a}	0.008	0.008	0.011
Grain weight (g/plant)	16.495^{a}	1.485^{b}	23.41^{a}	3.91^{b}	6.365	6.365	9.002
Total dry matter (g/plant)	34.84^{ab}	9.005^{b}	51.96^{a}	15.305^{b}	23.510	23.510	33.248
Nitrogen % in grain	1.08^{ab}	0.935^{b}	1.29^{a}	1.18^{ab}	0.208	0.208	0.295
Nitrogen % in straw	0.550^{ab}	0.46^{b}	0.5824^{a}	0.4914^{ab}	0.074	0.074	0.105
Nitrogen uptake in grain (kg/ha)	54.377^{a}	1.504^{a}	49.86^{a}	11.432^{a}	76.462	76.462	108.130
Nitrogen uptake in straw (kg/ha)	27.85^{ab}	8.612^{b}	46.582^{a}	11.948^{ab}	26.816	26.816	37.923
Total nitrogen uptake in grain + straw (kg/ha)	82.23^{b}	10.12^{c}	145.44^{a}	23.38^{bc}	41.743	41.743	59.033

Note: Each value represents mean of two genotypes in case of both high NUE and low NUE; Means followed by different letters for the same trait are significantly different.

genotypes was 1.2 times that of low NUE genotypes in both N treatments.

3.7. Characteristics of photosynthetic gas exchange

Both high NUE and low NUE genotypes exhibited significantly higher values for g_s, C_i and T all in N100 than those of in N0. In N0, the percent reduction in g_s, C_i and T were 43, 13 and 47 percent and 32, 15 and 38 percent in high and low NUE genotypes respectively. Significant differences for P_N, g_s, T and P_N/C_i were observed between genotypes in N0 and where as in N100 for g_s only. The peculiar observation in this study was Kolajoha-3, a low NUE genotype exhibited highest photosynthetic rates in both N100 and N0 (20.41 and 16.75 μmol m^{-2} s^{-1} in N100 and N0 respectively) among all four genotypes tested.

3.8. Agronomic and NUE traits

Mean value of high NUE genotypes showed more grain weight (than that of low NUE genotype in both N levels and the difference was more in N0 treatment (31 times more) where as in N100, it was only 7 times. Total dry matter (TDM) was 78.6% more in high NUE in N0 treatment and 73.3% more in N100 as compared with that of low NUE genotypes. Percent N content in grains of low NUE genotypes was 1.15 and 1.09 times that of high NUE genotypes in N0 and N100 respectively whereas % N content in straw was 16.36% and 15.63% more in high NUE genotypes in N0 and N100 respectively. High NUE genotypes exhibited more N uptake in both grain and straw than that of low NUE genotypes, irrespective of treatments, however the percentage increase was little more in case of low N conditions (88%) when compared with that of N100 conditions (84%). It was also observed that in case of high NUE genotypes, N uptake by grains was more in N0 treatment than in N100 though the difference was not significant (0.1 times). Significant differences were not observed between genotypes for all 5 types of efficiencies (AE, PE, APE, ARE and UE) related to NUE. However, AE, ARE and UE were recorded more (1–2 times) in case of high NUE genotypes where as remaining two efficiencies PE and APE were more (0.7–0.3 times) in low NUE genotypes (Fig. 4).

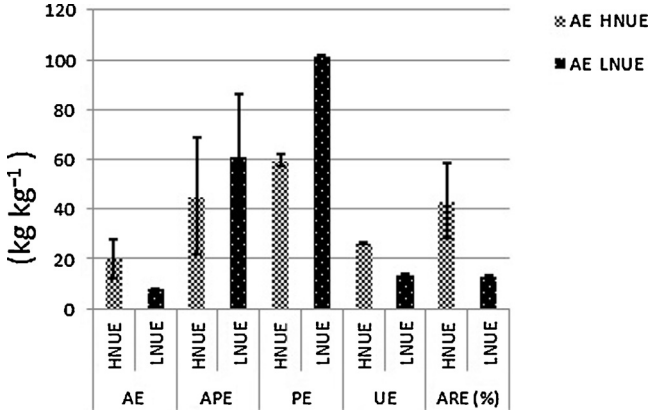

Fig. 4. Graph showing five efficiencies related to Nitrogen use efficiency in high NUE and low NUE genotypes.

4. Discussion

High NUE genotypes can be defined as genotypes which give more grain yield with minimal application of N fertilizer when compared with other recommended or standard N fertilizer conditions (Hawkesford, 2014). A total 78 genotypes, belonging to aromatic group, were screened for yield and its related traits under N0 and N100 conditions during wet (2011) and dry seasons (2012). Based on the cluster analysis, it was confirmed that Basmati 370 and Ranbir basmati as high NUE where as Kolajoha-3 and Ratnasundari as low NUE in low N environment. The present work studied biochemical, physiological and agronomic basis of NUE in these four selected rice genotypes. Total 32 traits related to nitrogen assimilatory enzymes activity, leaf pigments, leaf area and width, chlorophyll fluorescence and photosynthetic parameters and N content and uptake in grain and straw and different efficiencies of NUE were analyzed under N0 and N100 treatments during wet season 2012.

4.1. GS activity

GS is one of the key enzymes involved in the assimilation and recycling of mineral nitrogen which catalyses the ATP-dependent conversion of glutamine into glutamate utilizing ammonia as a substrate (Lea and Ireland, 1999; Cren and Hirel, 1999). In the current experiment, there is significant variation in GS activity between high NUE genotypes and low NUE genotypes. It was observed that low NUE genotypes had low GS activity in leaves and considerable proportion of N was not re-translocated to harvesting parts when compared to high NUE genotypes. There was proper coordinating mechanism for N uptake and assimilation in high NUE genotypes (Masclaux et al., 2001). Cao et al. (2008) reported that GS activity was two times more in plants growing in low N conditions than that in high N levels. The present results also showed that in N0 treatment, GS activity was almost doubled to that in N100 conditions. In case of low NUE genotypes there was not much change in GS activity with increased N levels. High NUE genotypes had the ability to utilize absorbed N and grew well under low N compared to low NUE genotypes. Under low N condition, the availability of N is limited and plant tends to take nitrogen source from other metabolic process like photorespiration, in which ammonia was released. Hence, GS activity may be increased to utilize this ammonia as substrate. Hirel et al. (2001) and Su et al. (1995) reported that leaf GS activity was positively correlated with grain yield and kernel number under low N-input. In low N, GS activity was increased mainly due to higher accumulation of cytosolic glutamine synthetase (GS1) (Thomas et al., 2008). The present results also in tune with these observations as GS activity was more in high NUE genotypes at N0 levels than in N100 conditions. Thus it may be inferred that high NUE genotypes can utilize more ammonia as alternate N source with help of more GS activity in N stress conditions.

4.2. NR activity

In case of NR activity, the trend was contrasting to that of GS. Hirel et al. (2001) and Reed et al. (1980) reported negative relationship between NR and GS activity suggesting that, when the rate of nitrate reduction was too high, GS activity was limited to cope with the stronger flux of reduced nitrogen. NR is the substrate inducible enzyme and its induction is closely dependent on the availability of nitrate. NR was known to be positively regulated by nitrate availability and nitrate reduction is the main limiting factor for nitrogen assimilation (Vincentz et al., 1993). The present research also showed large variation in NR activity between two treatments of N0 and N100 levels. NR activity, under N100 treatment, was 4–5 times to that of N0 treatment, which might be due to less available nitrogen in low N conditions. NR activity was lower in high NUE genotypes when compared with that of low NUE genotypes. It might specify that in lower NUE genotypes, NR had lower affinity for N and hence higher threshold level for NR. Hakeem et al. (2012) reported that high NUE genotypes showed more NR activity and consistent even with increase in N levels where as NR activity was increased with increase in N levels in low NUE genotypes. High NUE genotypes have low threshold level for NR activity and hence there was no change in NR activity with increase in N levels (Anjana et al., 2007; Chandna et al., 2012).

4.3. Photosynthetic pigments

Application of N fertilizer increased the chlorophyll content (Kitajima and Hogan, 2003; Verma et al., 2004; Pramanik and Bera, 2013). In the past two decades, SPAD or chlorophyll content was widely used to judge N demand at different growth stages to improve grain yield and to know N use efficiency in rice plants (Huang et al., 2008; Khurana et al., 2007; Peng et al., 2006). In addition, N availability to plants influences not only Chl content but also the composition of Chl pigment types, and ratio of Chl a/b (Kitajima and Hogan, 2003). The current results also are in agreement with the earlier reports as there was an increase in chlorophyll pigments content, chl a/b ratio, carotenoids, and leaf area with increase in N levels. The proportion of increase in chl a and carotenoids content was more when compared with that of chl b. Low NUE genotypes showed more significant chl a content in comparison to high NUE in both N100 and N0 treatments. Especially, Kolojoha-3 expressed maximum chl a content of 3.02 and 2.33 mg g^{-1} in N100 and N0 respectively. This genotype also showed maximum photosynthetic rate and stomatal conductance. These results are in harmony with the earlier report of higher leaf chlorophyll content increased the leaf transmittance and reflectance in the visible region and leading to more photosynthetic rate (Blackmer et al., 1996; Hansen and Schjoerring, 2003). The higher N content available to plant and the higher is chlorophyll content in plants and tended to absorb visible region more strongly, which resulted in the decrease of reflectance at blue, green, and red bands. There was also a report of distinct sub grouping of genotypes based on their different chl a/b ratios (Fritschi and Ray, 2007). Significant reduction of chlorophyll and carotenoid content under low nitrogen conditions was reported in wheat genotypes (Nouriyani et al., 2012).

4.4. Chlorophyll fluorescence and gas exchange parameters

Effect of slow nitrogen fertilizer release on photosynthetic mechanism of light absorption, its use and distribution and light suppression were studied in super hybrid rice (Long et al., 2013). Studies on various crops to determine the relation between nitrogen levels and photosynthetic parameters revealed that nitrogen fertilizer application increased the chlorophyll content, photosynthetic rate, electron transport capacity of PS I and PS II and photosynthetic production (Tang, 2000; Duan et al., 2007; Yang et al., 2002; Zhang et al., 2003). ETR and Φ_{PSI} values were changed with different levels of nitrogen in different genotypes of the same species and also across the species and their values were higher at optimum levels of nitrogen (Schreiber et al., 1994; White and Critchley, 1999; Sun et al., 2005). Low level nitrogen application in super hybrid rice supported for increase in ETR and the degree of the openness of PS II reaction centre, achieving higher Φ_{PSI} (Wu et al., 2003; Li et al., 2005). Long et al. (2013) reported that ETR and Φ_{PSI} were affected more by nitrogen after heading stage but by photosynthetic radiation before heading stage. There are several contradictory reports on maximum photochemical efficiency (F_v/F_m) in relation to nitrogen levels. Zhang and Shangguan (2007) and Shangguan et al. (2000) reported that nitrogen application significantly increased F_v/F_m ratio at flowering and grain

filling stage where as Zhang et al. (1997) and Guo et al. (2004) showed that F_v/F_m was reduced with increase in nitrogen fertilizer application. The current results did not show considerable change in ETR, Φ_{PSI} and F_v/F_m either between two nitrogen levels or between genotypes. Hence it is great challenge to determine the reason for variations in yield without affecting photosynthetic related characters. Photochemical quenching coefficient (q_P) and non-photochemical quenching (q_N) were found to be reduced with nitrogen application under water deficit conditions (Shangguan et al., 2000). q_N and q_P showed opposite trend under nitrogen treatments. q_P was increased at high N levels where as q_N was reduced (Long et al., 2013; Zhang and Shangguan, 2007). The present study showed significant decrease in both q_N and q_P with application of N fertilizer, however, there were no considerable differences between high NUE and low NUE genotypes. These results indicate increased photosynthetic efficiency at low levels of nitrogen. Many other experiments showed that enhanced q_N was recorded in N deficiency due to increased activity of the xanthophyll cycle (Verhoeven et al., 1997; Chen and Cheng, 2003; Kumagai et al., 2007). Several reports on rice suggested the existence of differences among rice cultivars in terms of both the susceptibility to photo inhibition and the activity of the xanthophyll cycle (Jiao and Ji, 2001; Jiao et al., 2003). N supply affects absorption and utilization of excitation energy in plant leaves (Chen and Cheng, 2003). Under N deficiency, the leaf Chl content was decreased, leading to decrease in light absorption (Chen and Cheng, 2003). Low N also decreased the light saturated photosynthetic rate, which was associated with the decrease in Rubisco content (Evans and Terashima, 1987). Rate of photosynthesis in leaf depends on many physiological and bio chemical processes such as stomatal conductance (g_s), internal carbon dioxide (CO_2) and activities of carbon fixation enzymes. Photosynthetic rate was highly correlated with stomatal conductance and/or carboxylation efficiency (Ding et al., 2014). The present research noticed decreasing P_N, g_s, C_i, T, E and C_i/C_a values under low N conditions. Similar conclusions of decreased P_N and other gas exchange characters with decreased N levels were also drawn in cassava crop by Cruz et al. (2003). Yong et al. (2009) reported that photosynthetic rate was increased with more N supply but CO_2 assimilation was restriction by RuBP restoration and chloroplastic CO_2 content. Chloroplastic size was the main favourable character for more carbon accumulation in chloroplast for high N utilization in rice genotypes. Low NUE genotypes, particularly Kolajoha-3 showed higher photosynthetic rate, stomatal conductance and transpiration rate among all genotypes. This result might be attributed to presence of more chlorophyll content. Despite this observation, photosynthetic, water and carboxylation efficiencies were not on the top and also it was low yielding and low NUE genotype according to earlier studies (Vijayalakshmi et al., 2015). This observation may signify either no relation or negative relation between photosynthetic characters such as chl content and photosynthetic rate etc. and nitrogen use efficiency of a genotype.

4.5. Agronomic and NUE traits

Cassman et al. (1996) reported that application of N increased the grain yield in comparison with low N application. Though the present results also tune with these observations of high grain yield and total dry matter in N100 levels than in N0 conditions, the increasing quantity was not up to significant level. In low N conditions, grain yield of high NUE genotypes was 32 times that of low NUE genotypes where as in N100 conditions, it was only 8 times, signifying better yield performance of high NUE genotypes in low N field. Apart from this, total N uptake by grain and straw was significantly more in high NUE genotypes when compared with low NUE genotypes in both N treatments, which supports better nitrogen use efficiency by high NUE genotypes.

Nitrogen accumulation in grain was the key indicator of NUE and N uptake or N utilization, or both components were more important for increasing NUE in plants (Hugins and Pan, 2003; Moose and Below, 2009). Anbessa et al. (2009) reported both genotypic variability and environment can account for differences in NUE in many spring-barley genotypes, grown under field conditions of two different N levels. In their study, they reported a genotype, which exhibited better NUE when grown on either high or low N soils, with a concomitant reduction in yield of 10% in low N soils. Here also, high NUE genotypes are superior agronomically in both N0 and N100 levels with reasonable decrease of 30% reduction in grain yield in N0 treatment.

The higher N uptake during grain filling stage for increasing leaf longevity and prolonging the capacity of the plant to absorb mineral nitrogen, achieved better yields in modern hybrids of maize (Tollenaar, 1991; Ma and Dwyer, 1998; Racjan and Tollenaar, 1999a,b). The present research also showed considerably higher N uptake by both grain and straw in case of high NUE genotypes in both tested N levels when compared with low NUE genotypes. Fageria et al. (2010) studied nineteen upland rice genotypes for N use efficiency. They reported quadratic relationship of grain yield with AE, APE and UE but negative correlation with PE. Here also high grain yielding high NUE genotypes showed higher AE, ARE and UE whereas low grain yielding low NUE genotypes had more values for PE and APE.

Even though there were differences in AE, PE, APE, ARE and UE between high NUE and low NUE genotypes, however these variations were not statistically significant. NUE related efficiencies are complex traits, signifying the importance of all the parameters viz., grain yield, straw yield, nutrient applied, nitrogen content of straw and grain and nitrogen uptake by straw and grain. Cumulative effect of all these traits might not give significant differences. It may be also due to complex and multiple ways of interaction of these traits with other metabolic pathways. Fagaria (2014) also reported that PE, APE and ARE were varied among rice genotypes but these differences were not statistically significant.

5. Conclusion

The activity GS and NR enzymes clearly indicate that high NUE rice genotypes were effective to assimilate and utilize nitrogen in low N conditions. High NUE genotypes also maintained sufficient values for leaf thickness and area, chlorophyll content, chlorophyll fluorescence parameters such as ETR, Φ_{PSI}, F_v/F_m and q_P even without application of N fertilizers. There are significant differences in photosynthetic characters and gaseous exchange parameters between two N treatments except for ETR, C_i/C_a, P_N/T and P_N/g_s. When compared with low NUE genotypes, high NUE genotypes performed better in both N0 and N100 treatments with regard to most of the observed traits of biochemical photosynthetic and agronomic parameters. High NUE genotypes with higher N uptake efficiency permit themselves to yield more under the same limited nitrogen conditions. These genotypes can be further utilized in breeding and molecular studies to develop varieties with high NUE and also to know basic molecular knowledge, at genomic level, behind genetic variations in crop varieties for NUE.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fcr.2015.04.012

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