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Physio-morphological and biochemical mechanism of nitrogen use efficiency in sugarcane (*Saccharum* spp.) genotypes under different growth stages and nitrogen levels

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ABSTRACT

Nitrogen is crucial for sugarcane crop to get economical sugar and biofuel throughout the world, and low nitrogen use efficiency is the main issue of sugarcane because of high nitrogen losses. In this experiment, physio-morphological, growth, and biochemical traits were observed for sugarcane genotypes (nitrogen inefficient-GT11 and efficient GXASF180-1-11) under 0.05 and 5 mMN levels at 5, 7, and 9 leaf stages. GXASF180-1-11 attained 46–58% and 16–23% more plant height and biomass than GT11, while photosynthesis and internal nitrogen use efficiency (iNUE) were 16 and 8% more at F2 fertilizer level, respectively. Highest key enzymes activity was showed in leaf whereas polynomial regression ranged between 0.53 and 0.98, 0.69 and 0.99 for iNUE and nitrogen use index. Principal component analysis explained 91.23–93.53% total variance across the stages. These results suggested that low nitrogen application rate with efficient germplasm may have the potential to improve iNUE for further cultivar development.

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Nitrogen; morphology; enzyme activities; polynomial regression and PCA

Introduction

Sugarcane (*Saccharum* spp.) has a good potential for sugar and bioethanol production, 110 countries worldwide involved in sugarcane cultivation. It contributes globally for sugar production approximately 80% (Islam et al. 2018; Sharma and Chandra 2018), while Brazil, India, China, and Thailand have 60% share in the total production (FAO 2014; Anas et al. 2020). The sugarcane approximately completes its crop production cycle once a year, and demands for higher amount of nutrients than the other short-duration crops. During the last century, crop improvement research in terms of agronomy focused on the yield enhancement (Conant and Grace 2013). High-yielding sugarcane cultivars which required more nutrients were released to compensate the food security (Ghaffar et al. 2012). The addition of depleted nutrients in the root zone especially nitrogen is a must for successful sugarcane production as well as sustainable high sugarcane yields.

Nitrogen (N) is important for plant growth, development, physiological, and metabolic changes, and also low nitrogen inhibits the plant performance and production of sugarcane (Wiedenfeld and Enciso 2008; Bassi et al. 2018). Sufficient N supply at a critical time period has a positive relation with plant growth, by increasing leaf area expansion, stalk girth, and inter-nodal distance (Bell et al. 2015). Excessive amount of N leads to enhance the economic cost, soil acidity, eutrophication, pollute the atmospheric air (Ju et al. 2009; Chen et al. 2016), and resulted in low cane yield (Muchow et al. 1996). Sugarcane N use efficiency is relatively low and lies between 20% and 40% (Meyer et al. 2007; Kingston et al. 2008; Franco et al. 2010) which can be improved by understanding physiology, morphology, and metabolism of different genotypes. Nitrogen use efficiency (NUE) on the bases of absorption and utilization is the possible solution to eliminate the inconsistencies of nitrogen (Hirel et al. 2001). The N use efficient genotypes are crucial for lower N fertilization and also have great significance to reduce N pollution under sufficient nitrogen supply. However, genotypic differences for physiological and morphological variations under different N supplies are less studied.

Many researchers had screened out high NUE genotypes, for example, Hajari et al. (2014) screened the sugarcane genotypes with high or low NUE according to their N uptake when different N concentrations applied and Robinson et al. (2007) found Q165A genotype performed better for iNUE and biomass at low and high N solutions. Both studies were related to sugarcane cultured with nutrient solutions. Liu et al. also screened 50 eight genotypes for low and high iNUE and found GXASF180-1-11 was N efficient and GT-11 was inefficient (Liu et al. 2019). However, to date according to our knowledge, no one studied for growth, physiological, and morphological behavioral differences between N efficient and inefficient genotypes. So, in this study, we chose two previously screened N use efficient and inefficient genotypes (Liu et al. 2019).

The objectives of our study were to understand the mechanism of N use efficient genotype with respect to in-efficient

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genotype through associated growth, physiological and morphological traits for sugarcane, relationships among these traits, and also for iNUE under low and high nitrogen levels. This study provides more information to understand the differences between nitrogen use efficient and inefficient sugarcane plants which may be helpful to improve NUE for new genotype development.

Material and methods

Experimental site and design

This experiment was conducted in the greenhouse at Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, China in plastic pots (30 cm diameter and 60 cm height). During the experiment, minimal day-to-day changes in climatic variables were monitored such as ambient air temperature, rainfall, average light exposure, and relative air humidity (Supplementary Figure 1).

The experimental design was a double-factor completely randomized design. Factor A was sugarcane genotypes GT11 (G1) and GXASF180-1-11 (G2) and factor B was 0.05 and 5 mM NH_4NO_3 solutions. Sugarcane setts from G1 (N-inefficient) and G2 (N-efficient) genotypes were surface sterilized with 5% (v/v) Bavistin solution for 5 min (Verma et al. 2020) and rinsed with deionized water thrice. Those setts were placed in plastic trays and put a thin layer of vermiculite and compost, and rest these trays in the greenhouse for germination. Each pot was filled with sand:soil at the ratio of 1:3 for transplanting of sugarcane seedling and watered 24 h before transplanting with tape water. After transplanting one seedling per pot, 100 ml nutrients solution (macronutrients; 4.5 mM K_2SO_4, 0.457 mM KH_2PO_4, 42.5 µM K_2HPO_4 and micronutrients; 2 mM $MgSO_4$, 2 mM $CaCl_2$, 100 µmol FeEDTA, 10 µM $MnCl_2$, 10 µmol H_3BO_3, 1 µmol $CuSO_4$, 2.5 µmol $ZnSO_4$, 0.35 µmol Na2MoO$_4$) with variable concentrations of NH_4NO_3 (F1 = 0.05 mM and F2 = 5 mM NH_4NO_3) was applied alternatively to each pot and also applied tape water on the other day to keep wet (Robinson et al. 2007).

At 5th leaf (S1), 7th leaf (S2), and 9th leaf stages of sugarcane genotypes, three random plants from each treatment combination (G1 × F1, G1 × F2, G2 × F1, and G2 × F2) were harvested for growth, root morphological, and enzymatic analysis. Plant parts like leaf blade, stem, and roots were frozen in liquid nitrogen and stored at −80°C for further analysis. The roots were rinsed in running tap water to remove soil and sand, and stored in 70% (v/v) ethanol solution to keep fresh and restrict their growth until further morphological observations (Arruda et al. 2016).

Growth and dry weight

The plant height, diameter, and green leaves count (GLC) were measured by measuring tape and digital Vernier scale. For dry biomass, the fresh green leaves, stems, and roots were placed in paper bags and oven-dried (65°C) until the constant weight, and dry biomass of the plant organs was determined. Leaf area was measured by Leaf Area Meter (CI-203 Area Meter, CID, Inc., USA).

Soil plant analysis development (SPAD)

The relative leaf greenness was measured by using a portable SPAD meter (SPAD 502, Minolta Camera, Osaka, Japan). Five readings were taken at each leaf of the three plants marked for leaf gas exchange measurements (Verma et al. 2020).

Root morphological observations

Root morphological traits included root length, root projection area, root surface area, average root diameter, and root volume. Root samples were taken from three biological replicates of each treatment, carefully cut the roots from plant, and washed carefully to remove soil dirt by running water. The measurements were done at S1, S2, and S3 stages after N application by using an Epson Expression 10000XL scanner and root analysis software WinRHIZO Prov. 2009c, Regent Instruments, Quebec, Canada (Iqbal et al. 2019).

Leaf gas exchange attributes

The photosynthetic leaf gas exchange parameters, i.e. net photosynthetic rate (Pn), transpiration rate (Tr), and stomatal conductance (gs) were determined at S1, S2, and S3 stages after N application. For each treatment, five different plants for flag leaves were selected randomly for the measurement of leaf gas exchange by using a portable photosynthesis system (Li-6400, Li-COR Inc., Lincoln, NE, USA). The data were collected on a sunny day (09:30–11:00) under the following inside leaf chamber conditions:, photosynthetic photon flux density (PPFD) was 1000 µmol m^{-2} s^{-1}, leaf temperature (25°C) and CO_2 concentration (400 µmol mol^{-1}) (Iqbal et al. 2019).

Determination of enzyme activities

The enzyme extract was prepared according to Ali et al. (2007) and the assay for nitrate reductase (NR) was prepared as 50 mM K2PO4 buffer (pH 7.5), 5 mM EDTA, 5 mM KNO3, and crude extract. The reactions were set up in triplicate at 25°C (20 min) by adding 0.6 ml sulfanilamide (1% w/v 3N HCl) and NED (0.1% w/v) in the ratio of 1:1. After the incubation at room temperature for 15 min to develop pink color and readings were taken at 540 nm wavelength. The nitrate concentration was measured by using standard curve (Hageman 1979).

Nitrite reductase (NiR) activity was quantified according to Wray and Fido (1990), the assay mixture of 50 mM potassium phosphate buffer (pH 7.5), 2.5 mM KNO2, 3 mM methyl viologen, 20 mM sodium dithionite was prepared in 290 mM $NaHCO_3$ and added 10 µl of enzyme extract. The reaction was started by the addition of Sodium dithionite at room temperature (25°C, 10 min). Then added water (0.7 ml), sulfanilamide (1% w/v, 3N HCl- 0.6 ml) and NED (0.1% w/v, 0.6 ml) to stop reaction and develop color, and further incubated for 15 min at room temperature. The measurements were taken at 540 nm and nitrite consumption was calculated from standard curve obtained from the known values of nitrite.

GS activity was measured according to Robinson et al. (2007), and one unit of GS activity was defined as one micro mole of GS/hour/g. The glutamine oxoglutarate aminotransferase (GOGAT) activity was determined according to Groat and Vance (1981). One unit of GOGAT was expressed as the oxidation of 1 nmol NADH per min. Glutamate dehydrogenase (GDH) activity was quantified

according to Yang et al. (2019), GDH activity was measured by the reduction of NAD or the oxidation of NADH. One unit of GDH was calculated in units of nmol (NAD reduced or NADH oxidized) per minute.

Determination of nitrogen uptake and iNUE

Sugarcane plant parts such as leaves, stems, and roots were collected and rinsed with running water. All plant samples were dried in an oven (65°C). Dry weight was calculated for the leaves, stem, and roots and then sum up to get the total plant dry weight. A mill (Shanghai Jingxin, Co., Ltd., China) was used to grind samples in fine powder and sieved by a 0.25-mm mesh. 500 mg of plant samples were digested with H_2SO_4-H_2O_2 at 260–270°C. According to Kjeldahl method reported by Cao et al. (2017), N content was determined by a Kjeltec 8200 type automatic azotometer (Foss, Denmark). N accumulation and internal nitrogen use efficiency (iNUE) were assessed in leaves, stems, roots, and total plant (Zhang et al. 2017; Liu et al. 2019).

Statistical analysis

All data were analyzed by Statistix 8.1 software to perform correlation test and linear regression analysis. The means of each factor were separated by least significant difference (LSD) values with $P < 0.05$. The principal component analysis (PCA) was performed in IBM SPSS Statistics 21 and graphs were designed by OriginPro 9.1.

Results

Growth and development

Growth attributes such as plant height, plant diameter, and number of green leave were significantly varied after N application. G2 showed the maximum growth with 52% plant height and 33% GLC than the G1as for F2 fertilizer applied while33% more plant height was observed for G1 in comparison of F2 and F1 fertilizer levels. For G2, F1 showed decreased plant height upto 30% as compared to F2, and F1 was statistically at par across the genotypes. Maximum green leaf count (10) was observed for F2 than F1 for G2 and G1 attained no significant difference for green leaf count under F1 level with G2. Across the fertilizer levels, G1 and G2 showed 72 and 68% more plant dry weight for F2 fertilizer level. All the sampling stages showed a similar pattern for treatment factors except plant diameter (Table 1).

Leaf SPAD, leaf area, and photosynthetic parameters

Genotypes and fertilizer levels significantly affected leaf SPAD, leaf area (LA), net photosynthetic rate (Pn), transpiration rate (Tr), and stomatal conductance (gs). Overall, leaf SPAD was highly affected by F2 across the fertilizer levels (Figure 1(b)) and also greater in G2 than G1 (Figure 1(a)). Between the genotypes, G1 had significantly lower SPAD than G2 (Figure 1(a)). Averaged SPAD of both genotypes was significant at F2 fertilizer level. The response of sugarcane leaf SPAD to low fertilizer level was non-significant, butG2 attained 31% higher SPAD than G1 at F2 level and 51% across the fertilizer levels (Figure 1(b)).

Leaf blade is the photosynthetic machinery and its area describes the effectiveness. The main effects of genotype and fertilizer levels on leaf area measured were highly significant (Figure 1(c,d)). Leaf area of G2 was 13% and 22% higher than G1 against both fertilizer levels (Figure 1(c)), respectively. Leaf area increased as the fertilizer level changes from low to high N levels. Maximum leaf area was noted in high N fertilizer level F2 (Figure 1(d)). At F1, leaf area was lowest than G1, G2, and F2 at all the sampling stages (Figure 1(c,d)).

The variations in net photosynthetic rate (Pn), transpiration rate (Tr), and stomatal conductance (gs) were observed as for as leaf SPAD and leaf area (Figure 1). Except for gs, Pn, and Tr were non-significant for both genotypes at S2 and S1 after N application (Figure 1(hj)). Pn, Tr, and gs for G2 were 23.5, 33.3, and 30% more than the genotype G1, respectively (Figure 1(e,g,i)). Plant growth stages mainly affect genotypic differences in net photosynthetic rate (Pn), transpiration rate (Tr), and stomatal conductance (gs). N rate had much more effect on Pn, Tr, and gs (Figure 1(f,h,j)). Maximum Pn, Tr, and gs were observed in F2 after N application (Figure 1(f, h,j)). Comparison within the genotypes, Pn, Tr, and gs was not different between the genotypes for F1 treatment factor (Figure 1(h,j)). These results indicating that NH_4NO_3 application improves the SPAD, leaf area, and gas exchange characteristics which resulted in more sugarcane plant growth and yield, and also the nitrogen efficient genotype G2 showed clear differences.

Root morphological attributes

N fertilizer rate greatly influenced the root length, surface area, volume, and average diameter, and the effect of genotypic variability was observed during the first stage. Root morphological attributes were significantly changed between fertilizer levels. However, these features were lower under F1 level for both genotypes. The genotypes G1 and G2 had a similar pattern for root length, surface area, and volume under F1 fertilizer level but root diameter was affected by genotypic variations. Root length was 19% higher under F2 level for G2 than G1. Across the fertilizer levels, F2 had 33% superior effect on root length of G2 than F1 level. Root surface area and volume were 18 and 16% less for G2

Table 1. Effect of genotypes and fertilizer levels on plant height, plant diameter, green leaf count, and plant dry weight for different sampling stages.

Genotype	Fertilizer level	Plant height (cm)			Plant diameter (mm)			GLC			Plant dry weight (g)		
		S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3
G1	F1	20.67d	23.67c	24.83c	7.71b	8.24c	8.60c	2.33c	3.67c	3.67c	4.78c	5.51d	5.78d
	F2	31.00c	46.67b	47.00b	11.00a	14.17a	14.26a	5.67b	7.33b	8.33b	14.51a	19.64b	21.30b
G2	F1	42.67b	46.33b	47.33b	6.99b	7.73c	7.91c	3.33c	4.00c	4.67c	6.12b	7.77c	8.55c
	F2	65.00a	83.33a	87.83a	8.59b	9.86b	10.34b	8.33a	10.0a	10.67a	15.70a	23.82a	26.80a
LSD value ($P < 0.05$)		8.025	8.385	10.205	1.894	1.403	1.563	1.718	1.215	1.438	1.232	1.368	2.149

Note: GLC; green leaf count, S1; sampling stage 1, S2; sampling stage 2, S3; sampling stage 3, G1; genotype 1 (GXASF180-1-11), G2; genotype 2 (GT-11) F1; 0.05 mM NH_4NO_3 solution.

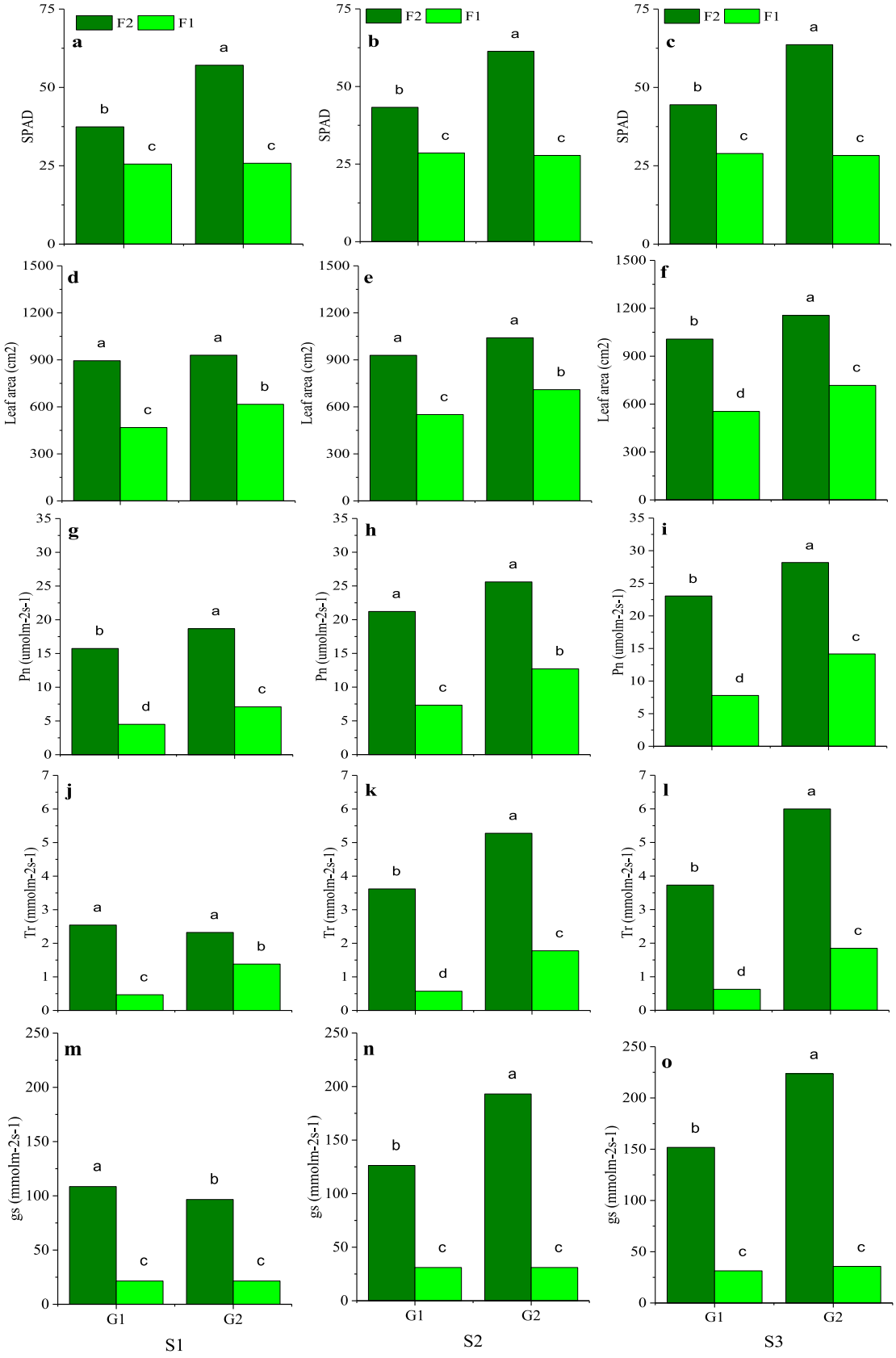

Figure 1. Effect of genotypes and fertilizer levels on SPAD (a–c), leaf area (d–f), net photosynthetic rate (Pn) (g–i), transpiration rate (Tr) (j–l), stomatal conductance (gs) (m–o) for different sampling stages and different letters on column show significant difference at $P < 0.05$.

than G1 under F1 fertilizer level, while root surface area of genotype G1 was 30% more for F2 than F1. The fertilizer level F2 showed 3 and 15% greater average diameter for G1 and G2, respectively. Similar behavior was observed across the stages and growth was higher for S3 than the other stages (Table 2).

Enzymes activity

The overall activities of NR, NiR, GS, GOGAT, and GDH were significantly higher in leaves than stem and roots against applied N. There was a significant difference among the enzymes activities in response to fertilizer

Table 2. Effect of genotypes and fertilizer levels on root length, root surface area, root volume and root average diameter for different sampling stages.

Genotype	Fertilizer level	Root length (cm)			Root surface area (cm^2)			Root volume (cm^3)			Average diameter (mm)		
		S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3
G1	F1	3743.5c	5031.9c	5406.8c	460.47c	463.22c	466.55c	6.81c	7.81c	8.31c	0.357b	0.429a	0.430a
	F2	5940.9b	7216.6b	7515.9b	549.67b	632.02b	665.62b	10.62b	12.99b	13.42b	0.427a	0.443a	0.445a
G2	F1	4417.0c	5236.6c	5528.8c	387.05d	495.04c	556.61c	5.52d	6.70c	7.13d	0.294c	0.338b	0.353b
	F2	7364.6a	8078.5a	8307.3a	717.96a	905.88a	959.04a	15.14a	18.76a	20.52a	0.387ab	0.398a	0.405a
LSD value ($P < 0.05$)		1010.0	502.47	758.96	39.51	67.87	94.12	1.16	2.94	1.16	0.049	0.048	0.052

Note: Length; root length, Surface area; root surface area, Volume; root volume, Average diameter; root average diameter, S1; sampling stage 1, S2; sampling stage 2, S3; sampling stage 3, G1; genotype 1 (GXASF180-1-11), G2; genotype 2 (GT-11) F1; 0.05 mM NH_4NO_3 solutions.

application levels. With the comparison of F1, F2 showed that the highest effect on the GDH and lowest on the GS activities, while leaves were more sensitive for low fertilizer level than stem and roots (Figure 2).

Significant differences were observed for sugarcane genotypes in response to fertilizer levels. The enzyme activities of NR, NiR, GOGAT, GS, and GDH were higher for the genotype G2 and F2 fertilizer level on the whole plant bases. However, it varied from different plant parts as leaves had more activities than stem and roots (Figure 2).

Lowest NR activity was observed in the root against low fertilizer application level F1 than leaf and stem. NR activity in leaf for G1 was 78% more than F1 fertilizer level and G2 had 32% more activity than G1 in stem (Figure 2(a)). NiR activity among genotypes had a significant difference, and F1 fertilizer level found lowest NiR enzyme activity for overall plant organs. The activity of NiR in G2 was 11.5 and 19.2% higher than G1 and F1, but 7.1% less than F2 fertilizer level. However, there was a 4.7% difference observed for leaf between F1 fertilizer level and G1 (Figure 2(b)).

The enzyme activity pattern for GOGAT was observed that F2 had greater GOGAT activity than G2, G1, and F1 fertilizer level. It was more influenced by fertilizer application rate, low fertilizer application rate (F1) had lowest activity in leaf and stem in G2 genotype (Figure 2(g–h)). The leaf, stem, and roots had similar activity against G1 and F1 fertilizer level (Figure 2(c)). All the plant parts had similar GS enzyme activity, G1 under F1 level of fertilizer showed minimum activity, and maximum was in leaf for both treatment factors. Stem showed 21 and 32% more GS activity than roots in G1 genotype under both fertilizer levels (Figure 2(k–l)). Highest GDH activity was noticed in leaf and the genotype G2 under F2 had 42% more GDH activity in leaf than G1 (Figure 2(m)). Minimum GDH activity was observed in root of G2 under F1 fertilizer level (Figure 2(o)).

There was a significant increase between the genotypes against N application levels (Figure 3) at S2 after N application. However, the increase in different plant parts (leaf, stem, and root) was variable. The overall enzyme activities of NR, NiR, GOGAT, GS, and GDH were 37.35%, 3.84%, 16.13%, 11.35%, and 21.17% higher at S2 stage on the whole plant bases (Figure 3(a–o)). The variability order of enzyme activities for plant parts was different among enzymes. The NR showed higher enzyme activity in G2 after F2 fertilizer application (Figure 3(a)). It was statistically insignificant for G2 under both fertilizer levels in roots and stem and also with G1 under F1 at S2 sampling stage (Figure 3(b,c)). NiR activity was significant for all the plant parts, higher in root than stem and F1 fertilizer application level decreased the enzyme activity (Figure 3(d–f)).

Leaf showed highest enzyme activity than stem and roots. The maximum GOGAT activity was observed in leaf of G2 genotype against F2 level. GOGAT enzyme activity was lowest in roots and it was 55% less than the leaf of G2 genotype as for as F2 applied (Figure 3(g–i)). The genotype G2 under F2 fertilizer had more GS enzyme activity as compared to the G1for both levels of fertilizer G2 showed 60%, 61%, and 73% more GS activity in leaf, stem, and root than G1 across the fertilizer levels (Figure 3(j–l)). GDH activity was highest in leave than stem and roots at S2 stage. G2 had 13 and 22% more GDH activity in leaf than stem and root under F2, respectively (Figure 3(m–o)).

Nitrogen uptake, internal N use efficiency, and N use index

Nitrogen uptake and its assimilation into the plant dry weight describe the efficiency of plant. The efficient plant can tolerate low N conditions and thus improve the yield in response to N, decrease the economic cost and environmental cost. Nitrogen uptake on dry weight basis under G2 was significantly higher than G1, indicating that sugarcane could absorb a higher percentage of N. Highest nitrogen uptake was observed in G2 across the genotypes. Genotype G2 had 17% more nitrogen uptake than G1 under F2 application level and both genotypes had insignificant difference under F1 fertilizer level (Table 3). Maximum NUI was observed for G2 in response to F2 level and G2 attained 24% more NUI of sugarcane plant against F2 level (Table 3).

The iNUE of sugarcane plant was significantly affected for both genotypes. On the whole plant basis, genotype G2 had 8% higher iNUE than G1 under F2. However, it was declined as the fertilizer level increased. G1 showed lower iNUE than G2 at the same level of applied fertilizer (Table 3). So, these results suggested that G2 may perform better for N uptake and iNUE in field under low nitrogen conditions.

Correlation of N uptake with iNUE and NUI

N uptake was positively correlated with N use index (NUI, R^2L 0.5223−0.9993, R^2 0.6866−0.9997) and iNUE (R^2L 0.5903−0.984, R^2 0.5326−0.9845) in different genotypes under different fertilizer levels at three sampling stages (Figure 5(a–l)). NUI was enhanced with increasing N uptake while the response of NUI and iNUE to F1 fertilizer supplies (Figure 4(d–f)) showed a significant correlation with N uptake, which indicated that up-regulated N uptake was closely associated with NUI and iNUE in the G2 sugarcane plants.

Biplot of the PCA explained 92.76%, 94.59%, and 93.99% of the total variation among the sugarcane genotypes for the observed parameters across all the growth stages (Figure 5 (S1–S3)). The length of vectors was the same, which

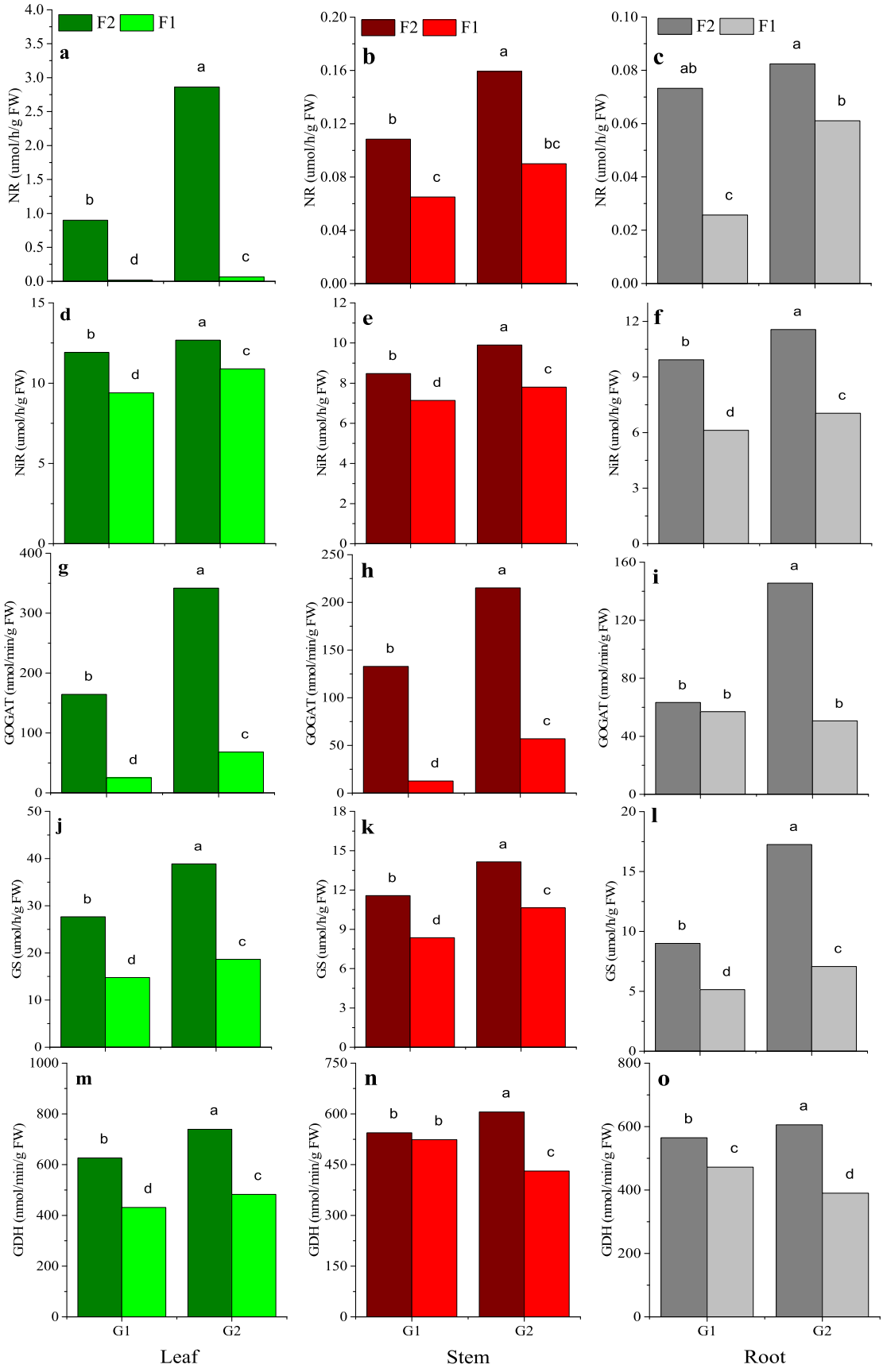

Figure 2. Effect of genotypes and fertilizer levels on nitrate reductase activity (a-c), nitrite reductase activity (d–f), glutamine synthetase activity (g–i) glutamine synthase activity (j–l) and glutamate dehydrogenase activity (m–o) in leaf, stem and root for sampling stage S1 and different letters on column show significant difference at $P < 0.05$.

indicated that all the traits had a parallel magnitude of variation. The vectors of the shoot, root, total dry weight, and N uptake had a close association. The head of each vector showed the contribution of traits for both genotypes at each fertilizer level. Thus, the iNUE and genotypes were the highest performing traits in PC 2, the leaf, stem, and root dry weights, SPAD, A, LA, GS, GDH, N uptake, and NUI were the highest performing in PC1. The iNUE was

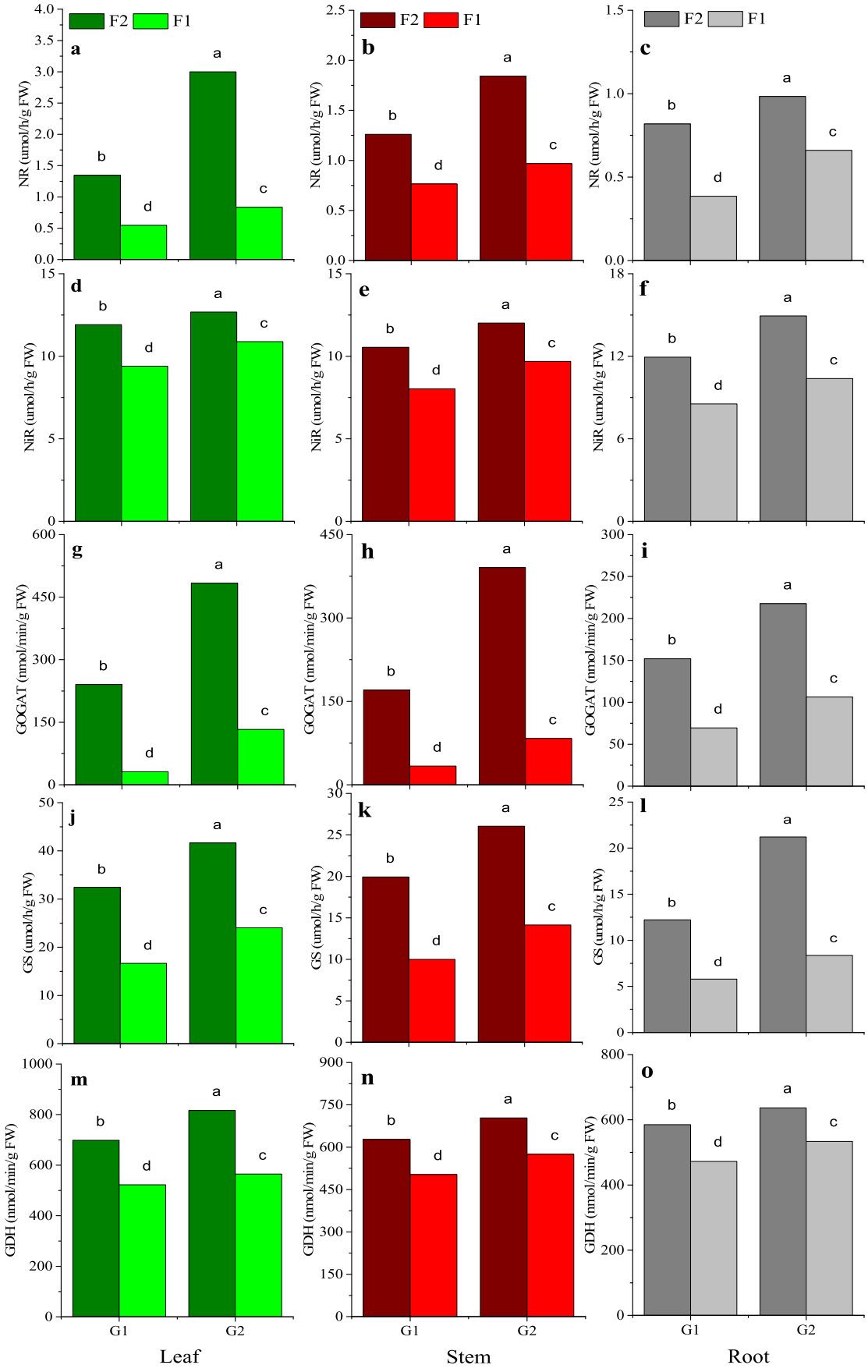

Figure 3. Effect of genotypes and fertilizer levels on nitrate reductase activity (a–c), nitrite reductase activity (d–f), glutamine synthetase activity (g–i) glutamine synthase activity (j–l) and glutamate dehydrogenase activity (m–o) in leaf, stem and root for sampling stage S2 and different letters on column show significant difference at $P < 0.05$.

the only trait at S1, S2, and S3 which was the highest performing trait in the PC2. The shapes and color in Figure 5are showing the same group of traits.

Discussion

Plants exhibit a remarkable ability to sense environmental variations, i.e. N application, and it is common to observe

Table 3. Effect of genotypes and fertilizer levels on nitrogen uptake, iNUE and nitrogen use index for different sampling stages.

Genotype	Fertilizer level	N uptake (mg/plant)			iNUE (mg/mg)			NUI		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
G1	F1	2.279c	3.009c	3.327c	2.013c	1.832b	1.738b	0.962d	1.010d	1.004d
	F2	12.574b	17.066b	19.703b	1.128d	1.152d	1.083c	1.637b	2.262b	2.305b
G2	F1	2.912c	3.947c	4.606c	2.103a	1.971a	1.860a	1.288c	1.531c	1.589c
	F2	13.280a	19.441a	23.684a	1.232c	1.225c	1.132c	1.935a	2.919a	3.032a
LSD value ($P < 0.05$)		0.702	1.080	2.338	0.029	0.062	0.069	0.167	0.211	0.211

Note: N uptake; nitrogen uptake, iNUE; internal nitrogen use efficiency, NUI; nitrogen use index, S1; sampling stage 1, S2; sampling stage 2, S3; sampling stage 3, G1; genotype 1 (GXASF180-1-11), G2; genotype 2 (GT-11) F1; 0.05 mM NH_4NO_3 solution.

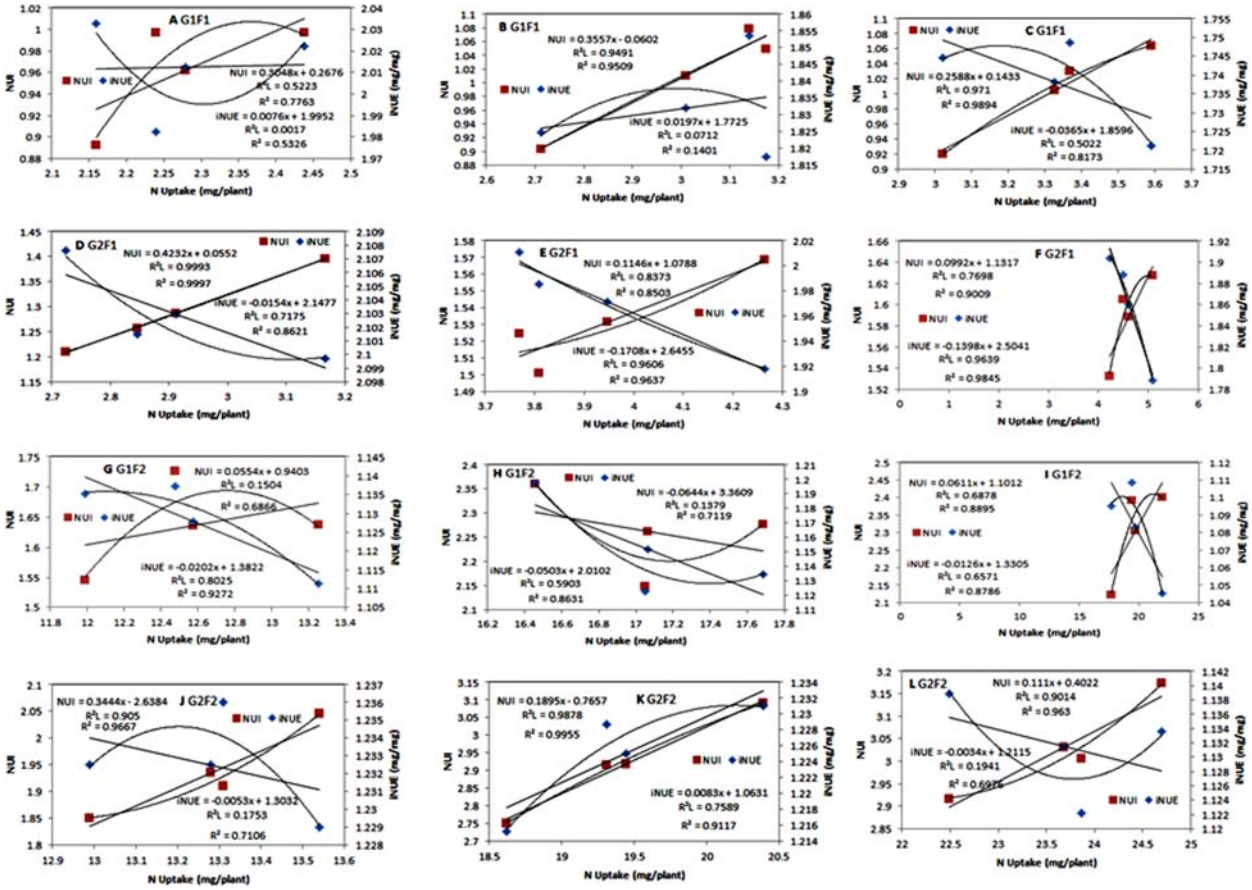

Figure 4. Correlations of N Uptake with N use index (NUI) and internal N use efficiency (iNUE) for sugarcane genotype 1 (G1) under F1 fertilizer level at sampling stages S1(A), S2 (B) and S3 (C), for genotype 2 (G2) under F1 fertilizer level at sampling stages S1(D), S2 (E) and S3 (F), for genotype 1 (G1) under F2 fertilizer level at sampling stages S1(G), S2 (H) and S3 (I), and for genotype 2 (G2) under F2 fertilizer level at sampling stages S1(J), S2 (K) and S3 (L), respectively. R^2L and R^2 are R square values in the graph for linear and polynomial regression types, respectively.

an array of genetic variation leading to different responses even within the species. Sugarcane plant height and number of nodes increased linearly as N increased (Zhao et al. 2014). In this study, the sensitivity of plant height was the most affected trait by fertilizer application and that for the GLC was least among plant height, number of green leaves, and plant diameter. The GLC as well as dry weight of leaf, stem, root, and total plant was significantly increased as N fertilizer application increased from F1 to F2. The application of nitrogen promotes the vegetative growth, and

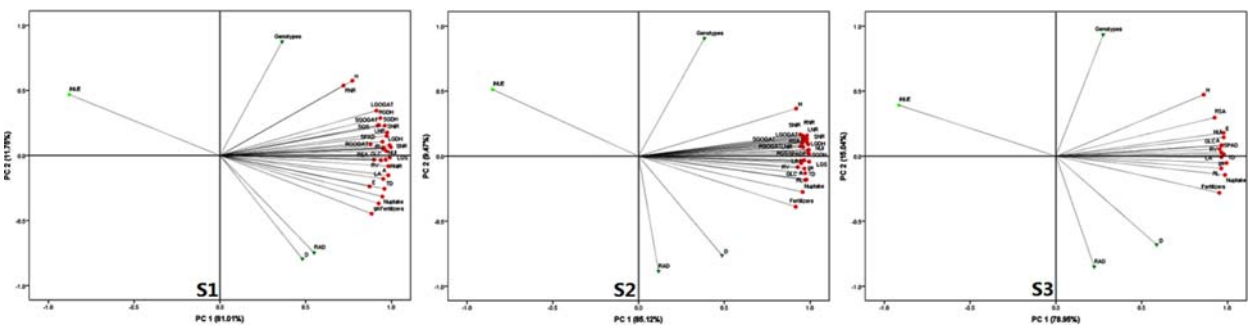

Figure 5. Principal component analysis of sugarcane genotypes for both N levels at S1, S2 and S3 growth stages.

high NUE of G2 genotype, might be the reasons for higher plant height. Sugarcane dry weight was suppressed due to F1, as it is mainly associated with reductions in leaf area expansion rather than Pn, Tr, and gs. Therefore, the nitrogen application has a significant positive effect on plant growth up to peak level.

Leaf SPAD and photosynthetic parameters

Specific sugarcane cultivars have been screened by applying various N levels to check their response for growth and yield (Abayomi 1987; Muchovej and Newman 2004; Ghaffar et al. 2012), but reports for sugarcane genotypic variability against N are not sufficient. In this study, two different sugarcane genotypes were tested for their physiological and growth behavior by applying different N fertilizer levels in this study. Among these two genotypes, G1 had the lowest leaf area, G2 had the highest leaf area development. It was reported that under low N supply, the leaf area, SPAD values, photosynthesis, transpiration, and stomatal conductance altogether decreased significantly in sugarcane (Meinzer and Zhu 1998; Kumara and Bandara 2001). Nitrogen is required for protein synthesis and its stress causes retarded growth. That is why leaf area was lower under F1 ferilizer level. Compared with SPAD values, Pn, Tr, and gs were less effective to differentiate two fertilizer levels on both sampling periods. Studies have indicated that Pn, Tr, and gs had a close relation with N supply and its contents in the leaves of maize (Wolfe et al. 1988; Muchow and Sinclair 1994) and sorghum (Zhao et al. 2005). In the present study, however, Pn, Tr, and gs showed a positive response to applied N. Although SPAD values, Pn, Tr, and gs of the fertilization application level were higher than those of the genotypes and SPAD had less values than Pn, Tr, and gs readings. The results of this study and KWONG et al. (2001) suggest that the SPAD units or Pn, Tr, and gs are not desirable traits to estimate low N stress and its status in sugarcane crop.

Although SPAD values, Pn, Tr, and gs were different for genotypes but their highness had shown near S3 (Figure 1), Pn, Tr, and gs were changed with the growth of sugarcane across genotypes compared to SPAD values. McCormick et al. (2008) claimed that the negative relation between source to sink ratio and Pn, Tr, and gs for sugarcane. The higher sink capacity might be elaborate the higher Pn, Tr, and gs of G2 compared with the G1, because higher fraction of stem was observed for G2 and smaller for green leaves than G1 (Table 1). Genotypic differences, divergence, age, and thickness of leaf are variant factors for SPAD index (Ruiz-Espinoza et al. 2010).

Crop growth and productivity improvement are mainly derived by Pn, Tr, and gs (Khan et al. 2017). It is highly dependent on water availability, N application levels, and its uptake (Makoto and Koike 2007). Our study also showed higher Pn, Tr, and gs under F2 compared with F1 (Figure 1 (c–h)). The higher gaseous exchange attributes due to F2 might be associated with enough available N from the applied NH_4NO_3 which increased the photosynthetic rate at both sampling periods (Yang et al. 2015).

Root morphological attributes

The important plant organ roots were involved in the uptake of nutrients and water, and also produce organic acids, amino acids, and phyto-hormones (Lanna et al. 2018). Root morphology and physiology have significant role in soil nitrogen uptake and plant development (Garnett et al. 2009; Lynch 2013; Wu et al. 2014). In contrast to F1, F2 level of fertilizer had significantly enhanced root growth, while across the genotypes; the G2 had higher all root traits (Table 2). It might be recognized to quick and easy consumption of N from F2 fertilizer level and it was exhilarating influence of F2 level on roots, possibly connected to enough nutrient availability and well-watered soil also delay root senescence and finally increasing root growth activities.

The application of sufficient amount of N and water will reduce the leaf water potential and causing stress, which can be mitigated by the production of ABA due to root based signal and resulted in stomatal opening, with improved photosynthetic activity due to high leaf water potential (Daszkowska-Golec and Szarejko 2013). The G2 improved the root growth in the present study (Table 2), which can enhance the ability of roots to uptake nutrients and water from the soil, gaseous exchange in leaves, and CO_2 fixation.

Enzyme activity

The assimilatory nitrate reduction pathway is two steps process that firstly reduces nitrate into nitrite by nitrate reductase (NR) in cytosol after energy-dependent uptake of nitrate from soil to roots and secondly nitrite into ammonium/nitrogen through nitrite reductase (NiR) in chloroplast. It is extensively synchronized because the metabolites (nitrite and ammonium) of the process are highly toxic and its dependency on photosynthesis for energy and reducing enzymes (Ali et al. 2007). The assimilated ammonium/nitrogen synthesizes glutamine (Gln) and glutamate (Glu) by the action of glutamine synthase (GS) and glutamine synthase (GOGAT) enzymes in a cyclic way. All the other amino acids get nitrogen from Gln and Glu during their synthesis.

The activity of NR and NiR is regulated by the application of nitrate based fertilizer application (Ali et al. 2007). Our results showed the similar pattern for NR activity, as the fertilizer level increased from F1 to F2, NR activity increased (Figure 2(a) and Figure 3(a)). The importance of nitrate varies across plant species and tissues (Ali et al. 2007). The NR activity was variable among plant organs (Figure 2(a) and Figure 3(a)). A similar mechanism was observed for synchronization of nitrate assimilation in various crops such as maize (Faure et al. 1991), rice (Ali et al. 2007), and spinach (Ogawa et al. 2000).

The toxic nitrite cannot accumulate into plant cells and its production depends on NR activity, due to these reasons, it is considered as a strong candidate for feedback mechanism (Oaks 1994). Balanced synchronization of NR and NiR also inhibits the accumulation of nitrite in plant cells (Ogawa et al. 2000). The activity of NiR across the plant organs was almost the same in our study indicating that there was no accumulation of nitrite (Figures 2(b) and Figure 3(b)). This might be due to inhibition of the NR activity by inhibitory nature of NiR and sudden reduction into ammonium.

The nature of applied N fertilizer, either it is ammonium or nitrate, or any other, also determines the crop growth (Xu et al. 2012). Normally, plants uptake nitrate and ammonium which is transformed into amino acids and then into

proteins that can be utilized in further processes. The metabolic rates are determined by the enzymes activity that involved in N assimilation (Shah et al. 2017). The strength of GS, GOGAT, and GDH enzymes activity showed the power of plant to assimilate organic N into amino acids. Their activities are affected by different N application doses (Shah et al. 2017). In this study, N assimilating enzymes showed more activity at F2 fertilizer application level in the leaf, stem, and roots at the S3 after N applied, and also high leaf area, SPAD, Pn, and chlorophyl subsequently increased the iNUE and total dry biomass. These results are in consistent with the earlier studies. In barley, activities of GS, GOGAT, and GDH in the roots or leaf of plantlets increased with increasing N content (0–2 mmol) under hydroponic culture (Shah et al. 2017). In a hydroponic study, GS, GOGAT, and GDH enzymes activity increased positively for N rate (0–2 mmol) in *Hordium vulgare*.

The activities of GS, GOGAT, and GDH were significantly lower at S1 under F1 fertilizer level as compared to the F2 fertilizer application level (Figure 2(c,d)). The plant organs sensitivity towards N is more in leaf and stem than roots (Chen et al. 2016). The activities of GS and GOGAT enzymes in sugarcane leaves and stem under F2 fertilizer level were higher at the S1 and S2 stages than the roots. The observed effect of F1 was smaller for GS activity in roots and higher for GS and GOGAT activities in leaf and stem (Figure 2(c, d)). The N metabolism (GS/GOGAT pathway) mainly occurs in the photosynthetic plant organ (leaf) and its product glutamine translocate to roots which may be the possible reason for low enzymes activity in the roots. The reduced GS and GOGAT activity in roots might be happening because the photosynthesis taking place in the leaf, where Gln production by GS/GOGAT pathway was higher and it transferred to roots via stem. Thus, the GS/GOGAT process was stronger in leaf than the root under F1 fertilizer level.

Nitrogen uptake, NUI and iNUE

Maximum nitrogen uptake and total dry mass were observed for high NH_4NO_3 fertilizer application level, which confirmed the linear behavior between the supply of N and its uptake in sugarcane. Applying NH_4NO_3 fertilizer in splits can enhance its efficiency by a better match of N supply to crop demand and also have an impact on crop NUE. Further, NUE can be enhanced by applying transgenic approaches, remote sensing techniques, and by the improvement of genetic characters and physiological features (Bell et al. 2015). However, screening of genotypes with high NUE and low N tolerance from currently cultivated crops is an effective method to get sustainable yield and lower cost of production (Zhang et al. 2017). The iNUE changes were monitored between genotypes under F1 and F2 conditions in this research period. G2 was identified to have high iNUE. The activities of key enzymes and high leaf chlorophyl improved the photosynthetic process might be the important aspects for its agronomic performance especially the total dry biomass. This study found that the G2 was more suitable for F1 and F2 fertilizer supply based on the enzyme activities, photosynthesis, iNUE, and total dry weight at all sampling stages. In addition, the G1 had low total dry weight and iNUE against F1 fertilizer level and showed as low iNUE genotype, which was consistent with Hajari et al. (2014). These results have the key importance for the sugarcane grower to grow N use efficient genotypes and promote the production with low cost.

It was observed from the principal component (PCA), the traits related to nitrogen uptake, enzymes activities, photosynthetic parameters, and plant dry weight were well discriminated and closely related to fertilizer. Granato et al. (2016) reported high discrimination of genotypes under low N conditions. So, a better evaluation of genotypes should be performed under low nutrients availability. In contrast, the mean of plant organ's dry weight was decreased under low N than high N. Therefore, high N level was found to be more reliable for distinguishing plant dry weight. Adu et al. (2018) reported that low N level could be the most sharp for the selection of low and high N use efficient genotypes.

Conclusions

The genotype G2 was found to have an efficient mechanism for N uptake, use index, iNUE, and dry weight under low and high nitrogen conditions. e. Leaf area, SPAD values, and enzymatic activities were higher for G2 even under low nitrogen level which might be the reason to enhance the photosynthetic process for G2 than the G1 and ultimately higher the plant dry weight. The observations associated with N uptake, NUI, and iNUE were differentiated well at low N rate which suggest efficient N mechanism can be identified under low nitrogen level. In another way, the high N level had better differentiation for the dry biomass and N uptake. So, the bigger difference for dry biomass and N uptake can be assessed for genotypes under high N conditions. In concern of the N uptake and iNUE, further suggestions from findings were that the N uptake by the roots should be considered at the early growing stage.

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Ethics approval and consent to participate

All applicable international, national, and/or institutional guidelines were followed.

Consent for publication

All authors of this paper consent for publishing manuscript, tables and figures in this Journal.

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article and are not used in any other published material.

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