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Study of enzymatic activity in rice (*Oryza sativa* L.) genotypes under different nitrogen levels

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Abstract
The field experiment was laid out in a split plot design with three nitrogen levels of T_1 (120 kg N ha^{-1}), T_2 (80 kg N ha^{-1}) and T_3 (120 kg N ha^{-1}) as main plot treatments and twenty screend rice genotypes as sub plot treatments. To identify nitrogen use efficient cultivars enzymatic activity play an important role in the selection criteria. Nitrate reductase, Nitrite reductase and Glutamine synthetase activities were increased with higher level of nitrogen except Glutamate synthase activity, which showed opposite pattern, Glutamate synthase activity decreases with higher nitrogen levels. Genotypes which were recorded maximum enzymatic activities are DXD (124)-3-28, DXD (124)-17-192, DXD (124)-17-193, DXD (124)-15-164, DXD (124)-17-210, DXD (124)-11-133 and DXD (124)-9-91.

Keywords: Rice genotypes, nitrogen levels, nitrate reductase, nitrite reductase, glutamate synthase and glutamine synthetase and nitrogen use efficiency

Introduction
Rice (*Oryza sativa* L.) one of the prime food for most of the world population belongs to the family Poaceae. Rice is cultivated over 433.8 Lakh hectare area with a production of 104.3 Mt (IGC, 2016-17). Total production of rice has increased significantly by 4.74 Mt (4.54%) than the production of 104.3 Mt during 2015-16 (in India). Consumption of rice was estimated to 581 Mt in 2015 against total consumption of 531 Mt in 2005 (Prasad, 2011) [13]. To fulfil this global rice demand, it was analysed that till 2035, there will be requirement of 114 Mt of extra milled rice to produce, this means that in upcoming 25 years, rice production have to increase 26% (Kumar and Ladha, 2011) [10]. The need for increasing agricultural production and productivity cannot be overstated. The use of chemical fertilizers becomes very important in this context. The balanced use of chemical fertilizer is important not only for increasing the agricultural productivity but also for sustaining soil fertility. Fertilizer is an essential component of modern agriculture. Though there has been substantial increase in production and consumption of fertilizers over the years. To meet the growing foodgrains need of the population, the only option available is increasing productivity through proper planning and optimum utilization of resources such as fertilizers, seeds, water, etc. Nitrogen (N) is a one of the primary nutrient for plant growth and development. It is reported that improvement in crop yields is attributed to the increase in fertilizer use, especially nitrogen fertilizer (Cassman *et al.,* 2003) [1]. India stands second in N fertilizer consumption and production (FAI, 2013). Nitrogen is not only expensive but also an essential nutrient to plant as the commercial N fertilizers show the major cost in plant production (Singh, 2005) [15]. Where, crop yields are increased by application of N fertilizers, there are also some environment hazards due to increased use of N fertilizers like effects of global N cycle, ozone layer depletion and nitrate leaching problems in soil. Nitrogen use efficiency is found low in rice due to leaching which effects economic efficiency of applied N (Hakeem *et al.,* 2012) [7]. It is need to select high NUE rice genotypes for production with low cost crop management practices (Lea and Azevedo, 2006). 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) (Vijayalakshmi *et al.*, 2015) [18]. The enzymes activity as one of the selection criteria to identify nitrogen use efficient cultivars and their use in developing mapping population for high NUE. Application of nitrogen alone is not a prescription to obtain higher yields. But there is need of selecting such genotypes which positively contributing to high nitrogen use efficiency.

Thus, the objectives of this study were to investigate the response of different rice genotypes at different nitrogen fertilizer levels.

Material and Methods

Experimental Details

A field experiment was conducted during *Kharif* season of 2017-18 at College Farm, College of Agriculture, Indira Gandhi Krishi Vishwavidyalya, Raipur. The experiment was laid out in a split plot design with three nitrogen levels of 40 kg N ha^{-1}, 80 kg N ha^{-1} and 120 kg N ha^{-1} as main treatments and twenty screend rice genotypes as sub treatments. The experiment was replicated twice. The rice genotypes were sown separately in raised bed nursery and thirty day old seedlings were transplanted into plots by adopting a spacing of 20 X 15 cm. The fertilizers were applied as per the recommended doses (40:60:40, 80:60:40 and 120:60:40 kg ha^{-1}) N: P: K applied in the form of Urea, SSP and MOP, respectively. The half dose of N was applied as basal dose and the rest half N was applied in two equal splits at 30 and 50 days after transplanting. Irrigation and weed management was done in time to time.

Nitrate reductase (NR) enzyme activity estimation

Extraction of NR was done by the method suggested by Hageman and Flesher (1960) [6]. A weighted quantity of plant material (root or shoot tissue) was homogenized with mortar pestle in a known volume of medium (6 ml for 1g fresh tissue) containing 1 mM EDTA, 25 mM cysteine and 25 mM potassium phosphate adjusted to final pH of 8.8 with KOH. All the contents were transferred to eppendorffs tubes and centrifugated at 15,000 rpm for 15 min at 4 ^{0}C to obtain supernatant. Supernatant was filtered through 4 layers of cheese cloth and decant through glass wool in black capped glass borosil bottle of 15 ml and used for assays. Entire extraction process was accomplished under ice-cold conditions.

Procedure for assay of NR activity

NR activity was measured by modification of the method described by Evans and Nason (1953) [3]. 0.5 ml phosphate buffer (pH 7.5) was pipetted out in a borosil glass bottle 0.2 ml potassium nitrate solution, 0.4 ml NADH solution and 0.7 ml water was added in bottles. Reaction was started by addition of 0.2 ml enzyme extract and for control, enzyme extract is replaced by water. Incubation was done at 30^{0}C for 15 min. Reaction terminated by rapid addition of 1 ml sulphanilamide followed by 1 ml NED. The pink colour developed was measured at 540 nm spectrophotometer. The amount of nitrite formed was calculated by a standard curve plotted using the A 540 values obtained from known amounts of nitrite.

Calculations

$$\text{Units/ml enzyme} = \frac{(\mu\text{mole Nitrite formed})}{(\text{df})(\text{T})(0.2)}$$

Where,
df = Dilution factor
T= Time of assay (in minutes)
0.2= Volume of enzyme (in millilitre) used

$$\text{Units/mg protein} = \frac{\text{units / ml enzyme}}{\text{mg protein/ml enzyme}}$$

Nitrite Reductase (NiR) enzyme activity estimation

Crude extract preparation for nitrite reductase (NiR) enzyme extraction, crude homogenates were prepared according to Gupta and Beevers (1984) [5]. 1g of root or shoot tissue was pulverized with mortar pestle in a known volume of 0.5 M Tris HCL buffer (10 ml for 1g fresh tissue) and adjusted to final pH of 7.5 with NaOH. All the contents were transferred to tarson tubes and centrifugated at 15,000 rpm for 15 min, 4 ^{0}C to obtain supernatant. Supernatant was filtered through 5 layers of cheese cloth and decant through glass wool in black capped glass borosil bottle of 15 ml and used for assays. Entire extraction process was accomplished under ice-cold conditions.

Procedure for assay NiR

Nitrite reductase (NiR) activity was assayed by using dithionite reduced methyl viologen as an artificial electron donor, procedure is adopted as given by Wray and Fido (1990). Assay mixture was prepared by adding 6.25 ml of 0.5 M Tris HCL, 2ml of 2.5 mM sodium nitrite, 2 ml of 3 mM methyl viologen in a volumetric flask and making up final volume of 20 ml by adding 14.75 ml distill water. The reaction was started by the addition of sodium dithionite-bicarbonate solution. The blanks contained all the assay components except methyl viologen. The reactions was carried out at Room Temperature (25 °C) and stopped after 10 minutes by the addition of 1.9 ml of reaction stopping (sulphanilamide) and colour developing reagent (NED). The reaction was incubated for a further 15 min at RT and the pink colour developed was measured at 540 nm in spectrophotometer (Biomate 5, Thermo Electron Corporation). The amount of nitrite formed was calculated from a standard curve plotted using the A 540 values obtained from known amounts of nitrite.

Calculations

$$\text{Units/ml enzyme} = \frac{(\mu\text{mole Nitrite disappeared})}{(\text{df})(\text{T})(0.2)}$$

Where,
df = Dilution factor
T = Time of assay (in minutes)
0.2 = Volume of enzyme (in milliliter) used

$$\text{Units/mg protein} = \frac{\text{units / ml enzyme}}{\text{mg protein/ml enzyme}}$$

Glutamine synthetase (GS) enzyme activity estimation

Crude extract preparation extracts used of GS was prepared according to Cooper and Beevers (1969) [2]. Extract was prepared at 0-4 ^{0}C. 1 g of respective tissue was homogenized with mortar in a 4 ml medium of 50 mM Tris HCL (pH=7.8) containing 15% v/v glycerol, 14 mM ß-mercaptoethanol, 1 mM EDTA and 0.1% Triton X-100. Homogenate was squeezed with 3 layers of cheese cloth and centrifugated at 15,000 rpm for 10 min. Further, supernatant was used as enzyme extract.

Procedure for assay GS

For determination of GS activity, method of Lillo and henriksen (1984) was followed. Standard reaction mixture containing L-glutamate (500 mM), hydroxylamine hydrochloride (200 mM), magnesium sulphate (300 mM), ATP (100 mM) in Tris-HCl (200 mM) in a total volume of 2

ml was prepared. Final pH was maintained 8.0. 0.1 ml of enzyme extract was added to start the reaction giving assay a total volume of 2 ml. After incubation at 27 ^{0}C for 30 min., reaction was terminated by adding 2.5% $FeCl_3$ and 5% trichloroacetic acid in 1.5 M HCL and after centrifugation absorbance of supernatant was recorded at 540 nm.

Glutamate synthase (GOGAT) enzyme activity estimation
For crude extract preparation method employed by Singh and Shrivastava (1986) [14] was employed. Tissue was extracted in a mortar in a medium containing 100 mM phosphate buffer (pH=7.5), 1mM EDTA, 1 mM dierythritol and 1% PVP. Ratio of tissue and medium was kept 1:5 (w/v). The homogenate was centrifuged for 15,000 rpm for 15 min, 4^{0}C to remove unbroken cells and cell fragments. The supernatant was filtered through cheese cloth and clear solution obtained was used for further assay.

Procedure for assay GOGAT
The GOGAT activity was determined by the method suggested by Singh and Shrivastava (1986 b) [14]. Assay mixture contained 1.8 ml of 50 mM Tris-HCL buffer (pH=7.6), 1ml of 5 mM L-glutamine, 1 ml of 5 mM 2-oxo glutarate, 1 ml of 0.25 mM (NADH) and 0.2 ml of enzyme preparation in a final volume of 5 ml. At these concentrations maximum level of activity was obtained with the extract of tissue studied. 2-oxoglutarate was omitted in blank sample. Sample was incubated at room temperature for 15-30 min at 37 ^{0}C. Change in absorbance was recorded at 340 nm.

Result and Discussion

Nitrate reductase enzyme activity under nitrogen treatments
The data on nitrate reductase activity have been presented in Fig. 1 and Table 1. Significant differences were noticed among different nitrogen treatments in relation to nitrate reductase activity. Under 40 kg N, rice genotypes DXD (124)-3-28 showed maximum (14.3 µmole min^{-1} mg^{-1}) nitrate reductase activity, whereas DXD (124)-1-14 showed minimum (2.9 µmole min^{-1} mg^{-1}) nitrate reductase activity. In 80 kg N, rice genotype DXD (124)-3-28 showed maximum (17.4 µmole min^{-1} mg^{-1}) nitrate reductase activity, whereas DXD (124)-1-14 found minimum (6.0 µmole min^{-1} mg^{-1}) nitrate reductase activity. Under 120 kg N, rice genotypes DXD (124)-3-28 showed maximum (19.7 µmole min^{-1} mg^{-1}) nitrate reductase activity, whereas DXD (124)-1-14 showed minimum (8.3 µmole min^{-1} mg^{-1}) nitrate reductase activity. Nitrate reductase enzyme activity increased by higher level of Nitrogen. NR is the substrate inducible enzyme and its induction is closely dependent on the availability of nitrate. Hakeem *et al.* (2012) [7] 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.

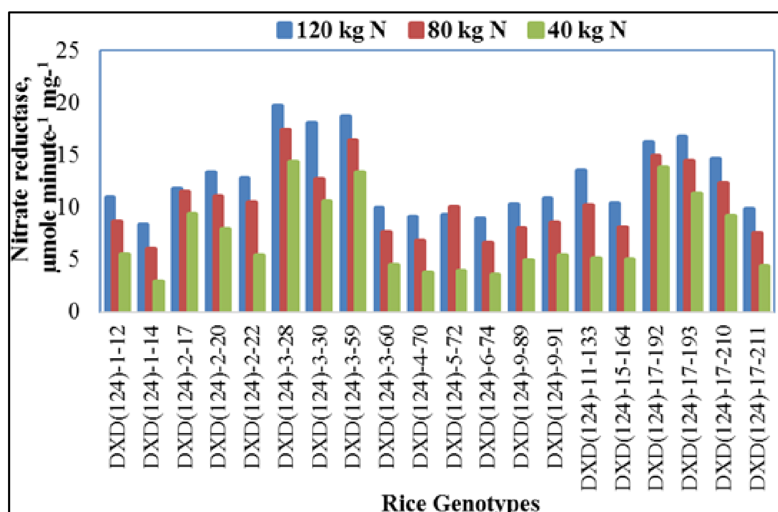

Fig 1: Effect of nitrogen levels on nitrate reductase enzyme of rice

Nitrite reductase enzyme activity under nitrogen treatments
The data on nitrite reductase activity have been presented in Fig. 2 and Table 1. Significant differences were noticed among different nitrogen treatments in relation to nitrite reductase activity. In 80 kg N, rice genotype DXD (124)-3-30 showed maximum (24.8 µmole min^{-1} mg^{-1}) nitrite reductase activity, whereas DXD (124)-1-12 found minimum (10.8 µmole min^{-1} mg^{-1}) nitrite reductase activity. Under 40 kg N, rice genotypes DXD (124)-3-59 and DXD (124)-17-192 similarly showed maximum (22.1 µmole min^{-1} mg^{-1}) nitrate reductase activity, whereas DXD (124)-1-12 showed minimum (9.9 µmole min^{-1} mg^{-1}) nitrate reductase activity, whereas. Under 120 kg N, rice genotype DXD (124)-3-30 showed maximum (25.9 µmole min^{-1} mg^{-1}) nitrite reductase activity, whereas DXD (124)-1-12 showed minimum (11.9 µmole min^{-1} mg^{-1}) nitrite reductase activity.

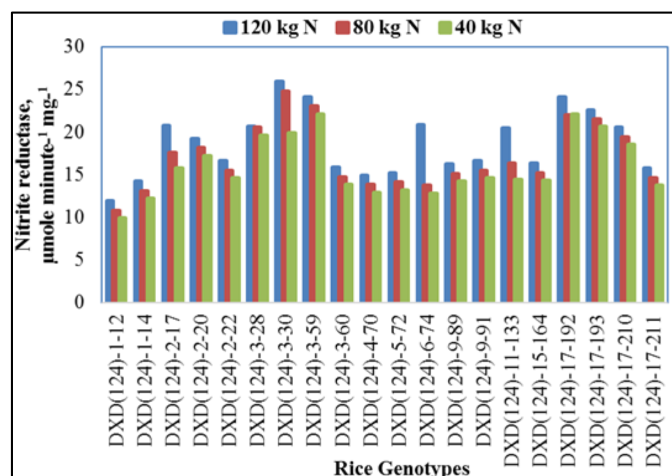

Fig 2: Effect of nitrogen levels on nitrite reductase enzyme of rice

Glutamine synthetase (GS) enzyme activity under nitrogen treatments

The data on GS activity have been presented in Fig. 3 and Table 1. Significant differences were noticed among different nitrogen treatments in relation to GS activity. Under 40 kg N, rice genotype DXD (124)-15-164 showed maximum (0.022 µmole min^{-1} mg^{-1}) GS activity, whereas DXD (124)-1-14 showed minimum (0.004 µmole min^{-1} mg^{-1}) GS activity. In 80 kg N, rice genotype DXD (124)-15-164 showed maximum (0.021 µmole min^{-1} mg^{-1}) GS activity and DXD (124)-1-14 found minimum (0.003 µmole min^{-1} mg^{-1}) GS activity. Under 120 kg N, rice genotypes DXD (124)-15-164 showed maximum (0.019 µmole min^{-1} mg^{-1}) GS activity, whereas DXD (124)-1-14 showed minimum (0.001 µmole min^{-1} mg^{-1}) GS. The present results indicated that in 40 kg N treatment, GS activity was higher to the 120 kg N conditions. 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) [8] and Su *et al.* (1995) [16] 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) [17].

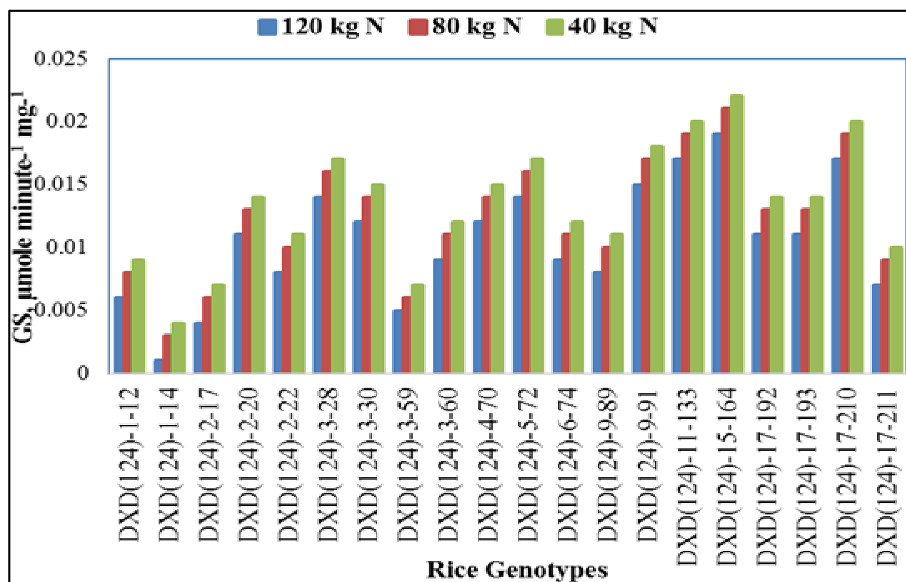

Fig 3: Effect of nitrogen levels on glutamine synthetase (GS) enzyme of rice

Glutamate synthase (GOGAT) enzyme activity under nitrogen treatments

The dada on GOGAT activity have been presented in Fig. 4 and Table 1. Significant differences were noticed among different nitrogen treatments in relation to GOGAT activity. In 80 kg N, rice genotypes DXD (124)-9-91 showed maximum (36.2 µmole min^{-1} mg^{-1}) GOGAT activity whereas DXD (124)-2-17 found minimum (17.4 µmole min^{-1} mg^{-1}) GOGAT activity. Under 40 kg N, rice genotypes DXD (124)-9-91 showed maximum (35.3 µmole min^{-1} mg^{-1}) GOGAT activity, whereas DXD (124)-2-17 showed minimum (16.5 µmole min^{-1} mg^{-1}) GOGAT activity. Under 120 kg N, rice genotypes DXD (124)-9-91 showed maximum (37.3 µmole min^{-1} mg^{-1}) GOGAT activity, whereas DXD (124)-2-17 showed minimum (18.5 µmole min^{-1} mg^{-1}) GOGAT activity.

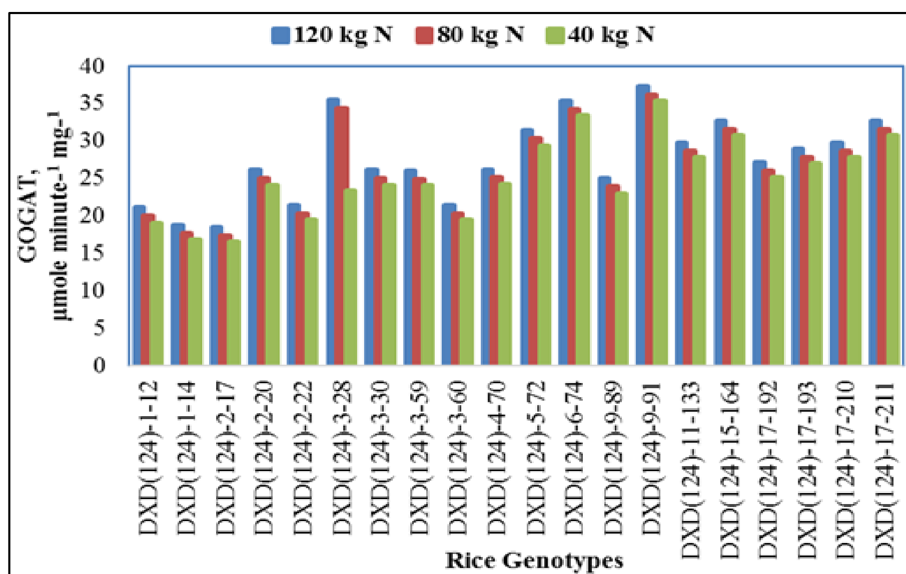

Fig 4: Effect of nitrogen levels on glutamate synthetase (GOGAT) enzyme of rice

Table 1: Effect of nitrogen levels on nitrate reductase, nitrite reductase, glutamine synthetase (GS) and glutamate synthase (GOGAT) enzymatic activity (μmole min^{-1} mg^{-1}) of rice (*Oryza sativa* L.) in leaves at panicle initiation stage

S.N	Name of rice genotypes	Nitrate reductase			Nitrite reductase			GS			GOGAT		
		T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3
1	DXD (124)-1-12	10.9	8.6	5.5	11.9	10.8	9.9	0.006	0.008	0.009	21.1	20	19.1
2	DXD (124)-1-14	8.3	6	2.9	14.2	13.1	12.2	0.001	0.003	0.004	18.8	17.7	16.8
3	DXD (124)-2-17	11.8	11.5	9.4	20.7	17.6	15.7	0.004	0.006	0.007	18.5	17.4	16.5
4	DXD (124)-2-20	13.3	11	7.9	19.2	18.1	17.2	0.011	0.013	0.014	26.1	25	24.1
5	DXD (124)-2-22	12.8	10.5	5.4	16.6	15.5	14.6	0.008	0.01	0.011	21.4	20.3	19.4
6	DXD (124)-3-28	19.7	17.4	14.3	20.6	20.5	19.6	0.014	0.016	0.017	35.4	34.3	23.4
7	DXD (124)-3-30	18	12.7	10.6	25.9	24.8	19.9	0.012	0.014	0.015	26.1	25	24.1
8	DXD (124)-3-59	18.7	16.4	13.3	24.1	23	22.1	0.005	0.006	0.007	26	24.9	24
9	DXD (124)-3-60	9.9	7.6	4.5	15.8	14.7	13.8	0.009	0.011	0.012	21.4	20.3	19.4
10	DXD (124)-4-70	9.1	6.8	3.7	14.9	13.8	12.9	0.012	0.014	0.015	26.2	25.1	24.2
11	DXD (124)-5-72	9.3	10	3.9	15.2	14.1	13.2	0.014	0.016	0.017	31.4	30.3	29.4
12	DXD (124)-6-74	8.9	6.6	3.5	20.8	13.7	12.8	0.009	0.011	0.012	35.3	34.2	33.3
13	DXD (124)-9-89	10.3	8	4.9	16.2	15.1	14.2	0.008	0.01	0.011	25	23.9	23
14	DXD (124)-9-91	10.8	8.5	5.4	16.6	15.5	14.6	0.015	0.017	0.018	37.3	36.2	35.3
15	DXD (124)-11-133	13.5	10.2	5.1	20.4	16.3	14.4	0.017	0.019	0.02	29.8	28.7	27.8
16	DXD (124)-15-164	10.4	8.1	5	16.3	15.2	14.3	0.019	0.021	0.022	32.7	31.6	30.7
17	DXD (124)-17-192	16.2	14.9	13.8	24.1	22	22.1	0.011	0.013	0.014	27.1	26	25.1
18	DXD (124)-17-193	16.7	14.4	11.3	22.6	21.5	20.6	0.011	0.013	0.014	28.9	27.8	26.9
19	DXD (124)-17-210	14.6	12.3	9.2	20.5	19.4	18.5	0.017	0.019	0.02	29.8	28.7	27.8
20	DXD (124)-17-211	9.8	7.5	4.4	15.7	14.6	13.7	0.007	0.009	0.01	32.7	31.6	30.7
	Treatment mean	12.3	10.6	7.4	17.6	17.1	16.5	0.011	0.013	0.013	27.6	26.5	25.6
	Factors	CD	SE (d)	SE (m)	CD	SE (d)	SE (m)	CD	SE (d)	SE (m)	CD	SE (d)	SE (m)
	Factor(N)	0.04	0.02	0.01	0.06	0.01	0.01	0.06	0.02	0.007	0.8	0.1	0.1
	Factor(G)	0.36	0.18	0.13	1.85	0.92	0.65	0.35	0.15	0.090	0.8	0.4	0.2
	GX N	0.51	0.31	0.02	1.91	1.60	0.04	0.47	0.25	0.020	0.9	0.5	0.3
	NX G	0.60	0.30	0.21	2.10	1.55	1.10	0.48	0.20	0.012	0.10	0.5	0.3

CD=critical difference, SE(m)=standard error of mean, SE(d)=standard deviation of mean

Conclusion

As the nitrogen treatment, enzymatic activity are significantly similar to each other, they shows similar pattern. Nitrate reductase, Nitrite reductase and Glutamine synthetase activities were increased with higher level of nitrogen except Glutamate synthase activity which showed opposite pattern, Glutamate synthase activity decreases with higher nitrogen levels. The enzyme activity showed minor differences according to genotypes. Nitrate reductase, nitrite reductase, GS and GOGAT enzyme differed from genotype to genotype. Genotypes which were recorded maximum enzymatic activities are DXD (124)-3-28, DXD (124)-17-192, DXD (124)-17-193, DXD (124)-15-164, DXD (124)-17-210, DXD (124)-11-133 and DXD (124)-9-91.

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