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Nitrogen metabolism enzymes activity in flag leaf and roots of pearl millet during grain development stage

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Abstract

Nitrate reductase (NR), Glutamine synthatase (GS), Glutamate dehydrogenase (GDH) and Aspartate aminotransaminase (AspAT) were assayed in flag leaves and roots of four pearl millet genotypes, started from ear emergence till grain maturity at four days intervals. All enzymes showed higher activities in flag leaf than that of roots irrespective of growth stage and genotypes. A slight increase in flag leaf NR, GS and AspAT activity till anthesis started decreasing while GDH increased till 12 days after anthesis (DAA) and almost constant till 18 DAA and then started decreasing. While in roots a steep declining trend was observed in NR, GS and AspAT and reached to almost non-detectable level till maturity while GDH increased up to 6 DAA and then decreased sharply irrespective of the genotypes. The almost stable GDH activity and decreasing trend in NR, GS and AspAT suggested that re-assimilation of amino nitrogen takes place in flag leaf instead of direct assimilation from the roots for nitrogen deposition in pearl millet developing grains.

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Introduction

Nitrogen metabolism is not only one of the basic processes of plant physiology, but also one of the important parts of global chemical cycle. Plant nitrogen assimilation directly takes part in the synthesis and conversion of amino acid through the reduction of nitrate. During this stage, some key enzymes, e.g., nitrate reductase (NR), glutamine synthetase (GS), glutamate dehydrogenase (GDH), glutamine synthase (GOGAT), aspargine synthetase (AS), and asparate aminotransferase (AspAT) participate in these processes. Nitrate is reduced to $NH4^+$ by sequential action of nitrate reductase (NR) and nitrite reductase (NiR). The conversion of NH_4^+ into glutamate proceeds via two pathways. In the GS/GOGAT pathway, $NH4^+$ is incorporated into glutamine by glutamine synthetase (GS), which is then converted with

2-oxoglutarate (2-OG) to glutamate by glutamate synthase (GOGAT). Glutamate dehydrogenase (GDH) catalyzes the incorporation of NH_4^+ into glutamate by reversible reductive amination of 2-OG (Inokuchi & Okada 2001; Cruz et al. 2004).

The cytosolic NR is the first enzyme in the pathway of nitrate assimilation, and its activity is highly regulated. Sufficient NR activity is a prerequisite for optimal utilization of soil nitrogen. NR has a major role in incorporation of nitrogen for plant yields under field conditions and it is widely known to be substrate inducible (Bergareche & Simon 1988). This is a rate limiting enzyme in the series of reactions whereby nitrogen is utilized for protein synthesis by plants and is known to be influenced by available soil nitrogen, the amount of light, environmental factors and genetic composition of plant (Ingle et. al. 1966; Kannangara et. al. 1967; Duffield et. al. 1972). Gupta et al. (2012) and Liyuan and Shi (2013) reported a positive response of NR activity in flag leaf after anthesis with nitrogenous fertilizer application in finger millet and winter wheat respectively. Similarly, Liyuan & Shi (2013) reported in winter wheat that soluble protein and nitrate reductase activity was very high at the beginning of the anthesis in flag leaf. However, flag leaf soluble protein content and NR activity continued to decrease in 7 days after anthesis and decreased rapidly in 14 days after anthesis under all treatments. Gupta et al. (2012) observed that the low grain protein genotypes reported higher NR in flag leaf at grain filling stage having better nitrogen use efficiency and responded well under higher nitrogen condition and its flag leaf was greener till maturity than that of high grain protein containing genotypes. The sequential decrease in NR during flowering and grain filling stages, in white and golden genotypes quickly transported the nitrogen from early stages of grain filling which continued till the end of grain filling.

After nitrate reduction, GS is the key enzyme responsible for the assimilation and re-assimilation of ammonia. In higher plants GS is one of the major enzymes responsible for the assimilation of ammonium absorbed from the growth medium, generated by nitrate reduction or re-assimilated after release of endogenous NH_4^+ by ammonium evolving processes such as photorespiration (Lea & Miflin 1974; Fentem et al. 1983; Claussen & Lenz 1999; Berwal et al. 2018). GDH is one of the few enzymes capable of releasing amino nitrogen from amino acids to give a keto-acid and NH_3 that can be separately recycled to be used in respiration and amide formation, respectively (Miflin & Habas 2002). GDH is expected to function in the de-aminating direction in tissues (germinating seeds and senescing leaves) that are converting amino acids into transport compounds with a low C/N ratio (Aubert et. al. 2001, Goyal et al. 2017). Srivastava & Singh (1987) reported that GDH level increased under various stressed conditions because plant may well need to give priority to carbon metabolism and keto-acid production over nitrogen metabolism. Many researchers observed an inhibited plant growth under stressed conditions in GDH *null* mutants in maize (Magalhaes et al. 1990; Pryor 1990) and in *Arabodopsis thaliana* (Melo-Olivera et al. 1996). It would happen

because GDH null mutants would be unable to convert glutamate to NH3, glutamine and oxoglutarate (Stewart et al. 1995). Liyuan & Shi (2013) found in winter wheat that GS activity was very high at the beginning of the anthesis in flag leaf. However, flag leaf GS activity continued to decrease in 7 days after anthesis and decreased rapidly after 21 days after anthesis under all treatments. Flag leaf GS activities increased with increasing amount of nitrogen application. It indicated that nitrogen fertilization improves the leaf NR and GS activity as well as leaf soluble protein content. Kichey et al. (2009) also observed similar results for GS and GDH with ageing flag leaf of wheat. Gupta et al. (2012) observed that the low grain protein (brown) showed higher GDH activity and lower GS activity in flag leaf at grain filling stage having better nitrogen use efficiency and responded well under higher nitrogen condition and its flag leaf was greener till maturity than that of high grain protein containing genotypes (Golden and Brown). The sequential increase in the activity of GDH and later GS during flowering and grain filling stages, respectively, in white and golden genotypes quickly transported the nitrogen from early stages of grain filling which continued till the end of grain filling. This might be a reason for high grain protein content in the white and golden genotypes. Contrastingly, in case of the brown genotype (low grain protein), GDH activity in the flag leaves was also found to be increased and it remained high till the grain filling stage. It indicates that both high GS activity along with high GDH activity is probably necessary at the time of flowering to grain filling to carry out the de-amination and transamination reactions during hydrolysis of flag leaves chloroplast initiated at the time of flowering.

Yong-Jian et al. (2009) had observed in rice flag leaves that GS activity increased from tillering stage to heading stages and started decreasing towards maturity stage at all irrigation and nitrogen fertilizer treatments. They observed that there were positive correlation between grain nitrogen content and functional leaves NR, GS and GOGAT enzymatic activities with the maximum correlation coefficient at heading stage, and also observed significant positive correlation between total nitrogen accumulation (TNA) and grain yield with activities of NR, GS and GOGAT at various growth stages except for tillering stage, this correlation was more closer with TNA than grain yield. Aspartate biosynthesis is mediated by the enzyme aspartate aminotransferase (AspAT; aspartate: 2-oxoglutarate aminotransferase; EC 2.6.1.1) which catalyses the reversible transamination between glutamate and oxaloacetate to generate aspartate and 2-oxoglutarate via a ping-pong bi-bi kinetic mechanism. The enzyme plays a key role in the metabolic regulation of C and N metabolism in all organisms. Nitrogen (N) is incorporated into carbon skeletons for the biosynthesis of the primary amino acids, glutamine and glutamate, which serve as N donors for the biosynthesis of major N compounds in plants, including other amino acids, nucleic acid bases, polyamines, and chlorophylls (Torre *et. al*. 2014). Under anaerobic condition i.e. under stressed conditions, accumulation of pyruvate leads to the alanine biosynthesis by upregulating the enzyme alanine

aminitransferase in plants (Streeter & Thompson 1972; Stewart & Larher 1980). Sempruch et al. (2012), observed under biotic stress (aphid feeding) which caused an increase in AlaAT activity and a decrease in AspAT with in tissues of winter triticale. Diestelfeld (2007) has reported a positive relationship between grain protein and Zn accumulation in wheat grain. No report is available in literature on pearl millet about such relationship. Keeping this view in mind the present experiment was designed to elucidate trend of nitrogen metabolism enzymes activity in flag leaf and roots of pearl millet during grain filling stage.

Materials and Methods

The present investigation was conducted with four pearl millet genotypes viz. HMS 14B, HMS 53B, HC 20 and WHC 901-445. The selected pearl millet genotypes were raised in 10 rows each with 10 x 45 cm intra and inter row spacing following the standared package and practices at research farm CCS HAU, Hisar during *kharif-2014*. Before growing of crop as well as after harvesting of crop, the Fe and Zn status of soil was determined. Four random soil samples were collected from each plot and mixed them properly to make it representative sample of the respective plots. The Fe content varied from 4.5 to 7 mg/kg and Zn content varied from 0.6 to 1.7 mg/kg. No significant variation was observed in Fe and Zn content before sowing and after harvesting of the crop.

The sampling time was fixed between 10.00 to 11.00 am in full sunshine. The plants were tagged on ear emergence and samples (flag leaf and roots) were collected on 25 days before anthesis (DBA) and 5 DBA, 0 DAA (days after anthesis) and on every forth day starting 12 DAA till 28 DAA and activities of nitrogen metabolism enzymes viz. NR, GS, GDH and AspAT were determined with following the standard protocols.

Nitrate reductase (NR) (E.C. 1.6.6.1)

In-vivo Nitrate reductase was determined using procedure of Sawhney and Naik (1972). The enzyme was assayed colorimetrically by monitoring the amount of nitrite produced. For determining *in-vivo* activity of nitrate reductase, 250 mg leaf discs were suspended in 10 ml of a medium constituting 50 mM phosphate buffer (pH 7.5), 20 mM KNO_3 and 3% (v/v) n-propanol and then vacuum infiltrated till all the discs sank to the bottom of solution. These tubes were incubated in dark in an incubator at 30^0 C. After 0, 10 and 30 min, 1/10 of aliquot was withdrawn and the amount of NO_2^- released was estimated by the method described by Fewson and Nicholas (1961). To 2 ml of properly diluted aliquot, 1 ml of 1% (w/v) sulfanilamide solution (prepared in 1N HCL) was added, the contents mixed thoroughly and then the colour developed with 1 ml of 0.02% (w/v) 1-Naphthyl ethylenediamine dihydrochloride (NED). The absorbance was recorded after 30 minutes on a UV–Vis spectrophotometer (Thermo Scientific, EVOLUTION 201) at 540 nm. The amount of nitrite was determined from the standard curve prepared with sodium nitrite (20-100 nmoles). The results of NR activities were expressed as µmoles of NO_2^- formed h^{-1} g^{-1} fresh weight.

Glutamine dynthetase (E.C.6.3.1.2)

The activity of glutamine synthetase (transferase activity) was assayed colorimetrically by estimation the amount of

□-glutamyl monohydroxamate (□ -GMH) produced according to the method of Kanamori and Matsumoto (1972). The plant tissues were extracted in 0.1 M phosphate buffer (pH 7.6) containing 2% polyvinylpyrrolidone (PVP), 1% β-mercaptoethanol and 10 mM dithiothreitol (DTT). Reaction mixture for assaying the enzyme activity, in the final volume of 2 ml, containing in µ moles: Tris-HCl buffer (pH 7.2), 150; glutamine, 150; hydroxylamine hydrochloride (neutralized), 70; ADP, 1.2; sodium arsenate, 80; $MnCl_2$, 1.5 and enzyme extract. Hydroxylamine was omitted from the blank. The tubes were incubated at 27 ^{0}C for 30 minutes and then the reaction was stopped by adding 2 ml of stop mixture containing 4 ml of 10% $FeCl_3$, 1 ml of 24% TCA, 0.5 ml of 6N HCl and 6.5 ml of distilled water. After 10 min, the protein precipitate was removed by centrifugation. The hydroxamic acid formed in the supernatant was measured on a UV–Vis spectrophotometer (Thermo Scientific, EVOLUTION 201) at 540 nm and its concentration was computed using -glutamyl monohydroxamate (-GMH) as standard (0.5 to 4 µmoles) (Annexure I, fig. M5). The results were expressed as µmoles of -GMH formed h^{-1} g^{-1} fresh weight.

Glutamate dehydrogenase (E.C.1.4.1.3)

Glutamate dehydrogenase (GDH) activity was assayed spectrophotometrically at 340 nm by following oxidation of NADH according to the method of Murrey and Kennedy (1980). The plant tissues were extracted in 0.1 M phosphate buffer (pH 7.6) containing 2% polyvinylpyrrolidone (PVP), 1% β-mercaptoethanol and 10 mM dithiothreitol (DTT). The reaction mixture, in a final volume of 3 ml, contained in µ moles: phosphate buffer (pH 7.5), 120; α-ketoglutarate, 18; ammonium sulphate, 150; NADH 0.4 and enzyme preparation. Rate of the reaction was followed by recording the change in absorbance at 340 nm in a UV–Vis spectrophotometer (Thermo Scientific, EVOLUTION 201). Background rates were also measured in the absence of α-ketoglutarate. The enzyme activity was expressed as µmoles NADH h^{-1} g^{-1} fresh weight.

Aspartate aminotransferase (EC 2.6.6.1)

Aspartate aminotransferase was assayed by following the method of Murrey and Kennedy, (1980). The plant tissues were extracted in 0.1 M phosphate buffer (pH 7.6) containing 2% polyvinylpyrrolidone (PVP), 1% β-mercaptoethanol and 10 mM dithiothreitol (DTT). The reaction mixture, in a final volume of 3 ml, contained in µ moles: phosphate buffer (pH 7.5), 120; α-ketoglutarate, 18; L-aspartate, 30; malate dehydrogenase, 3 IU; NADH 0.4 and appropriate volume of enzyme preparation. The reaction was started with α-ketoglutarate. Rate of the reaction was followed by recording the change in absorbance at 340 nm in a UV–Vis spectrophotometer (Thermo Scientific, EVOLUTION 201). Background rates were also measured in the absence of α-ketoglutarate. The enzyme activity was expressed as µmoles NADH h-1 g-1 fresh weight.

Results and Discussions

The results of the nitrogen metabolism enzymes activity in flag leaf and roots during grain filling stage are described here.

In vivo nitrate reductase (NR)

NR activity (*in vivo*) of the five pearl millet genotypes mentioned above was determined in flag leaf and roots at different plant growth stages indicated earlier. Profile of change in the activity of nitrate reductase in flag leaf and roots is depicted in figures 1& table 1. Magnitude of *in vivo* NR activity assayed at vegetative stage of growth on 25 DBA in flag leaf of the five genotypes was significantly different. Among the designated B-lines maximum activity was present in the leaf of HMS 14B (13.9 µmoles NO_2^- $g^{-1}h^{-1}$) followed by HMS 53B (7.16 µmoles NO_2^- $g^{-1}h^{-1}$) while *in vivo* NR activity of HC 20 and WHC 901-445 was closer to that of HMS 53B. The enzyme activity increased linearly in all the five genotypes till 0 DAA and reached at maximum level. After the start of anthesis activity of NR started declining in all the genotypes. Except in WHC 901-445, the enzyme activity decreased almost linearly till 20 DAA, to a level much lower compared to that was observed at the start of flowering. Decline in the enzyme activity in WHC 901-445 was very sharp. For example the activity in flag leaf of MHS 14B declined from 17.35 to 12.46 µmoles NO_2^- $g^{-1}h^{-1}$ between 0 and 16 DAA while that of WHC 901-445 declined from 13.47 to 4.50 µmoles NO_2^- $g^{-1}h^{-1}$ between the corresponding period. By 28 DAA activity of HMS 53B, HC 20 and WHC 901-445 fell to a minimum level of about 2 µmoles NO_2^- $g^{-1}h^{-1}$ while that of HMS 14B remained about two fold higher than that of the other genotypes.

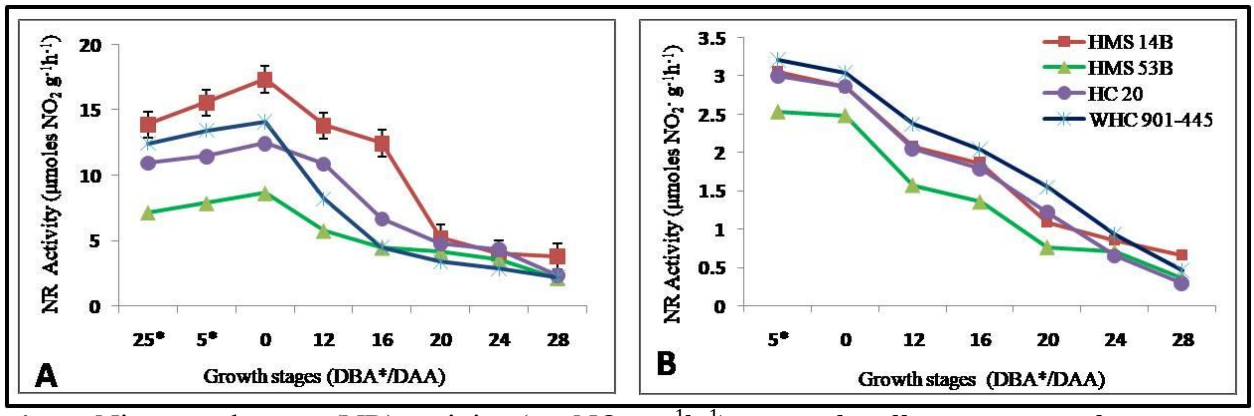

Fig 1. Nitrate reductase (NR) activity (µg NO_2^- $g^{-1}h^{-1}$) in Pearl millet genotypes during grain filling stages A. Flag leaf B. Roots. (**DBA-days before anthesis/DAA-days after anthesis*)

Table 1. Nitrate reductase (NR) Activity (µmoles NO_2^- $g^{-1}h^{-1}$) in flag leaf of pearl millet at different growth stages

Growth	HMS	HMS	HC	WHC
25 DBA	13.90	7.16	10.9	12.47
5 DBA	15.57	7.87	11.4	14.14
0 DAA	17.35	8.65	12.4	13.47
12 DAA	13.81	5.75	10.8	8.20
16 DAA	12.46	4.45	6.67	4.50
20 DAA	5.24	4.12	4.78	3.40
24 DAA	4.01	3.52	4.34	2.89
25 DBA	3.80	2.10	2.34	2.19

Table 2. Nitrate reductase (NR) Activity (µmoles NO2- g-1h-1) in roots of pearl millet at different growth stages

Grow	HMS	HMS	HC	WHC
5	3.05	2.53	3	3.21
0	2.85	2.48	2.86	3.04
12	2.08	1.57	2.05	2.37
16	1.86	1.35	1.79	2.04

20	1.09	0.76	1.22	1.55
24	0.85	0.7	0.65	0.93
25	0.66	0.35	0.29	0.46

Change in NR activity of roots of the grown four genotypes is presented in figure 1B & table 2. Compared with the flag leaf activity of the enzyme in root was significantly less in all the corresponding genotypes. NR activity continuously declined starting 5 DBA till maturity i.e. 28 DAA. Like flag leaf, in roots magnitude of NR activity of HMS 18B was higher while that of HMS 53B was lowest and other genotypes were more or less similar in respect of the level of activity.

Glutamine synthetase (GS)

Glutamine synthetase (GS) activity of above mentioned pearl millet genotypes was also monitored in flag leaf and roots starting from 25 days before anthesis (DBA) till maturity i.e. 28 days after anthesis (DAA) at the indicated time intervals (Fig. 2 & table 3, 4). Level of GS activity on 25 DBA in flag leaf of the tested genotypes was as follows: HMS 14B (697 µmoles -GMH $g^{-1}h^{-1}$), HMS 53B (309 µmoles -GMH $g^{-1}h^{-1}$), HC 20 (425 µmoles -GMH $g^{-1}h^{-1}$) and WHC 901-445 (377 µmoles -GMH $g^{-1}h^{-1}$).

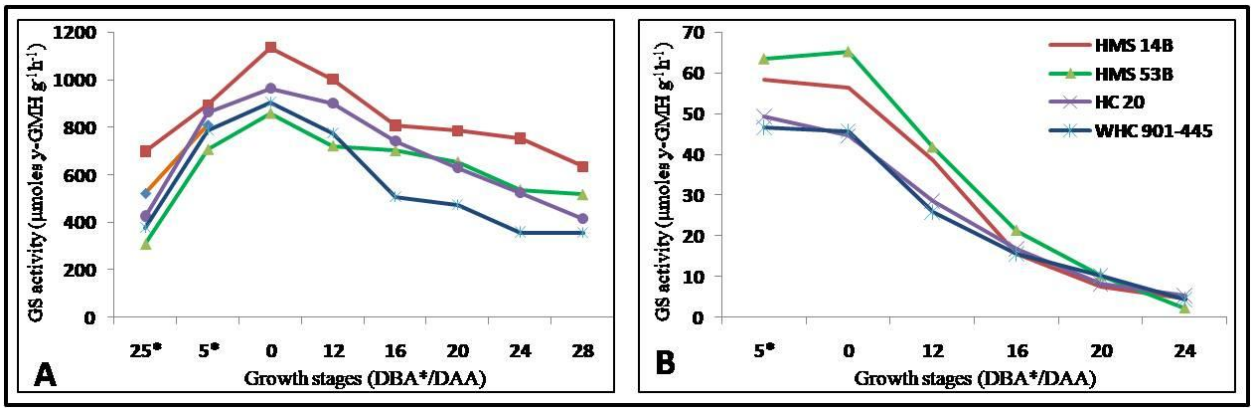

Fig 2. Glutamine synthetase (GS) activity (µmoles ϒ-GMH $g^{-1}h^{-1}$) in pearl millet during grain filling stages A. Flag leaf B. Roots. (**DBA-days before anthesis/DAA-days after anthesis*)

Table 3. Glutamine synthetase (GS) activity (µmoles gamma-GMH $g^{-1}h^{-1}$) in flag leaf of pearl millet at different growth stages

Grow	HMS	HMS	HC	WHC
25	697	309	425	377
5	893	707	863	789
0	1135	859	964	905
12	1002	722	901	775
16	806	702	741	507
20	784	654	631	474
24	752	538	524	357
25	634	518	415	356

Thus GS activity of the genotypes differed markedly among all the genotypes on 25DBA. GS activity was maximum in HMS 14B. Like NR, activity of this enzyme on the same day of plant growth in HMS 53B was lowest among the other genotypes. Level of activity of NR and GS in leaves of HC 20 and WHC 901-445 was contrasting, i.e. activity of NR was higher with lower level of GS activity in WHC 901-445 (compared to HC 20) whereas activity of NR was lower with a higher level of GS activity in HC 20. Activity of GS increased sharply along with growth of the plants till start of anthesis in each genotype but differentially and attained maximum level

in each genotype. For example, the enzyme activity increased by a maximum of more than two fold from 377 µmoles □-GMH $g^{-1}h^{-1}$ to 905 µmoles □-GMH $g^{-1}h^{-1}$ in WHC 901-445 whereas, in HMS 14B the enzyme activity increased from 697 µmoles gamma-GMH $g^{-1}h^{-1}$ to 1135 µmoles □-GMH $g^{-1}h^{-1}$. Except WHC 901-445, GS activity in the other genotypes declined sharply till 16 DAA and then gradually and reached to a minimum level by 28 DAA. On the last day GS activity in flag leaf of HMS 14B remained much higher than that of the other genotypes and on the last day, (28 DAA) of the sampling WHC 901-445 showed the least activity.

Table 4. Glutamine synthetase (GS) activity (µmoles gamma-GMH $g^{-1}h^{-1}$) in roots of pearl millet at different growth stages.

Grow	HMS	HMS	HC	WHC
5	58.23	63.36	49.2	46.64
0	56.32	65.12	44.5	45.76
12	38.48	41.66	28.5	25.83
16	15.55	21.27	16.8	15.59
20	7.58	10.25	8.23	10.215
24	4.55	2.21	5.32	4.25
25	ND	ND	ND	ND

Trend in changes in the activity of GS in roots of the pearl millet designated B-lines HMS 14B and HMS 53B and composites HC 20 and WHC 901-445 since vegetative stage starting 5 DBA till maturity is presented in figure 2B & table 4. The enzyme activity in root of each genotype analyzed on 5BDA was lower (ranging from $1/5^{th}$ to $1/10^{th}$) compared to that of flag leaf of the respective genotype. The level of activity observed on 5 DBA was maintained till stage of the anthesis by each genotype. A drastic reduction in the activity in roots of all the genotypes was recorded between 0 DAA and 16 DAA. And the enzyme remained minimal active and statistically similar in roots of all the genotypes on 20 DAA and 24DAA.

Glutamate dehydrogenase (GDH)

Figure 3 A & B shows pattern of change in activity of GDH in flag leaf and roots of five pearl millet genotypes. Like NR, NiR and GS, activity of GDH in flag leaf was higher than that of roots of all the genotypes during the entire growth period. GDH activity in flag leaf showed increasing trend during vegetative stage starting 25 DBA till start of anthesis i.e. 0 DAA (Fig. 3A & table 5) and in roots starting 5 DBA till 0 DAA (Fig. 3B & table 6). In general, level of GDH activity in flag leaf assayed on a particular day during the period between 0 and 20 DAA of HMS 14B, HMS 53B, HC 20 and WHC 901-445 remained either almost constant or decreased slightly.

Table 5. Glutamate Dehydrogenase (GDH) activity (µmole NADH $g^{-1}h^{-1}$) in flag leaf of pearl millet at different growth stages

Grow	HMS	HMS	HC	WHC
25	71.3	47.5	95.0	58.1
5	90.8	56.1	103.	72.6
0	101.6	72.6	107.	98.0
12	111.6	75.0	105.	94.9
16	104.6	73.0	98.0	80.5
20	101.0	64.7	78.0	78.0
24	81.8	59.4	73.0	73.0
25	75.5	56.8	60.7	72.6

For example the activity in flag leaf varied between 101 to 112 µmoles NADH $g^{-1}h^{-1}$ in HMS 14B and between 65 to 75 µmoles NADH $g^{-1}h^{-1}$ in HMS 53B during the reproductive period of 20 days, starting 0 DAA. After slow and gradual decrease in activity of the enzyme after 20 DAA, a substantial amount of enzyme remained active

on 28 DAA ranging from 57 to 76 µmoles NADH $g^{-1}h^{-1}$. From the pictorial presentation of data in fig. 3A, it is also clear that GHD activity in flag leaf during the entire reproductive period in respective genotype remained higher than the initial level of its activity recorded at 25 DBA. In contrast GDH activity in roots decreased sharply starting from 12 DAA and only trace of the enzyme remained active by 24th day of anthesis (Fig. 3B). Thus in flag leaf a significant level of GDH activity was retained throughout the vegetative and reproductive phase of growth of pearl millet genotypes tested.

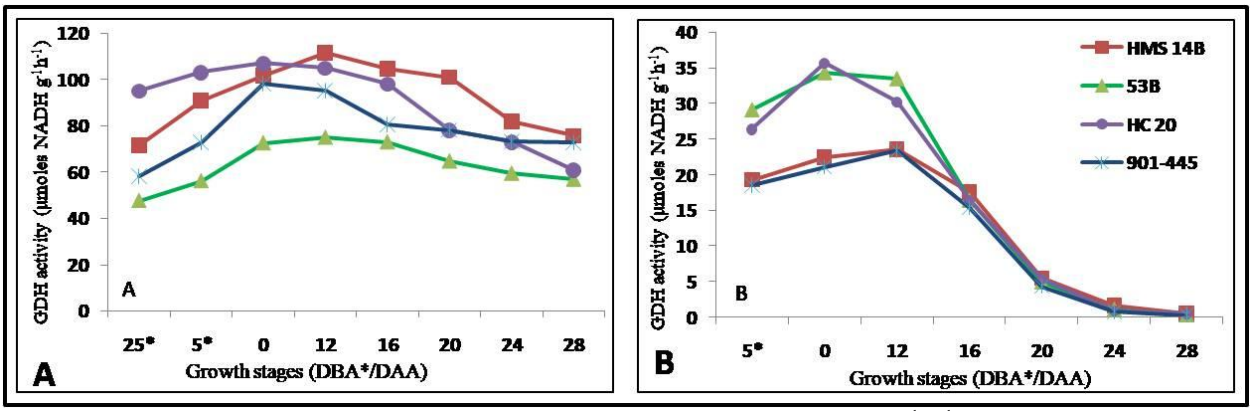

Fig 3. Glutamate dehydrogenase (GDH) activity (µmoles NADH $g^{-1}h^{-1}$) in flag leaf of pearl millet grain filling stages **A.** Flag Leaf **B.** Roots.

Table 6. Glutamate Dehydrogenase (GDH) activity (µmole NADH $g^{-1}h^{-1}$) in roots of pearl millet at different growth stages

Grow	HMS	HMS	HC	WHC
5	19.25	29.1	26.4	18.5
0	22.35	34.3	35.6	21.1
12	23.52	33.45	30.2	23.45
16	17.48	16.39	16.3	15.36
20	5.47	4.96	5.33	4.29
24	1.66	1.09	0.86	0.76
25	0.52	0.33	0.45	0.27

Aspartate aminotransferase (AspAT)

Qualitative and quantitative pattern of change in the level of activity of Aspartate aminotransferase in flag leaf and roots of the four genotypes is presented in figures 4 & table 7& 8. AspAT activity in flag leaf exhibited a profile similar to that of GDH activity in the respective tissues of the plant except that peak AspAT activity was detected at the onset of reproductive period i.e. 0 DAA instead of 12 DAA (Fig. 4). On 0 DAA flag leaf of HC 20 had maximum AspAT activity equivalent to 5861 µmoles NADH $g^{-1}h^{-1}$ followed by HMS 14B (5677 µmoles NADH $g^{-1}h^{-1}$), HMS 53B (4884 µmoles NADH $g^{-1}h^{-1}$) and WHC 901-445 (4512 µmoles NADH $g^{-1}h^{-1}$). Similar to GDH, a higher level of activity of AspAT was maintained during the period since flowering till physiological maturity of the grain compared to that recorded on 25 DBA.

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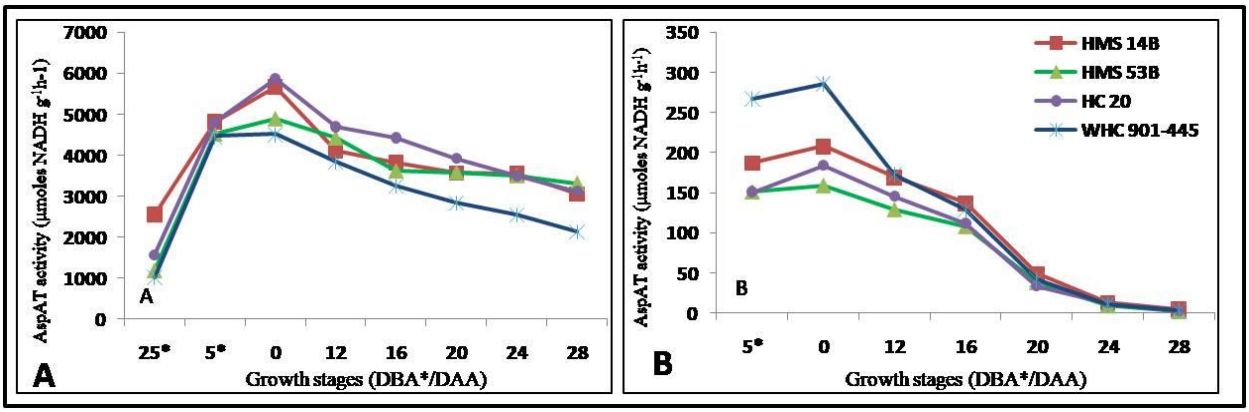

Fig 4. Aspartate aminotransferase (AspAT) activity (µmoles NADH $g^{-1}h^{-1}$) in flag leaf of pearl millet grain filling stages A. Flag Leaf B. Roots

Table 7. Aspartate aminotransferase (AspAT) activity (µmoles NADH $g^{-1}h^{-1}$) in flag leaf of pearl millet at different growth stages

Grow	HMS	HMS	HC	WHC
25	2554	1188	1582	1017
5	4817	4514	4806	4462
0	5677	4884	5861	4512
12	4119	4415	4686	3835
16	3828	3617	4429	3247
20	3571	3581	3927	2838
24	3557	3503	3498	2544
25	3056	3313	3122	2128

Table 8. Aspartate aminotransferase (AspAT) activity (µmoles NADH $g^{-1}h^{-1}$) in roots of pearl millet at different growth stages

Grow	HMS	HMS	HC	WHC
5	187	151	151	267
0	208	159	184	286
12	169	129	146	173
16	137	108	112	129
20	49	38	34	42
24	13	9	12	11
28	4.8	1.68	2.6	3.57

Even at 28 DAA substantial amount of activity of the enzyme (between 2000 to 3300 µmoles NADH $g^{-1}h^{-1}$) in flag leaf of the tested genotypes was still present. Compared to flag leaf, activity of AspAT was very low in root tissues (Fig.4B & Table 8). For example on 0 DAA activity of this enzyme in leaf of the respective genotype was at least 20 times higher than that present in roots. Compared to the other enzymes viz. NR, GS and GDH the difference in terms of folds in activity of this enzyme in leaf and root tissues was the highest. In contrast to flag leaf, AspAT in roots decreased sharply between 0 and 28 DAA. Among the designated B-lines maximum NR activity was present in the leaf of HMS HMS 14B (13.9 µmoles NO_2^- $g^{-1}h^{-1}$) followed by HMS 53B (7.16 µmoles NO_2^- $g^{-1}h^{-1}$). Flag leaf of the composites showed *in vivo* NR activity closer to that of HMS 53B. The enzyme activity increased linearly in all the four genotypes till 0 DAA and reached at maximum level. After the start of anthesis activity of NR started declining in the remaining four genotypes, while in roots, NR activity continuously declined starting 5 DBA till maturity i.e. 28 DAA. Like flag leaf, in roots magnitude of NR activity of HMS 14B was higher while that of HMS 53B was lowest and other genotypes were more or less similar in respect of the level of activity. These results are corresponded with the earlier published

reports. Wu et al. (2013), reported that NR activity in flag leaf of winter wheat peaked at 5DAA (Days after anthesis), decreased slowly until 15DAA and then dropped sharply under all irrigation and fertilizers treatments along with control. Liyuan & Shi (2013) reported in winter wheat that nitrate reductase activity was very high at the beginning of the anthesis in flag leaf and continued to decrease till 7th day after anthesis and decreased rapidly by 14th day after anthesis. Raimanova and Trckova, (2007) reported maximum NR activity in wheat flag leaf at flowering stages and decreased until 25 DAF. Similar results for flag leaf NR activity were also observed in finger millet by Gupta et al. (2012) and in maize by Narasimhan et al. (1987). No such reports are available on pearl millet in the literature. GS activity of the tested genotypes differed markedly. GS activity was maximum in HMS 14B. Like NR, activity of this enzyme on the same day of plant growth in HMS 53B was lowest among the other genotypes. Activity of GS increased sharply along with growth of the plants till start of anthesis in each genotype but differentially and attained maximum level in each genotype. GDH activity in flag leaf showed increasing trend during vegetative stage starting 25 DBA till start of anthesis *i.e.* 0 DAA and in roots starting 5 DBA till 0 DAA. In general, level of GDH activity in flag leaf assayed on a particular day during the period between 0 and 20 DAA of HMS 14B, HMS 53B, HC 20 and WHC 901-445 remained either almost constant or decreased slightly. After slow and gradual decrease in activity of the enzyme after 20 DAA, a substantial amount of enzyme remained active on 28 DAA. These results for GS and GDH enzyme activities are correspond with the earlier reports in wheat, rice and finger millets. Wu et al. (2013) reported that GS activity in flag leaf of winter wheat peaked at 5DAA (Days after anthesis), decreased slowly until 20DAA and then dropped sharply under all treatments along with control. Yong-Jian et al. (2009) had observed in rice flag leaf that GS activity increased from tillering stage to heading stages and started decreasing towards maturity stage at all irrigation and nitrogen fertilizer treatments. Liyuan & Shi (2013) reported in winter wheat that GS activity in flag leaf was very high at the beginning of the anthesis in flag leaf. However flag leaf GS activity continued to decrease in 7 days after anthesis and decreased rapidly after 21 days after anthesis under all treatments and also reported that flag leaf GS activities increased with increasing amount of nitrogen application. It indicated that nitrogen fertilization improves the leaf NR and GS activity as well as leaf soluble protein content. Kichey et al. (2005) also observed similar results for GS and GDH with ageing flag leaf of wheat. Ino-kuchi & Okada (2001) reported that GDH functions in the de-aminating direction in tissues (germinating seeds and senescing leaves) that are converting amino acids into transport compounds with a low C/N ratio. Srivastava & Singh (1987) reported that GDH level increased under various stressed conditions because plant may well need to give priority to carbon metabolism and keto-acid production over nitrogen metabolism. Gupta et al. (2012) had studied the relationship of nitrogen use efficiency with nitrogen metabolizing enzymes involved in nitrogen

uptake and assimilation in finger millet under different nitrogen inputs and observed that sequential increase in the activity of GS and GDH during flowering and grain filling stages, respectively, in white and golden genotypes quickly transported the nitrogen from early stages of grain filling which continued till the end of grain filling. Both high GS activity along with high GDH activity is probably necessary at the time of flowering to grain filling to carry out the de-amination and trans-amination reactions during hydrolysis of flag leaf chloroplast initiated at the time of flowering. It also indicated that not only high GDH activity but also high activity of GS in the flag leaves at the time of grain filling is necessary to achieve higher grain protein content. No such reports are available on pearl millet. AspAT activity in flag leaf exhibited a profile similar to that of GDH activity in the respective tissues of the plant except that peak AspAT activity was detected at the onset of reproductive period *i.e.* 0 DAA instead of 12 DAA. AspAT activity in flag leaf increases sharply till anthesis and reached to maximum and then started to decrease at a slower rate and was maintained more activity than its initial activity till maturity i.e. 28DAA. Before anthesis HMS 14B showed maximum activity but at anthesis HC 20 showed maximum activity in flag leaf. While in root at initial stage WHC 901-445 showed maximum activity. There was a little variation for AspAT activity among genotypes till 12DAA only after that all genotypes showed almost similar activity with sharply decreasing trend and reached to non detectable level at 24DAA.

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