RESEARCH ARTICLE

Effect of Nitrogen Nutrition Stress on Grain Weight, Starch Quality in Bread Wheat

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Abstract

Grain weight in wheat is influenced by genetics and the environment, with nitrogen (N) nutrition playing a crucial role by improving grain filling and dry matter accumulation. Starch constitutes 60 to 70% of wheat grain dry matter, consisting of amylose and amylopectin. Amylose/amylopectin ratio determines the resistance nature of the starch and glycaemic index, which are important for human health. Nitrogen nutrition affects dry matter accumulation in wheat grain by influencing starch biosynthesis and enzymes involved in it. In this study, four wheat genotypes (HB-208, VL-829, chotilerma and HD-2967) with varied grain weights were grown under optimum nitrogen (ON) and low nitrogen (LN) conditions. The effect of nitrogen stress on grain weight; starch, amylose and amylopectin content were studied in these genotypes. Further, the important enzymes linked to amylose and amylopectin biosynthesis activity were also assayed in grains during the active grain-filling stages. HB-208 showed the highest grain weight, followed by VL-829, HD-2967 and chotilerma. Under nitrogen stress, grain weight was significantly reduced in HB (29%) and VL (25%), whereas no reduction was observed in case of HD and CL. Genotypic difference in grain filling was observed, where maximum starch accumulation in HD was observed before 21 days after anthesis (DAA) whereas, after 21 DAA in VL. We observed an increase in grain protein content under nitrogen stress in all three genotypes except HD. Among the two components of starch, amylose content was significantly reduced across the genotype under N stress, whereas amylopectin was unchanged in HB and VL and increased in HD and CL. Reduction in amylose resulted to starch reduction in HB and VL, which also corroborated with grain weight reduction. AGPase enzyme activity at 21 DAA was reduced in HB and VL, resulting in decreased starch content.

Keywords: Wheat, Nitrogen, Starch, Enzyme activity, Grain weight.

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Introduction

Nitrogen nutrition is an indispensable factor for wheat production. During growth, especially during grain filling, nitrogen nutrition is a major limiting factor to wheat grain quality worldwide (Dupont and Altenbach, 2003). Studies show applying foliar nitrogen fertilizer during the later stages of wheat growth positively affects grain filling, particularly under drought conditions. Also, the omission of nitrogen fertilizer application in spring led to a significant reduction in both grain dry matter and grain nitrogen content by approximately 26 and 45%, respectively in winter wheat (Lv *et al.*, 2002; Blacklow and Incoll, 1981) and supplying nitrogen after flowering resulted in increased grain yield, (Madani *et al.*, 2010).

Wheat cultivars with different grain size exhibit variations in grain development. Endospermic cells are higher in larger grain type cultivar and take longer grain filling than small grain cultivar. Increased photosynthate production has been observed in larger gain type cultivars during mid grain grain-filling stage, providing ample substrate for grain development (Zhao *et al.*, 2003). Furthermore, enhanced conversion and utilization of assimilates in the sink tissues contribute to higher rates of starch accumulation

and filling in grains during the middle to late stages. The peaks of starch accumulation rate (SAR), photosynthetic rate, sucrose content, and activities of related enzymes in larger grain type typically occur during the middle to late filling stage and have a longer duration (Dai *et al.*, 2009; Wang, 2014)

Starch, which constitutes 60–70% of the wheat grain weight, is the most vital carbohydrate in wheat grain. Around 20–30% of the starch is contributed by amylose, while the remaining 70–80% is attributed to amylopectin (Cornell, 2003). Amylose is a linear polysaccharide with glucose residues linked solely by α-1,4 glycosidic bonds. On the other hand, amylopectin is a branched molecule with branch points connected by α-1,6 glycosidic bonds, and α-1,4 glycosidic bonds connect the linear portions. Composition of starch, i.e., amylose and amylopectin ratio have profound effect on health. High amylose content increases the proportion of resistant starch in cereal grains. Resistant starch is the portion of starch that resists digestion in the small intestine. It is a type of dietary fibre offering various health benefits like improvement in insulin response, colon health and fullness (Slade *et al.*, 2012). The amount and rate of accumulation of starch play a crucial role in determining the yield and quality of wheat. Starch synthesis primarily occurs in the endosperm cells by coordinated regulation of the series of enzymes (Ran *et al.*, 2020). Notably, adenosine diphosphate glucose pyrophosphorylase (AGPase), soluble starch synthase (SS), granule-bound starch synthase (GBSS), and starch branching enzyme (SBE) have significant functions in starch synthesis. AGPase catalyzes the reaction between glucose-1-phosphate (G-1-P) and ATP, resulting in the formation of adenosine diphosphate glucose (ADPG) and pyrophosphoric acid (PPi) which is also a rate limiting step in starch biosynthesis hance, critical in determining the rate of starch synthesis and accumulation in the endosperm (Bowsher *et al.*, 2007). SS and SBE is responsible for synthesizing amylopectin (Zhang *et al.*, 2010; Zhang *et al.*, 2011) and GBSS contribute to amylose synthesis by specifically binding to starch granules (He and Wei, 2020).

A study conducted on tartary buckwheat starch concluded that the activity of four key enzymes related to starch synthesis exhibited an initial increase followed by a decrease as nitrogen fertilization levels rose also, applying a moderate amount of nitrogen to rice crops resulted in a higher proportion of short amylopectin (DP 6–12), a lower proportion of long amylopectin (DP ≥ 37), an increased starch breakdown value, reduced SV (starch viscosity) and PT (pasting temperature) (Gao *et al.*, 2021). Overall, nitrogen fertilization influences enzyme activity and substance accumulation, subsequently impacting crop quality. Relations between grain weight, starch content and enzymatic activity have been investigated in various abiotic stress, soil type and irrigation conditions (Zhang *et al.*, 2021; Kim and Kim, 2021; Wang *et al.*, 2008). however, there are very limited studies investigating its relation with nitrogen nutrition.

With the above background, while on one hand the nitrogen application needs to be reduced, the effect on starch quality of wheat grains, mainly the amylose and amylopectin content and their ratio is very important under low nitrogen regime. To address this important area, in the present study, four genotypes with diverse grain weight was grown under optimum and low soil nitrogen regime and the effect of nitrogen stress has been studied on grain filling, grain weight, amylose content and amylopectin content. In addition to the grain weight, important enzymes involved in starch biosynthesis were also investigated during mid grain filling period of developing grains.

Material and Methods

Plant Material and Growth Condition
A total of 4 genotypes were selected for the study having different grain weight and size at optimum N input. HB-208 (HB) had bold type grain, VL-829 (VL) and HD-2967 (HD) had medium size grain and chotilerma (CL) had small grain. Plants were grown on net house pots under two nitrogen treatments, 120 kg/ha (ON) having soil N content 150 kh/ha and 0 kg/ha (LN) having soil available nitrogen content 100 kg/ha. Experimental design, growth condition and soil N status are mentioned in Bharati *et al.*, (2022). Grain dry weight was measured for a thousand grains in three replicates. Immature grains were harvested at 21 and 28 day after anthesis (DAA).

Starch Amylose and Amylopectin and Protein Estimation
Starch content was measured according to Kaufman *et al.*, (2015). To measure starch content, freeze dried immature grains and dry mature grains were ground to powder. For 100 mg of powdered grain were extracted 3 times by boiling in 1-mL of 80% ethanol to remove soluble sugar. Pellet was dissolved in 1-mL of 95% DMSO by incubating it on boiling water bath for 1-hour with intermittent vortexing. After complete dissolution of starch, 10 µL of aliquot of dissolved starch was removed into fresh tube and mixed with 990 µL of water. In 200 µL of diluted sample was mixed with 10 µL of Lugol's reagent and absorbance was recorded at 595 nm. Standard curve was prepared from potato starch. Amylose content was determined according to Zhu *et al.*, (2008) by dual wavelength iodine binding technique at 510 and 620 nm. Standard curve was prepared by potato amylose (A8515; Sigma–Aldrich). Amylopectin content was determined by subtracting amylose content to starch content. Total protein content was determined by measuring N content through Kjeldahl method followed by multiplying it with factor 6.2.

Enzyme Activity
100 mg grains were ground in 1-mL of extraction buffer containing 100 mM MOPS/NaOH (pH 7.2), 5 mM MgCl$_2$, 5% v/v glycerol, 5 mM dithiothreitol, 10 mg mL^{-1} BSA, 1%

(w/v) PVP. Samples were ground on ice cold mortar on ice followed by centrifugation at 10000 × g for 15 minutes at 4°C. Supernatant was collected for determining AGPase, UGPase, and SS activity. Pellet was again washed with 800 µL extraction buffer to remove any traces of SS. Pellets were suspended in 100 µL of extraction buffer for GBSS assay.

AGPase, UDPase and SS activity was assayed according to Nakamura *et al.*, (1989). For AGPase assay 40 µL of enzyme extract was mixed with 200 µL of reaction mixture containing 100 Mm HEPES-NaOH (pH 7.5), 3 Mm PPi, 4 mM Dithiothreitol, 1.2 mM ADP-Glucose and 5 mM $MgCl_2$. Mixture was incubated at 30°C for 20 minutes followed by placing in boiling water bath for 30 seconds to terminate the reaction. Mixture was centrifuged at 10000 × g for 10 minutes. To 200 µL supernatant 7 µL of 10 mM NADP was added and mixed well. Change in OD was recorded at 340 nm after addition of 1-µL of glucomutase (0.4 unit) and 1-µL of Glucose-6-phosphate dehydrogenase (0.35 unit).

UDPase assay was conducted in same manner as AGPase by only replacing ADP-Glucose to 1-mM UDP-Glucose in reaction mixture.

For assaying SS activity 20 µL of enzyme extract was combined with 120 µL reaction mixture (Mixture I) containing 50 mM HEPES-NaOH (pH 7.4), 0.7 mg amylopectin, 1.6 mM ADP-Glucose and 15 Mm dithiothreitol. Mixture was incubated at 30°C for 20 minutes followed by placing in boiling water bath for 30 seconds to terminate the reaction. To the 50 µL of solution containing 50 mM HEPES-NaOH (pH 7.4), 4 mM phosphoenol pyruvate, 10 mM $MgCl_2$, 200 mM KCl and 1.2-unit pyruvate kinase was added to mixture I and incubated at 30°C for 30 minutes followed by placing in boiling water bath for 30 seconds to terminate the reaction. Solution was centrifuged at 10000× g for 5 minutes and supernatant was collected (mixture II). 150 µL of mixture II was combined with 100 µL of solution containing 50 mM HEPES-NaOH (pH 7.4), 2 mM NADP, 20mM $MgCl_2$ and 10 mM glucose and incubated at 30°C for 10 minutes change in OD was measured after combining the above solution with 1-µL each of hexokinase (1.4 unit) and Glucose-6-phosphate dehydrogenase (0.35 unit).

GBSS activity was assayed according to Mukherjee *et al.*, 2015. Assay was conducted same as SS by using 40 µL of crude enzyme extract prepared from pellet and omitting amylopectin in 1st step.

Statistical Analysis

The standard error of means (SEM) for each mean value was calculated using standard methods using 3 replicates. Factorial analysis was performed using a completely randomized design (CRD) with treatment and genotypes as two factors to obtain ANOVA. The least significant difference at a significance level of 5% was calculated for the combination of treatment and genotype, and the range

was determined through the range test. Values expressed using alphabetical symbols. The field plot layout followed a randomized block design (RBD).

Results

Grain Weight
Under ON, thousand grain weight (TGW) was highest in HB (44 g), followed by VL and HD (36.8 and 36.22). TGW was least in CL, and was significantly lower than other genotypes (27.22). Out of four genotypes, HB and VL showed significant reduction in grain weight under LN (27 and 25% reduction respectively). TGW of HD and CL remains unaffected under LN (Figure 1).

Starch Amylose and Amylopectin Content
Starch is principal component of grain dry matter of wheat, and hence, to understand starch biosynthesis and its two components viz. amylose and amylopectin is important. Starch amylose and amylopectin content was measured in immature (21 DAA, 28DAA) as well as mature grains (Table 1). In immature grains, starch content ranges from 332 to 616.2 mg. g^{-1} dry weight; whereas, in the mature grains this range was from 709 to 767 mg. g^{-1} dry weight. Under ON, HD had maximum starch at 21 DAA followed by HB, CL and VL. During 28 DAA starch content of HB, VL and HD became equivalent and CL showed significantly higher starch then other three genotypes. At maturity HB had maximum starch followed by VL and HD. Starch content of CL was least. Under LN all genotypes, except CL, exhibited a significant reduction in starch content at all growth stages. HB and VL experienced an approximate 3% reduction in starch content, while HD experienced a 2% reduction. Per grain reduction in starch was however drastic in HB and VL (25 and 29%). On the other hand, chotilerma demonstrated an increase in starch content at maturity. It is worth noting that the starch content in immature grains of CL was significantly lower under LN
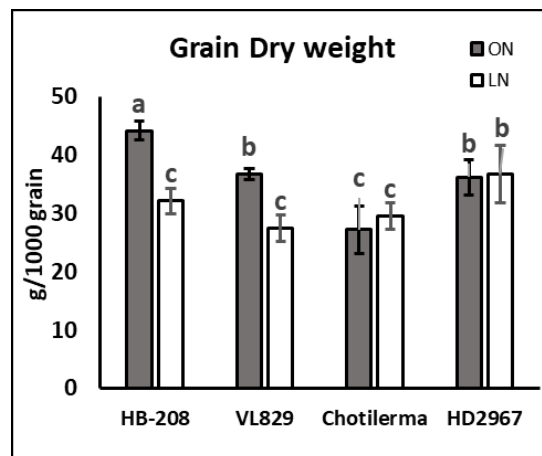

Figure 1: Grain weight per thousand grains under optimum and low nitrogen condition.

Table1: Starch, amylose, and amylopectin content at 21 and 28 DAA and maturity.

Component	Growth stage	HB-208		VL-829		Chotilerma		HD-2967	
		ON	LN	ON	LN	ON	LN	ON	LN
Starch (mg/g dry weight)	21 DAA	548.18 ± 0.99[b]	332.37 ± 3.7[f]	334.4 ± 1.46[f]	210.5 ± 1.64[g]	537.7 ± 1.05[c]	403.4 ± 1.61[e]	616.2 ± 1.66[a]	516.5 ± 1.27[d]
	28 DAA	557.7 ± 1.22[b]	357.3 ± 2.89[e]	557.2 ± 1.96[b]	487.2 ± 3.10[d]	565.8 ± 1.43[a]	504.8 ± 1.80[c]	552.0 ± 2.37[b]	497.2 ± 1.43[c]
	Mature	767.66 ± 2.6[a]	738.67 ± 2.3[c]	731.16 ± 2.1[cd]	709.69 ± 4.8[e]	712.13 ± 1.4[e]	739.54 ± 3.1[c]	754.39 ± 2.3[b]	735.23 ± 0.7[b]
Amylose (mg/g dry weight)	21 DAA	91.58 ± 2.43[e]	79.37 ± 1.2[f]	115.2 ± 2.02[d]	33.3 ± 1.05[g]	130.8 ± 3.34[c]	163.3 ± 2.09[d]	231 ± 4.37[a]	164.9 ± 3.82[b]
	28 DAA	123.43 ± 2.71[e]	104.73 ± 3.89[g]	114.67 ± 1.43[f]	36.80 ± 0.7[h]	137.51 ± 1.5[c]	128.41 ± 2.2[d]	261.82 ± 3.5[a]	175.64 ± 2.7[b]
	Mature	195.2 ± 0.58[d]	169.53 ± 1.2[f]	208.13 ± 0.24[b]	191.6 ± 1.8[e]	214.4 ± 0.95[a]	203.66 ± 0.2[c]	201.3 ± 0.31[c]	193.6 ± 1.1[de]
Amylopecti (mg/g dry weight)n	21 DAA	456.5 ± 3.4[a]	252.9 ± 2.8[d]	219.1 ± 4.2[e]	177.1 ± 1.9[f]	406.8 ± 2.9[b]	240.03 ± 4.9[d]	385.1 ± 3.4[b]	351.5 ± 4.1[c]
	28 DAA	434.2 ± 4.2[ab]	252.5 ± 1.8[e]	442.5 ± 3.2[a]	450.3 ± 3.6[a]	428.2 ± 2.8[ab]	376.3 ± 2.2[b]	290.1 ± 2[d]	321.5 ± 1.9[c]
	Mature	572.46 ± 4.7[a]	569.14 ± 4.2[a]	540.03 ± 3.2[b]	501.6 ± 4.1[bc]	497.73 ± 2.1[c]	535.88 ± 3.9[b]	553.09 ± 3.5[a]	541.63 ± 2.9[a]

conditions, suggesting an increase in the rate of starch synthesis after 28 DAA. Under ON, HD had highest amylose content in immature grain followed by CL, VL and HB. At maturity amylose content ranges from 195 to 214 mg. g^{-1} dry weight, with CL having the highest amylose content, followed by VL, HD, and HB. Throughout all growth stages, HB consistently had the lowest amylose content. Conversely, under LN conditions, all genotypes experienced a significant reduction in amylose, with HB experiencing the highest reduction of 13%, followed by VL with a reduction of 7%. At 21 and 28 DAA, HD exhibited the highest amylose content under both ON and LN conditions. Amylose content decreased as the grain matured, indicating that the maximum amylose synthesis occurred in HD at these stages. On the other hand, VL had relatively lower amylose compared to the other genotypes at 21 and 28 DAA. However, the amylose content significantly improved by maturity, at least under ON condition, suggesting that a substantial amount of amylose synthesis occurred after 28 DAA. Under ON condition, HB exhibited the highest amylopectin content at all growth stages. At 21 DAA, a reduction in amylopectin was observed under low nitrogen (LN) conditions in all genotypes. However, at 28 DAA, only HB and HD had significantly lower amylopectin content under LN compared to ON. In contrast to amylose, at maturity, HB compensated for amylopectin production and became on par with ON regime. VL also did not show any significant difference in amylopectin between the nitrogen treatments during maturity.

Amylose/amylopectin in mature grain ranges between 1: 2.3 to 1:2.9 under ON. Under LN condition range was found between 1:2.6 to 1:3.3 (Table 2). Under ON, CL had maximum amylose/amylopectin followed by VL, HD and HB in mature grains where difference between VL and HD was insignificant. Under LN condition HB and CL experienced decrease in amylose/amylopectin while changes in VL and HD was insignificant. Ratios was quit variable among genotypes throughout development phase. HB, VL and CL showed gradual increase in amylose/amylopectin whereas HD showed gradual decrease.

Protein Content

Protein content in the mature grain was found highest in HD followed by CL, VL and HD under ON. Under LN, all the genotypes except HD showed significant increase in on protein content whereas HD had significant reduction (Figure 2)

Enzyme Activity

In 4 key starch synthesizing enzymes adenosine diphosphate glucose pyrophosphorylase (AGPase), GBSS and soluble SS as well as uridine diphosphate glucose pyrophosphorylase (UGPase) were assayed at 21 and 28 DAA under ON and LN input to understand the role of enzyme in variation of starch, amylose, and amylopectin content under different nitrogen regime. All the enzyme activities were expressed as change in absorbance (OD) per minute per gram of fresh weight.

AGPase

AGPase is a rate limiting enzyme in starch biosynthesis pathway. AGPase activity in HB under ON was found to be highest at both the stages. Under LN, CL showed higher activity during 21 DAA. At 28 DAA VL and HD had highest activity under LN. At 21 DAA, only HB and VL showed a significant reduction in activity under LN, while at 28 DAA, all genotypes experienced a significant reduction. VL displayed a remarkable increase in AGPase activity at 28 DAA (9.4 and 7.6 min^{-1} g. f.wt.$^{-1}$ under ON and LN, respectively) compared to 21 DAA (5.6 and 4.9 min^{-1} g. f.wt.$^{-1}$ under ON and LN,
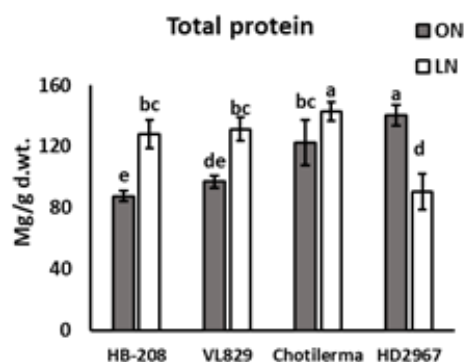

Figure 2: protein content in mature wheat grain under optimum and low nitrogen condition.

Table 2: Amylose/amylopectin ration in wheat grains

Genotype		ON			LN		
	Stage	Amylose percentage	Amylopectin percentage	Ratio	Amylose percentage	Amylopectin percentage	Ratio
HB-208	21 DAA	16.7[e]	83.2[a]	1: 4.9	23.8[d]	76.11[b]	1: 3.1
	28 DAA	22.1[e]	77.8[bc]	1: 3.5	29.31[c]	70.68[d]	1: 2.4
	Mature	25.4[c]	74.5[b]	1: 2.9	22.9[d]	77.04[a]	1: 3.3*
VL-829	21 DAA	34.4[c]	84.1[d]	1.9	15.82[e]	84.17[a]	1:5.3
	28 DAA	20.5[e]	92.4[b]	1:3.8	7.55[f]	92.44[a]	1:2.2
	Mature	27.8[b]	72.3[bc]	1:2.5	27.63[b]	72.36[c]	1:2.6
Chotilerma	21 DAA	24.3[d]	59.5[b]	1:3.1	40.48[a]	59.51[e]	1:1.4
	28 DAA	24.3[d]	74.5[cd]	1:3.1	25.44[d]	74.55[bcd]	1:2.9
	Mature	30.1[a]	72.4[d]	1:2.3	27.53[b]	72.46[c]	1:2.6*
HD-2967	21 DAA	37.4[b]	68[d]	1:1.6	31.93[c]	68.06[c]	1:2.1
	28 DAA	47.4[a]	64.6[f]	1:1.1	35.33[b]	64.66[e]	1:1.8
	Mature	26.4[b]	73.6[bc]	1:2.7	26.33[b]	73.66[bc]	1:2.7

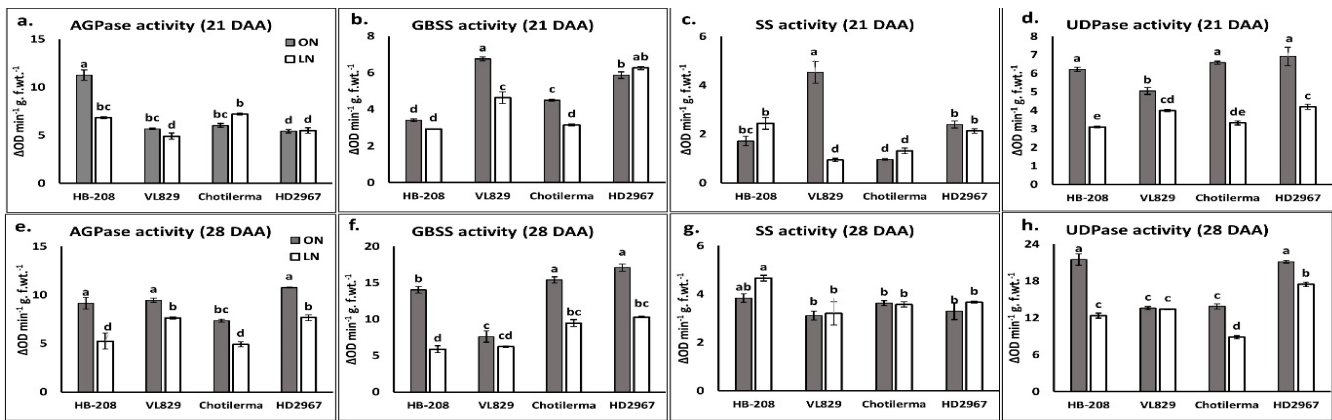*Significant difference between ON and LN at *p-value* 0.5

Figure 3: Activity of starch synthesizing enzyme at 21 and 28 DAA under optimum and low nitrogen condition.

respectively), providing an explanation for the increase in starch content following a similar pattern. The AGPase activity in HD under ON and LN was similar at 21 DAA but significantly reduced at 28 DAA under LN (Figure 3a, e).

GBSS

GBSS is the key enzyme determining amylose content in grains. GBSS activity ranges from 2.9 to 6.7 min^{-1} g.f.wt.$^{-1}$ during 21 DAA and 5.8 to 17 min^{-1} g. f.wt.$^{-1}$ during 28 DAA showing substantial increase in later growth period. At 21 DAA, the GBSS activity was similar between ON and low nitrogen LN conditions in HB and HD. However, VL and CL showed a significant decrease. A drastic reduction in GBSS activity was observed at 28 DAA under LN in HB, CL, and HD. In contrast, VL did not show a significant difference. HD consistently maintained significantly higher GBSS activity at both stages under ON, which explains its higher amylose content compared to other genotypes at these stages (Figure 3b, f).

SS

Like GBSS, an increase in SS activity was observed at 28 DAA compared to 21 DAA. The SS activity under both ON and LN conditions was on par in HB, CL, and HD at 21 DAA. Only VL exhibited a significant decrease in SS activity under LN. However, there was no significant difference in SS activity between N treatments at 28 DAA. Furthermore, at 28 DAA, HB displayed significantly higher SS activity compared to other genotypes. The SS activity in VL, CL, and HD was similar at 28 DAA (Figure 3c, g).

UGPase

UGPase is an important enzyme works in coordination with sucrose synthase and AGPase to maintain equimolar ration of ADP-glucose (precursor of starch synthesis) in cereal grains and hence plays a crucial role in determining starch content in grains. Notable increase in UGPase activity was observed at 28 from 21 DAA. HB and HD had highest activity

under ON. All the genotypes at 21 DAA and HB, CL, and HD at 28 DAA had significant reduction in UGPase activity under LN (Figure 3d, h).

Discussion

Grain weight accumulation in wheat is influenced by genetic traits such as grain filling rate and grain filling duration (Pržulj and Mladenov, 1999; Yu *et al.*, 2022) as well as environmental factor like soil properties, temperature, soil nutrition status and N nutrition (Teng *et al.*, 2023; Zheng *et al.*, 2022; Dias and Lidon, 2009; Madani *et al.*, 2010; Duan *et al.*, 2018). Genotypes under investigation in this study exhibited variations in grain weight under different nitrogen regime. HB, characterized by bold seeds, displayed the highest TGW under optimum nitrogen (ON) conditions. However, under low nitrogen (LN), the TGW of this genotype decreased by 29%. Similarly, VL also experienced a reduction in grain weight by 25%. On the other hand, the TGW of the other two genotypes, CL, and HD, remained stable. Grain filling also varied among these genotypes both under ON and LN. at 21 DAA, grain filling was found maximum in HD (81%-ON to 70%-LN) and least in VL (45%-ON to 29%-LN). However, VL showed the maximum rate of starch accumulation from 21 DAA to maturity and HD showed least accumulation during this period. Therefore, genotypes like HD was efficient in grain filling at early stage of grain development, which has a relevance with climate change induced terminal heat stress; and it is likely that these genotypes will have less effect due to the stress (Shirdelmoghanloo, 2022). Studies has shown that N nutrition improves grain filling (Langer & Liew, 1973) and grain dry matter accumulation (Lv *et al.,* 2002; Blacklow and Incoll 1981). On the other hand, genotytpes with larger grain size do better photosynthesis during mid grain filling stage, which provides ample substrate for grain development (Zhao *et al.*, 2003). Grain filling rate is an important determinant of final grain weight in various crops (Xie *el al.*,2015) and difference in final grain weight have been attributed to difference in grain filling rate in bread wheat (Xie *el al.*,2015) durum wheat (Gebeyehou *et al.*, 1982) corn (Daynard *et al.*, 1971) rice (Jones *et al.*, 1979) and pear millet (Fussell *et al.*, 1978)

In various abiotic stresses, a reduction in grain weight is often accompanied by a decrease in starch content and a reduction in the activity of starch metabolism enzymes (Zhang *et al.*, 2010; Zhang *et al.*, 2021). In our study, we observed both types of genotypes, one that displayed a reduction in grain weight and other one, which did not exhibit any significant change in grain weight under N-stress. This allowed us to investigate the reason with respect to accumulation of starch and its two components – amylose and amylopectin

Starch is the predominant component of wheat grains, accounting for around 60 to 70% of the grain's dry matter. Two types of alpha-glucan chain amylose and amylopectin forms starch in wheat. Among the two components of starch, amylose content was significantly reduced across the genotype. However, amylopectin content was unchanged in case of HB and VL, but increased in case of HD and CL. The ultimate effect was the reduction in total starch content in case of HB and VL, whereas no change in case of HD and CL. This is also corroborated with the grain weight reduction in case of HB and VL under LN condition. Previous studies also highlighted that various environmental stress as well as N input affects starch content in wheat grain (Kim and Kim., 2021; Asthir *et al.*, 2017). These results not only highlight the quantity of starch and grain weight, but also exhibit the changes in starch quality (amylose: amylopectin ratio) under N stress condition. We observed, under LN conditions, all genotypes experienced a significant reduction in amylose, at all stages with HB and VL experiencing and highest reduction of (13 and 7%.) in mature grain. Amylose/ amylopectin ratio in the mature grains is crucial parameter for starch quality determination. Starch with high amylose/ amylopectin is beneficial for health (Slade *et al.*, 2012). In our study, CL had the highest amylose/amylopectin ratio in the mature grain under ON conditions, while HB had the least. Under N stress condition, the amylose: amylopectin ratio in mature grain was significantly decreased in case of two genotypes, HB and CL.

Total grain protein in most of the genotypes were increased under N stress, that is mainly due to reduction in yield. The inverse relation between grain yield and grain protein content is already reported in wheat (Zörb *et al.*, 2018).

Previous studies have indicated that the activities of enzymes involved in starch biosynthesis in wheat are influenced by both developmental factors and environmental conditions (Jenner *et al.*, 1991; Hawker and Jenner, 1993; Jenner, 1994). It has been observed that the maximum activities of AGPase, starch synthase (SS) and granule-bound starch synthase (GBSS) in winter wheat grains are reached during the middle stage of grain filling (Xu *et al.*, 2003). Hence, in our study, the activity of starch synthesizing enzymes was assayed at 21 and 28 DAA to investigate their role during these key developmental stages. AGPase, a rate-limiting enzyme in the starch biosynthesis pathway, responsible for catalyzing the first enzymatic step, which is the synthesis of ADP-glucose (Saripalli and Gupta, 2015), hence this enzyme is very important for both amylose and amylopectin biosynthesis. In our study the reduction in starch content as well as grain weight in HB and VL under N stress might be due the reduction in AGPase activity in these two genotypes at early grain filling stage (21 DAA). It is also reported that low AGPase activity is a major cause of low starch content in waxy wheat grains (Zi *et al.*, 2018). GBSS enzyme, which is related to amylose biosynthesis (Zi *et al.*, 2018), showed the reduction of its activity in all the genotypes, and probably the reason for low amylose

content under N-stress. Two other enzymes related to starch biosynthesis, SS and UDPase did not show any effect under N-stress.

Conclusion

Nitrogen stress not only affects yield and starch accumulation but also impacts starch quality. We observed a reduction in amylose content across all genotypes under N stress, while amylopectin content increased in some genotypes, maintaining the overall starch content. Consequently, the balance between amylose and amylopectin content determines whether the grain weight of a specific genotype will decrease or remain unchanged under N-stress condition. Our findings revealed that two of the genotypes, HB and VL experienced a decrease in both starch content and grain weight, primarily due to the reduction in amylose content. In the case of the HD and CL genotypes, the reduction in amylose was compensated by an increase in amylopectin content in the grain, resulting in no change in grain weight. Furthermore, we observed differential grain filling among the genotypes e.g., HD showed early grain filling and VL showed late grain filling under both ON and LN.

Acknowledgments

Conflicts of Interest

The authors declare that they have no conflict of interest

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