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Understanding the effect of elevated carbon dioxide and heat stress on wheat grain quality under differential nitrogen dose

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Abstract
The increase in atmospheric carbon dioxide (eCO_2) concentrations and rising temperature has emerged as a significant concern due to its potential implications for humankind, particularly in terms of jeopardizing food and nutritional security. The present study aimed to investigate the effects of eCO_2 and terminal heat stress (HS) on grain quality in wheat. We assessed the grain quality in four different wheat cultivars: HD2967 (thermotolerant), HD2329 (thermo susceptible), HI1500, and GW322 (nitrogen responsive). HD2967 demonstrated a more favourable response to heat stress compared to the other genotypes. Additionally, we observed a positive impact of eCO_2 on starch content, maximum in HD2967 (74%), whereas heat stress led to a decrease in starch content across all genotypes, albeit with minimum starch content in the thermo susceptible genotype i.e., HD2329 (57%). Moreover, our study demonstrated that eCO_2 enhanced the accumulation of total soluble sugar and reducing sugar. Additionally, a significant increase in total soluble protein and free amino acid content were observed in all genotypes under combined stress. These findings highlight the potential of these genotypes, particularly the thermotolerant one, for breeding programs aimed at developing high-yielding and thermotolerant wheat cultivars under eCO_2 and heat stress conditions.

Keywords: eCO_2, heat stress, starch, reducing sugar, free amino acid

Introduction
Wheat is a crucial cereal crop globally, providing substantial nourishment for humans and animals. With a rising population and demand for food, it is essential to explore ways to enhance crop productivity and quality. One factor that significantly affects crop growth and development is the concentration of carbon dioxide (CO_2) in the atmosphere. Currently, CO_2 levels are at 415 parts per million (ppm), which is the highest recorded in the last 800,000 years (Friedlingstein *et al.*, 2022) [11]. Human activities, such as burning fossil fuels, deforestation, and land-use changes, have caused this increase in CO_2 concentration (eCO_2). Research indicates that eCO_2 levels can positively or negatively impact plant growth and development, and the effect on grain quality in wheat is not yet fully understood. Grain quality is vital in determining the value of wheat for human consumption and animal feed, and various factors, including genetics, environmental conditions, and management practices, influence it (Lamichaney *et al.*, 2021) [19]. eCO_2 levels affect grain quality by altering the concentration and composition of proteins, carbohydrates, and other nutrients, leading to both positive and negative outcomes depending on the cultivar, environmental conditions, and management practices (Li *et al.*, 2023) [20]. An important impact of elevated CO_2 on grain quality is changes in the protein content and composition, especially the gluten fraction. eCO_2 levels can also impact the concentration and composition of carbohydrates in wheat grains, particularly starch, leading to changes in texture and flavor, with positive or negative implications for their nutritional value and acceptability (Bhargava & Mitra, 2021) [4]. The impact of eCO_2 on grain quality is also influenced by other environmental factors such as temperature, water availability, and nutrient availability.

Heat stress is another significant concern for wheat farmers and researchers as it affects the physiological and biochemical processes of wheat plants, leading to a reduction in grain yield and quality. High temperatures during grain filling can result in lower yields, grain weight, and size, and damage to the cellular structure of the grain, resulting in reduced starch content and altered protein composition. Heat stress can also affect the sensory quality of the grain, making it less desirable to consumers and reducing its nutritional value (Kumar *et al*., 2022; Narwal *et al*., 2022) [16, 30]. Terminal heat stress is a prevalent and debilitating issue in wheat farming, particularly during the reproductive phase, which encompasses grain filling and development (Kumar *et al*., 2021) [14]. Starch, constituting approximately 75% (w/w) of the wheat grain, comprises amylose (25%) and amylopectin (75%) (Liu *et al*., 2021) [22]. Amylose is a linear glucose polymer linked via α-1, 4 linkages, whereas amylopectin is a branched polymer with α-1, 6 linkages. Starch biosynthesis occurs in both the photosynthetically active green parts, i.e., chloroplasts, and the non-photosynthetic amyloplasts of plant (Bürgy *et al*., 2021) [5]. This metabolic pathway necessitates the involvement of several enzymatic catalysts, including ADP-Glucopyrophosphorylase (AGPase), Starch synthase (SS), starch branching enzyme (SBE), and starch debranching enzyme (DBE). AGPase is the foremost enzyme responsible for initiating this process by catalysing the conversion of glucose-1-phosphate and ATP into ADP- glucose, a crucial precursor molecule for starch biosynthesis.

Very limited information is available on the effect of differential nitrogen, eCO_2 and HS on grain quality in wheat. Here, we studied the effect of eCO_2 (650 ppm) and HS (38 ± 2°C) on grain quality mineral composition of contrasting wheat cvs. HD2967 (thermotolerant), HD2329 (thermo susceptible), GW322, and HI1500 (nutrient responsive) under two different nitrogen level (Nn and Ns). In order to study the effect of differential dose of nitrogen on selected genotypes under the different types of treatment like elevated CO_2, heat stress and combination of elevated CO_2 and heat stress, the following study has been conducted to insight biochemical and ionomic changes occurring in selected genotypes under different stress.

Materials and Methods

The experiment was conducted in green house located at ICAR-IARI (Indian Agricultural Research Institute), New Delhi. Four different wheat genotypes were selected in this experiment out of which two are heat responsive i.e., HD2967, HD2329 and two are nutrient responsive i.e., HI1500, GW322. The experiment was set up in a completely randomized block design with three replications using a split plot arrangement. The plots were irrigated at regular intervals, and other agricultural operations were carried out in accordance with standard farm methods. Whole experiment is divided in two different group one with normal dose of nitrogen and another with 25% extra dose of nitrogen. For elevated CO_2 treatment, pots containing seedling were placed inside growth chamber at National Phytotron Facility, IARI (Indian Agricultural Research Institute), New Delhi maintaining the optimum environmental condition for growth like 22 °C/18 °C Day/night temperature with relative humidity of 80-90%. For heat.

Estimation of starch content estimation

The Anthrone technique was used to calculate the starch content. Mature endospermic tissue that had been oven-dried was extracted using 70% hot ethanol and centrifuged for 15 minutes at 12,000 rpm. The resulting residue was re-suspended in water and perchloric acid (52%), centrifuged, and the supernatant was collected. The supernatant was collected, and water was added and kept for starch estimation. Using the anthrone reagent, the aliquot (100 µL) was utilized to estimate the glucose level. At 620 nm, the degree of colour formation was seen. The standard curve was used to estimate glucose, and the measured value was multiplied by 0.9 to obtain the starch yield.

Estimation of amylose and amylopectin

The iodine binding method as reported by Ohwada *et al*. (1970) [32] was utilized for the determination of amylose. Endospermic tissue was homogenized in 0.5 N potassium hydroxide, followed by vortexing for 5 minutes and dilution to a final volume of 100 mL with distilled water. To a 10 mL aliquot of the homogenate, 5 mL of 0.1 N hydrochloric acid and 0.5 mL of iodine reagent were added and the volume was made up to 50 mL. The absorbance was measured at 625 nm, and amylose content (%) was determined using a standard curve prepared with amylose and amylopectin blends. The amylopectin content was obtained by subtracting the amylose content from the total starch content.

Estimation of reducing sugar content

The 3, 5-Dinitrosalicylic acid (DNS) colorimetric method was used to determine reducing sugars from grain (Miller, 1959) [26]. Grain samples were homogenized in 80% ethanol before being centrifuged at 12000 rpm for 15 minutes at room temperature. The supernatant was concentrated in an 80°C water bath. The reducing sugar concentration was determined by placing the unknown OD values on a graph with glucose as a reference.

Estimation of total soluble sugar

Total soluble sugar was estimated using method of Dubois *et al*. (1956) [9] with slight modification. The grain material was homogenized in ethyl alcohol, centrifuged at 10000rpm, and extracted again in ethyl alcohol. With extraction medium, the final volume of pooled supernatants was diluted. Phenol was added to the extract followed by adding reagent B and constantly mixed it. The OD of the produced greenish brown color was measured in a spectrophotometer at 490 nm. The concentration of sugars was determined using a standard curve produced with pure glucose (10-100g/ml) and expressed as mg g^{-1} dry weight.

Estimation of total soluble protein and free amino acid

Grain sample were crushed into fine powder with liquid nitrogen and transferred to the extraction buffer (Tris-HCl 100 mM, pH 6.8). The homogenate was centrifuged for 20 minutes at 4°C, and the supernatant was utilized for protein quantification using the Bradford *et al*. (1976) [27]. Free amino acid was estimated by the method of Moore & Stein (1954) [28] with slight modification.

Statistical analysis

The analysis of variance of different biochemical parameters were analysed by MINITAB statistical package using linear model of variance. Difference in grain quality parameters under different treatment were calculated using the least significant difference (LSD) at $p \leq 0.05$ based on two-way variance analysis.

Results and Discussion

Wheat is extremely heat-sensitive, and even a small change in the ambient temperature at key growth and development stages can have a significant impact on the crop's growth, development, and yield. Despite their significance, most of the important mechanisms linking sources (photosynthesis) and sinks (starch biosynthesis) under eCO_2 and heat stress have not yet been fully uncovered. Since starch biosynthesis is the main factor affecting sink strength, concentrating on this pathway will help researchers better understand how eCO_2 and heat stress affects the grain quality and starch biosynthesis pathway. Here, we screened selected genotypes using different biochemical parameters like total soluble sugar, starch content, amylose and amylopectin content, reducing, non-reducing sugar, total soluble protein, free amino acid, enzyme activity associated with starch biosynthesis, iron and zinc content under eCO_2 (650 ppm) and heat stress (36 ± 2°C) condition as compared to normal {CO_2 conc. (400 ppm) and ambient temperature (34 ± 2°C)}. In the current investigation, we have examined the different biochemical parameters and ionome parameters which are subjected to change during eCO_2 and heat stress treatment in four wheat cvs. i.e., HD2967 (thermotolerant), HD2329 (thermo susceptible), HI1500 and GW322 (Nitrogen responsive).

Effect of eCO_2 and heat stress on AGPase activity

We observed significant difference in AGPase activity in all selected genotypes in response to eCO_2 and heat stress treatment. The specific activity of AGPase was found maximum in HI1500 (2.04 U/mg) under eCO_2 treatment while comparatively lower activity was found in HD2967 (1.84 U/mg) while minimum activity found in HD2329 (1.77 U/mg) compared to control. Heat stress significantly declined the AGPase activity in all selected genotypes and maximum % decrease was observed in HD2329 (23%). Compared to a normal dose of nitrogen supply, a higher dose of nitrogen supply reduced the AGPase activity but least effect was observed in N responsive genotypes i.e., HI1500 and GW322 (Fig. 1a). Higher AGPase activity in HI1500 and HD2967 showed the presence of thermostable AGPase compared to susceptible genotypes. AGPase activity is mainly regulated by concentration of 3-PGA (activator) and PPi (inhibitor). Increased CO_2 concentration enhanced the photosynthetic rate resulting more accumulation of 3-PGA hence stimulate the AGpase activity (Saripalli & Gupta, 2015) [33]. AGPase is thermolabile enzyme hence its activity significantly reduced under heat stress (38±2 °C) (Bansal *et al*., 2013) [3].

Effect of eCO_2 and heat stress on soluble starch synthase activity

We observed significant difference in soluble starch synthase (SSS) activity among the selected genotypes under different treatment. HD2967 showed the highest SSS activity (2.55 U/mg), whereas HD2329 showed the lowest (2.23 U/mg) activity under both adequate and normal nitrogen conditions. Heat stress (HS) had a stronger repressive effect on SSS activity and percentage decrease was higher in HD2329 compared to HD2967 while eCO_2 had synergistic effect and maximum activity was observed in HD2967 (2.85 U/mg) followed by HI1500 (2.59 U/mg) and minimum in HD2329 (1.65 U/mg) under heat stress. Higher dose of nitrogen supply decreased the SSS activity compared to normal dose of nitrogen supply but effect was insignificant in N responsive genotypes i.e., HI1500 and GW322 (Fig. 1b). These enzymes being heat labile, are involved in starch biosynthesis and increase in temperature significantly affect their activity which is in conformity with previous observation (Kumar *et al*., 2019; Kumari *et al*., 2020) [17, 18].

Effect of eCO_2 and heat stress on starch content

The grain of HD2967 under elevated CO_2 exhibited 10% more starch than the control showing maximum accumulation among selected genotypes. Elevated carbon dioxide enhanced the cytosolic AGPase activity resulting in higher accumulation of starch (Kang *et al*., 2013). It was observed that heat stress significantly reduced the starch content and maximum reduction (23%) was observed in HD2329 compared to control. HD2967 showed 14% reduction as compared to control under heat stress treatment. Increase in temperature significantly affect grain growth because it limits assimilate supply, grain-filling duration and pace, and starch biosynthesis and deposition (Farooq *et al*., 2011) [10]. Elevated CO_2 alleviated the heat stress effect in all selected genotypes under combined eCO_2 and heat stress treatment (Fig. 1a). Despite the benefit of eCO_2 on vegetative tissue, temperature is the most important factor influencing wheat grain production and quality when subjected to both eCO_2 and heat stress throughout the reproductive phase (particularly anthesis and grain-filling). This is because high temperatures cause irreversible harm to the anabolic and metabolic processes in wheat flowers and grains (Chavan *et al*., 2019.; Kadam *et al*., 2014; Wang *et al*., 2013) [6, 12,]. It was also observed that higher dose of nitrogen supply reduced the total starch content as compared to normal dose of nitrogen supply showing its negative impact on starch content.

Effect of eCO_2 and heat stress on amylose and amylopectin content

Amylose and amylopectin content was significantly influenced by heat stress and eCO_2. In this study, heat stress reduced the amylose content in all selected genotypes and the effect was more pronounced in HD2329 showed 12% reduction while HD2967 showed only 7% decrease in amylose content. GW322 showed maximum amylose accumulation (30.43%) under eCO_2 treatment while HD2329 showed minimum amylose content (26.29%) under eCO_2 treatment. The amylose content increased by an average of 5.15% under eCO_2 conditions compared to the control group. The increase in amylose content under eCO_2 conditions is likely due to the stimulation of photosynthesis, which results in an increase in the availability of photosynthetic products for amylose and starch synthesis (Sakamoto *et al*., 2007). Some studies have indicated that under eCO_2 conditions, the activity of starch synthase, an enzyme responsible for amylose synthesis, may be enhanced. This increased enzyme activity can contribute to the higher accumulation of amylose in plants. Wang *et al*. (2013) also reported higher accumulation of amylose in elevated carbon dioxide concentration. In general, higher dose of nitrogen supply enhance the amylose content across all the genotypes. Similarly, amylopectin content was significantly affected under eCO_2 and heat stress treatment while extra dose of nitrogen application reduced the amylopectin content compared to normal dose of nitrogen supply (Fig. 2b & 2c). It is reported that high temperature reduced the amylopectin content in heat stress (Kumari *et al*., 2020; Liu *et al*., 2011) [18, 21].

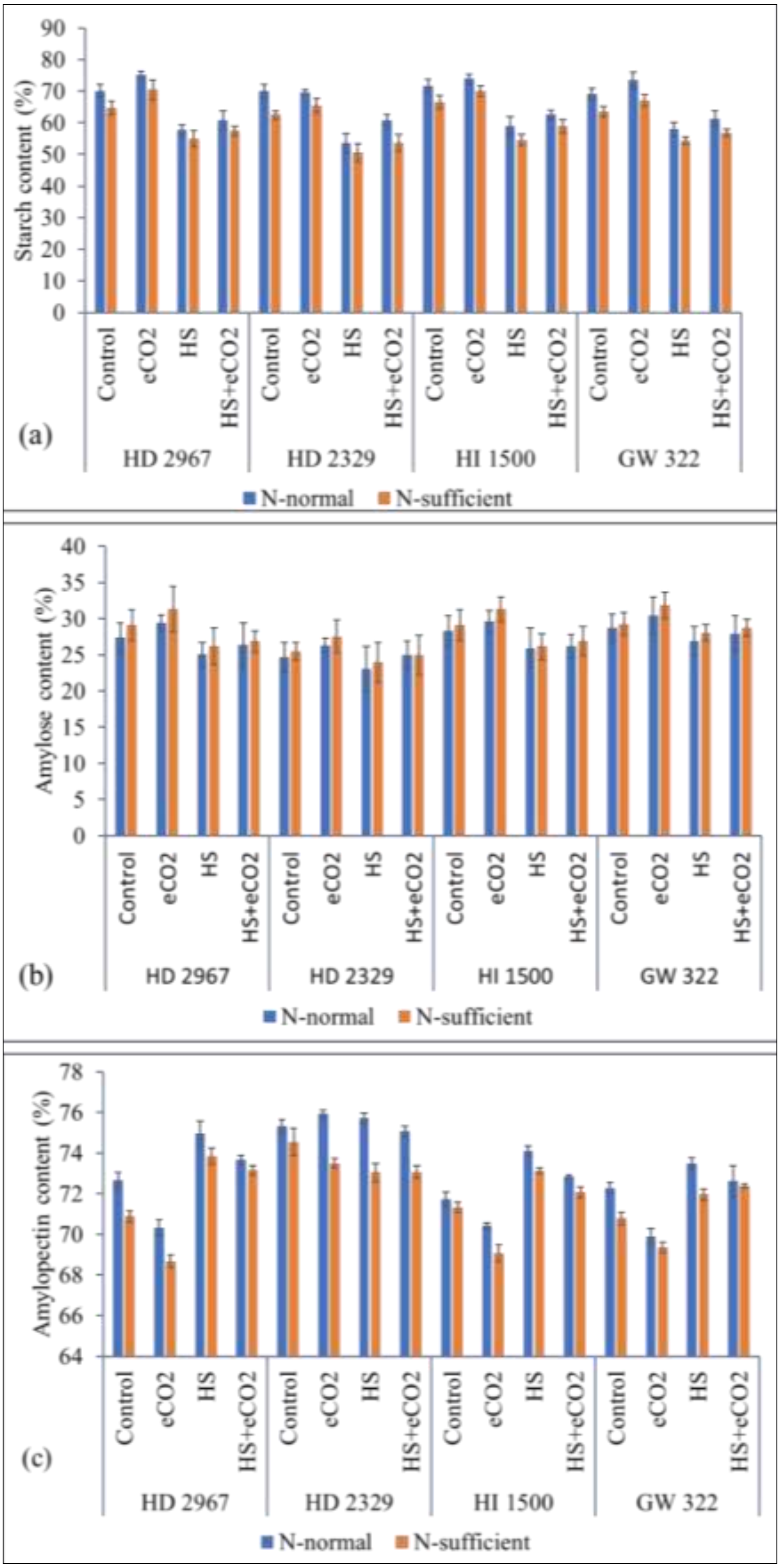

Fig 1: Effect of eCO_2 and HS on grain quality related parameters (a) Starch content (b) Amylose content (c) Amylopectin content in wheat *cvs* HD2967, HD2329, HI1500 and GW322 under two different nitrogen doses; means for varieties, treatments and interaction are significant at $p<0.05$, vertical bars indicate s.e. (n=3)

Effect of eCO_2 and heat stress on total soluble sugar and reducing sugar: It was observed that reducing sugar content significantly increased in grain and higher accumulation was observed in thermotolerant genotype compared to thermo susceptible genotype. Maximum accumulation of total sugar in leaves was observed in HD2967 (39.9 mg/g) under terminal heat stress, as compared to eCO_2 and control condition. Total soluble sugar accumulation was least observed in GW322 under control condition (27.5 mg/g). Effect of higher dose of nitrogen application significantly increased the total soluble sugar content among selected genotypes and the effect was observed more in HD2967 (42.6 mg/g) under heat stress treatment and least observed in HD2329 (37.4 mg/g) (Fig. 2a). This is due to more accumulation of soluble carbohydrates, mainly sucrose, since higher nitrogen dose stimulate sugar biosynthesis and reduces their conversion to starch (Asthir & Bhatia, 2014; Almodares *et al*., 2009) [2, 1]. The enhanced CO_2 fixation under eCO_2 encourages the synthesis of triose phosphate in leaves, which can then be converted into other carbohydrates like glucose, fructose, and sucrose (Dong *et al*., 2018) [8].

Reducing sugar was significantly increased in heat stress, eCO_2 and combined eCO_2 and heat stress. An increase in reducing sugar content was observed in all the selected genotypes. The reducing sugar content was maximum in HD2967 (9.3 mg/g) and minimum found in HD2329 (7.2 mg/g) under eCO_2 and heat stress treatment. Effect of extra dose of nitrogen supply further enhanced the reducing sugar content in all genotypes and maximum effect was observed in HD2967 (11.3 mg/g) under eCO_2 and heat stress treatment and minimum observed in HD2329 (6.4 mg/g) under control condition. Similar effect was observed in non- reducing sugar content in selected genotypes and different treatment and maximum accumulation was observed in HD2967 followed by HI1500, HD2329 and GW322 (Fig. 2b). Higher dose of nitrogen application increased the triose phosphate/ phosphate translocation activity and sucrose phosphate synthase activity which led to more accumulation of reducing sugar rather than conversion into starch (Mariem *et al*., 2020; Ning *et al*., 2018) [24, 31].

Effect of eCO_2 and heat stress on total soluble protein and free amino acid content

We observed significant increase in total soluble protein in spike of different genotypes exposed to heat stress. Maximum accumulation was observed in HI1500 (9.03 mg/g FW) under heat stress treatment while minimum observed in HD2329 (7.11 mg/g FW) under eCO_2 treatment. Similarly, higher dose of nitrogen application significantly enhanced total soluble protein content across all the genotypes and maximum accumulation was observed in HI1500 (9.97 mg/g FW) under HS treatment. Under terminal HS conditions, the spike of HI1500 showed significantly higher accumulation of total soluble protein (Fig. 2c). Our findings are consistent with the findings of (Kumar *et al*., 2017) [15] who observed an increase in the accumulation of total soluble protein content in the grains of different wheat cultivars under HS (Wang & Liu, 2021) [36].

They stated that the grain may have a compensating effect on nitrogen metabolism in response to the HS-mediated change in the carbon assimilatory pathway.

Free amino acid was significantly decreased in spikes of all selected genotypes under eCO_2 and heat stress treatment. Maximum free amino acid accumulation was observed in HD2967 (8.59 mg/g FW) under control condition while minimum accumulation was observed in HD2329 (6.4 mg/g FW) under combined eCO_2 and heat stress treatment. Higher dose of nitrogen application significantly enhanced the free amino acid content among all selected genotypes and maximum effect was observed in HI1500 (9.68 mg/g FW) under controlled condition (Fig. 2d). Our findings are consistent with the findings of (Leonardis *et al*., 2015; Kumar *et al*., 2017) [7, 15].

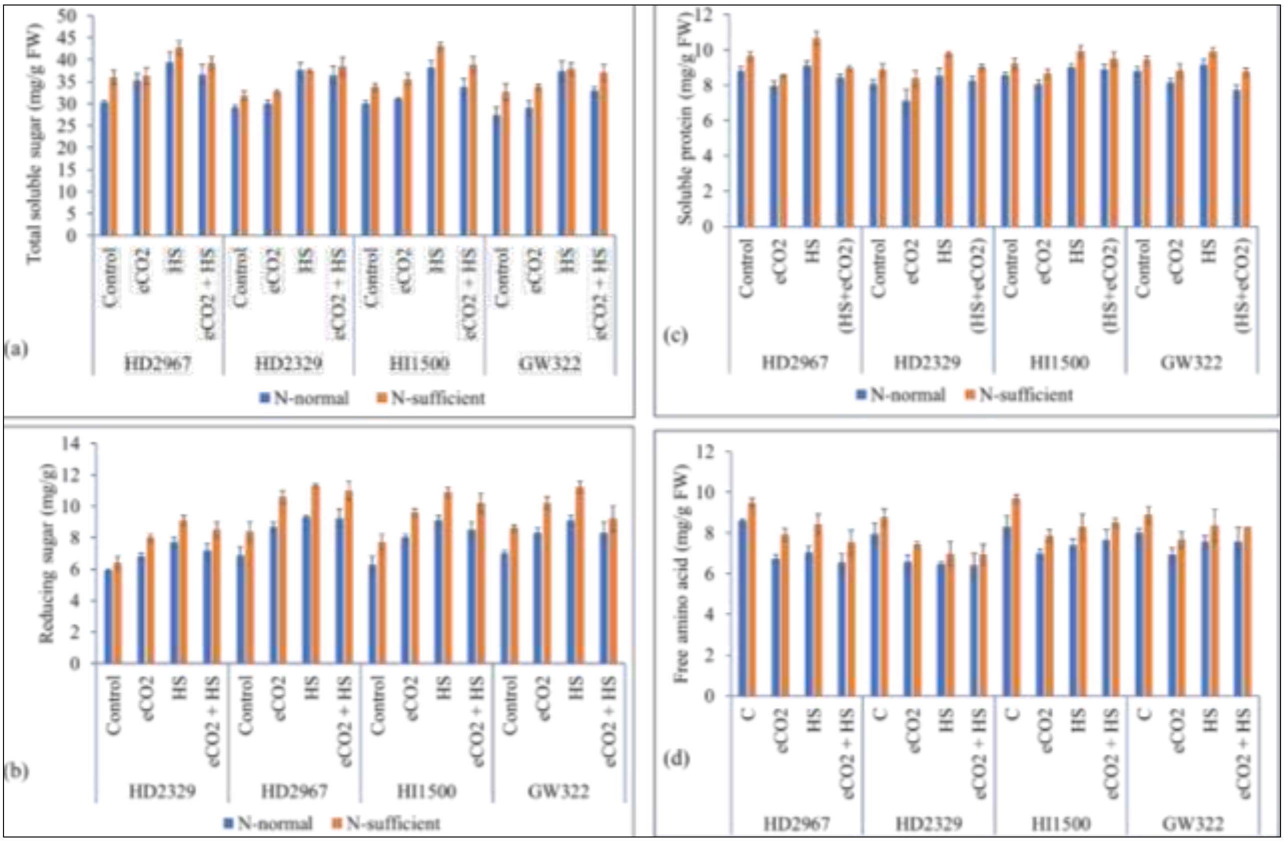

Fig. 3. Effect of eCO_2 and HS on grain quality related parameters (a) Total soluble sugar (b) Reducing sugar (c) total soluble protein and (d) Free amino acid content in wheat *cvs* HD2967, HD2329, HI1500 and GW322 under two different nitrogen doses; means for varieties, treatments and interaction are significant at $p<0.05$, vertical bars indicate s.e. (n=3)

Conclusion

To conclude, elevated carbon dioxide concentration and terminal heat stress significantly modulate the grain quality in wheat. Here, we have assessed the change in biochemical and mineral composition in wheat cvs. HD2967 (thermotolerant), HD2329 (thermo susceptible), HI1500 and GW322 (nitrogen responsive) under elevated carbon dioxide and heat stress to understand its impact on grain quality. We observed positive impact of eCO_2 on starch content while heat stress decreased the starch content across all the genotypes but thermotolerant genotype showed lesser reduction in starch content and mineral composition. Our study showed that eCO_2 enhance the accumulation of total soluble sugar, reducing sugar but reduce the level of iron and zinc content in grain. These genotypes showed better response and can be used for breeding program to develop high yielding and thermotolerant genotypes under eCO_2 and heat stress.

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