ORIGINAL ARTICLE

Variation in nonstructural carbohydrates and antioxidant metabolism in wheat leaf and spike under changing CO_2 and nitrogen supply

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Abstract
Nonstructural carbohydrates and antioxidants affect the yield of any plant. In this study, changes in nonstructural carbohydrates and antioxidant metabolism in leaf and spike, as well as their effects on grain yield, were examined in relation to elevated CO_2 and nitrogen supply. For this, a wheat (*Triticum aestivum*) was grown at two levels of CO_2, i.e., ambient 400 ppm (T1) and elevated 800 ppm (T2), with two levels of nitrogen supply, i.e., 0 gN (N1) and 1 gN (N2). In the sink, elevated CO_2 and nitrogen caused a several-fold increase in glucose content. Fructose showed an increase of 53% and 60% in N_2 treatment under both carbon levels. At the same time, sucrose content decreased by 112% and 100% with an increase in nitrogen doses under 400 ppm and 800 ppm. Higher N decreased the superoxide dismutase activity at ambient CO_2, while higher N at elevated carbon levels increased the superoxide dismutase activity. Elevated CO_2 decreased the catalase activity, while the peroxidases activity increased. In the spike, catalase activity increased at a higher N level. Grain yield was significantly enhanced at elevated CO_2. The correlation analysis showed that catalase has a strong positive correlation with grain yield. The changes in nonstructural carbohydrates and antioxidant enzyme activities are associated with the altered leaf-spike relationship under N availability at high CO_2 levels, which could be a key factor contributing to variable yield. Differential response of nonstructural carbohydrates and antioxidant enzymes in leaf and spike is responsible for changes in grain yield.

Keywords Elevated CO_2 · Nitrogen · Nonstructural carbohydrates

Abbreviations
CO_2	Carbon dioxide
$a[CO_2]$	Ambient carbon dioxide
$e[CO_2]$	Elevated carbon dioxide
SOD	Superoxide dismutase
CAT	Catalase
APX	Ascorbate peroxidase
GR	Glutathione reductase
POX	Peroxidases
PVPP	Polyvinylpolypyrrolidone

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Introduction

The increase in atmospheric carbon dioxide (CO_2) is currently at a very concerning and dangerous level. It has been assumed that CO_2 levels would exceed 800 ppm by 2100 (Wang et al. 2013). Its concentration has increased significantly and unpredictably, making it one of the most alarming causes of global warming (FAO et al. 2018), which could further cause water shortage, resulting drought stress on plants (Ulfat et al. 2021; VanDerSleen et al. 2015). According to numerous studies (Liu and Stützel 2004; Yan et al. 2017), drought can inhibit plant development and physiology, including non-structural carbohydrates, antioxidants, and other processes. Nonstructural carbohydrates (NSCs), are pivotal in plant metabolism, providing a consistent supply of glucose and starch, while also governing growth status, ecophysiological functions, and reactions to external stresses (Liu and Huang 2018). To achieve high crop yields,

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it is important to improve the output and reuse capability of NSCs.

Beyond the fact that elevated levels of atmospheric CO_2 positively impacts the plant growth by stimulating several important factors such as increased photosynthesis rate, improved water use efficiency, and reduced stomatal conductance (Wang and Wei 2013). However, the contents of plant nutrients were found to be negatively impacted by elevated CO_2 (e[CO_2]) in various research (Li et al. 2019, 2020a, b). According to Radin and Eidenbock (1986) and Schurr et al. (2000), the lower nutrient contents, particularly nitrate, may also act as a signal to cause changes in nonstructural carbohydrates and antioxidant activities. Nitrogen is one of the essential nutrients that affects plant growth and physiology. According to Takashima et al. (2004), and Zhang et al. (2020), nitrogen is a crucial component of amino acids, proteins, nucleic acids, and chlorophyll, which helps to control the metabolism and assimilation of carbon (Boussadia et al. 2010). This decrease in nitrogen concentration can be attributed to various factors. First, it results from the dilution effect (Reich et al. 2006; Myers et al. 2014). The diluted nitrogen content caused by e[CO_2] levels can be counteracted by enhancing or optimising nitrogen levels through applied nitrogen fertilizer (Lam et al. 2012; Walker et al. 2017). Second, the reduced flow of nutrients from the soil to the plant can be attributed to decreased stomatal conductance and transpiration (McGrath and Lobell 2013). Along with changes in the rhizosphere environment, other factors that contribute to the fall in nitrogen concentration include lower levels of the Rubisco enzyme and hindered nitrate assimilation (Long et al. 2004; Reich et al. 2006; Bloom et al. 2012; Myers et al. 2014).

In response to e(CO_2), plants undergo photosynthetic processes to convert CO_2 into carbohydrates and other organic compounds (Whitehead et al. 1995). CO_2 fertilization offers a promising way to enhance crop yield and ultimately contribute to sustainable food production in the forthcoming decades. Further, applying nitrogen (N) can enhance the photosynthetic rate, leading to improved plant NSCs (Zhang et al. 2013). Ensuring an adequate nitrogen supply is essential as it influences the establishment and activity of the leaf and spike tissue processes (Ning et al. 2018).

Under e(CO_2) conditions, plant growth experiences a significant boost, resulting in higher yields (Long et al. 2004). The grain yield of plants can be seen as a balance between the source activity, which relates to the supply of carbohydrates, and the sink strength, which refers to the capacity of grains to store the available carbohydrates (Zhang et al. 2013). The significant increase in nitrogen uptake seen in crop and other plant species under e[CO_2] (Ainsworth and Long 2005; Bloom et al. 2012; Weigel and Manderscheid 2012; Mcgrath and Lobell 2013; Vicente et al. 2016; Dier et al. 2018). The increase in carbon availability induced by

e[CO_2] has a direct impact on starch synthesis and carbohydrate metabolism in both leaf and spike (MacNeill et al. 2017). Under e[CO_2] conditions, starch accumulation is enhanced due to increased Rubisco carboxylation activity, in contrast to a[CO_2] conditions (Ainsworth and Rogers 2007). Moreover, e[CO_2] affects various enzymes involved in carbohydrate metabolism in distinct ways. It has been proposed that enzymes within the same metabolic pathway might respond differently to e[CO_2] (Jammer et al. 2015). However, the overall trends in the alterations of major carbohydrate metabolism enzyme activities in leaf and spike organs, in response to both e[CO_2] and nitrogen fertilization, are not yet well understood.

Moreover, meeting the future food demand has emerged as a significant challenge in the twenty-first century. According to some predictions, the present food output would need to be doubled by 2050 to meet the task of feeding a global population of 9.7 billion people (FAO et al. 2018). Globally, wheat (*Triticum aestivum* L.) holds a prominent position as one of the primary food sources. Its outstanding qualities and compatibility for processing into different food products may help meet the need for food in the future and referred to as a bread cereal in many countries (Biel et al. 2020). Over the past decade, breeders have consistently focused on enhancing wheat grain yield while maintaining its quality (Igrejas et al. 2020). Among all crops, wheat has been extensively studied in terms of its response to e[CO_2]. The stimulation of plant growth under high CO_2 concentrations has been found to significantly enhance wheat yield (Long et al. 2004).

Hence, the extent of the plant productivity response greatly relies on the plant's capacity to uptake nitrogen under e[CO_2] conditions. Therefore, the objective of the ongoing study was to investigate the effects of e[CO_2] coupled with additional nitrogen supplementation, on non-structural carbohydrates and antioxidant enzymes.

Materials and methods

Experimental layout

Seeds were grown under ambient a[CO_2] (400 mmol L^{-1}) and elevated e[CO_2] (800 mmol L^{-1}) concentration in two greenhouse sections at the University of Copenhagen, Denmark. The experiment was performed in a randomized block design with four replicates, and each replicate was consisted of four 4-L pots (17 cm diameter; 16.5 cm height). Each pot was filled with 4.8 kg of sandy loam (EC = 0.45 ms cm^{-1}, pH 5.6–6.4, 1.0 g kg^{-1} of total N and 10.3 g kg^{-1} of total C Sphagnum, 32% organic matter). In each greenhouse section, equal numbers of pots as control (N1; without NH_4NO_3) and with the 1 g of N (N2, addition of 1 g NH_4NO_3) were

cultivated. In both greenhouse cells, one set of pots were kept control as no N was added and the 2nd set of pots was amended with 1 g N (1 g/pot) in the form of NH_4NO_3. Levels of CO_2 in the greenhouses were maintained by releasing CO_2 from bottle tanks. Throughout the entire experimental period, the concentration of CO_2 in the greenhouse was continuously monitored using a CO_2 transmitter (GMT220, Vaisala, Helsinki, Finland) at six-second intervals. Inside the greenhouse, the photoperiod spanned 12 h, providing a consistent duration of light exposure. The photosynthetic active radiation (PAR) level was maintained at 400 µmol m^{-2} s^{-1}, and the relative humidity was kept at a steady 70%.

Estimation of nonstructural carbohydrates (NSCs) activities

Nonstructural carbohydrates such as fructose, glucose, and sucrose were quantified from wheat leaf and spike samples following the method of Yemm and Willis (1954) with some modifications. Leaf and spike samples were wrapped in aluminum foil, snap-frozen in liquid nitrogen, and stored at -80 °C. Extraction was made with 80% ethanol and 5 mM HEPES solution by Debruyne et al. (1994) method. Ion chromatography (anion) was used for analysis using 200 mM NaOH as an eluent and pulsed amperometric detector (PAD) with a gold electrode (Dionex, ICS 3000, Sunnyvale, Canada).

Estimation of antioxidant (a) enzyme activities

For the determination of antioxidant enzymes activities, the plant samples were ground in 1 mL extraction buffer consisting of 40 mM TRIS–HCl pH 7.6, 3 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF, 1 mM benzamidine, 14 mM β-mercaptoethanol, and 24 µM NADP, using a semi-high-throughput analytical platform described by Jammer et al. (2015). After extraction, samples were centrifuged at 8000 rpm and then dialyzed. Dialyzed aliquots were prepared and kept at -800 °C for further analysis. Aliquots of the extracts, supplemented with respective reaction mixes for individual enzymes, were incubated in a 96-well plate reader (Ascent Multiskan; Thermo Fisher Scientific) at 30 °C for 40 min in UV-transmissive flat-bottom 96-well plates (UV-Star; Greiner Bio One, Kremsmünster, Austria) in a total reaction volume of 160 µL. The change in absorbance per second during the linear phase of substrate conversion was used as the basis for the calculation of specific enzyme activity in nkat gFW–1. Estimation of antioxidant enzymes activities including ascorbate peroxidase (APX) at 290 nm, catalase (CAT) at 240 nm, peroxidases (POX) at 450 nm, glutathione reductase (GR) at 340 nm, and superoxide dismutase (SOD) at 550 nm were determined photometrically

with the semi-high throughput analytical platform described by Fimognari et al. (2020).

Grain yield

The grain yield was calculated after crop harvesting at physiological maturity, using the algorithm proposed by Zadoks et al. (1974).

Statistical analysis

Analysis of variance for two factors (two-way ANOVA) was done using R Studio 1.0.153.exe, R software package "Agri-oclae" and their significance was tested at 0.05%. Similarly, R software package "corrplot" was used to compute correlation matrix in MetaR-v6.0_BASE_setup.exe software.

Results

Non-structural carbohydrates (NSCs)

In the leaf, the content of NSCs was significantly varied under both levels of CO_2 and nitrogen (N) as shown in Table 1. Glucose, fructose and sucrose contents were exponentially higher at e[CO_2] compared to a[CO_2], accounting for several-fold increase. When N was added, the amount of glucose under e[CO_2] level significantly increased. Fructose contents were increased up to 53% and 60% in N1 treatments under e[CO_2], compared to N2. Simultaneously, sucrose content was significantly increased with N2 dosage under both levels of CO_2.

In spike, NSCs content showed a varied response in different treatments. Glucose and sucrose contents were higher under e[CO_2] in comparison to a[CO_2]. Further, glucose, fructose and sucrose content was exponentially increased by 112%, 100% and 56% in N2 dose under e[CO_2]. However, starch content was greater with N2 dose under a[CO_2] level (Table 1).

Antioxidant enzyme activities

Antioxidant enzyme activities in leaf and spike have distinct patterns under different treatments (Table 2). In leaf, in comparison to a[CO_2], activities of SOD and CAT were significantly higher under e[CO_2]. However, APX and GR activities were greater under a[CO_2]. Furthermore, SOD, CAT and APX activities showed a marked increase with N2 dose under both levels of CO_2 (Table 2). In spike, compared to a[CO_2], activities of CAT and POX were higher under e[CO_2]. However, APX and GR activities were greater under a[CO_2]. Activities of CAT, GR and POX showed increased pattern with N2 dose under different level of CO_2 (Table 2).

Table 1 Analysis of variance (ANOVA), mean squares and standard deviation of nonstructural carbohydrate enzymes in wheat leaf and spike grown under ambient and elevated CO_2 with and without influence of nitrogen

Samples	Metabolite	400 ppm		800 ppm		Statistics
		N1	N2	N1	N2	
Leaf	Glucose	119.98 ± 12.57	87.20 ± 15.71	19.89 ± 4.60	131.34 ± 13.73	CO N* CO: N
	Fructose	3.33 ± 0.32	1.44 ± 0.22	4.65 ± 1.47	0.74 ± 0.16	CO N** CO: N
	Sucrose	4.68 ± 1.42	9.77 ± 1.46	0.59 ± 0.28	10.00 ± 1.74	CO N*** CO: N
Spike	Glucose	1.26 ± 0.20	0.82 ± 0.25	469.50 ± 69.59	1096.88 ± 230.98	CO*** N* CO: N*
	Fructose	2.27 ± 0.19	3.48 ± 0.58	2.37 ± 0.19	3.63 ± 0.31	CO: NS N** CO: N
	Sucrose	4.68 ± 1.07	2.21 ± 0.59	3.53 ± 2.02	4.75 ± 0.31	CO* N* CO: N
	Starch	27.28 ± 1.33	31.50 ± 1.80	27.03 ± 0.63	28.20 ± 2.49	CO N* CO: N
	Yield	66.55 ± 14.05	98.70 ± 11.37	87.95 ± 12.69	101.36 ± 15.31	CO* N CO: N

Ambient (400 ppm) and elevated (800 ppm) CO_2 level, N1 = without NH_4NO_3, N2 = with NH_4NO_3

$*P < 0.05$, $**P < 0.01$, $***P < 0.001$

Grain yield

Grain yield was significantly changed in response to different level of CO_2. The plants grown at e[CO_2] had 10.8% and 24% higher grain yield than those grown in a[CO_2] under N1 and N2 treatment, respectively (Fig. 1). In addition, yield was greater with N2 dose under both levels of CO_2 (Table 1).

To see the association of all the parameters of spike and source with yield, a Pearson correlation was also computed, and results are shown in Fig. 2a, b. In both the organs, namely leaf and spike, sucrose contents showed a strong negative correlation with yield accounting for $r = -0.33$ and $r = -0.21$, respectively. Regarding antioxidants, spike SOD showed a negative correlation with yield, while source CAT showed higher positive values of correlation with yield (Fig. 2a, b).

Discussion

Future projections indicate that the nutritional quality of wheat may decline under elevated CO_2 conditions. This potential decline could possibly be mitigated by enhancing or optimizing fertilizer usage, a strategy that could also lead to increased grain yield and reduce stress. On a global scale, wheat (*Triticum aestivum* L.) holds a prominent position as a C3 crop, and most of the research work was done on wheat

with regard to elevated CO_2 concentrations. Among the various atmospheric nutrients, nitrogen (N) is consider as the most critical and important element, as it plays a pivotal role in regulating wheat growth, yield, and quality. The interaction between nitrogen and CO_2 has been shown to significant effects, and further research in this direction needs consideration (Fangmeier et al. 1999; Hawkesford 2017). Under elevated CO_2 conditions, there is an increased demand for nitrogen, which could possibly impact overall plant growth (MacNeill et al. 2017). Considering these features, particularly in the context of future climate scenarios, adopting eco-friendly nitrogen management practices becomes imperative for ensuring the sustainable wheat production.

In recent study, grain yield was increased in e[CO_2] along with nitrogen treatment compared to a[CO_2] (Fig. 1). Nitrogen fertilizer application often improves wheat yield (Sudderth et al. 2005; Belete et al. 2018; Xu et al. 2020). It has been already recognized that low nutrient availability restricts the fertilization effect of CO_2 and yield would be enhance with the increasing dose of N under e[CO_2] (Long et al. 2006; Wang et al. 2013). Our findings agree with those of Kim et al. (2003) that rice (*Oryza sativa*) grain yield increased while moving from a low to a moderate N application rate, but that a high N application rate had no further effect on the CO_2 response ratio. Similarly other researchers worked on rice against e[CO_2] in combination with three levels of N application in a FACE experiment and conclude

Table 2 Analysis of variance (ANOVA), mean squares and standard deviation of antioxidant enzymes activities of wheat leaf and spike grown under ambient and elevated CO_2 with and without influence of nitrogen

Samples	Metabolite	400 ppm		800 ppm		Statistics
		N1	N2	N1	N2	
Leaf	SOD	0.91 ± 0.04	1.06 ± 0.04	1.24 ± 0.24	1.26 ± 0.06	CO N CO: N
	CAT	0.60 ± 0.20	1.31 ± 0.28	0.58 ± 0.14	1.41 ± 0.15	CO N ** CO: N
	APX	0.44 ± 0.15	0.49 ± 0.12	0.39 ± 0.08	0.46 ± 0.12	CO N CO: N
	GR	3.50 ± 0.17	2.74 ± 0.36	2.78 ± 0.35	3.34 ± 0.28	CO N CO: N
	POX	0.03 ± 0.01	0.02 ± 0.00	0.01 ± 0.01	0.03 ± 0.01	CO N CO: N
Spike	SOD	0.25 ± 0.02	0.17 ± 0.01	0.17 ± 0.01	0.20 ± 0.01	CO* N CO: N*
	CAT	0.22 ± 0.05	0.22 ± 0.02	0.27 ± 0.03	0.38 ± 0.07	CO* N CO: N
	APX	1.87 ± 0.15	1.83 ± 0.05	1.33 ± 0.37	1.56 ± 0.09	CO* N CO: N
	GR	0.32 ± 0.07	0.64 ± 0.22	0.38 ± 0.04	0.37 ± 0.06	CO N CO: N
	POX	0.02 ± 0.00	0.03 ± 0.00	0.05 ± 0.00	0.07 ± 0.01	CO ** N CO: N

Ambient (400 ppm) and elevated (800 ppm) CO_2 level, N1 = without NH_4NO_3, N2 = with NH_4NO_3

SOD superoxide dismutase, *CAT* catalase, *APX* ascorbate peroxidase, *GR* glutathione reductase, *POX* peroxide

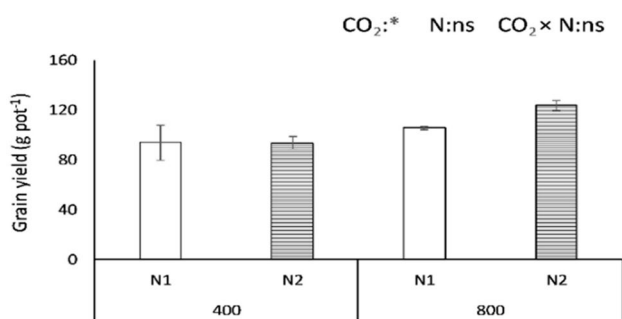$*P < 0.05$, $**P < 0.01$, $***P < 0.001$

Fig. 1 Grain yield of wheat grown under ambient and elevated CO_2 with and without influence of nitrogen. Ambient (400 ppm) and elevated (800 ppm) CO_2 level, N1 = without NH_4NO_3, N2 = with NH_4NO_3, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

that the medium level of N application led to the highest yield (Yang et al. 2009), which is also in line with the results of our study. Moreover, e[CO_2] significantly increased N uptake of wheat (Butterly et al. 2016). It seems that sufficient N supply may lead to optimization of photosynthetic processes and enhanced productivity under e[CO_2] (Ainsworth and Long 2005).

Numerous studies recorded that e[CO_2] levels lead to an increased wheat grain yield, primarily by increasing grain weight and the number of grains per spike (Ainsworth and Long 2005). This increase in grain yield under e[CO_2] was due to maximum starch accumulation in grains compared to a[CO_2] conditions (Högy et al. 2009), a phenomenon strongly linked to alterations in starch biosynthesis enzyme activities within spikes. The additional nitrogen (N) supply boosts the growth of tillers in wheat grown under e[CO_2] due to increased N availability. Moreover, the increased N supply has a direct impact on enzymes involved in carbohydrate metabolism which in turn, leads to modifications in carbohydrate metabolism within both leaf and spike when exposed to e[CO_2] conditions (Tausz-Posch et al. 2020). These modifications are primarily attributed to the increased

Fig. 2 **A** Correlation matrix of nonstructural carbohydrates and antioxidant activities of leaf with yield. **B** Correlation matrix of nonstructural carbohydrates and antioxidant activities of spike with yield. *SOD* superoxide dismutase, *CAT* catalase, *APX* ascorbate peroxidase, *GR* glutathione reductase, *POX* peroxide
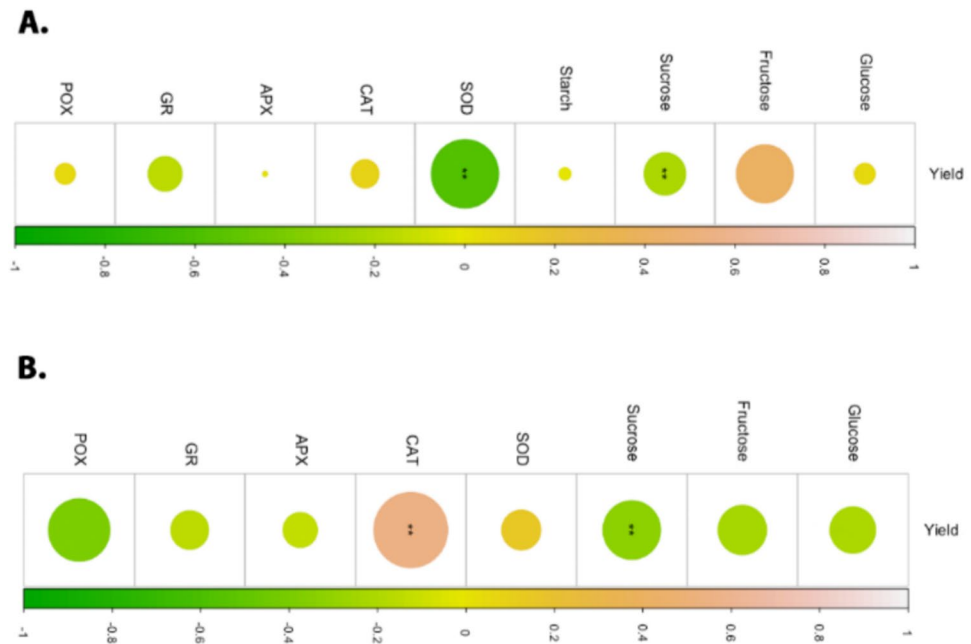
activities of sucrose enzymes, alongside decreased activities of fructose in leaves when subjected to greater N supply in recent findings. Additionally, it has been proposed that optimizing the balance between leaf and spike could offer a potential way for regulating wheat grain yield responses to e[CO_2] levels.

In this study, among the nonstructural carbohydrates, glucose and sucrose content were higher at e(CO_2) in both leaf and spike (Table 1). Possibly it was due to higher photo-assimilation under e[CO_2] which enhanced the activity of sucrose synthase and sucrose phosphate enzymes. In the previous studies, it was found that nonstructural carbohydrates increased under e[CO_2] (Li et al. 2020a, b; Yang et al. 2009). Correlation analysis also showed the strong positive correlation of sucrose with yield (Fig. 2a). Variations in results could be explained by the fact that sucrose first disintegrates and then move to the green organs of plants (Gesch et al. 2007). Moreover, a higher dose of nitrogen resulted in a reduction of nonstructural carbohydrates (fructose, sucrose and carbohydrates) in both leaf and spike, which was similar with previously published studies (Liu et al. 2018; Cao et al. 2020). In addition, opposing results has also been reported earlier under different experimental setup and plant species used. For example, when *Nicotiana plumbaginifolia* grown in hydroponic culture in high nitrate, e[CO_2] led to a significant increase of sugars where starch did not change significantly. Furthermore, when the plants were grown in pots on a lower nitrogen supply, it led to a significant increase of starch and a slighter increase of sugars (Ferrario-Méry et al. 1997). This is due to lower activities of starch metabolism enzymes such as starch synthase, secondary branch enzymes, and adenosine diphosphate-glucose

pyrophosphorylase under high nitrogen application (Li et al. 2018).

Antioxidant enzymes play a pivotal role in mitigating the damage caused by reactive oxygen species (ROS) (Mittler 2002). The action of these enzymes relies on not only in scavenging the free radicals but blocking the reactions leading to generate ROS, and repairing the damages caused by ROS. In the spike, SOD activity was higher in the N2 treatment under e[CO_2]. In the leaf, CAT activity increased with greater nitrogen levels under both carbon concentrations, while in the spike, CAT activity increases in response to elevated CO_2 in both nitrogen treatments (Table 2). Additionally, POX activity was higher in the spike under e[CO_2]. Previous research has shown mixed effects, such as reduced ZnSOD and POD activities but increased GR and Mn-SOD activities in soybeans under e[CO_2] (Badiani et al. 1993). Similarly, decreased SOD activities in spruce, pine, and oak (Polle et al. 1993; Schwanz et al. 1996) and reduced CAT activities in spruce and tobacco (Havir and McHale 1989; Polle et al. 1993) were observed. Interestingly, CAT activities in the leaves of orange, oak, and pine were unaffected by e[CO_2] (Schwanz et al. 1996).

Differential findings exist regarding the relationship between nitrogen (N) levels and antioxidant enzymes. For instance, in wheat (*Triticum aestivum* L.) leaves, low N conditions resulted in significantly increased activities of SOD, APX, and CAT (Polesskaya et al. 2004), whereas coffee (*Coffea arabica* L.) leaves exhibited higher SOD and APX activities under low N (Ramalho et al. 2018). However, in rice and *Arabidopsis thaliana*, low N conditions led to reduced CAT and APX activities (Kandlbinder et al. 2004). Notably, a positive correlation was observed

between SOD and CAT activities and grain yield under stress conditions, as depicted in Fig. 2b, which aligns with our study's results, where SOD and CAT displayed a positive correlation with grain yield (Tabarzad et al. 2017). Veronica et al. (2017) found that increased catalase (CAT) activity under phosphorus stress conditions was correlated with higher grain yield.

Globally, climate change results in significant wheat yield losses. Our research highlights the impact of (e[CO_2]) and nitrogen supply on wheat crop yield. Elevated CO_2 maintains antioxidant activities, aiding ROS scavenging and carbohydrate metabolism under e[CO_2] and nitrogen supply. Alterations in nonstructural carbohydrates and antioxidant enzymes in leaves and spikes likely contribute to grain yield maintenance.

Data availability Not applicable.

Declarations

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