

Enhancing Drought Resilience in *Calligonum mongolicum* through Nitrogen-Mediated Amelioration of Metabolic Stress: A Comprehensive Exploration of Phytohormones, Sugar Metabolism and Antioxidants Responses

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Abstract

Groundwater resources sustain phreatophytes in arid ecosystems. Nevertheless, how phreatophyte seedlings respond to topsoil water and nutrients before reaching groundwater remains elusive. This study unraveled the effects of three irrigation levels (well-watered, medium-drought, and severe-drought) and N-fertilization on multiple physio-biochemical responses in *Calligonum mongolicum* seedlings. Drought-stressed seedlings significantly enhanced reactive oxygen species, lipid peroxidation, and oxidized ascorbate-glutathione in shoots and roots, leading to impaired chlorophyll pigments, water status, and biomass, compared to control. They displayed higher abscisic acid, salicylic acid, jasmonic acid, and strigolactones but reduced indole acetic acid (IAA), cytokinin (CTKs), and zeatin riboside (ZR) in shoots and roots, and gibberellic acid (GA) and brassinosteroids (BR) in shoots. Lower starch and higher fructose, glucose, and sucrose, are possibly due to dynamic changes in carbohydrate metabolizing enzymes. Further, significantly upregulated superoxide dismutase (SOD), catalase, and ascorbate peroxidase (APX) in shoots, while glutathione-peroxidase and glucose-6-phosphate dehydrogenase observed in shoots and roots under either stress. Lower SOD and APX in roots; PPO in shoots while other enzymes of the ascorbate-glutathione cycle in shoots and roots following either stress, suggesting the sensitivity of the anti-oxidant mechanism. Conversely, N-addition enhanced the productivity of drought-stressed seedlings by improving their chlorophyll pigments, and endogenous hormones (IAA, GA, CTK, BR, and ZR), which may account for their better growth. Moreover, upregulated $O_2^{\cdot-}$ - H_2O_2 -scavenging mechanism, and soluble sugar, resulting in better status and biomass. Hence, N-supplementation could be an effective strategy to enhance drought-resistance in *Calligonum* seedlings to restore their communities in hyper-arid conditions under future climate change.

1. Introduction

Arid lands are characterized by frequent severe drought conditions, occasional floods, high soil pH levels, and limited nutrient availability (Tariq et al. 2022). In arid desert environments, phreatophytes take advantage of groundwater resources through their long and extensive root systems (Arndt et al. 2004; Canham et al. 2015). Thus, rapid root elongation is necessary for their successful establishment and restoration. Young seedlings are more sensitive to water deficit conditions (McDowell et al. 2008). Moreover, their roots are small and are incapable of utilizing groundwater. Due to this, the spontaneous emergence of phreatophytes is limited, and the regeneration of natural vegetation is inhibited (Tariq et al. 2022). This emphasizes the need to study the response of phreatophyte seedlings to water and nutrient availability, particularly before they reach groundwater sources. Water deficit is a key limiting factor affecting metabolism, growth, development, species distribution, and overall ecosystem productivity. Several studies have shown that drought stress can directly cause a variety of plant injury symptoms, including inhibiting plant photosynthesis (Ohashi et al. 2006), increasing oxidative stress (Tariq et al. 2019a), and altering metabolism (Valliyodan and Nguyen 2006).

Plant responses to abiotic stress depend on a variety of factors, however, phytohormones are the most important endogenous molecule which plays a critical role in the stress response. They adjust the cellular, molecular, and physiological activity of plants, which are essential for their survival (Fahad et al. 2015). Despite classifications that may differ slightly, there are commonly recognized phytohormones such as abscisic acid (ABA), gibberellins (GA), brassinosteroids (BR), jasmonic acid (JA), auxins (IAA), salicylic acid (SA), cytokinins (CK), ethylene (ETH) and strigolactones (SL) (Rai et al. 2023). These phytohormones play key roles in enhancing stress tolerance, including drought (Fahad et al. 2021). Plants typically respond to abiotic stresses such as salinity and drought by increasing abscisic acid levels. This is due to abscisic acid being the plant's defense against abiotic stresses (Zhang et al. 2006). For instance, the upregulation of abscisic acid (Fleta-Soriano et al. 2015) and strigolactones (Tariq et al. 2023) is associated with drought resistance. Furthermore, SLs are newly discovered hormones that regulate shoot-and-shoot architecture in response to nutrient limitations. (Yoneyama et al. 2012; Andreo-Jimenez et al. 2015). It is suggested that higher SL accumulation promotes lateral roots in a P-limited environment by facilitating P uptake (Ruyter-Spira et al. 2011). Substantial evidence exists demonstrating the interaction between ABA, SA, JA, and ET, with auxins, CKs, and GAs, in the regulation of plant defense responses (Iqbal et al. 2022; Navarro et al. 2008; Rai et al. 2023). They play a pivotal role in the response to diverse biotic and abiotic stresses, as well as orchestrating and regulating a range of developmental and growth processes in plants (Jiang and Asami 2018). Their complex interaction regulates processes such as stomatal closure, root growth, osmolyte adjustment, and stress-responsive genes (Fleta-Soriano and Munné-Bosch 2016; Liu et al. 2022; Fahad et al. 2021; Iqbal et al. 2022). For an in-depth understanding of plant defense mechanisms, it is imperative to study the intricate interaction between phytohormonal profiles and their interactions with physiological functions under drought conditions.

Drought stress diminishes photosynthesis and carbon fixation, resulting in disruptions of carbohydrate metabolism and dry matter partitioning (Chaves et al. 2002). Due to a reduction in photosynthesis, photoassimilates are reduced, resulting in a decrease in starch reserves (Galmés et al. 2007). The result is an imbalance in the accumulation and utilization of photoassimilates (Abid et al. 2016). Several studies have demonstrated that plants under dehydration produce significantly more fructose, glucose, and sucrose than they do under normal conditions (Fàbregas and Fernie 2019; Krasensky and Jonak 2012; Urano et al. 2009) to optimize osmotic potential and minimize the risk of oxidative stress damage. Moreover, several tree species reduce transpiration rates by closing their stomata, resulting in a reduced diffusion rate of CO_2 , and this in turn reduces growth (Cowan 1978; Vesala et al. 2017). Additionally, the stress effect is also dependent on the plant's ability to increase water uptake with minimal loss (Chaves et al. 2003). This can be accomplished by developing deeper roots, reducing leaf area and/or stomatal conductance, enhancing osmolyte concentration, and implementing other physiological modifications (Munné-Bosch and Alegre 2004; Tariq et al. 2017, 2022).

Plants produce excessive reactive oxygen species (ROS) such as superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) during periods of water shortage, resulting in oxidative stress damage (Nahar et al. 2015; Zhang et al. 2020). Several studies have shown that plant species have evolved

antioxidative defense systems and osmolyte accumulation strategies in response to water deficits to reduce the effects of cellular damage and improve osmotic adjustment (Liu et al. 2015; Tariq et al. 2019a). Nevertheless, shifting assimilatory products from growth to stress acclimation or tolerance explains why drought tolerance has an adverse effect on growth and biomass. As a result, alternative strategies must be developed to minimize this metabolic cost as much as possible by improving the tolerance mechanisms of economically and ecologically important species to ensure vegetation restoration.

Nitrogen is an important component of proteins and nucleic acids, where it plays a variety of functions, such as assisting in photosynthesis, respiration, storing and converting assimilated substances, and other functions (Vrede et al. 2004). In arid and hyper-arid regions, where water scarcity further restricts their availability and uptake, it is the primary factor limiting tree and shrub growth and productivity (Tariq et al. 2022). Drought conditions can severely reduce nitrogen mobility, resulting in a nitrogen deficiency that ultimately limits tree growth (Mahieu et al. 2009). Drought can also hinder plants' ability to metabolize nitrogen by disrupting enzyme activities that are involved in N metabolism. It is clear from this scenario that there is a strong correlation between nitrogen availability and drought since drought severely restricts nitrogen access and mineralization. Studies suggest that exogenous nitrogen can play a significant role in plants experiencing water deficiency (Tariq et al. 2019a; Zhang et al. 2021, 2020; Zhou et al. 2011) by upregulating antioxidant potentials, regulation of photoassimilates and osmolyte productions (Li et al. 2020; Tariq et al. 2019a). Yet, little attention has been paid to how nitrogen supplementation affects drought resistance functions and metabolic changes in xerophytic shrubs such as *C. mongolicum*.

C. mongolicum (Family *Polygonaceae*) is a perennial sand-fixing pioneer native to the arid deserts of central Asia. It is commonly utilized in programs for stabilizing dunes and ameliorating salinity in degraded soils, including areas of the hyperarid Taklimakan Desert in northwestern China that is experiencing agricultural expansion, overgrazing, overharvesting, and vegetation due to rapidly increasing populations. Consequently, revegetation and restoration of this species are urgently needed. Nevertheless, water availability is crucial to xerophytes' growth and establishment (Gui et al. 2013). As a result, it is extremely difficult to plant and establish seedlings for revegetation and restoration in hyper-arid conditions (Tariq et al. 2022). In this regard, it is imperative to improve their drought stress tolerance to establish successful seedling plantations.

As previously reported, *C. mongolicum* has been studied in terms of its resilience under P supplementation (Ullah et al. 2022b), biomass and NP allocation patterns, and few physiological indicators following drought, and N application (Zhang et al. 2020). Considering that plant adaptation is a complex process, data derived from a limited set of indicators may not be reliable. Consequently, it is imperative to evaluate multiple comprehensive indices to understand plant adaptation strategies. For instance, the ability of plants to cope with abiotic stresses is largely associated with the upregulation of anti-oxidant potential (Hasanuzzaman et al. 2012), phytohormones (Fleta-Soriano et al. 2015; Fleta-Soriano and Munné-Bosch 2016; Iqbal et al. 2022; Neves et al. 2017), osmolytes (Fang and Xiong 2015; Sami et al. 2016), and regulation of sugar metabolism in above- and belowground plant parts (Du et al. 2020). Further, drought resistance is a complex trait influenced by the inherent strategies of species regarding drought severity and duration, which reflect the multifaceted nature of plant responses to drought. The physiological adaptation mechanisms of *C. mongolicum* seedlings based on their regulation of anti-oxidant potential, hormones, and sugar metabolism in above- and belowground parts have not yet been assessed under different drought stress levels and nitrogen supplementation. This study unraveled (a) the effects of well-watered and drought stress levels [medium (MD), and severe drought (SD)] on assimilating shoot relative water content, biomass and regulation of physiological metabolism *viz.* photosynthetic pigments, phytohormones, anti-oxidant potential and sugar metabolism in *C. mongolicum* roots and assimilating shoot, and (b) to decipher how nitrogen supplementation regulate these physiological responses under water deficit conditions.

2. Materials and Methods

2.1.1 Seedling establishment

We conducted this study at Cele National Station of Observation and Research for Desert-Grasslands Ecosystem, located on the southern fringes of the saline, hyperarid Taklimakan Desert (37°00'N, 80°43'E). Within the nearby oases, there is sparse vegetation, with mostly shrubs and subshrubs such as *Calligonum mongolicum*, *Alhagi sparsifolia*, *Tamarix ramosissima*, and *Karelinia caspica*. A pot experiment was conducted from May to September 2022 in an outdoor nursery. Healthy seeds of *Calligonum mongolicum* were collected from the nearby desert and sown in plastic pots (42-liter, 35- and 30 cm in diameter at the top and bottom respectively, with a bottom small hole) containing approximately 38 kg of homogenized topsoil (0–30 cm depth; aeolian loamy sand with the following properties: organic C, 2.99 g kg⁻¹; total N, 0.23 g kg⁻¹; total P, 0.60 g kg⁻¹) and total K, 23.11 g kg⁻¹. Within the first 30 days of the experiment, water was supplied on a three-day cycle to each pot (n = 1 seedling) to the field capacity (18% w/w).

2.1.2. Drought-stress and nitrogen application

One-month-old seedlings with uniform height were exposed to three different levels of water deficit and nitrogen supplementation: (controlled, medium-drought (MD), and severe-drought (SD)] and nitrogen supplementation (0- and 4.0 gN·m⁻² yr⁻¹). Each treatment was repeated 12 times. There were 12 replications of each treatment. Based on the following equation, pots were maintained at a soil-relative water content (the ratio of soil water to water at water-holding capacity) of 70–75% (well-watered, CK), 45–50% (medium drought), and 25–30% (severe drought) (Xu et al. 2009).

$$SRWC = [(W_{\text{soil}} - W_{\text{pot}} - DW_{\text{soil}}) / (W_{\text{FC}} - W_{\text{pot}} - DW_{\text{soil}})] \times 100$$

In this equation W_{soil} represents the current soil weight (soil + pot + water), W_{pot} is the weight of the empty pot, DW_{soil} represents the weight of dry soil, and W_{FC} is the weight of soil at field capacity (soil + pot + water).

To minimize the effect of potential environmental heterogeneity, the pots were rearranged randomly. When it rained, transparent plastic film was used as a canopy to protect the pots. The application of N (solid urea) was made once per month to the upper surface of the pots after watering. Each harvested fresh plant was separated into roots and leaves, which were wrapped in tinfoil, immediately immersed in liquid nitrogen, and stored at -80°C for physiological analysis.

2.2. Measurements of assimilating shoot relative water content (ASRWC), height, and biomass

We measured the fresh weights (FW) of the assimilating shoots, and then immersed the samples in distilled water at 4°C for 4 hours in the dark, to obtain their turgid weights (TW). Next, all the samples were placed in an oven at 70°C for 24 hours to obtain their dry weights (DW). For each sample, the ASRWC values were determined using the following equation:

$$ASRWC = [(FW - DW) / (TW - DW)] \times 100\% \text{ (Eq. 1)}$$

We then separated each plant into above-ground (shoot) and below-ground (root) parts and dried them for 24 hours at 70°C before calculating their dry weight (g).

2.3. Estimation of concentrations of photosynthetic pigments

We determined chlorophyll a (Chl a), and chlorophyll b (Chl b) concentrations using 80% acetone extracts of fresh assimilating shoots (0.1–0.3 g) following A standard method (Lichtenthaler and Wellburn 1983). A spectrophotometer was used to measure the absorbance of the filtrate at 663 and 646 (Chl a and Chl b, respectively) (Hitachi, Tokyo, Japan). Based on the following equations, chlorophyll content was calculated as mg g⁻¹ FW.

$$\text{Chl a} = 13.98 A_{665} - 6.88 A_{649} \text{ (Eq. 2)}$$

$$\text{Chl b} = 24.96 A_{649} - 7.32 A_{665} \text{ (Eq. 2)}$$

2.4. Determination of metabolites and enzymes of sugar metabolism

All biochemical parameters were determined using fresh samples of assimilating shoots and roots. We measured the concentrations of glucose and fructose in fresh samples using a previous method (Johnson et al. 1964). Furthermore, van Handel's (1968) method was used to determine sucrose concentration (van Handel 1968). For the determination of citric acid, approximately 0.5 g of samples were ground in 10 ml of 0.6 M perchloric acid. A solution of 2 ml of 2 N KOH was added to the supernatant following centrifugation and kept on ice for 15 minutes. The reaction solution contained 2 ml of 0.1 M triethanolamine, 0.01 ml of 0.03 M ZnCl₂, 0.01 ml of 0.01 M NADH, and 0.1 ml of the extraction solution. Finally, the absorbance was monitored for five minutes at 366 nm (Moellering and Gruber 1966). Following the calorimetric analysis, the starch concentration of the samples was determined with a slightly modified anthrone method at 620 nm (Hansen and Møller 1975).

In leaf and root extracts, enzymatic activities of sugar metabolism were assessed using appropriate ELISA kits according to the manufacturer's instructions (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China): sucrose phosphate phosphatase (SPP; YJ550955), Sucrose synthase (SS; YJ302947), sucrose phosphate synthase (SPS; YJ302949), α -amylase (AMY; YJ190046), β -amylase (BAM; YJ190047), 6-phosphogluconate dehydrogenase (6-PGDH; YJ241016), glucose-6-phosphate dehydrogenase (G6PDH; YJ513908), glucose-6-phosphate (G6P; YJ550940), Hexokinase (HK; YJ530492), Fructokinase (FK; YJ364900), invertase (Inv; YJ306942) and phosphoenolpyruvate carboxylase (PEPC; YJ224906).

2.5. Estimation of ROS and malonaldehyde concentrations

The production of superoxide anion ($\text{O}_2^{\cdot -}$) was estimated by monitoring the formation of nitrite from hydroxylamine was monitored in the presence of $\text{O}_2^{\cdot -}$. Fresh samples were homogenized in phosphate buffer solution (pH 7.8) and centrifuged for 10 minutes at $5000 \times g$ (Elstner & Heupel 1976) (Elstner and Heupel 1976). The incubation mixture consisted of 65 mM phosphate buffer (pH 7.8), 0.1 ml of hydroxylammonium chloride (10 mM), and 1 ml of supernatant. The incubation mixture was added to 17 mM sulfanilamide and 7 mM α -naphthylamine and incubated at 25°C for 20 minutes. Following this, ethyl ether was added to the same volume and centrifuged for 5 minutes at $1,500 \times g$ and measured the absorbance at 530 nm.

Hydrogen peroxide (H_2O_2) was measured by monitoring the titanium-peroxide complex absorbance at 410 nm (Patterson et al. 1984). We homogenized the fresh samples in acetone (5 ml) and centrifuged at $3000 \times g$ for 10 minutes. The reaction mixture, which contained 1 ml of supernatant, 0.1 ml of titanium reagent, and 0.2 ml of ammonia, was centrifuged for 10 minutes at $3000 \times g$. The precipitate was then washed five

times with acetone and centrifuged for five minutes at $10\,000 \times g$. Using 1-M H_2SO_4 (3 ml), the precipitate was dissolved, and the absorbance was determined at 410 nm.

Malonaldehyde (MDA) content was determined in fresh root and assimilating shoot samples following a standard procedure (Zhou et al. 2007). The MDA assay is conducted on 0.25 grams of fresh samples that have been ground in 5 mL of 1% trichloroacetic acid (TCA) and centrifuged at $3,000 \times g$ for 10 minutes in a refrigerator centrifuge. In 4 ml of a 20% TCA solution (containing 0.5% TBA), 1 ml of the supernatant was added. The mixture was then boiled for 30 min in a water bath at 95°C . Afterward, the reaction mixture was quickly cooled in an ice bath, and MDA content was determined by reading the absorbances at 450, 532, and 600 nm using a spectrophotometer. MDA concentration was calculated using the following equation.

$$\text{MDA (mol g}^{-1}\text{ FW)} = 6.45(\text{OD}_{532} - \text{OD}_{600}) - 0.56\text{OD}_{450}$$

2.6. Determination of reduced and oxidized ascorbate and glutathione and antioxidant enzyme activities

To determine the concentrations of reduced and oxidized ascorbate-glutathione and the enzymatic activities of antioxidant enzymes, approximately 1.0 grams of fresh samples of assimilating shoots and roots were rinsed, frozen in liquid nitrogen, ground to powder, and mixed with 0.1 moles of phosphate-buffered saline (PBS) (pH 7.0). We then centrifuged the extracts at $10\,000 \times g$ at 4°C for 20 minutes to obtain supernatants for analysis using appropriate ELISA kits by the instructions of the manufacturer (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China): ascorbate (mlsh0046), glutathione (YJ299073), dehydroascorbate (YJ880342), oxidized glutathione (YJ302944), ascorbate peroxidase (YJ320415), monodehydroascorbate reductase (YJ103677), glutathione reductase (YJ203471), dehydroascorbate reductase (YJ803460), glutathione peroxidase (YJ203440), catalase (YJ203416), peroxidase (YJ203419), superoxide dismutase (YJ203409) and polyphenol peroxidase (YJ203490).

2.7. Determination of concentrations of endogenous phytohormones

We measured phytohormone concentration in fresh samples using ELISA kits. We ground frozen roots and leaves into powders with liquid nitrogen and mixed them with 5 mL of 0.1 mol L^{-1} PBS (pH 7.0). The concentration of jasmonic acid (JA; YJ803408), strigolactones (SLs; YJ803429), abscisic acid (ABA; YJ803413), salicylic acid (SA; YJ803461), cytokinin (CTK; YJ803406GA), zeatin riboside (ZR; YJ803411), gibberellic acid (GA; YJ803421), indole acetic acid (IAA; YJ803465), and brassinosteroids (BR; YJ803463) were measured using appropriate ELISA kits by the instructions of the manufacturer (Shanghai Enzyme-linked Biotechnology Co., Ltd. Shanghai, China), respectively (Wu et al. 2020).

Statistical analysis

SPSS (Chicago, IL, United States) was used to analyze variance (ANOVA) test to examine the differences between treatments in terms of biomass, photosynthetic pigments, sugar metabolism, and antioxidant mechanisms. Duncan's multiple range tests were used to compare the means ($p > 0.05$). Figure graphics were created using GraphPad Prism 8. We conducted Pearson correlation analyses on the growth and physiology of roots and leaves separately using OriginPro (Version 2024, OriginLab Corporation, Northampton, MA, USA). To determine the overall complexity of drought and nitrogen response, and to identify traits showing similar trends, principal component analysis (PCA) of standardized data was conducted using OriginPro (Version 2024, OriginLab Corporation, Northampton, Ma, USA). The results of this analysis provided us with an insight into the physiological response to stress and the integration of all responses to drought stress and nitrogen treatment in leaves and roots.

3. Results

3.1 Changes in growth and chlorophyll concentration under drought and N fertilization

We observed that *C. mongolicum* seedlings significant reductions in growth metrics including shoot dry weight (SDW), root dry weight (RDW), and assimilating shoot relative water content (ASRWC) by 46, 30, and 14% in MD stressed seedlings and 70, 58 and 25% in SD seedlings, compared to CK. Moreover, the concentrations of Chl a and Chl b showed a reduction of 25, and 36% under MD stress, and 39, and 44% under SD stress. In contrast, the Chl-a/Chl-b ratio and root/shoot ratio (RSR) increased up to 1.17 and 1.30-fold under MD and 1.10 and 1.42-fold under SD stress, respectively.

However, N application considerably decreased the negative impact of both stress levels by improving ASRWC, SDW, and RDW i.e. up to 1.16, 1.76, and 1.52 under MD and up to 1.19, 1.52, and 1.79-fold under SD stress. Moreover, Chl-a and Chl-b displayed significant upregulation following N addition under either stress level. Nevertheless, the N application reduced Chl-a/Chl-b ratio (18%) and RSR (14%) under MD whereas significantly increased RSR under SD stress (Table 1). In comparison to non-fertilized seedlings, N application significantly improved SDW (1.44-fold), ASRWC (1.08-fold), and RDW (1.30-fold) of CK seedlings, while there was no significant impact on Chl-a and Chl-b ratio and RSR (Table 1).

Table 1

Changes in growth metrics and chlorophyll pigments in fresh assimilating shoots of *Calligonum mongolicum* seedlings in response to different drought regimes and nitrogen (N) application.

Parameters	Well-watered		MD stress		SD stress	
	- N	+N	- N	+N	- N	+N
Shoot dry weight (g)	13.01 ± 0.26b	18.78 ± 0.94a	7.03 ± 0.41d	12.39 ± 0.17b	3.86 ± 0.28d	5.89 ± 0.47e
Assimilating shoot RWC (%)	80.48 ± 0.99b	86.71 ± 0.95a	69.24 ± 1.11c	80.60 ± 2.06b	60.13 ± 1.38d	71.36 ± 1.07c
Root dry weight (g)	14.60 ± 0.25b	19.00 ± 0.81a	10.23 ± 0.58c	15.53 ± 1.08b	6.15 ± 0.82d	10.99 ± 0.66c
Root/shoot ratio	1.12 ± 0.05d	1.01 ± 0.06d	1.46 ± 0.17bc	1.25 ± 0.10cd	1.60 ± 0.25b	1.87 ± 0.09a
Chl- a (mg/g)	1.80 ± 0.04b	2.15 ± 0.07a	1.36 ± 0.07d	1.69 ± 0.07c	1.10 ± 0.03e	1.42 ± 0.02d
Chl- b (mg/g)	0.97 ± 0.02b	1.23 ± 0.04a	0.62 ± 0.03c	0.95 ± 0.01b	0.54 ± 0.05d	0.66 ± 0.05c
Chl-a/ Chl-b ratio	1.86 ± 0.03bc	1.76 ± 0.08c	2.18 ± 0.18a	1.78 ± 0.05c	2.05 ± 0.18ab	2.15 ± 0.16a
Data are presented as means ± SD (n = 3). different letters indicate treatment differences at p < 0.05 (Duncan's method).						

3.2. Responses of sugar metabolism to drought stress and N addition

In our study, starch levels decreased but fructose and sucrose increased in leaves and roots under either stress; while glucose increased in leaves under MD and in roots under SD stress (Tables 2 and 3). Moreover, G6P significantly increased in leaves under either water stress, or in roots under MD stress. Furthermore, SD stress significantly increased CA in leaves but decreased in roots. The concentration of F6P increased in leaves and roots, but it was statistically significant only in leaves under MD stress (Tables 2 and 3). Moreover, N fertilization significantly increased F6P and CA in leaves under either stress and F6P in roots under MD stress. In addition, fructose, glucose, and sucrose in leaves while starch in roots increased significantly following either water stress level compared to their unfertilized peers. In roots N-mediated improvements in starch in leaves while glucose, sucrose, and fructose in roots were statistically significant under MD stress. As compared to CK, the enzymatic activities of AMY, BAM, SPP, and G6PDH significantly increased in both leaves and roots while SuSy and Frk in leaves under either stress level. Further MD stress caused a significant elevation in SPS and HK in leaves, whereas PEPC in roots. Moreover, SD stress increases SuSy and decreases Frk and HK in roots. While in leaves SD stress increased 6PGDH compared to CK. Under either water stress regime, N fertilization significantly increased INV, SPP, and PEPC activity in leaves, while HK activity in roots, compared to non-fertilized seedlings (Tables 2 and 3). In addition, HK in leaves while FRK in leaves and roots increased significantly under MD stress following N fertilization. In roots, SPP and SPS increased under MD and SD stress respectively. Following N supply, leaves displayed lower AMY, G6PDH, and 6PGDH under either stress, while SuSy and BAM were lower under MD stress. In roots, it decreased AMY under either stress, while BAM under MD and SuSy under SD stress (Tables 2 and 3). However, the root displayed higher G6PDG and 6PGDH under MD stress following N fertilization.

Table 2

Changes in metabolites and enzymatic activities of sugar metabolism in fresh leaf samples of *Calligonum mongolicum* seedlings in response to different drought regimes and nitrogen (N) application.

Parameters	Well-watered		MD stress		SD stress	
	- N	+N	- N		- N	+N
Sucrose (mg/g)	4.17±0.09e	5.74±0.13d	7.06±0.01c	8.15±0.22a	6.90±0.06c	7.82±0.03b
Glucose (mg/g)	13.32±0.22de	13.56±0.07d	15.23±0.13c	19.37±0.23a	13.14±0.25d	15.69±0.08b
Fructose (mg/g)	19.63±0.38e	22.56±0.28d	31.41±0.17b	32.94±0.38a	26.88±0.24c	31.00±0.68b
Sucrose phosphate synthase (U/L)	88.58±2.26d	105.37±0.28b	106.86±1.20b	113.10±1.49a	88.03±1.76d	87.70±1.80d
Sucrose synthase (U/L)	173.84±4.24c	163.22±4.66d	208.59±2.69a	197.25±3.53b	208.40±4.59a	208.44±1.42a
Sucrose phosphate phosphatase (U/L)	79.01±3.40e	83.73±0.57d	89.09±2.32c	107.96±0.81a	87.15±1.02c	96.01±0.90b
Starch (mg/g)	10.86±0.12b	12.12±0.15a	8.24±0.20d	10.33±0.10c	7.86±0.20e	7.94±0.18e
α-Amylase (U/L)	3.94±0.02d	3.63±0.08e	4.92±0.03b	4.08±0.06c	5.10±0.07a	4.89±0.08b
β-Amylase (U/L)	2.49±0.01d	2.76±0.02c	3.52±0.14a	2.79±0.06c	3.18±0.04b	3.14±0.04b
Hexokinase (U/L)	183.80±3.06d	203.66±2.89c	212.30±3.33b	245.47±1.87a	184.56±2.88d	185.55±3.83d
Invertase (U/L)	93.46±0.18de	91.95±0.58e	94.60±0.60c	112.08±2.29a	95.40±1.63c	105.29±1.56b
Glucose-6-phosphate (ng/L)	34.73±0.91c	35.02±0.91c	44.67±0.92a	44.40±0.99a	42.48±0.65b	44.26±0.42a
Glucose-6-phosphate dehydrogenase (U/L)	188.41±4.36c	179.94±4.58d	229.47±3.95a	208.86±5.55b	224.62±3.12a	201.69±2.42b
6-Phosphogluconate dehydrogenase (U/L)	247.17±2.56d	226.87±3.29e	249.48±3.50d	258.42±0.75c	278.74±2.05a	267.11±4.30b
Fructose-6-phosphate (ng/L)	86.05±2.87d	117.55±1.54a	89.70±1.47c	98.73±1.38b	88.95±2.53cd	101.73±1.38b
Fructokinase (U/L)	41.27±0.50e	44.17±0.47c	46.50±0.67b	49.17±0.31a	42.70±1.17d	43.82±0.70cd
Phosphoenolpyruvate carboxylase (U/L)	25.01±0.38c	25.20±0.40c	28.93±0.34b	33.91±1.54a	26.83±0.44c	30.29±2.02b
Citrate acid (µg /g)	225.29±6.59de	222.59±8.05e	234.34±3.64d	297.14±3.28a	249.60±3.11c	272.24±4.76b
Data are presented as means ± SD (n = 3). different letters indicate treatment differences at p < 0.05 (Duncan's method).						

Table 3

Changes in metabolites and enzymatic activities of sugar metabolism in fresh root samples of *C. mongolicum* seedlings in response to different drought regimes and nitrogen (N) application.

Parameters	Well-watered		MD stress		SD stress	
	- N	+N	- N	+N	- N	+N
Sucrose (mg/g)	5.43±0.16c	5.42±0.21c	6.58±0.39b	7.30±0.75a	6.43±0.22b	6.75±0.16ab
Glucose (mg/g)	8.91±0.36b	9.22±0.9b	9.33±0.58b	12.47±0.92a	12.96±0.74a	13.46±0.11a
Fructose (mg/g)	14.60±0.97c	17.00±0.95b	18.37±0.93ab	14.21±1.27c	19.48±0.56a	19.27±1.09a
Sucrose phosphate synthase (U/L)	75.52±1.08d	74.09±1.02d	87.20±2.35c	89.10±2.22c	105.90±2.84b	120.05±2.33a
Sucrose synthase (U/L)	145.66±6.02c	149.00±3.45c	147.00±3.34c	145.86±2.60c	162.23±1.61a	155.46±2.58b
Sucrose phosphate phosphatase (U/L)	86.12±3.94d	115.45±3.99a	96.55±3.15c	110.79±1.68a	102.86±2.19b	101.56±3.66bc
Starch (mg/g)	12.68±0.37b	10.94±0.66cd	10.00±0.85d	14.43±1.00a	9.59±1.27d	11.73±0.71bc
α -Amylase (U/L)	4.28±0.18c	4.55±0.20c	5.81±0.22b	4.35±0.16c	6.42±0.42a	5.73±0.09b
β -Amylase (U/L)	2.99±0.21d	2.82±0.17d	4.47±0.31b	3.82±0.51c	5.03±0.18a	4.87±0.10ab
Hexokinase (U/L)	201.61±5.39c	207.63±0.81b	197.28±0.92c	229.96±2.59a	167.22±3.41e	173.87±2.52d
Invertase (U/L)	77.11±2.58d	78.68±3.72d	96.22±1.90c	97.91±5.26bc	103.46±0.97b	114.98±3.45a
Glucose-6-phosphate (ng/L)	27.57±1.31c	35.50±1.05a	32.46±0.76b	34.32±1.81ab	20.56±1.64d	21.41±1.32d
Glucose-6-phosphate dehydrogenase (U/L)	159.45±2.27c	160.55±1.69c	193.63±3.23b	211.80±7.39a	191.82±3.69b	190.63±3.57b
6-Phosphogluconate dehydrogenase (U/L)	217.15±1.96c	195.31±3.03d	218.08±1.48c	232.68±2.17a	222.12±2.68bc	226.60±5.71b
Fructose-6-phosphate (ng/L)	81.29±1.91b	91.17±3.41a	83.88±1.20b	95.58±1.03a	86.03±1.76b	84.20±4.78b
Fructokinase (U/L)	55.03±4.50b	57.80±1.00ab	55.87±0.66b	61.30±4.03a	46.18±2.60c	46.90±1.74c
Phosphoenolpyruvate carboxylase (U/L)	31.32±2.78b	31.40±1.12bb	38.85±2.23a	37.10±1.07a	31.68±0.71b	32.80±0.69b
Citrate acid (μ g /g)	217.37±4.49bc	229.94±2.77a	226.30±3.21ab	228.11±3.75ab	202.26±13.43d	207.60±3.09cd
Data are presented as means $\pm$ SD (n = 3). different letters indicate treatment differences at p < 0.05 (Duncan's method).						

3.3. Responses of oxidative stress indicators and anti-oxidant enzymes under drought and N application

In our study, the levels of O_2^- , H_2O_2 , and MDA, showed an up-regulation of 1.24, 1.29, and 1.68-fold in leaves, while 1.27, 1.38, and 1.13-fold in roots under MD, whereas 1.31, 1.38 and 1.80-fold in leaves and 1.32, 1.62 and 1.54-fold in roots of *C. mongolicum* seedlings under SD stress, respectively (Fig. 1a-c). However, the nitrogen supplementation significantly reduced their concentration in leaves up to 12, 27, and 32% following MD stress, and 9, 18, and 7% under SD stress. A similar pattern was observed in roots under MD stress, whereas in SD stress downregulation was only observed in MDA levels, compared to their unfertilized peers (Fig. 1a-c). Moreover, N fertilization significantly reduced O_2^- in the leaves and MDA in the roots of CK seedlings, while it had no significant effect on H_2O_2 levels in both leaves and roots. The enzyme activity of SOD, CAT, and GPX increased in leaves up to 1.25, 1.57, and 1.34 under MD and up to 1.21, 1.30, and 1.43-fold under SD, compared to CK (Fig. 1d-f). In roots, SOD decreased under either stress (26 and 33%) while CAT and GPX increased (1.66-fold 1.44-fold) following MD and SD stress. Furthermore, both MD and SD stress reduced POD (27 and 22%) and PPO (11 and 9%) in leaves, whereas increased PPO in roots (Fig. 1gh). Nitrogen application resulted in improved SOD and CAT in leaves and roots under both stresses, while GPX was improved in leaves under MD and in roots under SD. Moreover, N-induced upregulation of POD and PPO was significant in leaves under both stress and in roots under MD stress, compared to their unfertilized peers. N supply significantly improved CAT and PPO in the leaves of CK seedlings, but did not affect them in the roots. Furthermore, it had no impact on POD but increased GPX in leaves and roots and SOD in roots, when compared with unfertilized seedlings (Fig. 1ah).

3.4. Responses of the ascorbate-glutathione cycle to drought stress and N addition

The concentration of AsA and GSH decreased by 15, and 18% in leaves, while 24, and 15% in roots under MD, whereas 35, and 31% in leaves and 31 and 35% in roots under SD stress, respectively (Fig. 2ab). Moreover, DHA and GSSG significantly increased in both leaves and roots, leading to reduced ratios of AsA-DHA and GSH/GSSG compared to CK (Fig. 2c-f). APX activity was upregulated (1.23 and 1.20-fold) in leaves under either stress level (Fig. 3a). However, APX in roots and MDHAR, DHAR, and GR in both leaves and roots displayed downregulation following either stress (Fig. 3a-d). N fertilization-induced reduction in GSSG and DHA led to improvement in AsA and GSH levels. This resulted in an improved ratio of GSH/GSSG in leaves and roots and that of AsA/DHA in leaves under both stress levels compared to their untreated counterparts; whereas in roots AsA/DHA ratio increased only under MD stress following N fertilization (Fig. a-f). Under both MD and SD, N application improved enzymatic activity of APX and MDHAR in leaves and roots; and those of DHAR and GR in leaves under both stress levels, compared to the unfertilized seedlings. Moreover, N addition also upregulated GR and DHAR in roots only under MD stress but had no significant effect on under SD stress (Fig. a-d).

3.5. Responses of phytohormones production to drought stress and N addition

In our study, the concentration of ABA, JA, and SA in leaves and roots and SLs in roots displayed significant elevations under MD and SD stress, compared to CK (Fig. 4a-c). In leaves, SLs only increased under MD stress. Further, we observed significant reductions in IAA, CTK, and ZR concentrations in leaves and roots, as well as GA and BR concentrations in leaves following MD and SD stress compared to CK (Fig. 4d-e and Fig. 5a-c). In roots, BR did not demonstrate significant changes, while GA decreased under SD stress. Compared to their unfertilized counterparts, N fertilization significantly improved ABA in leaves under either stress whereas ABA and SA in roots under MD. In both leaves and roots, N fertilization increased SLs under SD, while IAAs and BR under MD. Moreover, the N-mediated improvement in CTK and GA levels was significant in leaves under MD and in roots under SD. While, ZR improved in leaves under either stress and roots under SD stress (Fig. 4a-e and Fig. 5a-c).

3.6. Relationships between growth and physiology

We performed a Pearson correlation analysis on growth metrics and physio-biochemical metabolism of *Calligonum* seedlings (Fig. 6a-d). We observed that ASRWC, GR, MDHAR, DHAR, and ASA had a significant positive correlation ($p \leq 0.05$) with the phytohormones (ZR, BR, CTK, GA, and IAA). On the other hand, reactive oxygen species (H_2O_2 , and O_2^-) and antioxidant enzymes (CAT and SOD) showed a negative correlation with GSH, GSH/GSSG, while a positive correlation with ABA, JA, and SA (Fig. a). However, the correlation of root analysis showed that RSR, O_2^- , MDA, and H_2O_2 showed a negative correlation ($p \leq 0.05$) with the phytohormones (ZR, BR, CTK, GA, and IAA) (Fig. B). The correlation analyses displayed that, ASRWC, F6P, and SPS showed a strong negative correlation ($p \leq 0.05$) with SuSy, AMY, BAM, G6P, and G6PDH (Fig. C). A positive relation can be seen among the SPS, SuSy, AMY, BAM, sucrose, glucose, and fructose (Fig D).

3.7. Principle component analysis

The principal component analysis (PCA) was executed to explore the response of shoot and root sugar metabolism including phytohormones and osmolytes (Fig. 7a and b) respectively, and stress tolerance (hormones and antioxidant enzymes; Fig. 7c and c, respectively) under different nitrogen and drought conditions. The first two principal components (PCs) described 72% of the phytohormones and osmolytes variations in shoot under WW + N and SD + N (Fig. 6a). However, the PCA results of roots were 53% of the first two axis displaying that, SPP and FRK were negatively associated with BAM and AMY under SD (severe drought) condition (Fig. 6c). On the other hand, the PCA of antioxidants and hormones showed 77% of the first two axis in shoot and in root 47% under different nitrogen and drought treatments. The results described that phytohormones were closely correlated with each other under WW + N conditions and antioxidant activities were positively correlated under SD + N conditions in shoot (Fig. 7b). However, in roots the phytochromes had a positive correlation with antioxidant activities under SD + N and WW + N conditions, while under SD condition antioxidants (MDA, DHA, GSSG, O_2^- , and H_2O_2) depicted negative correlation with DHAR, GSH, IAA, and GA (Fig. 7d).

4. Discussion

4.2. Responses of chlorophyll concentration to drought and N fertilization

The concentrations of chlorophyll a (Chl a), and chlorophyll b (Chl b) were significantly reduced under both drought conditions, irrespective of N availability, underscoring their sensitivity to drought conditions in xerophytic shrubs (Ullah et al. 2022b; Zhang et al. 2021, 2020). Photosynthetic pigments exhibit high sensitivity to water deficits, and their concentrations are directly linked to N availability because it is an integral structural component of photosynthetic machinery (Farooq et al. 2009). In response to drought, pigment concentrations may be reduced due to oxidative stress, chlorophyll degradation, reduced pigment synthesis, or nutrient availability, particularly N; all of which negatively impact photosynthesis and inhibit plant growth (Table 1). Chl-a and Chl-b levels were significantly improved by nitrogen fertilization under drought and well-watered conditions compared to non-fertilized peers (Table 1). According to previous research, nitrogen (N) promotes the formation of photosynthetic pigments by elevating protein levels in the stroma and thylakoids (Cooke et al. 2005) as well as chlorophyll synthesis during leaf development (Li et al. 2012) which subsequently increases net photosynthesis (Tariq et al. 2019a).

4.3. Responses of sugar metabolism to drought stress and N addition

In normal and stressful conditions, plant growth and development are dependent on coordinated carbon supply and utilization (Muller et al. 2011; Sami et al. 2016). Drought stress inhibits photoassimilates production and disrupts the carbon balance, resulting in physiological and metabolic alterations and biomass reduction (Du et al. 2020). In our study, the changes in carbohydrate composition, with enhanced fructose and sucrose and reduced starch, indicate a dynamic response of the *Calligonum* seedling's carbohydrate metabolism to either drought stress regime. Moreover, glucose increased in leaves under MD and in roots under SD may point to distinct regulatory mechanisms operating in leaves and roots under different stress intensities. Reduced starch levels may indicate mobilization of stored energy reserves to raise soluble sugar levels, possibly acting as osmoprotectants or energy sources (Rosa et al. 2009; Sami et al. 2016). Moreover, soluble sugar accumulation can protect cells by replacing the OH group with water, maintaining hydrophilic interactions between proteins and membranes, and preventing membrane damage under water deficit conditions (Hoekstra et al. 2001).

Moreover, it has been suggested that soluble sugar accumulation by plants (primarily sucrose) is associated with greater resistance to abiotic stresses (Van den Ende and Valluru 2009). Increased soluble sugar levels in our study may be the result of increased enzyme activities involved in sugar metabolism. For instance, the activities of AMY, BAM, and SPP upregulated in both leaves and roots while SuSy and Frk in leaves and INV in roots under either water stress (Table 3, 4). The upregulation of starch-degrading genes and the subsequent acceleration of starch degradation has been reported in *Arabidopsis thaliana* (Thalmann et al. 2016) and *Glycine max* (Du et al. 2020), which supports our findings. The observed changes in starch and soluble sugar levels (fructose, sucrose, glucose) and the corresponding alterations in the activity of sugar metabolism enzymes indicate a dynamic metabolic adjustment of *Calligonum* seedlings in response to water deficit conditions. The addition of N fertilization not only enhanced soluble sugar but also increased the accumulation of starch in *Calligonum* seedlings. This could be attributed to their metabolizing enzymes (Tables 2 and 3). Our results demonstrate that *Calligonum* seedlings are dependent on soluble sugar accumulation for osmotic balance, regardless of nitrogen availability.

HK and FRK further facilitate the phosphorylation of hexoses through additional enzymatic processes (Renz and Stitt 1993). According to a recent study, HK and FRK activity is reduced following drought stress (Shokat et al. 2020). In our study, FrK activity was significantly higher in leaves under either stress and HK under MD. In roots, their activities significantly decreased under SD stress compared to CK. This implies that the assimilating shoot and root demonstrate distinct responses in hexose phosphorylation under drought stress, reflecting the tissue-specific adaptation strategy of *Calligonum* to meet its metabolic needs during drought stress. Moreover, FRK in leaves and roots under MD, as well as HK in leaves under MD and in roots under either stress, exhibit significant upregulation following N supply. Fulda et al. (Fulda et al. 2011) suggested that drought-tolerant sunflower plants upregulated SIFRK3, an essential protein involved in FrK activity, under water deficit conditions. However, Whittaker et al., (Whittaker et al. 2001) reported that the higher activity of HK in *Sporobolus stapfianus* might confer drought tolerance. Hence, the observed increase in FrK and HK following N supplementation seems to play a role in conferring drought resistance to *Calligonum* seedlings.

As compared to CK, the enzymatic activity of G6PDH significantly increased in leaves and roots under either stress while that of 6PGDH leaves under SD. Upregulation of cytosolic G6PDH activity and transcripts has been suggested to be a cellular response to enhance NADPH production. This enzyme is responsible for controlling carbon flow in the pentose phosphate pathway and generating NADPH, a reducing equivalent that functions as an antioxidant to manage reactive oxygen species (ROS) and maintain redox balance and stress tolerance mechanisms (Dal Santo et al. 2012; Naliwajski and Skłodowska 2018) (Fig. 8). Moreover, it is clear that the leaves of stressed plants exhibit a high demand for the reduced form of NADPH; a demand that has been reduced under N fertilization in the form of decreased activity of G6PDH under either stress and 6PGDH under SD stress. Contrary to this, N-addition increased G6PDH and 6PGDH in roots under MD, thus satisfying the need for this nucleotide. Such responses of these enzymes to N addition might be associated with the N-induced alleviation of physiological responses.

4.4. Changes in oxidative indicators and antioxidant enzymes to drought and N fertilization

The measurement of MDA levels serves as a reliable biomarker for oxidative stress-induced damage to cell membranes in plants. In our study, both leaves and roots of *Calligonum* seedlings exhibited higher MDA levels under either stress, due to an increased accumulation of ROS (H_2O_2 , and O_2^-) in both leaves and roots. Excessive ROS levels result in the degradation of proteins, lipids, and DNA, resulting in the death of cells (Apel and Hirt 2004). In addition to oxidizing the pigments, they may also elevate the concentrations of ABA which induces stomatal closure and therefore compromises photosynthesis and ultimately reduces biomass production (Carvalho 2008; Tariq et al. 2018). Both ASA and GSH are integral components of the cellular antioxidant system. They work together with enzymes of the AsA-GSH cycle to neutralize ROS and maintain cellular health (Shan et al. 2020). The redox buffering functions of AsA and GSH can trigger stress adaptation (Apel and Hirt 2004). In our study, DHA and GSSG led to reduced AsA and GSH in leaves and roots under either stress. Previous studies suggest that drought causes the AsA and GSH pools to decrease and become more oxidized (Jiang et al. 2022; Sarker and Oba 2018). Plants produce antioxidant enzymes in response to various environmental factors, including drought, to combat oxidative stress (Sunil et al. 2013; Tariq et al. 2019b; Ullah et al. 2022a).

The induction of antioxidant enzymes is a crucial process that plants employ to eliminate excessive ROS maintain cellular redox balance and reduce oxidative stress. The dynamic regulation of antioxidant enzyme activities in response to drought stress is a complex and highly fine-tuned process. In our study, both stress levels increased SOD, APX, CAT, and GPX in leaves under either stress and PPO in roots. Moreover, MD stress elevated CAT and GPX in roots, and POD in leaves. Previous studies reported that water deficit increases antioxidant defense mechanisms in trees and shrubs (Tariq et al. 2018, 2019a; Ullah et al. 2022b; Zhang et al. 2020), which is consistent with our findings. Under stress, antioxidant enzymes may decrease or increase, depending on the nature and duration of the stress exposure and the specific tissue of the plant. The variations observed between roots and assimilating shoots in our study suggest tissue-specific regulatory mechanisms. For example, APX and SOD decreased in roots but increased in assimilating shoot tissues; whereas PPO and POD decreased in assimilating shoots whereas in roots PPO enhanced but POD remained unchanged. This suggests that assimilating shoots and roots of *Calligonum* seedlings may imply a different strategy or reliance on alternative antioxidant systems to cope with oxidative stress.

The differential response between leaves and roots illustrates the complexity of the plant's overall stress adaptation strategy. The priorities of different organs may vary according to their specific functions and stress exposure (Du et al. 2020). The different responses of antioxidant enzymes to drought stress suggest a differential resource allocation or regulatory mechanisms of *Calligonum* seedlings to cope with oxidative stress. In challenging conditions, this organ-specific adaptation contributes to the plant's overall resilience and survival. In the AsA-GSH cycle, MDHAR, DHAR, and GR play pivotal roles in recycling and maintaining AsA and GSH pools. In our study, the reduced enzymatic activities of these enzymes suggest a compromised ability of *Calligonum* seedlings to regenerate and sustain the optimal concentration of AsA and GSH under drought conditions (Jiang et al. 2022).

In plants, an adequate nutrient supply is necessary for maintaining optimal enzyme activity, ensuring that essential metabolic pathways continue to function even under drought conditions (Li et al. 2020). Nitrogen application significantly upregulated SOD and CAT in leaves and roots, while POD and PPO in leaves under either stress. Moreover, PPO and POD in roots and GPX in leaves were significantly elevated by N addition upregulated MD stress. Our findings suggest that N supply improves the O₂⁻ and H₂O₂-scavenging ability of *Calligonum* seedlings by improving their enzymatic antioxidant mechanism, which helps the plants better cope with the oxidative stress associated with elevated levels of reactive oxygen species. Our findings are in line with previous studies on trees (Tariq et al. 2019a) and xerophytic shrubs (Zhou et al. 2011; Zhang et al. 2020). Studies have shown that high activities of APX, MDHAR, and DHAR are associated with greater tolerance to stress (Jiang et al. 2022). In contrast, their low activity leads to a greater degree of membrane lipid peroxidation (Shao et al. 2008).

A higher AsA/DHA ratio generally indicates a more reduced state and a better capacity for ROS scavenging. The N-mediated enhanced MDHAR and DHAR activities suggest an enhanced capacity of *Calligonum* seedlings to regenerate AsA from DHA, contributing to a higher AsA/DHA ratio, compared to unfertilized seedlings. In addition, GSH is critical for regenerating AsA and metabolizing H₂O₂ during the AsA-GSH cycle under normal and stressful conditions (Hojati et al. 2011). In stressful conditions, increased GR activity promotes the removal of H₂O₂, thereby maintaining a high GSH/GSSG ratio (Verma et al. 2015), which contradicts our findings. However, N supplementation restored the GSH concentration in *Calligonum*, which resulted in a high GSH/GSSG ratio. This could be attributed to increased GR activity which increases GSH biosynthesis and reduces its degradation (Szalai et al. 2009). High GSH/GSSG ratios indicate stress tolerance in plants. A transformation of GSSG into GSH enhances the ability of plants to resist environmental stresses (Verma et al. 2015).

Moreover, N addition has been reported to upregulate the APX activity for maintaining adequate H₂O₂ balance (Chang et al. 2016), which is in line with our results. Therefore, N fertilization contributes to the balancing of the AsA-GSH redox balance. This balance is crucial for maintaining the antioxidant defense system in plants, and its enhancement suggests an improved ability to counteract oxidative stress. Several studies have demonstrated that N fertilization significantly reduces the concentrations of O₂⁻ and H₂O₂ as a result of the upregulation of both enzymatic and nonenzymatic antioxidant mechanisms in trees and xerophytes (Tariq et al. 2019a; Zhang et al. 2021, 2020), which results in lower oxidative stress and greater membrane stability. The coordinated mechanism observed in response to N fertilization suggests an adaptive response, enabling *Calligonum* seedlings to better cope with the oxidative stress associated with water deficit conditions.

3.6. Responses of phytohormones production to drought stress and N addition

As compared to CK, we observed significant increases in the concentration of ABA, JA, SA, and leaves and roots while SLs in roots under either stress. Furthermore, drought stress levels reduced IAA, CTK, and ZR in leaves and roots, as well as GA and BR in leaves. Phytohormones play crucial roles as signaling molecules, influencing various physiological mechanisms, growth, and development under normal and stressful conditions. Thus, the imbalance in hormone regulation might have resulted in a marked reduction in growth and biomass in our study (Li et al. 2018). Since hormone regulation is closely related to drought stress, our results may also be interpreted as a strategy to cope with drought through differential hormone regulation (Li et al. 2018). Additionally, phytohormones affect plant responses to oxidative stress through their interactions with ROS, resulting in distinct transcriptomic and physiological responses. This interaction is mediated by respiratory burst oxidase homologs (RBOHs) in plants. Multiple mechanisms have been demonstrated to influence the production of ROS and RBOH by stress (Devireddy et al. 2021) (Fig. 8). JA levels increase under drought conditions, modulating antioxidant mechanisms and osmolyte production, contributing to drought tolerance (Dhakarey et al. 2017). There is evidence that JA concentrations increase rapidly in *Citrus* (de Ollas et al. 2013) and *Arabidopsis* plants

(Balbi and Devoto 2008), which supports our findings. Our study revealed that drought exposure increased ABA, which is involved in a wide variety of mechanisms for coping with stress, including antioxidant activity and the prevention of oxidative stress (Iqbal et al. 2022).

Compared to their unfertilized peers, N fertilization significantly improved ABA in leaves under either stress and in roots under MD stress. An increase in ABA concentration regulates several stress-related mechanisms (Danquah et al. 2014), including regulation of turgor pressure (Iqbal et al. 2022), protection of xanthophyll cycle and photosynthetic machinery (Du et al. 2010; Zhu et al. 2011), modulation antioxidant and osmotic potentials (Iqbal et al. 2022) leading to improved drought tolerance and growth responses.

Moreover, SA increased in leaves and roots under either stress. The SA accumulation improves drought resistance in plants by modulating several physiological responses including stomatal regulation, activation of defense responses, protection of photosynthetic machinery, and prevention of electron leakage (Iqbal et al. 2022). An increasing body of evidence suggests that SLs, which are carotenoid-derived phytohormones, can play a critical role in regulating plant response to stress conditions (Tariq et al. 2023). In our study, SLs increased in leaves under MD and in roots under either stress. The dry lands experience nutrient limitations, especially P and N availability, and the drought further aggravated the situation (Gao et al. 2022; Tariq et al. 2022). Indeed, there is evidence suggesting that SLs play a crucial role in modulating shoot-and-shoot architecture in response to nutrient limitations, which promotes the accumulation of SLs (Yoneyama et al. 2012; Andreo-Jimenez et al. 2015). Ruyter-Spira et al. (Ruyter-Spira et al. 2011) suggested that increasing SLs promotes lateral roots in a P-limited environment by facilitating P uptake. Hence, the upregulation of strigolactones in *Calligonum* seedlings appears to be a part of a multifaceted strategy to improve drought resilience, facilitate root growth, and improve nutrient uptake in the challenging conditions of the hyper-arid Taklamakan Desert. The increase in SL levels in response to N fertilization under SD conditions suggests a dynamic and adaptive response of *Calligonum* seedlings. SD conditions may have prevented seedlings from absorbing N optimally, which could have triggered the upregulation of SL biosynthesis.

Drought interaction with other hormones can also affect IAA activity, synthesis, metabolism, and transport in a variety of plants (Iqbal et al. 2022). Furthermore, GA has been reported to alter the regulation functions of several genes in tomato plants exposed to drought, resulting in smaller cells, fewer internodes, shorter shoots, and lower biomass (Litvin et al. 2016). Furthermore, CTK concentrations may increase or decrease in response to drought conditions. In addition to regulating cell division, CTK is involved in apical meristem support, and several physiological responses, which allow plants to adapt to rapid changes in the environment (Yadav et al. 2021). The observed decrease in the concentrations of IAA, GA, and CTK under drought stress in our study suggests that these hormonal imbalances could contribute to the sensitivity and severe reduction in shoot and root growth and biomass in *Calligonum* seedlings. In both leaves and roots, N fertilization increased IAA and BR under MD while CTK and GA in leaves under MD and roots under SD. Moreover, ZR improved in leaves under either stress or roots under SD stress. Since these hormones play a role in stimulating plant growth (Tiwari et al. 2017); their increased concentration following N supplementation under drought might result in improved biomass of *Calligonum* seedlings compared to their non-fertilized peers.

5. Conclusion

In our study, both medium and severe drought stress levels reduced biomass and impaired metabolism, compared to control. Drought-induced overproduction of soluble sugar resulted in lower starch which could be the result of increased enzymatic activities of sugar metabolism. Moreover, the enzymes and G6PDH and 6PGDH increased following drought stress, which indicates the potential role of the pentose phosphate pathway in satisfying the demand for NADPH; the antioxidant-reducing equivalent. Moreover, drought-stressed seedlings exhibited reduced enzymatic activities of SOD and APX in roots, and PPO in shoots whereas MDHAR, DHAR, and GR in shoots and roots following either stress, suggesting the sensitivity of anti-oxidant mechanism. This phenomenon resulted in a higher accumulation of oxidized ascorbate (DHA) and glutathione (GSSG), indicating the overall failure of the ascorbate-glutathione cycle to properly eliminate the H_2O_2 . In contrast, N-supplemented drought-stressed seedlings upregulated their H_2O_2 and O_2 -scavenging mechanisms, resulting in reduced levels of ROS and lipid peroxidation and improved AsA and GSH redox status. They also displayed higher growth hormone production, and soluble sugars which may account for their improved biomass and water status. Moreover, starch degradation was reduced in drought-stressed seedlings supplemented with N which led to increased starch levels. Consequently, our findings emphasize the intricate and flexible nature of *Calligonum* seedling adaptive mechanisms, as well as the positive effect of N-supplementation on changing the physiological responses of the shoot and root dynamically, resulting in improved drought resistance. Our study reveals physiological stress adaptation strategies of phreatophyte seedlings to water deficits and nutrient availability before their roots reach groundwater resources and have practical implications for vegetation restoration and management in hyperarid and nutrient-deficient ecosystems.

Declarations

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Author Contributions AU, and AT conceived and designed the research; AU conducted the experiments; AU, AT, JN, JP, and JS analyzed the data and prepared the figures; AU wrote the first draft; JP, JS, ZF, JN, AT, and MAA helped edit and review the manuscript; ZF supervised the research. All authors read and approved the manuscript.

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Figures

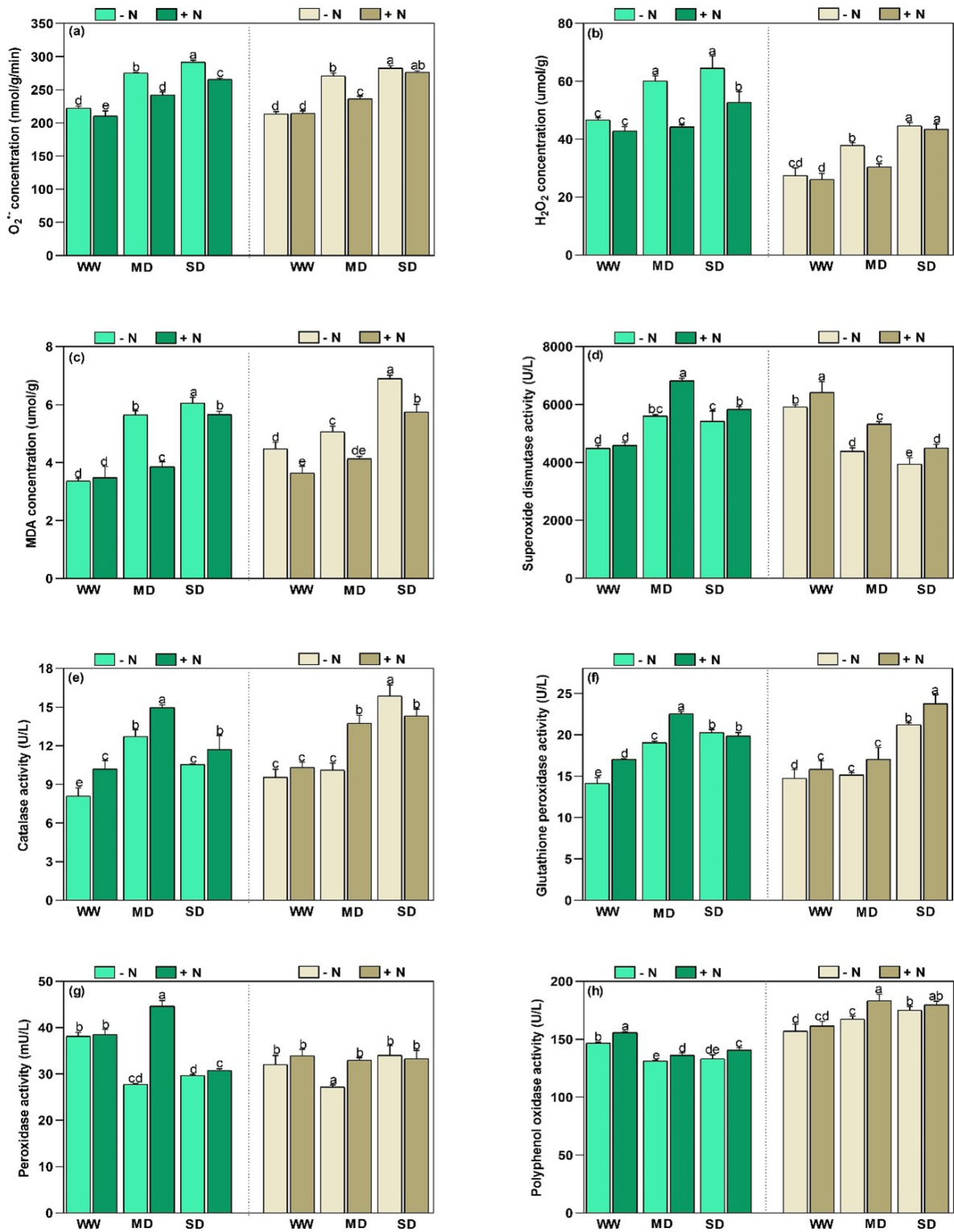


Figure 1

Changes in the concentration of (a) malondialdehyde (b) superoxide anion (c) hydrogen peroxide, and enzyme activity of (d) superoxide dismutase (e) catalase (f) glutathione peroxidase (g) peroxidases and (h) polyphenol oxidase in assimilating shoots and roots of *C. mongolicum* seedlings in response to different drought regimes and nitrogen (N) application. Bars represent means $\pm$ SD (n=3). different letters indicate treatment differences at $p < 0.05$ (Duncan's method). WW: well-watered; WW+N: well-watered + nitrogen; MD: medium drought; MD + N: medium drought + nitrogen; SD; severe drought; SD+N: severe drought + nitrogen.

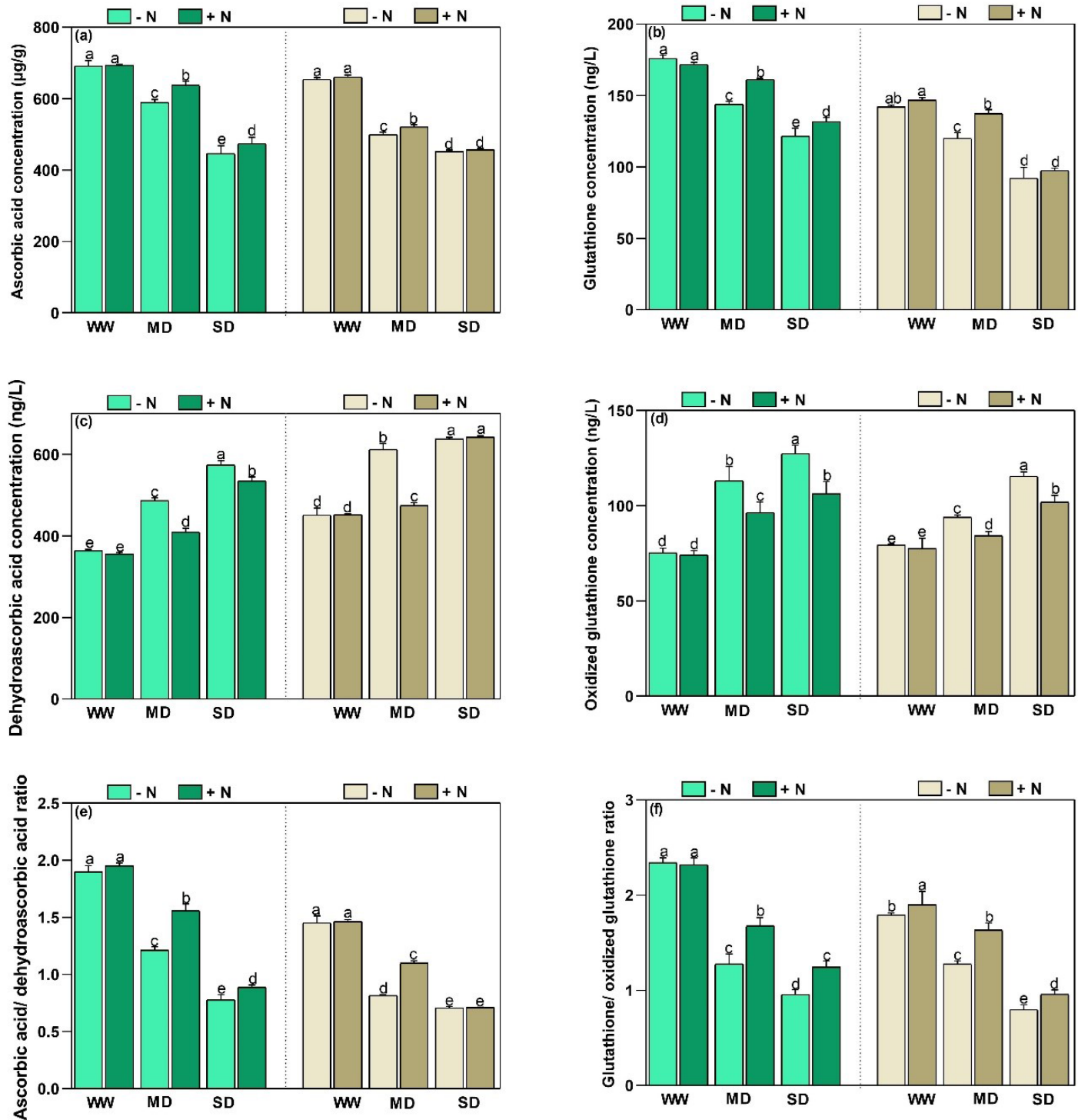


Figure 2

Changes in the concentration of (a) ascorbic acid, (b) glutathione, (c) dehydroascorbic acid (d) oxidized glutathione (e) ascorbic acid/dehydroascorbic acid ratio, and (f) glutathione/oxidized glutathione ratio in assimilating shoots and roots of *C. mongolicum* seedlings in response to different drought regimes and nitrogen (N) application. Bars represent means $\pm$ SD (n=3). different letters indicate treatment differences at p< 0.05 (Duncan's method). WW: well-watered; WW+N: well-watered + nitrogen; MD: medium drought; MD + N: medium drought + nitrogen; SD; severe drought; SD+N: severe drought + nitrogen.

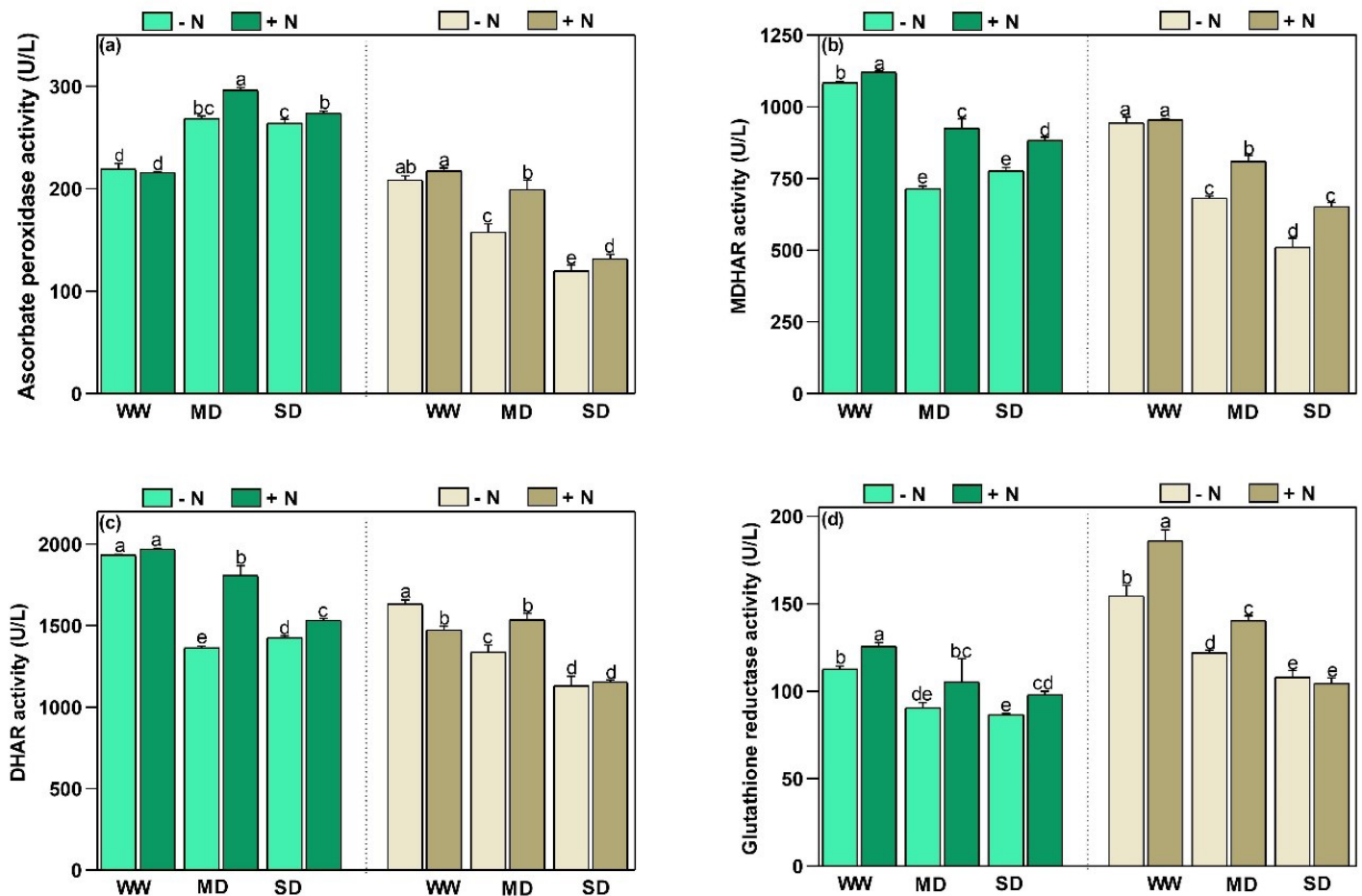


Figure 3

Changes in the enzyme activity of (a) ascorbate peroxidase (b) monodehydroascorbate reductase (c) glutathione reductase and (d) dehydroascorbate reductase in assimilating shoots and roots of *C. mongolicum* seedlings in response to different drought regimes and nitrogen (N) application. Bars represent means $\pm$ SD (n=3). different letters indicate treatment differences at $p < 0.05$ (Duncan's method). WW: well-watered; WW+N: well-watered + nitrogen; MD: medium drought; MD + N: medium drought + nitrogen; SD: severe drought; SD+N: severe drought + nitrogen.

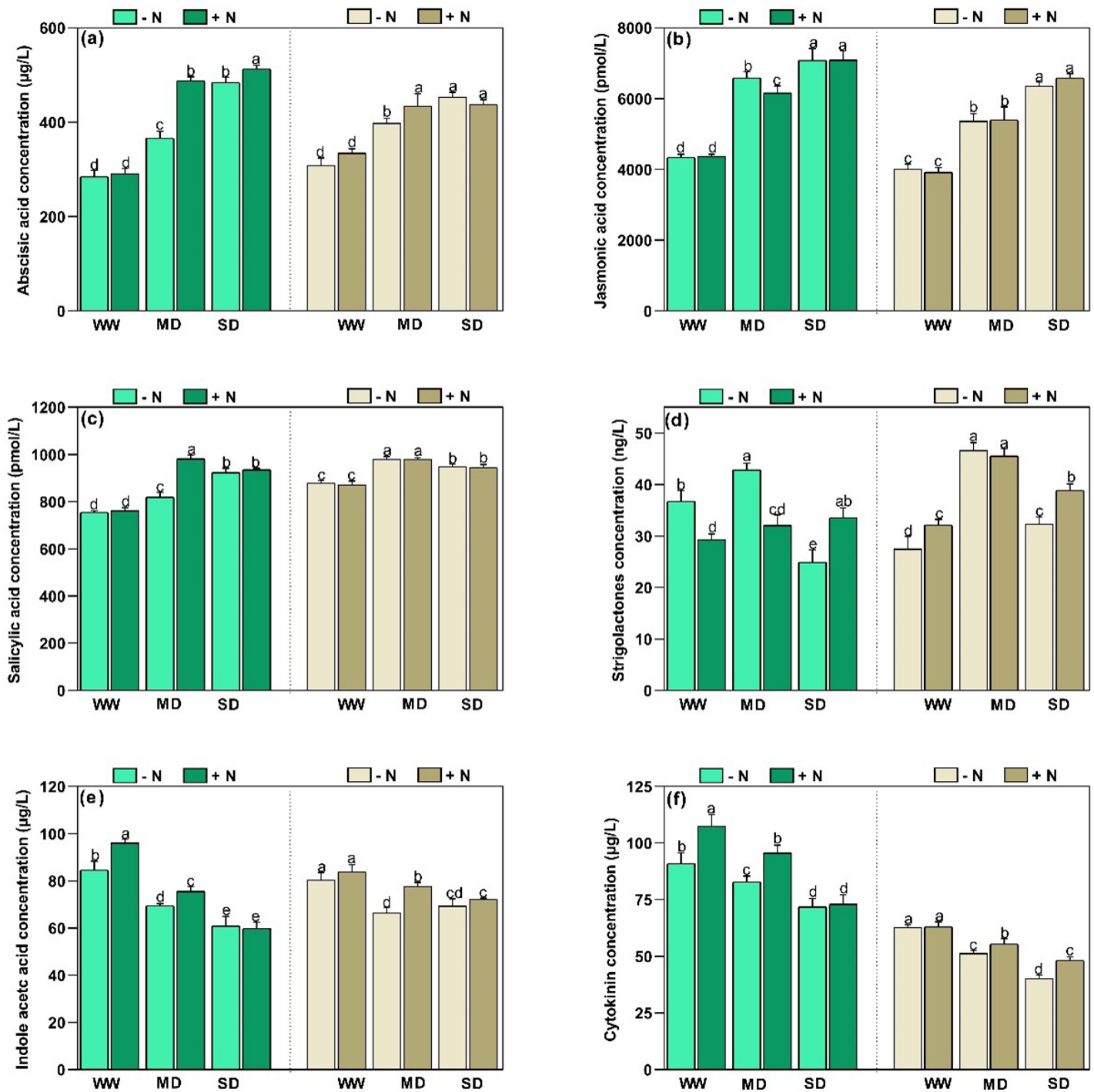


Figure 4

Changes in the concentration of (a) abscisic acid, (b) jasmonic acid (c) salicylic acid (d) strigolactones (e) indole acetic acid, and (h) gibberellic acid in assimilating shoots and roots of *C. mongolicum* seedlings in response to different drought regimes and nitrogen (N) application. Bars represent means $\pm$ SD (n=3). different letters indicate treatment differences at $p < 0.05$ (Duncan's method). WW: well-watered; WW+N: well-watered + nitrogen; MD: medium drought; MD + N: medium drought + nitrogen; SD; severe drought; SD+N: severe drought + nitrogen.

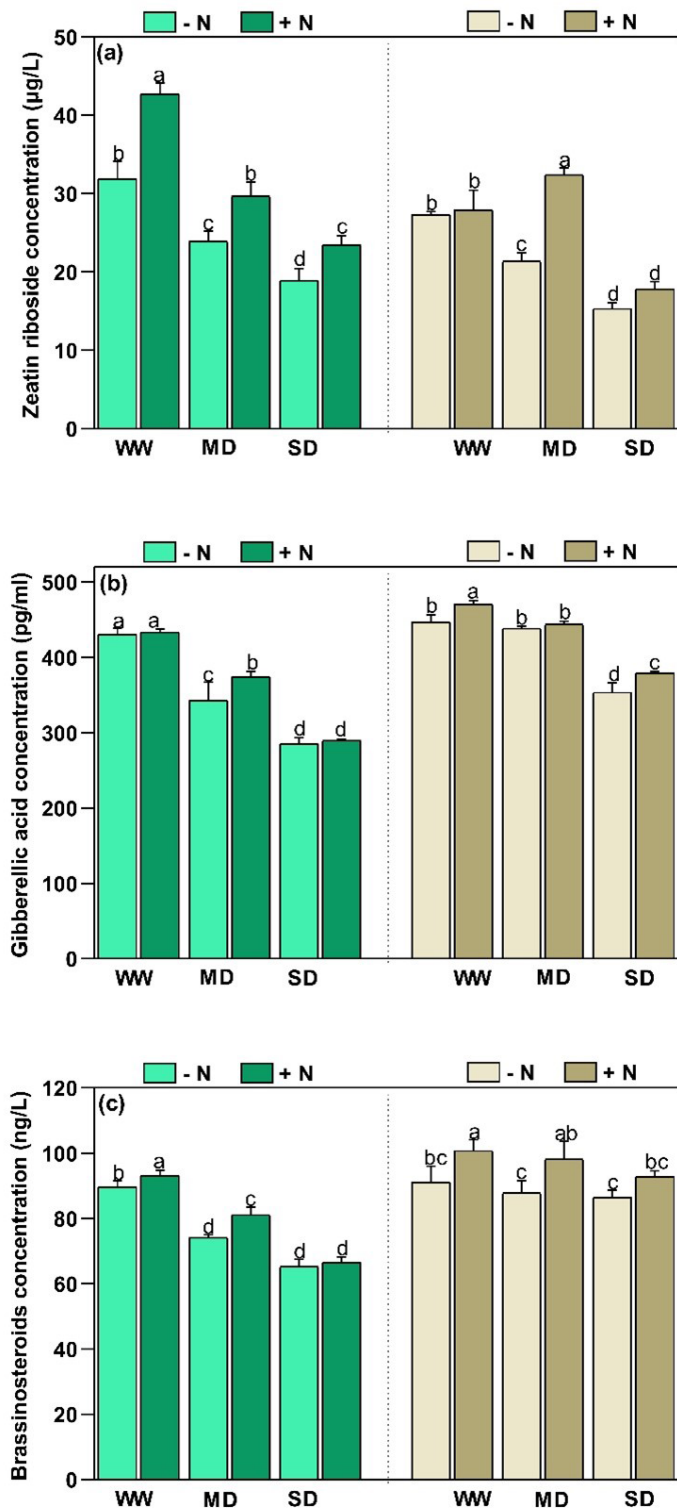


Figure 5

Changes in the concentration of (a) cytokinin (b) zeatin riboside, and (c) brassinosteroids in assimilating shoots and roots of *C. mongolicum* seedlings in response to different drought regimes and nitrogen (N) application. Bars represent means $\pm$ SD (n=3). different letters indicate treatment differences at $p < 0.05$ (Duncan's method). WW: well-watered; WW+N: well-watered + nitrogen; MD: medium drought; MD + N: medium drought + nitrogen; SD; severe drought; SD+N: severe drought + nitrogen.

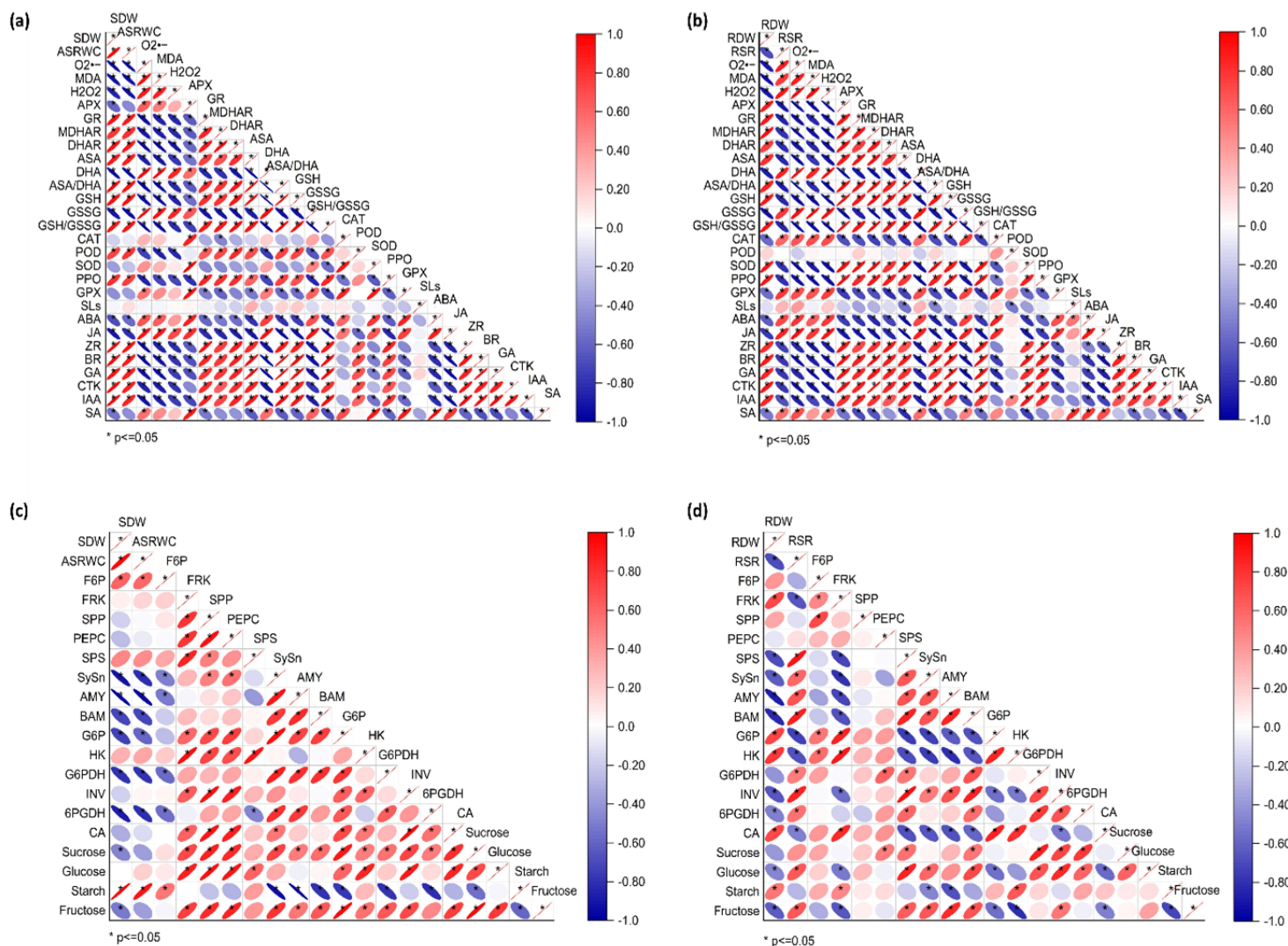


Figure 6

Associations between growth metrics and stress and stress tolerance indicators in assimilating shoot (A, C) and roots (B, D). ASRWC: Assimilating shoot relative water content; SDW: shoot dry weight; RDW: root dry weight; RSR: Root/shoot ratio; JA: jasmonic acid; ZR: zeatin riboside; SLs: strigolactones; GA: gibberellic acid; IAA: indole acetic acid; ABA: abscisic acid; CTK: cytokinin; BR: brassinosteroids; SA: Salicylic acid; AsA: ascorbate; DHA: dehydroascorbate; GSH: glutathione; GSSG: oxidized glutathione; APX: ascorbate peroxidase; GR: glutathione reductase; MDHAR: monodehydroascorbate reductase; DHAR: dehydroascorbate reductase; MDA: malondialdehyde; H₂O₂: hydrogen peroxide; O₂⁻: superoxide anion; SOD: superoxide dismutase; POD: peroxidases; GPX: glutathione peroxidase; CAT: catalase; and, PPO: polyphenol oxidase; SS: Sucrose synthase; SPP: sucrose phosphate phosphatase; SPS: sucrose phosphate synthase; AMY: α -amylase; BAM: β -amylase; G6PDH: glucose-6-phosphate dehydrogenase; 6PGDH: 6-phosphogluconate dehydrogenase; G6P: glucose-6-phosphate; FRK: Fructokinase; HK: Hexokinase; PEPC; phosphoenolpyruvate carboxylase; INV; invertase.

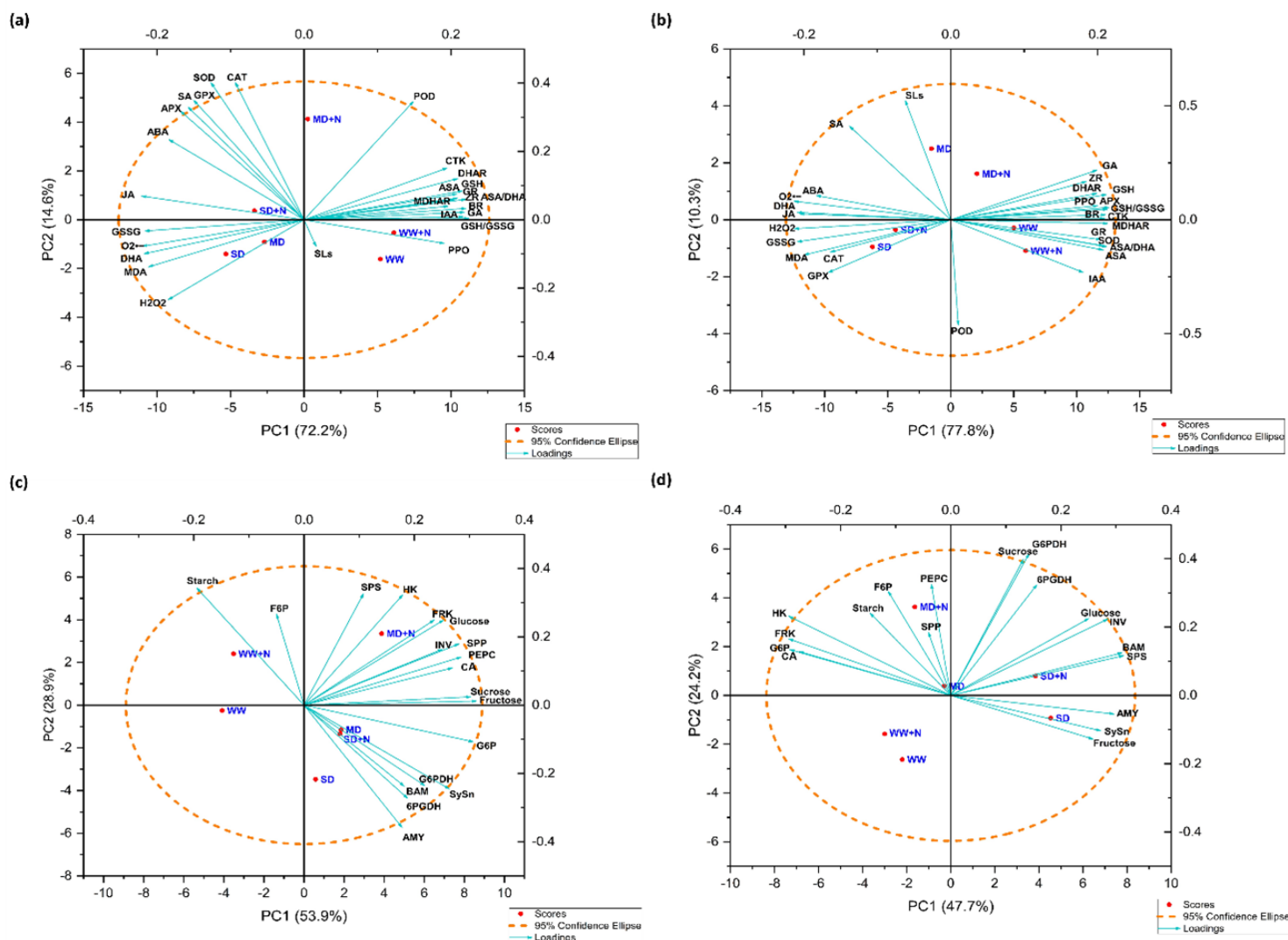


Figure 7

Principle component analysis (PCA) of responses of sugar metabolism in assimilating shoot (A) and root (B) and phytohormone and antioxidant indicators of stress tolerance in assimilating shoot (C) and roots (D) in response to different drought regimes and nitrogen (N) application in *C. mongolicum* seedlings. WW: well-watered; WW+N: well-watered + nitrogen; MD: medium drought; MD + N: medium drought + nitrogen; SD: severe drought; SD+N: severe drought + nitrogen. JA: jasmonic acid; ZR: zeatin riboside; SLs: strigolactones; GA: gibberellic acid; IAA: indole acetic acid; ABA: abscisic acid; CTK: cytokinin; BR: brassinosteroids; SA: Salicylic acid; AsA: ascorbate; DHA: dehydroascorbate; GSH: glutathione; GSSG: oxidized glutathione; APX: ascorbate peroxidase; GR: glutathione reductase; MDHAR: monodehydroascorbate reductase; DHAR: dehydroascorbate reductase; MDA: malondialdehyde; H₂O₂: hydrogen peroxide; O₂⁻: superoxide anion; SOD: superoxide dismutase; POD: peroxidases; GPX: glutathione peroxidase; CAT: catalase; and, PPO: polyphenol oxidase; SS: Sucrose synthase; SPP: sucrose phosphate phosphatase; SPS: sucrose phosphate synthase; AMY: α-amylase; BAM: β-amylase; G6PDH: glucose-6-phosphate dehydrogenase; 6PGDH: 6-phosphogluconate dehydrogenase; G6P: glucose-6-phosphate; FRK: Fructokinase; HK: Hexokinase; PEPC: phosphoenolpyruvate carboxylase; INV: invertase.

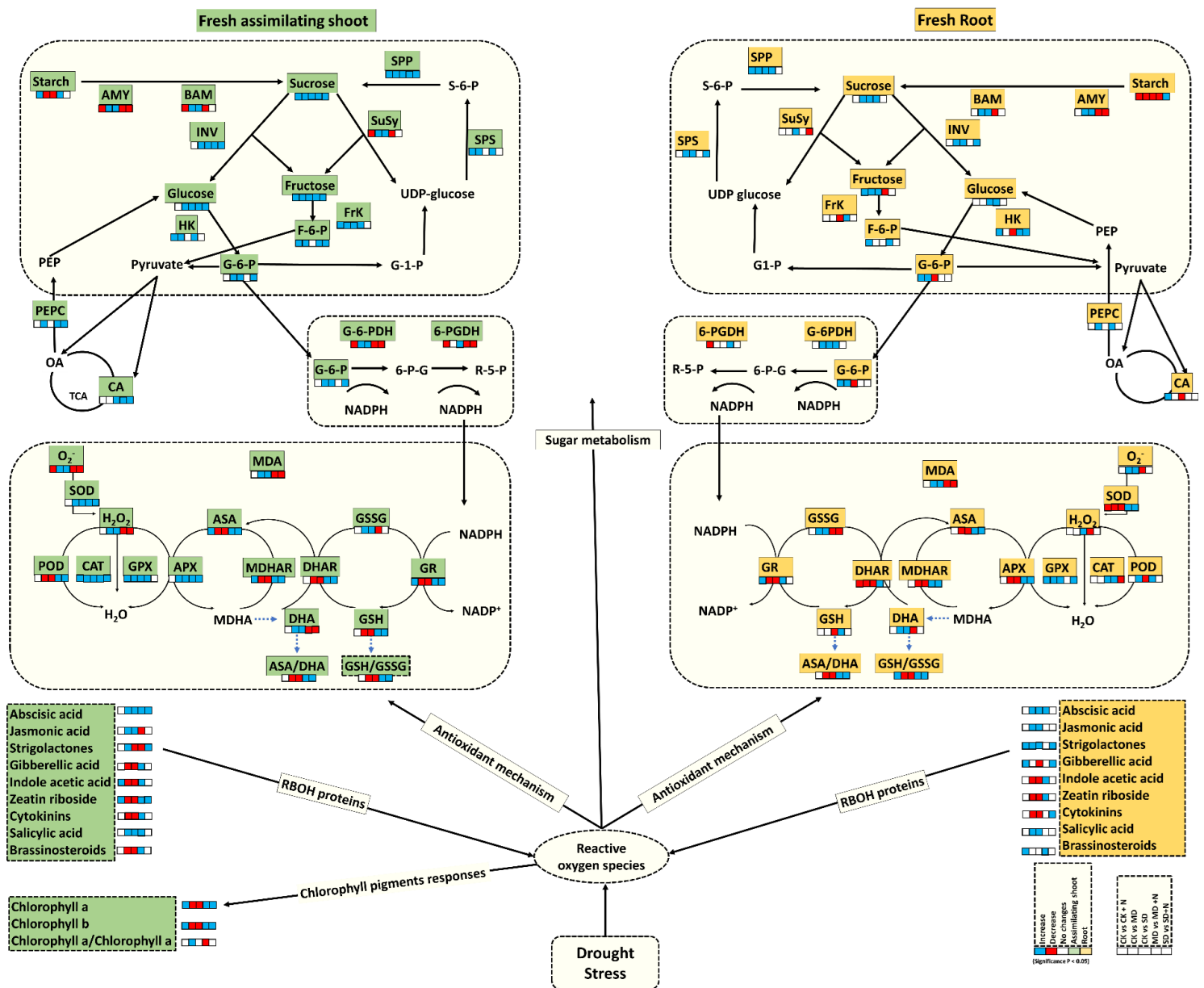


Figure 8

Schematic representation of the interplay between assimilating shoot and root content of chlorophyll pigments, phytohormones, and sugar metabolism and antioxidant mechanism in *C. mongolicum* seedlings under different drought regimes and nitrogen (N) application. WW: well-watered; WW+N: well-watered + nitrogen; MD: medium drought; MD + N: medium drought + nitrogen; SD: severe drought; SD+N: severe drought + nitrogen. RBOH: respiratory burst oxidase homolog; Chl-a: chlorophyll a; Chl-b: chlorophyll b; JA: jasmonic acid; ZR: zeatin riboside; SLs: strigolactones; GA: gibberellic acid; IAA: indole acetic acid; ABA: abscisic acid; AsA: ascorbate; DHA: dehydroascorbate; GSH: glutathione; GSSG: oxidized glutathione; APX: ascorbate peroxidase; GR: glutathione reductase; MDHAR: monodehydroascorbate reductase; DHAR: dehydroascorbate reductase; MDA: malondialdehyde; H_2O_2 : hydrogen peroxide; O_2^- : superoxide anion; SOD: superoxide dismutase; POD: peroxidases; GPX: glutathione peroxidase; CAT: catalase; and, PPO: polyphenol oxidase; SS: Sucrose synthase; SPP: sucrose phosphate phosphatase; SPS: sucrose phosphate synthase; AMY: α -amylase; BAM: β -amylase; G6PDH: glucose-6-phosphate dehydrogenase; 6PGDH: 6-phosphogluconate dehydrogenase; G6P: glucose-6-phosphate; FRK: Fructokinase; HK: Hexokinase; PEPC: phosphoenolpyruvate carboxylase; INV: invertase.