Redox control of metabolism in wheat

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List of abbreviations

AAA: alfa-aminoadipic acid

ADH: alcohol dehydrogenase

Asn: asparagine

Arg: arginine

ASA: ascorbic acid

APX: ascorbate peroxidase

CAT: catalase

Cit: citrulline

Cys: cysteine

CySS: cysteine-disulphide

CBF: C-repeat binding transcription factors

DHA: dehydroascorbate

DHAR: dehydroascorbate reductase

DLD: dihydrolipoamide dehydrogenase

FAO: Food and Agriculture Organization

FR: Far-red

GABA: gamma-aminobutyric acid

Glu: glutamic acid

GluCys: gamma-glutamylcysteine

GPX: glutathione peroxidase

GR: glutathione reductase

GSH: reduced glutathione

GST: glutathione transferase

HL: high light

hmGSH: hydroxymethylglutathione

IDH: isocitrate dehydrogenase

hmGSH: hydroxymethylglutathione

IDH: isocitrate dehydrogenase

MDA: malondialdehyde

MDH: malate dehydrogenase

MDHA: monodehydroascorbate

MDHAR: monodehydroascorbate reductase

Met: methionine

NaHS: sodium hydrosulfide

NR: nitrate reductase

NO: nitric oxide

$^{1}O_{2}$: singlet oxygen

$O_{2}^{\bullet -}$: superoxide radical anion

O_{3}: ozone

$OH^{\bullet}$: hydroxyl radical

Orn: ornithine

PARP: poly(ADP)-ribose polymerase

PEP: phosphoenolpyruvate

PGM: phosphoglycerate mutase

PGK: phosphoglycerate kinase

PPFD: photosynthetic photon flux density

POD: peroxidase

PRX: peroxiredoxin

ROS: reactive oxygen species

SCS: succinyl-CoA synthetase beta subunit

SOD: superoxide dismutase

TBA: thiobarbituric acid

TCA: trichloroacetic acid

TIM: triosephosphate isomerase

1 Introduction

Wheat (*Triticum aestivum* L.) is the world's largest frequently grown cereal, with over 220 million ha cultivated annually under a wide range of climatic conditions [1]. Wheat grains contain 8-20% protein and trace amounts of antioxidant enzymes, minerals, and vitamins such as thiamine, riboflavin, niacin, and vitamin E [1,2]. Environmental stressors that caused oxidative stress induce various biochemical, molecular, and physiological changes in the plant's primary metabolism, leading to reduced yield. These effects, however, are mostly dependent on the severity and length of the stress conditions.

Among the inducers of oxidative stress, changes in light intensity or spectrum are very important, the alteration of which may result in pronounced modifications of wheat metabolism [3–7]. It is widely reported, that high light-induced oxidative stress modified the redox status of plants [8–13]. Similar to extreme light conditions, low temperature may also lead to redox imbalance and oxidative stress in wheat [14–16]. The C-repeat binding transcription factors (CBF) are important regulators of transcriptional and metabolic changes in cereals and other plant species during cold acclimation [17,18]. Like changes in light and temperature conditions, the excess of oxidants (for example hydrogen peroxide: H_2O_2) and reductants (for example ascorbate: ASA) could also disturb the redox homeostasis, thereby influencing the metabolism in plants. It was revealed that the exogenous application of H_2O_2 could induce oxidative stress in plants depending upon the concentration and duration of its application [19,20]. The application of a reductant (dithiothreitol) to *Arabidopsis* stimulated organic and amino acid synthesis while reducing sucrose synthesis [21]. Furthermore, foliar ASA treatment induced the deposition of free amino acids and soluble carbohydrates in flax cultivars [22]. The regulating effect of oxidants has been studied more extensively in plants; however, the potential deleterious effect of reductants applied in high concentrations or for a prolonged period has only been evaluated in a few studies and has not been clearly understood, yet.

Although the influence of the environmental factors on the redox system and metabolism was examined in several plant species, the effect of light intensity and spectrum on the glutathione metabolism under optimal and low temperature was not compared earlier in wheat genotypes with different freezing tolerance. In addition, an extensive comparative study about the effect of an oxidant and reductant on wheat metabolism was not carried out until now. The aim of this thesis was the investigation of the aforementioned scientific questions.

2 Review of literature

2.1 Redox system

Reduction-oxidation (redox) state of the plant cells and tissues serves as an essential modulator of the subcellular and extracellular metabolism in plants [23]. It is mainly determined by the antioxidant system controlling the level of the reactive oxygen species (ROS) [23,24]. Cellular redox homeostasis is referred as an ‘‘integrator’’ of the information from metabolism and environment regulating plant growth and development. The redox status not only modulates the cellular metabolism but also participates in the signalling process both under optimal and stress conditions [25]. Its changes during oxidative stress lead to the reprogramming of the global metabolome [21,26–28]. The changes in the cellular redox environment result in thiol/disulfide conversions, which in turn modify the conformation and activity of proteins leading to alterations in gene expression and metabolism (Fig. 1).

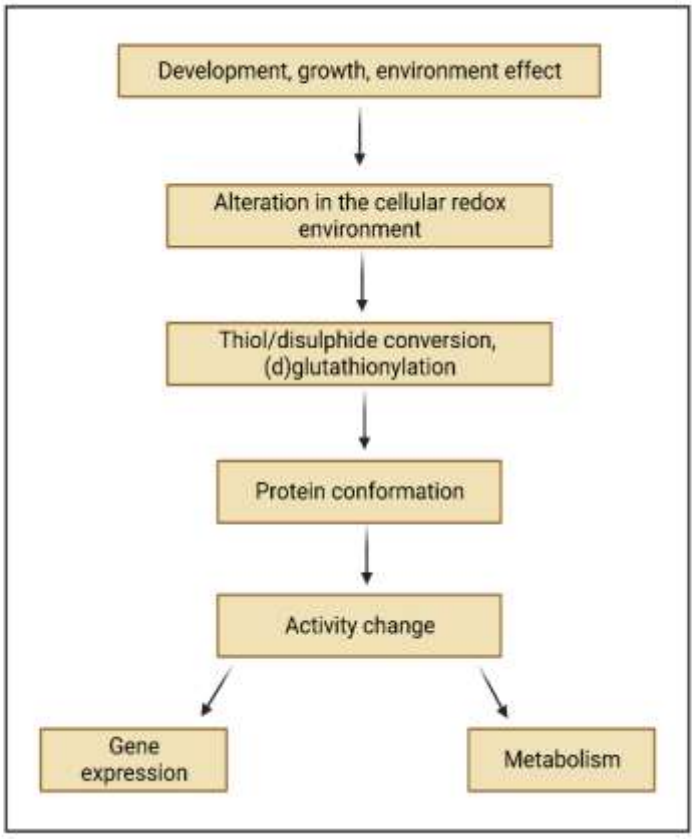

Fig. 1. Effect of environmental conditions on redox regulation in plants. This figure was prepared with the Biorender software.

During oxidative stress, increased production of ROS occurs, which are removed by the antioxidant components of the redox system. Among them, the ascorbate-glutathione (ASA-GSH) cycle is crucial in maintaining proper redox balance in plant cells and tissues. It mitigates oxidative damage in the cells (Fig. 2) [29]. The regeneration of the ASA, GSH, and nicotinamide adenine dinucleotide phosphate (NADPH) after the removal of the excess H_2O_2 is ensured by the enzymes of the ASA-GSH cycle which are ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) [30].

2.1.1 Reactive oxygen species

The various ROS are produced in excess amount under stress conditions. Their main source is the partial reduction of the molecular oxygen (O_2) by the high-energy state electrons [31,32]. ROS are the most prevalent oxidants, which include hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$), superoxide radical anion ($O_2^{\bullet-}$), and singlet oxygen ($^1O_2$). As by-products of aerobic metabolism, ROS are synthesized in various plant cellular compartments. In addition to the over-reduction of the components of the electron transport chain, the limited CO_2 fixation is the main reason for ROS generation in the chloroplasts. In mitochondria, the over-reduction of the electron transport chain may only induce high ROS levels under oxidative stress [33]. Due to their high reactivity, ROS may interact with various cellular components, such as lipids, nucleic acids, and proteins, and damage them [34]. However, besides their harmful effects, ROS are also considered to be integral signalling molecules that modulate plant growth both under optimal and stress conditions [34,35]. The production of ROS is greatly influenced by environmental stressors, and its rate could be even elevated when the photon flux density is higher than necessary for CO_2 assimilation [36]. The rapid increase of ROS content is the most common indicator signal in plants for oxidative stress. To maintain crop growth and development, the balance between production and detoxification of ROS must be kept. In addition, ROS has the potential to act as a signalling molecule in the readjustment of plant growth [24,37]. They are also involved among others in the crosstalk of light and temperature signals to control circadian clocks and calcium-dependent regulation in plants [38].

In the signalling processes, H_2O_2 has an important role due to its longer half-life compared to other ROS. The mitochondria, chloroplast, apoplast, and peroxisome are major production sites in plants. The dismutation of $O_2^{\bullet-}$ by superoxide dismutase (SOD) is the main source of H_2O_2 formation in all these compartments. It could be also produced in various enzymatic reactions such as that one being catalyzed by glycolate oxidase in the peroxisome

[39,40]. At low concentrations, the H_2O_2 serves as a secondary messenger for stress signaling [41], fruit ripening, and postharvest processes [42]. Under stressful conditions, ROS signaling mainly depends on the balance between ROS synthesis and detoxification. To metabolize the oxidants such as H_2O_2, the plants have various ROS-detoxifying non-enzymatic antioxidants and antioxidant enzymes [35]. They keep the level of H_2O_2 and other ROS under control (Fig. 2).

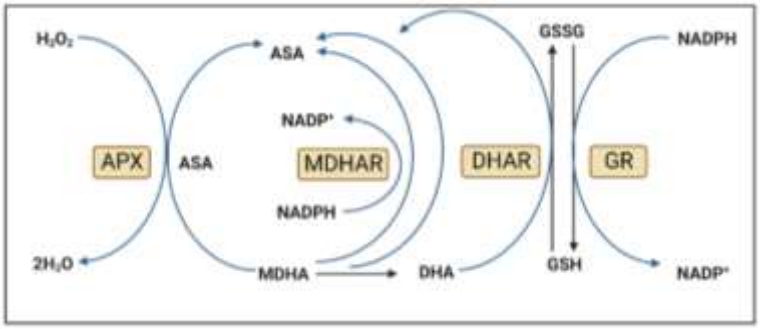

Fig. 2. Ascorbate-glutathione cycle (Foyer-Halliwell-Asada pathway). ASA: ascorbic acid, APX: ascorbate peroxidase, DHA: dehydroascorbate, DHAR: DHA reductase, GR: glutathione reductase, GSSG: oxidized glutathione, GSH: reduced glutathione, MDHA: monodehydroascorbate, MDHAR: MDHA reductase, $NADP^+$: an oxidized form of nicotinamide adenine dinucleotide phosphate, NADPH: reduced form of $NADP^+$ [43]. This figure was prepared with the Biorender software.

2.1.2 Antioxidants

The main ROS-detoxifying enzymes are SOD, APX, catalase (CAT), DHAR, MDHAR, glutathione peroxidase (GPX), GR, peroxiredoxin (PRXs), and glutathione S-transferases (GSTs) [24,39,44–47]. These enzymes are found in various cellular compartments and interact together to scavenge ROS. The ASA-GSH cycle comprises APX, MDHAR, DHAR, and GR which are involved in the elimination of H_2O_2 (Fig. 2).

APX (class I haem-peroxidase) is referred to as an ASA-dependent peroxidase. APX enzyme acts as a detoxifier of H_2O_2, and its increased activity is a good indicator of the redox imbalance which is an indication of the redox imbalance [48]. The APX utilizes ASA as an electron donor to convert H_2O_2 into water (Fig. 2). The APX has been found in different subcellular compartments including cytosol [49], mitochondria [50], chloroplast [51], peroxisome [52] and glyoxisome [53]. The overexpression of *OsAPX2* in rice resulted in increased APX activity under oxidative stress conditions [54]. Short-term exposure of wheat cultivars to low-temperature considerably elevated the APX activity [55]. The decline in the

red:far-red (R:FR) ratio resulted in higher transcript levels of the gene encoding APX enzyme in tomato [56]. The APX activity exhibited a declining tendency in the wheat genotypes following high light-induced oxidative stress [57]. In another study investigating light adaptation, increased APX activity and protein levels were found, while the transcription inhibitor did not show any remarkable effect [58]. This result indicates the post-transcriptional control of APX

Even though GSH oxidation is mainly regulated by some GSTs and PRXs [59], it is also evidenced that the DHAR as being an important player in ensuring GSH oxidation during oxidative stress [60,61]. The DHAR regulates the reduction of dehydroascorbate (DHA) using reduced GSH, producing ASA and GSSG (Fig. 2) [62]. Hence, the DHAR activity is an important determinant of the redox environment in the plant cells. It is demonstrated that DHAR is an integral part of a large protein complex and acts as a signal for activating the plant's defense mechanisms against oxidative damage [63], as was shown after one-week cold acclimation in wheat [64]. The DHAR enzyme showed lower activities in *Phaseolus vulgaris* L. following lower R:FR ratios [65], which indicates the regulation of its activity by light spectrum.

The MDHAR is involved in the reduction of MDHA to ASA by utilization of NADH or NADPH, suggesting its importance in keeping the redox balance in plants (Fig. 2) [62]. MDHAR is present in various cellular compartments such as mitochondria, chloroplast, cytosol, and peroxisome [66–68]. Similar to other enzymes, MDHAR plays a critical role in the stress response, since its over-expression improved tolerance against salt-, ozone- and PEG-induced oxidative stress [67]. Following cold stress, the MDHAR genes were up-regulated in the white clover [69]. The decreasing temperatures increased the transcript level and activity of MDHAR in wheat [70]. Low light also enhanced MDHAR activity in wheat [71]. Cucumber plants showed considerably elevated transcription of MDHAR under low-intensity light [72]. In another study, the high light-induced oxidative stress substantially increased the MDHAR activities in wheat plants [73].

In the ASA-GSH cycle, the GR reduces the GSSG to GSH (Fig. 2). Although its greatest activity was found in the chloroplasts, it can also be found in the nucleus, cytosol, peroxisomes, and mitochondria [74]. A relationship between the activity of GR and the amount of GSH was observed, and they are differentially regulated by various stresses [75–80]. Thus, following cold-induced osmotic stress, the rapid elevation of the activities of GR and GSH metabolism-associated enzymes was observed in maize seedlings. Moreover, low light intensity also

resulted in the up-regulation of GR activity in wheat seedlings [71]. However, lower activity of GR enzyme was found in *Phaseolus vulgaris L.* following low R:FR treatment [65].

GSTs are involved in the conjugation and detoxification of herbicides, these are enzymes of secondary metabolism and form one of the most crucial families of H_2O_2-detoxifying enzymes [81–84]. The genome-wide investigation of the GSTs in *B. oleracea* signified the possible roles of GSTs under cold stress [85]. Similarly, upon exposure to 2 days of cold acclimation, the GST activity was elevated in *S. commersonii* and somatic hybrid SH9A [86]. The important role of the GST in light-dependent cold acclimation was also observed in spring and winter wheat genotypes [87] which corroborates the role of this enzyme in response to low temperature.

CAT does not require any reductant for its catalytic activities [62]. The low light-dependent induction of CAT activity was associated with the increment of drought stress tolerance in the soybean seedlings [88]. Under low R:FR, CAT was induced at transcriptional levels, which ensured the detoxification of excessive H_2O_2 [56]. Contrarily, high light stress led to lower activity of CAT in wheat [57].

Non-enzymatic antioxidants such as ASA, GSH, flavonoids, carotenoids, and tocopherols are also important regulators of ROS levels in the plants [77]. The two important redox couples among non-enzymatic antioxidants are ASA/DHA and GSH/GSSG [89,90].

The ASA, commonly known as vitamin C, has multifaceted roles in both plants and animals. It was found in the mitochondria, chloroplasts, vacuoles, cytosol, and cell wall [91]. The ASA and its oxidized form, DHA play critical roles in the redox state-based regulatory mechanisms [92]. The ASA /DHA couple's half-cell reduction potential can be utilized to characterize the ASA-dependent redox environment [93]. During redox regulation, the ASA interacts with GSH [91,94,95]. The ASA, together with GSH make a functional entity via the ASA-GSH cycle (Fig. 2), and they are involved in the control of protein functions and gene expressions [96]. Along with the ASA-GSH cycle-associated enzymes, the ASA considerably contributes to ROS elimination [97]. It is evident that ASA has a key role in redox signaling during oxidative stress induced by pathogen attack [98], ozone [99], salt [100], drought [101], lead [102], cadmium [103], cold [104] and high light stresses [57]. Although the ASA has protective roles against oxidative damage in plants, its higher concentration and prolonged supplementation can also be detrimental to plants [105]. The ASA and GSH are not only active ROS-detoxifiers but also serve as co-factors of oxidases as well [106]. In addition, ASA is

involved in a variety of physiological processes, for instance, cell elongation, cell division, and cell defense against stresses [107,108].

GSH consists of three amino acids, i.e., cysteine, glycine, and glutamate. It exists in two forms, i.e., GSH and GSSG, and the latter one is reduced to GSH by GR [62]. In its homolog, hydroxymethylglutathione (hmGSH) occurring in cereals, glycine is replaced by serine. hmGSH is regenerated from its oxidized form (hmGSSG) [109]. As an antioxidant, GSH is an important redox buffer in both plants and animals [110–112]. Alterations in the GSH/GSSG couple's half-cell reduction potential (EGSSG/GSH) are important determinants of the intracellular redox system [93]. Numerous defense mechanisms are initiated through GSH-dependent redox transduction pathways [24,113–115]. Along with the stimulation of stress-responsive genes [116,117], GSH also affects the redox-dependent division of the cells [118–120]. In response to cold, heat, drought, salinity, heavy metals, and UV radiation, GSH activates various defense mechanisms [121]. During cold acclimation, the redox potential of the GSH/GSSG couple altered, which could initiate the detoxification of ROS and other defense mechanisms leading to the improved freezing tolerance of wheat plants [122]. Additionally, another study revealed that GSH is involved in cold tolerance-dependent modifications in wheat seedlings [123]. GSH not only aids in defense activities but also controls growth and several developmental processes, including gametogenesis and seed development [124]. It is also evidenced that the GSH is a signalling molecule. GSH (either alone or in combination with H_2O_2) governs adaptive or cell death mechanisms via an intercellular signalling system under various oxidative stress conditions [125,126]. It also regulates protein translation, post-translational protein modification, metabolic process regulation, and gene expression [115,127].

Besides GSH, one of its precursors, cysteine (Cys), and its oxidized form cysteine disulfide (cystine, CySS) are also important in the regulation of the cellular redox state [128]. It is mainly involved in the synthesis of various defense molecules, such as GSH, protective proteins, thionins, glucosinolates, phytoalexins, phytochelatins, and metallothioneins [129–133].

2.2 Effect of environment on the redox system

The various adverse environmental conditions induce oxidative stress. It can cause a cascade of phenological, physiological, genetic, biochemical, metabolic, and molecular changes that have a deleterious impact on plant growth and development (Fig. 3) [134–137]. Extreme (low or high) temperatures and light stress (intensity and spectrum) are often linked and may induce

similar modifications in the redox system of plants. For instance, temperature and light stress are manifested primarily as oxidative stress, which leads to the disruption of the building blocks of proteins and optimal ion distribution [138–140]. Consequently, these oxidative stressors often initiate similar regulatory mechanisms and cellular responses, such as up-regulation of the antioxidants, production of stress-responsive proteins and transcripts, modification of hormone metabolism, and rapid production of the compatible solutes which led to altered redox and osmotic state of the plants (Fig. 3) [141–145]. Among the inducers of oxidative stress, light (intensity or spectrum) is one of the most important ones [3–7]. It is widely reported that exposure to high light intensities resulted in altered redox status of plants which may lead to great metabolic modifications as observed in wheat [8–13]. Similar to high light intensity, low temperature can also induce oxidative stress in wheat [14–16]. In addition, the excess of an oxidant (for instance: hydrogen peroxide: H_2O_2) or a reductant (for instance ascorbate: ASA) could impair the redox homeostasis and influence the metabolism in plants, too. Exogenous application of H_2O_2 caused oxidative stress in plants depending on the amount and duration of treatment [19,20]. Although the ASA has protective roles against oxidative damage in plants, its higher concentration and prolonged supplementation can also be detrimental to plants [105].

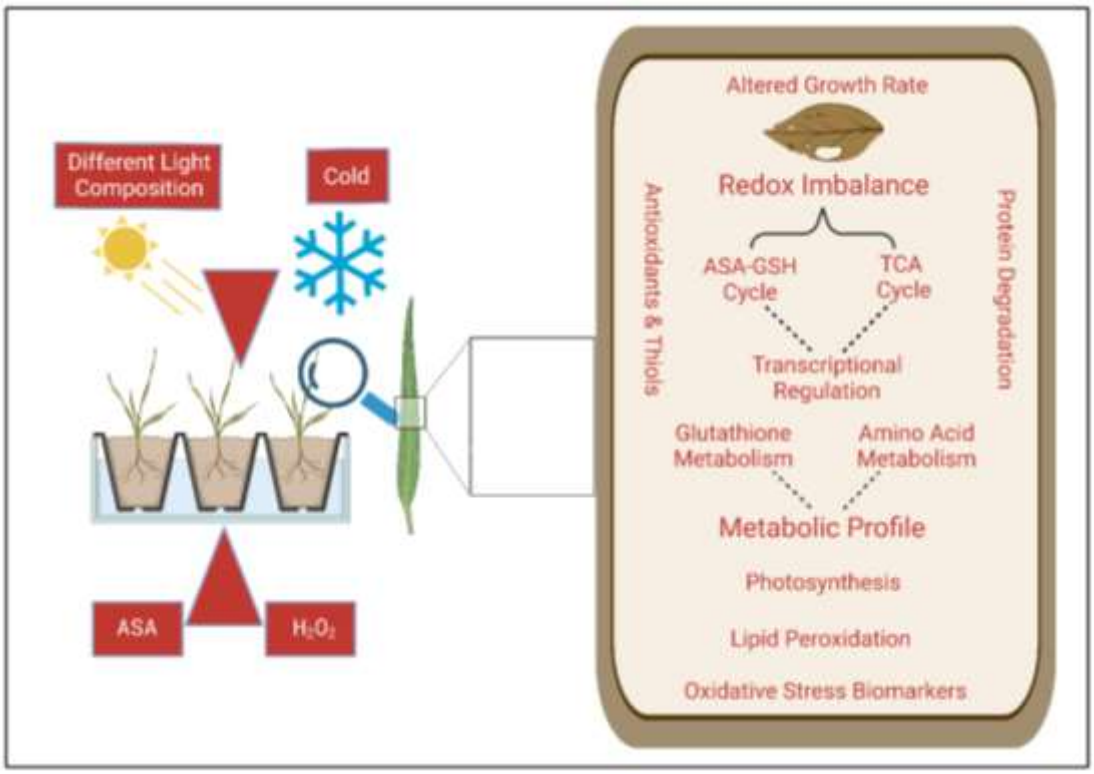

Fig. 3. Model figure elucidating the effect of light, cold, oxidant (H_2O_2), and reductant (ASA) on the physio-biochemical, metabolic, and molecular regulatory mechanisms in wheat seedlings. This figure was prepared with the Biorender software.

2.2.1 Modulation of the redox system by light

Sunlight (quality and quantity) is a key factor determining crop productivity since it influences plant growth via photosynthesis. Plants utilize sunlight for the conversion of water and carbon dioxide into sugars, and photosynthetic pigments are essential for changing light energy into chemical energy. The light conditions exhibit spatial and temporal changes. Lower R:FR ratios are associated with higher latitude, while higher ratios of ultraviolet-B (UV-B) light, photosynthetically active radiation (PAR), and blue light (BL) occur at increasing altitudes. The spectral composition of sunlight also shows seasonal alterations [146–148]. It has been revealed that, the lower R:FR ratio is experienced during twilight and it remains constant during daylight and cannot be influenced by cloud cover [149]. When cultivated under dense vegetation, the existence of the neighboring plants alters light conditions by decreasing the R:FR. A low R:FR also indicates the below-canopy environment, because during transmission of light through the leaves, the chlorophyll absorbs the blue and red regimens of the spectrum, but not the far-red one. Reduced R:FR and blue/green ratios, as well as lower red, blue, and UV irradiance, are crucial factors in detecting nearby vegetation. Several light sensors, such as red and far-red light-absorbing phytochromes, blue/UV-A light-absorbing phytochromes, and UV-B-sensing UVR8, can detect these spectrum changes.

The variations in the light quality lead to modifications in the redox environment in the plants (Fig. 3) [88,150]. Each light spectral component can induce certain responses in plants. For example, electron excitation in photosynthetic apparatus is mainly influenced by the red and blue light regimens, while the accumulation of anthocyanins and carotenoids is associated with blue and UV lights [151,152]. Another study found that lettuce (*Lactuca sativa* L.) plants with a low red: blue light ratio (1:3) had elevated levels of ASA [153]. Contrary to red light, blue light increased buckwheat's (*Fagopyrum esculentum* L.) total phenolic and flavonoid content as well as the activity of antioxidant enzymes [154]. It has been extensively reported that the different light compositions substantially influenced ROS production in plant tissues [155]. Relative to normal white light, the low R:FR ratio resulted in the rapid production of ROS in the mitochondria and chloroplasts of soybean plants [156]. The reduction of protochlorophyllide to chlorophyllide, the immediate precursor of chlorophyll, is catalyzed by protochlorophyllide oxidoreductase (POR) and requires light absorbed by protochlorophyllide [157,158]. This is significant because, when exposed to light, free chlorophyll and numerous regulators in the biosynthesis of chlorophyll, including protochlorophyllide, can stimulate the production of reactive oxygen species. Reduced ROS levels were reported in chloroplasts of

Arabidopsis plants growing under continuous far-red light treatment as a result of restricted chlorophyll production [161]. Long-term far-red light supplementation (shade) produced higher levels of ROS in chloroplasts due to the limited occurrence of assimilates and reductants (NADPH and ATP). It is suggested that ROS (H_2O_2) are initially produced in the peroxisomes and chloroplasts and subsequently in the vacuoles and cytosol [157,159–161]. Similar to the regulation of ROS and glutathione metabolism by light spectrum in leaf tissues, their subcellular control by spectral composition is also of paramount importance. The GSH amount was not significantly affected by the lower R:FR in wild-type *Arabidopsis thaliana*, however, the GSH-deficient mutant showed an increment of the total GSH content in the nuclei (200%), peroxisomes and cytosol (100%) [10]. These results indicate the potential involvement of the R:FR ratio in the control of the subcellular redox environment. The changes in the subcellular GSH distribution were similar after the low R:FR ratio in wheat and *Arabidopsis* (*pad2-1*, GSH-deficient mutant) [10], which may indicate the general existence of this regulatory mechanism in the various plant species. Although various investigations have been done to elucidate the light spectrum-related physio-biochemical modifications in the various organs of plants, the lower R:FR ratio-mediated redox regulatory mechanisms are poorly understood. Therefore, certain experiments of this thesis were focussed on the low R:FR ratio-induced variations in the redox environment of wheat shoots.

As observed for the light spectrum, the light intensity also influences the redox status of plants by the modulation of ROS generation and the activities of antioxidant enzymes [162]. Fascinatingly, the ROS production was described in the leaves of the model plant *Arabidopsis* after light deprivation (2 days) [163]. It was suggested that this alteration was brought on by the decrease in GSH and the activities of APX, GR, DHAR, and CAT enzymes. However, it was noticed that long-term darkness (4 days) induced the SOD, CAT, and APX enzymatic activities in *Pelargonium zonale* L. [164]. Similar to darkness, the high light intensity can also influence the redox regulatory hub of plants. Although H_2O_2 is a key signaling ROS [165–167], the $^1O_{2-}$ and $O_2^{\bullet-}$-responsive genes were also up-regulated in *Arabidopsis* following highlight application [167,168]. The higher $O_2^{\bullet-}$ accumulation was exhibited by *Arabidopsis* [169] and pea [170] leaves after exposure to high light intensity for 3-6 hours. Similar stress symptoms were shown by the wheat plants subjected to prolonged high light-induced oxidative stress [57]. These results are corroborated by other authors observing the excessive levels of ROS after high light-induced oxidative stress which helps the *Arabidopsis* plants to activate the protective mechanisms against oxidative damage [166]. Generally, the pronounced influences of the light

intensity were reported in the activation of several antioxidants in different organs of various plant species including wheat [170,171]. This stimulation of antioxidant activities was primarily due to the transcriptional modulation of the ASA-GSH cycle components [165,171].

The influence of light intensity on the redox regulatory system was also demonstrated at the subcellular level. Its fluctuations caused rapid modulations in ROS generations in various plant cell compartments. For instance, higher accumulation of H_2O_2 was detected in the chloroplast, cytosol, stroma, and nuclei of genetically modified tobacco after excessive PPFD (1000 μmol m^{-2} s^{-1}) [172], indicating g imbalance in the transport of electrons. The high light-regulated H_2O_2 generation can be reduced by various antioxidants in the various cellular compartments i.e., the chloroplast (APX, ASA, and GSH), in cytosol and peroxisomes (ASA, APX, CAT, and GSH), and in the vacuoles (ASA and peroxidase: POD) [10,173–175]. The low resistance of the *cry1phyAB1* mutant to high light may be because of the low activities of key antioxidant enzymes and their related genes [176]. Low light or shade condition generally diminishes the GST activity, whereas high or excess light intensity considerably raises both the activity and the expression levels of GSTs and GSH. Similar changes were found in *Arabidopsis* leaves where high or excess light (2,500 μmol m^{-2} s^{-1}) remarkably enhanced the GST activity [166]. A similar pattern was found in micro-propagated *Phalaenopsis* plantlet leaves following high light (300 μmol m^{-2} s^{-1}) [177]. In addition, high light (500 μmol m^{-2} s^{-1}) stress-induced a rapid up-regulation of the *PgGST* within 1 h in *Panax ginseng* [178]. It was also noticed that the high light (1,200 μmol m^{-2} s^{-1}) profoundly elevated the expression of *GST5* and *GST13* and increased the activity of GST in *Arabidopsis* tissues [179]. Higher light intensity is also responsible for an increase in ASA concentration, and ASA subcellular recycling has been identified in the cytosol, mitochondria, and peroxisomes [180]. Similarly, higher PPFD stimulated the GSH amounts in chloroplasts and cytosol [173]. The compartment-specific enhancement of GSH in wheat was associated with the up-regulation of cytosolic GR [10]. In addition, with increasing light intensity, an increment in GSH redox potential (E_{GSH} was detected in potato chloroplasts [181]. These studies indicate that changes in light intensity differently modulate the redox system in various organelles.

2.2.2 Interactive effect of light with other abiotic stresses

Changes in light spectrum and intensity can alter the stress response of plants. For instance, growing wheat seedlings at low R:FR ratios changed the antioxidant enzymes involved in the ASA-GSH cycle's response to cold treatment [182]. The detrimental effects of salt stress were reduced by a low R:FR ratio in tomato since it reduced the levels of H_2O_2 and malondialdehyde

(MDA) [183]. In another study on tomatoes, a low R:FR ratio prevented salinity-induced damage by promoting the activities of SOD, POD, CAT, and APX and their corresponding transcriptional levels [56,184]. Under drought stress, CySS and GSSG levels, as well as CySS/Cys and GSSG/GSH ratios, were shown to be lower in cold-tolerant wheat genotype seedlings grown in far-red light compared to those grown in white, blue, or pink light. These results suggested that the redox environment was adjusted in more reducing directions, which can ensure the effective functioning of various proteins. Moreover, red light-induced the production of ROS (H_2O_2), which resulted in cell death in *Arabidopsis* [185]. During low-temperature stress, a low R:FR ratio had a favorable effect on the stress response by maintaining the equilibrium between the formation and detoxification of ROS in wheat and barley [17] and *Arabidopsis thaliana* [186]. In addition, under cadmium-induced oxidative stress, blue light increased the activity of several antioxidant enzymes in cucumbers (*Cucumis sativus* L.) compared to red light [187]. Thus, the cultivation of plants under special spectral conditions can improve their stress tolerance due to the modulation of the redox environment.

High light can be also beneficial for the reduction of stress-induced damage. Pre-treatment with high light induced the accumulation of GSH in drought-stressed wheat seedlings, thereby improving oxidative stress resistance [5,168]. At the transcriptional level, similar effects of light were observed for a variety of enzymes including APX, GR, and GST. In *Anacardium occidentale* L., drought and high light were observed to positively correlate with the activation of ASA, APX, GSH, and SOD activities [188]. A negative correlation was observed between H_2O_2 content and APX activity under combined high-light and low-temperature stress [189]. Intriguingly, after a 14 h treatment of high PPFD, the GSH levels in the chloroplasts were able to return to a normal state during the recovery period. However, it was not the case when high light was coupled with the cold stress (3 °C) [181], indicating the sensitivity of the chloroplasts to a simultaneous effect of light intensity and cold stress (or other oxidative stresses) which can occur at natural habitats of the plants. These results demonstrate that the appropriate adjustment of the light intensity could enhance stress resistance in plants.

2.2.3 Influence of low temperature on the redox system

A sudden drop in temperature is one of the most detrimental environmental cues that severely limit crop yield. Winter hardiness is the ability of plants to withstand winter challenges including cold temperatures, frost, and snow mold, which have an impact on their growth [190]. Below +10 °C, the plants start acquiring freezing tolerance, which process is enhanced when the temperature decreases to +5 °C or even 0 °C [191–193]. Because of the falling temperature

and day length in autumn, appropriate acclimatization increases freezing resistance and reduces damage to vegetative tissues [194,195]. This process is very crucial for winter wheat since damage during the winter (winter kill) and the first few weeks of spring can be quite disastrous. During cold acclimation, the redox defense system, including the ASA-GSH cycle, is also activated [196,197]. This cycle activates several redox-sensitive transcription factors involved in the improvement of freezing tolerance [122,195,197]. During cold acclimation, the balance between the synthesis and degradation of GSH is a very important phenomenon to ensure the optimal redox status of plants, as experienced in wheat plants [123]. Other antioxidant system components, including SOD and CAT, also had substantial effects on keeping the ROS levels under control at low temperatures [14]. During cold stress conditions, the inhibition of ROS production was detected in chickpea after the induction of antioxidants by polyamine treatment [198]. Following cold stress, there was a notable activation of the APX, GPX, SOD, CAT, and GR activities in wheat [199]. In another study, higher APX activities were also observed in winter wheat plants during acclimation at low temperature [200]. Under frost, higher ASA and GSH levels and greater GR, APX, DHAR, and MDHAR activities were detected in cold-primed plants as compared to non-primed ones [201]. Compared to the control plants, the AMF (ABA-mimicking ligand) wheat plants showed higher activities of APX under low temperature [202]. The maintenance of the optimal proline concentration is also a crucial factor in the cold resistance of plants since proline is largely involved in the ROS homeostasis and consequently in low-temperature tolerance of the plants such as rice [203], maize [204], and wheat [205,206]. Taken together, the redox system plays a significant role in plant adaptation to low temperatures due to redox environment modifications.

2.3 Redox regulation of the metabolism

2.3.1 Photosynthesis

The process through which plants use sunlight to create the metabolic energy that is then needed to sustain practically all living things on Earth is known as photosynthesis. As the global population is expected to increase significantly over the next few decades, photosynthesis—the process by which plants grow and develop—can be improved to help ensure greater food security and sustainable agriculture [207]. Excessive amounts of antioxidants (reductants) and oxidants have been found to have the potential to disrupt photosynthesis and redox homeostasis, which are crucial for the maintenance of plants' metabolism (Fig. 3). The oxidative stress caused by methyl viologen resulted in the reprogramming of carbon metabolism in *Arabidopsis*, as evidenced by the inhibition of anabolic pathways (e.g., the Calvin cycle) and the stimulation of

catabolic ones (e.g. oxidative pentose phosphate pathway, OPPP) [208]. The comparison of wild-type *Arabidopsis* with *Rcd1* (radical-induced cell death1) mutant being tolerant to methyl viologen further revealed the adjustment of adequate metabolic pathway adjustment during herbicide treatment [209]. Exogenous ASA treatment improved chlorophyll concentration while declining the electrolyte leakage in tomato [210]. These later findings demonstrate the diverse impacts of ASA on the stability of the photosynthetic membrane and pigments in several plant species, which are also dependent on the concentration and length of the treatments. Likewise, ASA, the influence of H_2O_2 on various photosynthesis-related parameters differed in different plant species, as evidenced by the unchanged chlorophyll level and net photosynthetic rate in H_2O_2-treated cucumber [211]. Furthermore, chlorophyll content and chlorophyll fluorescence in *Ficus deltoidae* leaves did not alter after a 3-week treatment with 8 mM H_2O_2 [212]. A study on tomato (*Solanum lycopersicum* L.) showed that the H_2O_2 application (0.5 mM for 4 h) even promoted growth, photosynthesis, and metabolic state of the plants which assisted them to enhance the tolerance against copper stress by activating the protective mechanisms against ROS [213]. Another study on *Vigna radiata* reported that spraying with H_2O_2 (2.5 mM) strongly favored the growth and photosynthetic rate by maintaining the redox state [214]. Seed priming with H_2O_2 in wheat relieved the drastic impacts of water-deficit conditions on the photosynthetic pigments by modulating the osmolytes accumulation (GB and Pro) and the antioxidative defense mechanism which led to maintaining the redox homeostasis under control [215]. Soursop seedlings also exhibited improvement in growth and photosynthetic efficiency following H_2O_2 treatment (20 μM) [216]. Collectively, it is indicated that the redox treatments have a substantial impact on the photosynthetic rate of the plants, whereas, the intensity of impact mainly depends on the length and the duration of the treatments.

2.3.2 Oxidative pentose phosphate pathway

The OPPP is a key source of the reducing power and metabolic intermediates for many biosynthetic processes [217]. Varying amounts of the fructose-6-phosphate and triose phosphate, are possibly recycled by the OPPP pathway followed by their conversion to glucose 6-phosphate by the glucose-6-phosphate isomerase [218,219]. The OPPP pathway has paramount importance in cellular metabolism since it is potentially involved in the maintenance of amino acid biosynthesis, anabolism, carbon homeostasis, and ultimately oxidative stress regulation. It participates in several downstream processes with Entner–Doudoroff pathway and Calvin cycle and splits into an oxidative and non-oxidative branch. Glucose 6-phosphate is

transformed into carbon dioxide, ribulose 5-phosphate, and NADPH in the oxidative branch. The latter is very crucial in maintaining the redox balance during stressful environments [220]. The decrease of the triosephosphate isomerase (TPI) by glycolytic intermediate phosphoenolpyruvate (PEP) maintains higher OPPP pathway activity [221].

It has been widely reported that a number of substances that cause oxidative stress, such as methyl viologen, have a significant effect on the readjustment of carbon metabolism, which is evidenced by the stimulation of catabolic pathways (such as the OPPP) and the inhibition of anabolic ones (e.g. Calvin cycle) [208]. The metabolic changes caused by oxidative stress indicate the rerouting of the glycolytic carbon flow into the OPPP pathway for the provision of NADPH for the antioxidant defense system [222]. The OPPP pathway is significantly influenced by the redox treatments. For instance, following H_2O_2 addition, the decline in glucose 6-phosphate content was a signal of the enhanced glycolysis and/or OPPP [NAD(P)H production], which was noticed in various plant species under oxidative stress [208,223]. This alteration led to increased levels of NAD(P)H, which could ensure the effective speed of the H_2O_2 scavenging in the ASA-GSH cycle, during which enough $NADP^+$ was synthesized for accepting the electrons from photosynthesis, leading to inhibit ROS production. This change resulted in enhanced NAD(P)H levels, which could assure the effective speed of H_2O_2 scavenging in the ASA-GSH cycle, during which enough $NADP^+$ was produced to receive electrons from photosynthesis, resulting in suppressed ROS formation.

2.3.3 Glycolysis

Being a catabolic anaerobic process, glycolysis evolved to serve two main functions: it oxidizes hexoses to make ATP, reductant, and pyruvate, and it creates the building blocks for anabolism. As an amphibolic pathway, glycolysis can play a significant role in energy-dependent gluconeogenesis by creating hexoses from a variety of low molecular weight compounds [224]. The cytosolic glycolytic route is a highly complicated network of parallel enzymatic reactions at the levels of sucrose, fructose 6-phosphate, glyceraldehyde 3-phosphate, and PEP metabolism [225]. In plants, glycolysis occurs in the cytosol and at least the first half of the glycolytic pathway is also present in the plastid [226]. The functioning of glycolysis can be influenced by the redox treatments. Consequently, salt-induced oxidative stress lowered the activity of the enzymes involved in the glycolysis/Krebs cycle pathway, whereas ASA pretreatment increased it in wheat [227]. Moreover, it was discovered that ASA-induced H_2O_2 accumulation resulted in DNA damage that activated poly(ADP)-ribose polymerase (PARP). The following NAD^+ depletion caused the inhibition of glycolysis [228]. Under hypoxia-

induced oxidative stress, 0.1 mM sodium hydrosulfide (NaHS) affected glycolysis by up-regulating the transcript levels of alcohol dehydrogenase (ADH) in maize seedlings [229]. It was also demonstrated that hydrogen sulfide (H_2S) modulated the glycolysis pathway by regulating the glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the central enzyme of this pathway [230]. In *Nannochloropsis oceanica,* enzyme analysis showed that the application of H_2S (NaHS) enhanced the glycolysis pathway [231]. The H_2S treatment increased the expression of three genes for 6-phosphofructokinase, one of the rate-limiting enzymes in glycolysis. Hence, it is postulated that ATP synthesis uses a substrate provided by H_2S-regulated glycolysis [232].

Similarly to reductants, oxidants are also involved in the modification of the glycolysis pathway. For instance, exogenous potassium nitrate (KNO_3) treatment facilitated plant growth and development through controlling energy metabolism by inhibiting glycolysis. The activity of glycolysis-associated proteins such as GAPDH increased, while that of phosphoenolpyruvate carboxylase decreased following KNO_3 treatment [233]. Compared with NaCl stress alone, glycolysis was enhanced when plants were treated with nitric oxide (NO) under saline conditions, which was reflected by the enhancement in glucose 6-P content [234]. In addition, NO treatment raised the activity of GAPDH, a crucial glycolysis enzyme, which reduced the amount of glucose [235]. Ozone (O_3), as an oxidant resulted in the modulation of the glycolysis pathway by regulating the enzymes which are involved in the energy production phase in glycolysis: phosphoglycerate mutase (PGM), triosephosphate isomerase (TIM), GAPDH, and phosphoglycerate kinase (PGK) in Fiskeby III roots [236]. These findings suggested that the significant amount of free energy generated by glycolysis adds to the ability to sustain a normal redox state and other energy-intensive metabolic pathways under oxidative stress.

2.3.4 Citrate cycle

In the cytosol, the citric acid cycle/tricarboxylic acid cycle (TCA) turns PEP into malate and/or pyruvate. These organic acids are then transported into the mitochondria by several mitochondrial carrier family members. Subsequently, interconversion of these organic acids takes place there, which yields 15 ATP equivalents per pyruvate molecule [224]. The mitochondrial pyruvate dehydrogenase is the first enzyme in the TCA cycle and is responsible for catalysing the irreversible process that turns pyruvate into acetyl-CoA. This enzyme is considered as the key regulatory point for fluxes into the cycle [237]. The TCA-generated reducing equivalents were then utilised by the electron transport chain to power the ATP production in mitochondria [224]. Likewise glycolysis, the TCA is also affected by the redox

treatments (Fig. 3). As previously noted, the TCA cycle's organic acids (the malate and citrate valves) play a crucial part in the integration of redox metabolism [209,238].

The exogenous application of reductants confirmed the redox control of the TCA cycle. H_2S treatment suppressed the TCA cycle in apples, as indicated by the down-regulation of the related genes [232]. Another study discovered that H_2S acts downstream of NO to stimulate citrate secretion through the increase of plasma membrane (PM) H^+-ATPase-coupled citrate transporter cotransport mechanisms [239]. The reversible conversion of malate to oxaloacetate in the TCA cycle is catalysed by malate dehydrogenase (MDH), and MDH overexpression raises the concentration of organic acids. Salt stress increased the amount of MDH, and subsequent NaHS treatment increased it further in rice seedlings [240].

Likewise reductants, the oxidants could also modulate the functioning of the citrate cycle. For example, H_2O_2 application resulted in the modulation of several proteins (O80577, Q9FJQ8, and Q9SKL2) related to the TCA cycle in tomato, which can induce tolerance against oxidative stress caused by low temperature [241]. Many enzymes such as pyruvate dehydrogenase E1 component beta subunit (PDHE1), pyruvate dehydrogenase E2 component (PDCE2), dihydrolipoamide dehydrogenase (DLD), isocitrate dehydrogenase (IDH), dihydrolipoamide succinyltransferase (DLST), and succinyl-CoA synthetase beta subunit (SCS) were down-regulated in O_3-treated Mandarin (Ottawa) Numerous enzymes, including dihydrolipoamide succinyltransferase (DLST), isocitrate dehydrogenase (IDH), pyruvate dehydrogenase E1 component beta subunit (PDHE1), pyruvate dehydrogenase E2 component (PDCE2), and succinyl-CoA synthetase beta subunit (SCS), were down-regulated in Mandarin (Ottawa) after exposure to O_3 [236]. NO enhanced metabolic flux via the OPPP and glycolysis, thus providing pyruvate for the TCA cycle to enhance energy production [242]. The application of sodium nitroprusside (NO donor) led to metabolic reprogramming in white clover, which may lead to improved stress tolerance via participation in the TCA cycle for energy supply, osmotic adjustment, antioxidant defense, and signal transduction for stress defense. The authors further noticed that the pretreatment of mannose, α,β-aminobutyric acid, and nitric oxide affects the metabolic profiles of white clover under water stress. [243]. Collectively, based on the diverse effect on the redox pools, it may be proposed that the different redox compounds modulate the redox homeostasis through the different control of the TCA cycle.

2.3.5 Amino acid metabolism

Many studies showed that oxidative stress altered the metabolic profile in various plant species [223,244,245]. During oxidative stress, some plant species experienced an increase in the

various free amino acids. With stress, however, the levels of methionine (Met), glutamic acid (Glu), and asparagine (Asn) reduced [222]. In another study, it was found that menadione-induced oxidative stress enhanced the number of amino acids [246]. The CAT-deficient *Arabidopsis* mutants with higher H_2O_2 levels were shown to have higher amino acid levels than wild-type plants [247]. Moreover, the protein and transcript levels of H_2O_2-treated rice and wheat demonstrated oxidative stress-dependent alterations in amino acid metabolism [19,20]. Furthermore, foliar ASA treatment increased the accumulation of free amino acids in flax cultivars [22]. This change in turn might regulate several metabolic pathways. Furthermore, arginine (Arg), a precursor to polyamines with numerous regulatory functions, was increased by ASA treatment [248].

Thiourea (reductive stress-inducing agent) boosts amino acid metabolism (glutamate, asparagine, isoleucine, phenylalanine, tryptophan, and glutamine) to provide arsenic stress tolerance in rice [249]. In *Medicago sativa*, H_2O_2 significantly influenced the proline metabolism in order to ensure tolerance against drought-mediated oxidative stress [250]. The H_2O_2 profoundly alleviated the salinity-induced oxidative damage by increasing the proline production in wheat seedlings [251]. The H_2O_2 priming substantially enhanced the levels of asparagine and tyrosine under salinity-induced oxidative stress as compared to the control plants. At the germination stage, the exogenous application of H_2O_2 resulted in a higher accumulation of amino acids in *Chenopodium quinoa* Wild. [252]. The exogenously applied H_2O_2 is involved in the modulation of proline levels in tomato to enhance the tolerance against oxidative damage produced by low-temperature environments [241]. Taken together, the redox modulation of amino acid levels can contribute to the improvement of stress tolerance in plants since some of the amino acids can directly improve stress tolerance and other ones can serve as precursors for protective compounds.

3 Aims

We aimed to investigate the modulating effect of light conditions on cold acclimation and the regulation of the metabolism by reducing and oxidising compounds in wheat. Therefore, this study was done to get answers to the following questions:

1) What is the role of light (intensity and spectrum) in the regulation of redox homeostasis in wheat seedlings?

2) How do the light-dependent redox changes affect the biochemical processes during the subsequent cold period in wheat seedlings?

3) How does the exogenous application of an oxidant (H_2O_2) and a reductant (ASA) modify the redox environment and the expression of the redox-responsive genes in wheat?

4) How do the changes in the redox environment modulate the primary metabolite profile in wheat?

4 Materials and methods

4.1 Plant material and treatments

4.1.1 Study of the influence of light conditions on the response to cold treatment

Four wheat (*Triticum aestivum* L.) genotypes were used in the current experiments, among them two freezing-tolerant (Cheyenne – Ch, winter growth habit; Miranovskaya 808 – Mir, winter growth habit) and two freezing-sensitive (Cappelle Desprez – CD, winter growth habit; Chinese Spring – CS, spring growth habit) [253,254]. The seeds of the different wheat genotypes were germinated at 25 °C for 1 day, then at 4 °C for 1 day, and again at 25 °C for 2 days between wet filter papers. Afterward, the wheat seedlings were transferred to a modified half-strength Hoagland solution (changed every 2nd day) for 14 days at 20/17 °C day/night temperature and 75% relative humidity using a 16 h illumination period [123]. The three light conditions (in the same growth chamber with the division of one LED panel into three parts) used in this experiment are as follows: 1) white light; 250 μmol m^{-2} s^{-1} (denoted as normal light intensity: N; red/far-red ratio – 15:1; blue/red ratio – 1:2), 2) white light; 500 μmol m^{-2} s^{-1} (denoted as high light intensity: H, blue/red ratio – 1:2; red/far-red ratio - 15:1) and 3) far-red light: FR; (blue/red ratio of 1:2, red/far-red ratio - 10:1) with 250 μmol m^{-2} s^{-1} (Table S1, Fig. S1) [255]. The light modules were furnished with three narrow spectrum LED armatures with dominant wavelengths of 750 nm (Edison Edixeon, 2ER101FX00000001, New Taipei, Taiwan), 655 nm (Philips Lumileds, LXZ1-PA01, San Jose, CA, USA), and 448 nm (Philips Lumileds, LXZ1-PR01, San Jose, CA, USA) and a continuous wide spectrum LED (Philips Lumileds, LXZ2-5790-y, San Jose, CA, USA).

The following cold treatment at 5 °C continued for a week and the recovery period lasted for 3 weeks at 20/17 °C. The duration of the cold treatment was selected based on the earlier findings, in which the freezing-tolerant wheat genotypes reached the highest freezing tolerance level after 7 days at low temperature, whereas the sensitive wheat genotypes reached it only after 6 weeks (Kocsy et al., 2000b; Vágújfalvi et al., 1999). The sampling for biochemical analysis was done before (14 days in Hoagland solution) and after (21 days in Hoagland solution) the cold treatment by taking fully developed second leaves in the middle of the photoperiod, in 3 biological replicates, with three parallels. Additionally, electrolyte leakage and fresh weight data were also determined after a 3-week recovery period (42 days in Hoagland solution). The developmental stage of the wheat seedlings was evaluated by checking the morphology of the shoot apex after a 3-week recovery period (Fig. S2). Even with the 16 h

photoperiod, all the wheat genotypes cultivated under normal or high light intensity or in supplementation of far-red light were still in the vegetative phase as indicated by their single-ridge phenotype.

4.1.2 Investigation of the effect of ASA and H_2O_2 on primary metabolism and redox homeostasis in wheat

The wheat (*Triticum aestivum* L.) cultivar (Chinese Spring) was involved in this experiment. The seeds were germinated at 25 °C for 1 day, then at 4 °C for 1 day, and again at 25 °C for 2 days between wet filter papers. The wheat seedlings were then moved to a modified half-strength Hoagland medium for 10 days at 75% relative humidity and 20/17 °C day/night temperature, with 16 h illumination (250 μmol m^{-2} s^{-1}) in a growth chamber (Poleco, Poznan, Poland) (Kocsy et al. 2000). Then 0, 5 and 20 mM ASA (reductant) and H_2O_2 (oxidant) were added to the nutrient solution (Fig. S3). The concentrations of both chemicals (ASA and H_2O_2) were determined based on the growth indices and gene expression data in the preliminary evaluations, in which the two chemicals (ASA and H_2O_2) were applied with the following concentrations: 0, 0.5, 1, 5, 10 and 20 mM. The sampling for physio-biochemical and molecular determinations was done following 0-, 3- and 7-day treatments in the middle of the photoperiod. The experiment was repeated thrice, and, in each experiment, three parallel samples were taken for the subsequent analyses. The results of control samples in the case of all the measured physio-biochemical parameters did not alter substantially throughout the experiment, consequently, they are not presented after 3 days and 7 days of applications of ASA or H_2O_2.

4.2 Physiological, biochemical, and molecular biological measurements

4.2.1 Measurement of membrane injury, chlorophyll pigment contents, and photosynthetic parameters

The membrane injury was measured by determining the electrolyte leakage of the leaf segments (1 cm long) with a conductometer after their shaking in 4 mL of deionised water for 2 h. The conductivity was determined again after destroying cell membranes by incubating the plant samples at 100 °C for 30 min. Then, relative electrolyte leakage was then calculated as the ratio of the first and second recordings [258].

For the determination of the chlorophyll (*a* and *b*) and carotenoid contents, the homogenization of the wheat leaf tissues of (50 mg) was done with 1 mL of 80% acetone. This step was followed by centrifugation at 12,000 × g for 10 min. These steps were repeated until the pellet turned into white colour. The supernatants were then taken and diluted to 12 mL. The

absorbance was measured spectrophotometrically (Varian, Middelburg, The Netherlands) at 470, 646, 664, and 750 nm. Arnon's equations were used to calculate the chlorophyll a, b, and carotenoid contents [259].

The fully developed and detached leaves were used to determine the maximum quantum efficiency of photosystem II (F_v/F_m) after 20-min dark adaptation by using a pulse amplitude modulated fluorometer (PAM) (Imaging-PAM MSeries, Walz, Effeltrich, Germany). The F_v/F_m reveals the maximal quantum efficiency of PSII.

The photosynthetic activity of the wheat seedlings was measured by using Ciras 3 Portable Photosynthesis System (PP Systems, Amesbury, MA, USA) using a narrow (1.7 cm^2) leaf chamber. From each treatment, five fully developed attached leaves were selected for the photosynthetic measurements. The net photosynthetic rate (P_n), stomatal conductance (g_s), transpiration rate (E), and internal CO_2 concentration (C_i) were measured at a steady state level of photosynthesis using the light intensity (250 μmol m^{-2} s^{-1}) and CO_2 level (390 μL L^{-1}).

4.2.2 Determination of the H_2O_2 content and lipid peroxidation

The H_2O_2 levels of the wheat shoots were measured by the FOX1 method in a colorimetric reaction using a spectrophotometer as mentioned previously [260]. Wheat shoot samples (200 mg) were homogenized in 1 mL H_3PO_4 (10%). In the reaction, the H_2O_2 oxidases the ferrous ion to the ferric ion, and the latter one was detected by xylenol orange [261].

The lipid peroxidation was measured by the analysis of MDA levels. The samples (200 mg) were ground in 600 μl of 0.1% (w/v) trichloroacetic acid. Afterward, the centrifugation was done at 12,000 × g for 10 min. Then 2 mL of 0.5% (w/v) thiobarbituric acid, which was prepared in 20% (w/v) trichloroacetic acid, was added to the 300 μl supernatant. The samples were then incubated at 90 °C for 30 min. This step was followed by centrifugation at 12,000×g for 10 min. The MDA levels were then measured spectrophotometrically at 532 nm with the subtraction of non-specific absorption at 600 nm. The lipid peroxides concentration was then calculated in terms of MDA levels using an extinction coefficient of 55 mM^{-1} cm^{-1} [262].

4.2.3 Measurement of the activity of antioxidant enzymes

For the measurements of enzyme activities, the wheat shoot samples (200 mg) were ground in a mortar with liquid nitrogen followed by the addition of 1 mL of 50 mM MES/KOH (pH 6.0) buffer having 2 mM $CaCl_2$, 40 mM KCl and 1 mM ASA [263]. For the determination of APX enzyme activity, the reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.25 mM ASA, 5 mM H_2O_2, and 50 μl extract in a total volume of 1 mL [263]. The other three

enzyme activities of the ASA-GSH cycle were measured by using 50 mM Hepes buffer (pH 7) and 50 µl plant extract in a total volume of 1 mL [263]. In addition to these constituents, the reaction mixture contained 0.2 mM DHA, 0.1 mM EDTA, and 2.5 mM GSH for DHAR; 1 U ascorbate oxidase, 2.5 mM ASA and 0.25 mM NADPH for MDHAR and 0.5 mM GSSG, 0.5 mM EDTA and 0.25 mM NADPH for GR. The GST and CAT activities were analysed as described previously [264,265]. For the measurement of the GST enzyme, the reaction mixture consisted of the following chemicals: 0.1 M KH_2PO_4 (pH 6.5) buffer, 50 mM GSH, and 10 mM CDNB. The measurement of CAT was done in the reaction mixture, which contained 0.5 M phosphate buffer (pH 7.5) and 15 mM H_2O_2 (30%). The enzyme activities are presented on a protein basis which was measured by the method of an earlier study [266]. For all the measurements, a Cary 100 UV–visible spectrophotometer (Varian, Middelburg, The Netherlands) was used.

4.2.4 Determination of thiols

The fresh wheat shoots (200 mg) were crushed with liquid nitrogen. This step was followed by the addition of 1 mL of 0.1 M HCl to the powder. The non-protein thiol levels and their disulphide forms were measured by the method of a previously published study [267]. The total thiol amount was analysed after reduction with dithiothreitol and derivatisation with monobromobimane. For the determination of the oxidised thiols, the blockage of reduced thiols was done by N-ethylmaleimide, and toluene was used to remove its surplus. The amounts of oxidised thiol were then measured similarly to the total thiol analysis. Reverse-phase HPLC (Waters, Milford, MA, USA) was used to separate the cysteine (GSH precursor), GSH, and hmGSH (a homologue of GSH in *Poaceae),* and their amounts were measured by a W474 scanning fluorescence detector (Waters, Milford, MA, USA). The levels of the reduced thiols were then calculated as the difference between the total and oxidised thiols amounts.

4.2.5 Ascorbate and dehydroascorbate analyses

The fresh wheat shoot samples (500 mg) were pulverized with liquid nitrogen. This step was followed by the extraction with 3 mL of 1.5% meta-phosphoric acid [268]. After centrifugation of the supernatants, the reduced (ASA) and total ascorbic acid (the latter after reduction by dithiothreitol) amounts were determined by HPLC using an Alliance 2690 system equipped with a W996 photodiode array detector (Waters, Milford, MA, USA) [268]. The DHA concentration was calculated by subtracting the amount of ASA from the total ascorbic acid content.

4.2.6 Metabolite profiling

The shoot samples of wheat (100 mg) were extracted twice with 0.5 mL 60 v/v % MeOH, after that, twice with 0.5 mL 90 v/v % MeOH following the addition of internal standard (30 µL 1 mg mL^{-1} ribitol solution) as defined formerly [269]. The extraction was continued with 2 mL 75 v/v % MeOH using a vortex for 30 s, and this step was followed by an ultrasonic bath for 5 min at room temperature. Successively, the centrifugation of the samples was carried out at 10,000 g for 5 min at 4 °C. The supernatants were then taken, and aliquots (150 µL) were dried under a vacuum. The derivatization for GC measurements was carried out with the methoxyamine hydrochloride (20 mg mL^{-1} in pyridine) at 37 °C for 90 min. Afterward, the *N*-Methyl-*N*-trimethylsilyl-trifluoroacetamide was added followed by the incubation of the samples at 37 °C for 30 min. Then, they were injected into the LECO Pegasus 4D GCxGC TOFMS (LECO, Benton Harbour, MI, USA) equipped with a 1.5 m column (Rxi-17Sil MS phase) and 30 m column (Rxi-5MS phase). One µL of the measuring sample was injected into the column in a split mode at 230 °C. The constant flow rate was 1 mL min^{-1} and carrier gas (He) was used. Additionally, the ion source and transfer line were maintained at 250 °C. Initially, the temperature for the thermal program was 70 °C which was kept for 3 min, then raised to 320 °C at 7 °C min^{-1} rate. The high temperature was kept for 5 min with 3.25 s modulation period in 2D GC mode. For the identification of metabolites, the standards, and Kovats retention index were used. For each metabolite, the GC analyses and data evaluation, and normalization were carried out by the LECO ChromaTOF program. During calculation, the ChromaTOF 4.72 was used with Finn and Nist databases.

4.2.7 Measurement of free amino acids

The fresh wheat shoots (300 mg) were extracted with 2 mL cold 10% trichloroacetic acid in the course of 1 h agitation on a shaker at room temperature [270]. The filtration of samples was carried out by a 0.2 µm pore membrane filter, and this step was followed by the examination of the samples using an automatic amino acid analyser (Ingos Ltd., Praha, Czech Republic) equipped with an Ionex Ostion LCP5020 cation exchange column. The stepwise separation of free amino acids was carried out by a Li^{+}-citric buffer system (Ingos Ltd., Praha, Czech Republic).

4.2.8 Gene expression studies

The fresh wheat shoots (50-100 mg) were pulverized in liquid nitrogen followed by the addition of 600 µl TRIzol reagent for the extraction of the samples. The total RNA was extracted from the samples by Direct-zolTM RNA Miniprep Kit (Zymo Research) following the guidelines of

the supplier. The reverse transcription (RT) was accomplished by oligo(dT)15 primer (Promega) and M-MLV reverse transcriptase as defined by the supplier. For cDNA synthesis, the final volume of the template was 11 µl which contained 9 µl DEPC-treated water, 1 µg RNA in 1 µl H_2O, and 1 µl oligo-dT primer (0.5 µg/µl). 1.25 µl dNTP mix (100 mM), 5 µl M-MLV RT 5x buffer, 7.25 µl DEPC-treated water, 0.5 M-MLV RT (200 U/µl), and 11 µl template (prepared above) were used for the master-mix (25 µl) preparation. The following conditions were maintained for RT: 25 °C for 5 min, 42 °C for 60 min, 70 °C for 15 min, and then the samples were retained at 4 °C. After adding 75 µl sterilized MQ water, the final volume of cDNA solution was 100 µl. The gene expression levels were then measured by the quantitative real-time reverse-transcription PCR **(**qRT-PCR) using a CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with specific primers (Table S2) [5,12,258,271–274]. The qPCR reaction mixture contained 1 µl cDNA, 0.4 µl each of the primers, 5 µl qPCRBIO SyGreen blue mix, and 3.2 µl MQ water. The following conditions were established for the qPCR program: 95 °C for 3 min, 95 °C for 0.05 min, 60 °C for 0.30 min, GOTO 2 for 39 times, 65 °C for 0.05 min, and 95 °C for 0.5 min. The shoot samples were examined in triplicates. The *Ta30797* gene was used as a reference to calculate the relative gene expressions [$2^{-\Delta\Delta Cq}$, where $\Delta Cq = Cq(ref) - Cq(target)$] [271,275].

4.2.9 Statistics

The significant differences were calculated by the analysis of variance (ANOVA) using SPSS statistics (16.0) software. To compare the means, the least significance difference (LSD) test was performed at a 5% probability level. The correlation analysis was done according to a previously published method [276].

5 Results

5.1 Influence of light conditions on the cold-dependent changes in glutathione metabolism

5.1.1 Influence of light conditions on growth during cold treatment

The results of freezing tests confirmed that the CS and CD are freezing-sensitive wheat genotypes, while Ch and Mir are freezing-tolerant ones and these observations are consistent with the earlier published studies [253,254] (Table S3). The light treatments (intensity or spectrum) could not induce a significant change in shoot fresh weight except for high light treatment in the CD genotype (Fig. 4A). During cold, the obvious difference was recorded in the growth between the wheat genotypes, because the two tolerant wheat genotypes (Ch and Mir) exhibited increment in shoot fresh weight only remarkably in this period. After recovery from cold stress, 2- to 3-fold changes were found in shoot fresh weight in all the studied wheat genotypes. Moreover, the light treatments (intensity or spectrum) did not have a considerable effect on the fresh weight of the root except for high light in the CD genotype during recovery and in the Ch genotype under cold (Fig. 4B). The cold treatment did not exhibit a significant impact on root fresh weight, which displayed a 2- to 3-fold elevation after a 3-week recovery period.

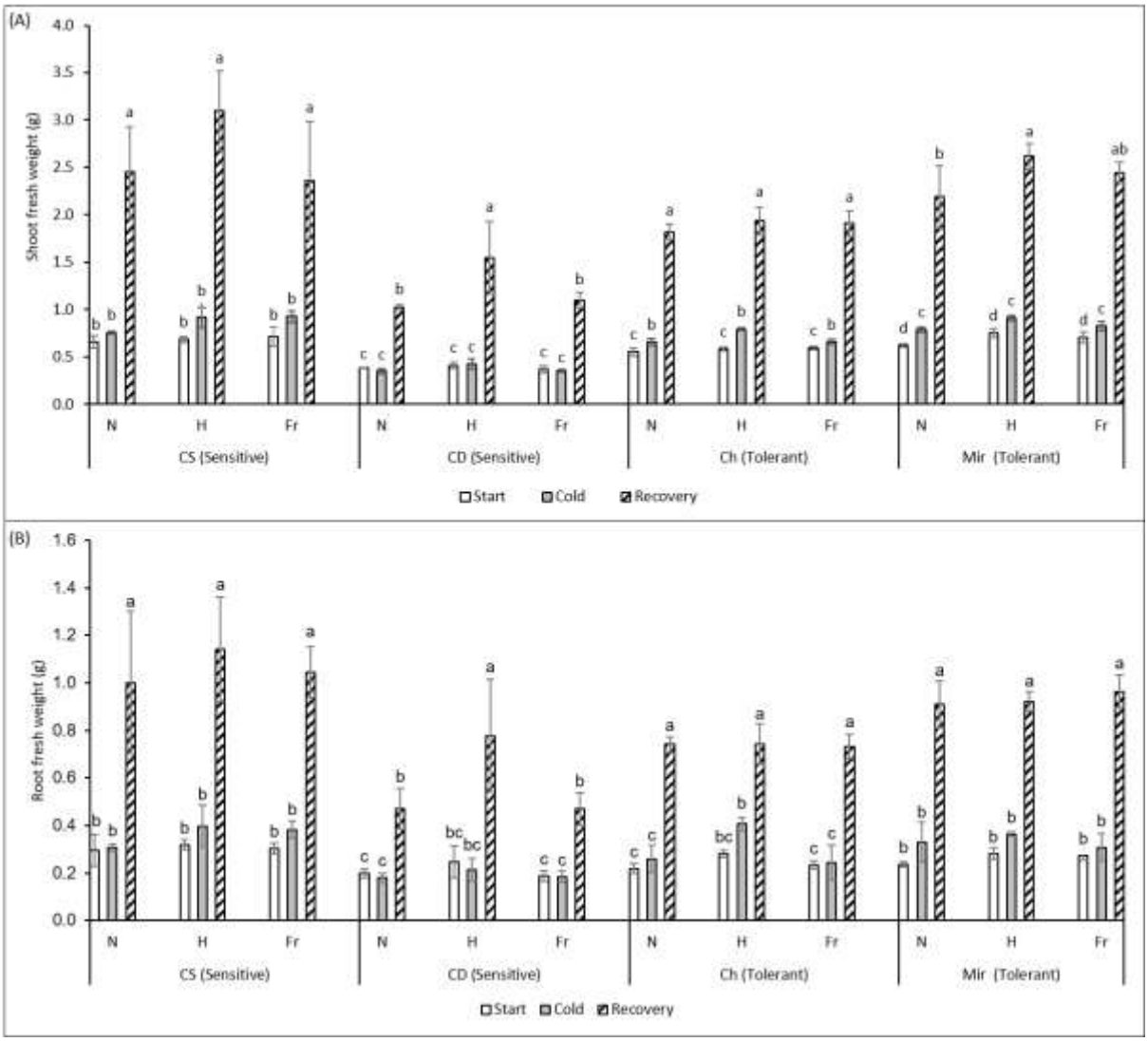

Fig. 4. Influence of the high light intensity and supplementary far-red light on the shoot and root fresh weight during cold acclimation and recovery. The CS (Chinese Spring) and CD (Cappelle Desprez) are freezing-sensitive wheat genotypes, and Ch (Cheyenne) and Mir (Miranovskaya 808) are freezing-tolerant wheat genotypes. (A): Shoot fresh weight, (B): Root fresh weight. The seedlings were cultivated for two weeks at 20/17°C, for one week at 5°C, and as a recovery period, again at 20/17°C for three weeks under three different light conditions. N: normal light intensity, H: high light intensity, FR: far-red light. In the case of each genotype, the different letters showed significant differences from each other at $p \leq 0.05$.

5.1.2 Influence of light conditions on cold-induced alterations in the electrolyte leakage

High light intensity significantly reduced the electrolyte leakage after the one-week cold acclimation in two freezing-sensitive genotypes (Mir, Ch), but not in the sensitive ones (Fig. 5). Interestingly, high light increased this parameter before the cold treatment and after the

recovery in CS. Cold-induced membrane injury manifested in increased electrolyte leakage in all the genotypes, but after a recovery period, it decreased back again to the control level even in the sensitive genotypes.

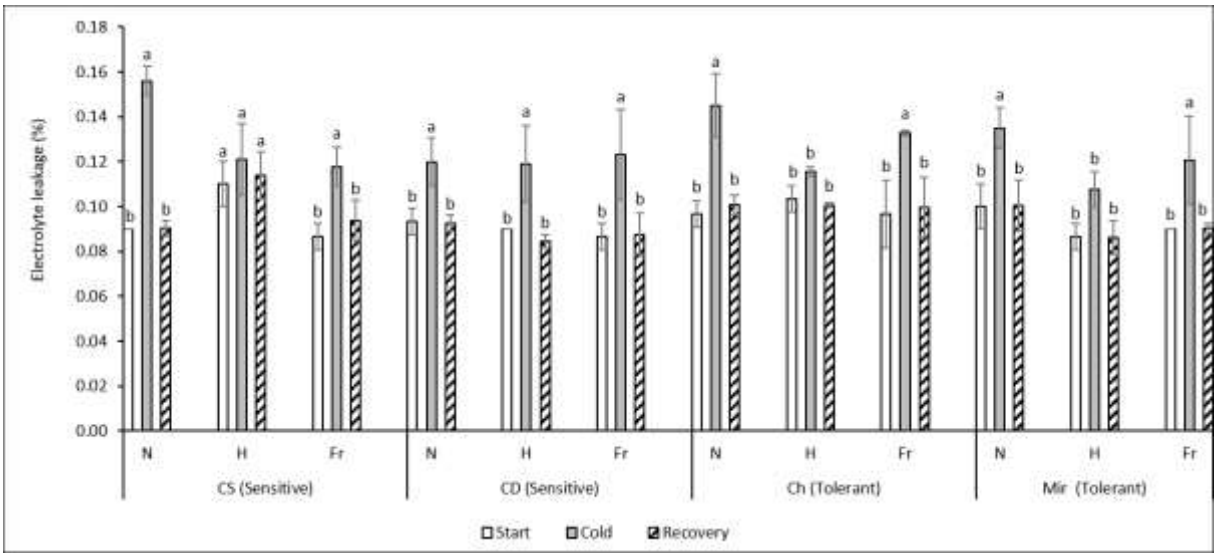

Fig. 5. Influence of the high light intensity and supplementary far-red light on the electrolyte leakage during cold acclimation and recovery. The CS (Chinese Spring) and CD (Cappelle Desprez) are freezing-sensitive wheat genotypes, and Ch (Cheyenne) and Mir (Miranovskaya 808) are freezing-tolerant wheat genotypes. The seedlings were cultivated for two weeks at 20/17 °C, for one week at 5 °C, and as a recovery period, again at 20/17 °C for three weeks under three different light conditions. N: normal light intensity, H: high light intensity, FR: far-red light. In the case of each genotype, the different letters showed significant differences from each other at $p \leq 0.05$.

5.1.3 Influence of light conditions on glutathione metabolism during cold treatment

Light spectrum and intensity had a slight influence on the Cys and CySS contents and their ratio (CySS/Cys) (Fig. 6). However, the cold substantially elevated the Cys and CySS levels under the most investigated light conditions. For Cys, there was an exception in high light intensity in the CS wheat genotype and normal light intensity (white light) in the Ch wheat genotype. In addition, the cold treatment did not have a significant influence on CySS under high light intensity in the CD genotype and in both light intensities in the Ch genotype, as well as in the Mir genotype for both thiols irrespective of light intensity and spectrum, too (Fig. 6A&B). Cold did not induce a significant change in CySS/Cys under the investigated light regimens except

for CD in normal and high light intensity (Fig. 6C). Taken together, it was noticed that the redox state and amount of cysteine was more sensitive towards the cold treatment in the sensitive wheat genotypes relative to the tolerant ones.

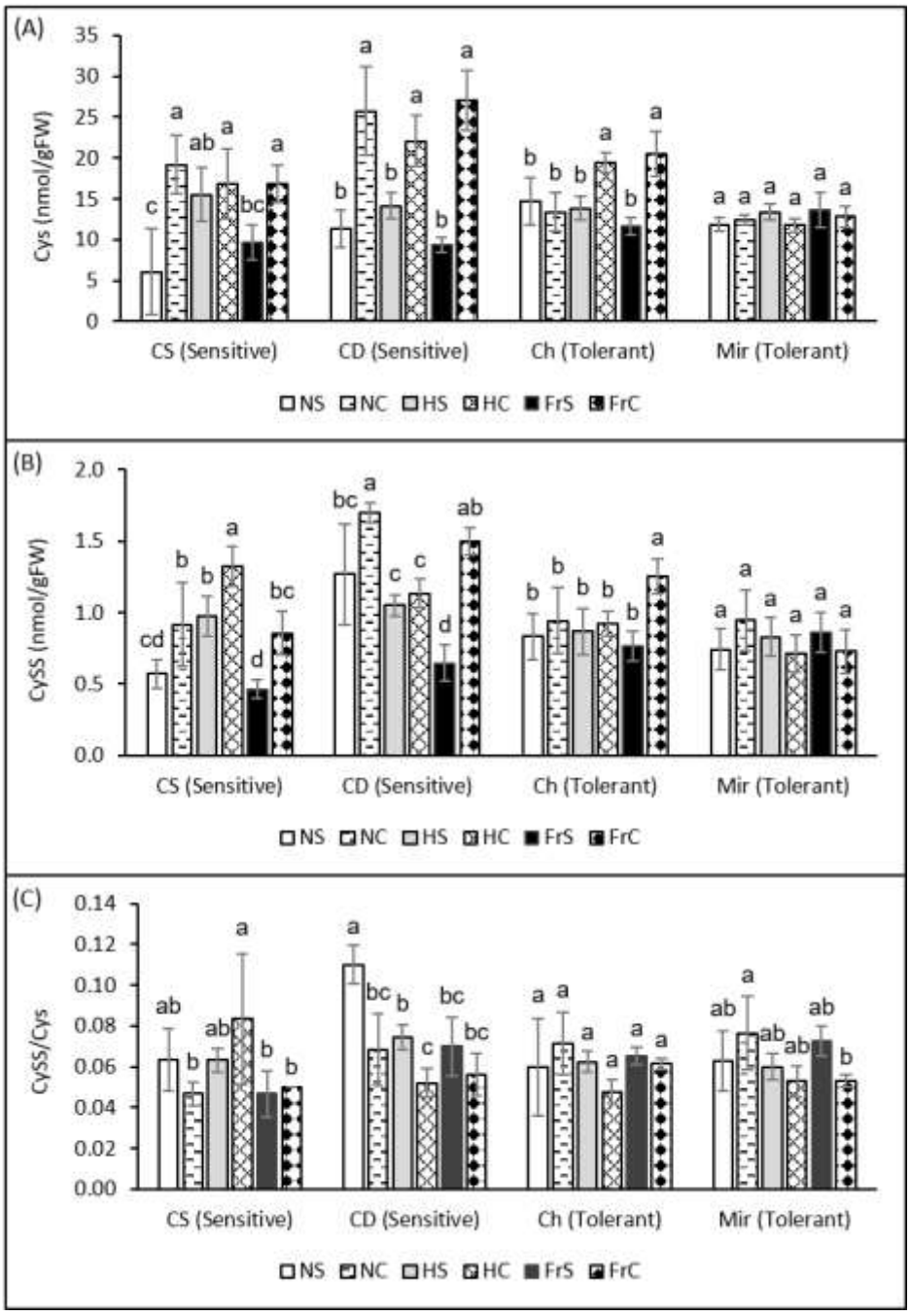

Fig. 6. Influence of the high light intensity and supplementary far-red light on the redox state and amount of the cysteine during cold acclimation. The CS (Chinese Spring) and CD (Cappelle Desprez) are freezing-sensitive wheat genotypes, and Ch (Cheyenne) and Mir (Miranovskaya 808) are freezing-tolerant wheat genotypes. The seedlings were cultivated for two weeks at

20/17 °C, for one week at 5 °C, and as a recovery period, again at 20/17 °C for three weeks under three different light conditions. (A): Cys: cysteine, (B): CySS: cystine, (C): CySS/Cys: ratio of two forms. N: normal light intensity, H: high light intensity, FR: far-red light. In the case of each genotype, the different letters showed significant differences from each other at $p \leq 0.05$.

Cold acclimation caused a two- to five-fold up-regulation in the γEC amount of the plants cultivated under high and far-red lights, however, such an effect was not noticed in the plants grown in white light (Fig. 7A). However, the γESSE amount displayed a larger cold-induced stimulation in normal light as compared to high light intensity and far-red light (Fig. 7B). Whereas the ratio of γESSE/γEC was enhanced or was not modified after cold treatment under normal light, it was reduced by the high light intensity and supplementary far-red light (Fig. 7C). There were variances in the amount and redox state of γ-glutamylcysteine between the studied genotypes, but they could not be allied with freezing tolerance level.

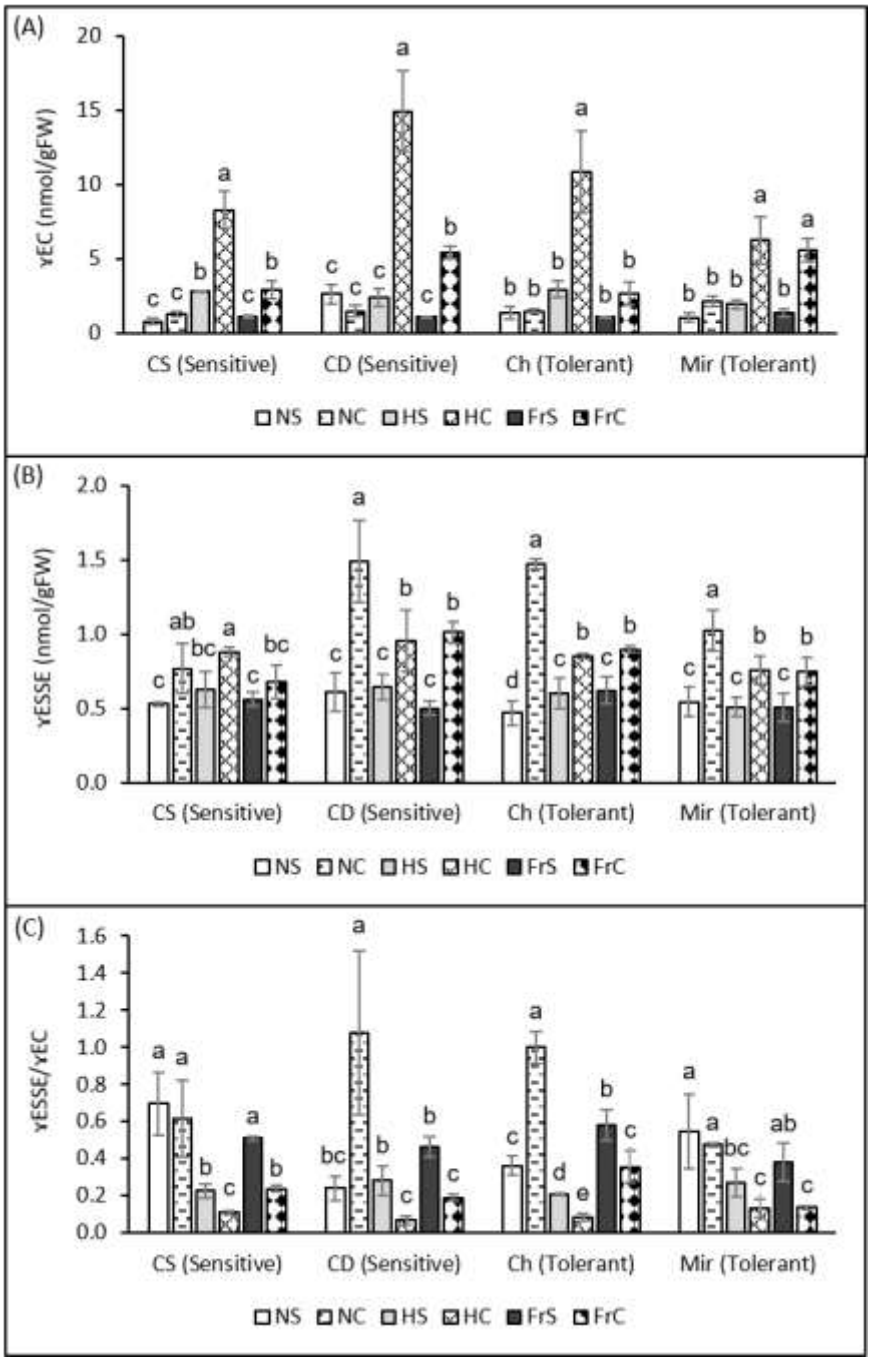

Fig. 7. Influence of the high light intensity and supplementary far-red light on the redox state and amount of the γ-glutamylcysteine during cold acclimation. The CS (Chinese Spring) and CD (Cappelle Desprez) are freezing-sensitive wheat genotypes, and Ch (Cheyenne) and Mir (Miranovskaya 808) are freezing-tolerant wheat genotypes. The seedlings were cultivated for two weeks at 20/17 °C, for one week at 5 °C, and as a recovery period, again at 20/17 °C for three weeks under three different light conditions. (A): γEC: γ-glutamylcysteine, (B): γESSE: γ-glutamylcystine, (C): γESSE/γEC: ratio of the two forms. N: normal light intensity, H: high

light intensity, FR: far-red light. In the case of each genotype, the different letters showed significant differences from each other at $p \leq 0.05$.

At normal temperature, the light intensity and spectrum had a significant influence on the amount and redox state of GSH (Fig. 8). Except for Ch, a substantial increase (2- to 3-fold) in GSH content was detected in high light-grown plants relative to those cultivated in normal light (Fig. 8A). This alteration in GSH content was not substantial in case of far-red light condition except for Ch. At low temperature, CS, CD, and Ch showed a greater increase in the GSSG level, along with GSSG/GSH ratio in Ch genotype under normal light conditions. The freezing-sensitive genotypes (CS and CD) had a 2- to 4-fold enhancement in the content of GSSG and the GSSG/GSH ratio, while no or only weaker variations were noted in two tolerant wheat genotypes in the case of far-red light at low temperature (Fig. 8B&C).

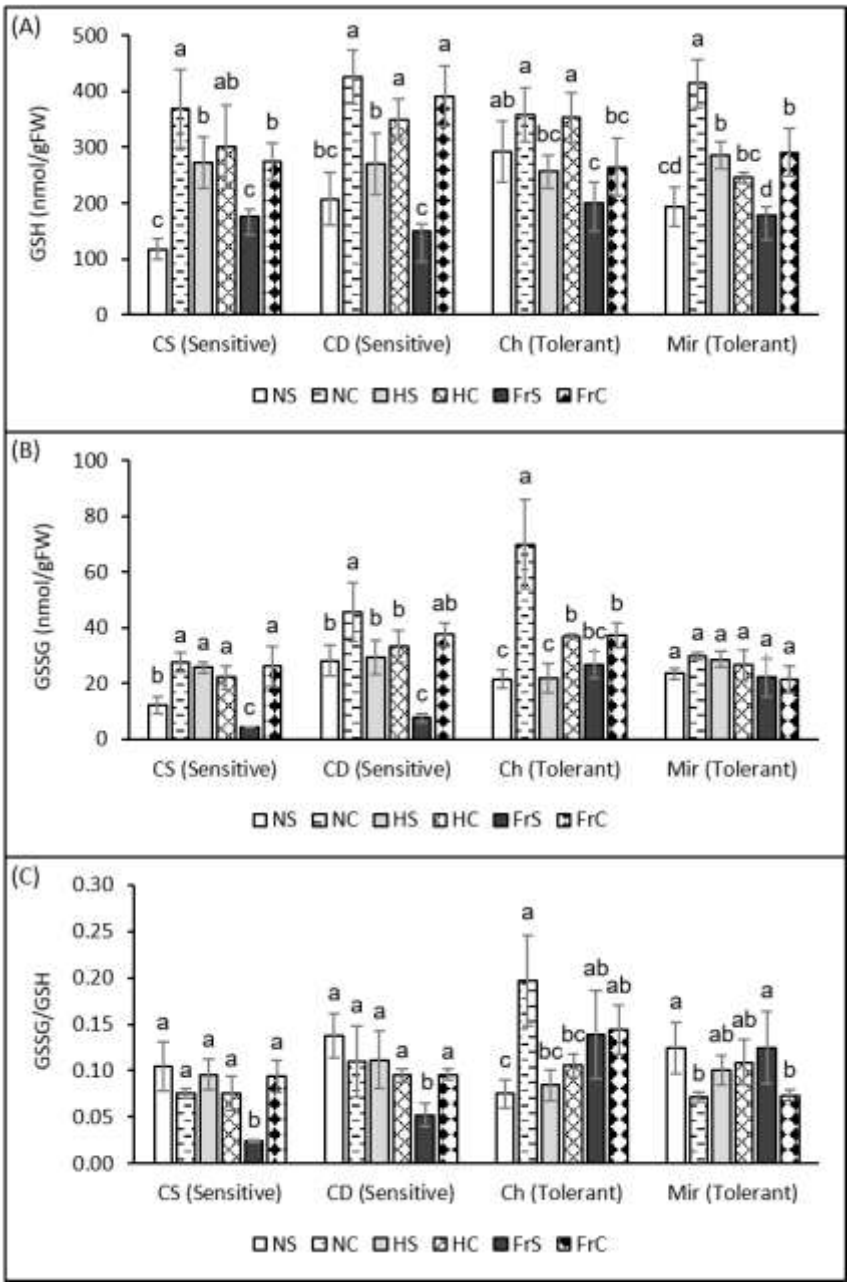

Fig. 8. Impact of the high light intensity and supplementary far-red light on redox state and amount of glutathione during cold acclimation. The CS (Chinese Spring) and CD (Cappelle Desprez) are freezing-sensitive wheat genotypes, and Ch (Cheyenne) and Mir (Miranovskaya 808) are freezing-tolerant wheat genotypes. The seedlings were cultivated for two weeks at 20/17 °C, for one week at 5 °C, and as a recovery period, again at 20/17 °C for three weeks under three different light conditions. (A): GSH: glutathione, (B): GSSG: glutathione disulphide, (C): GSSG/GSH: ratio of the two forms. N: normal light intensity, H: high light intensity, FR: far-red light. In the case of each genotype, the different letters showed significant differences from each other at $p \leq 0.05$.

At normal growth temperature (20/17 °C), the amounts of hmGSH and hmGSSG and their ratio (hmGSSG/hmGSH) were affected by the high light intensity and supplementary far-red light in almost all cases in the studied wheat genotypes when compared with normal light (Fig. 9). Following cold acclimation, the hmGSH and hmGSSG amount exhibited a two- to four-fold enhancement under far-red light in the two freezing-sensitive wheat genotypes (CS, CD), nevertheless such profound effect was not noticed in the two tolerant ones (Fig. 9A&B). Moreover, the amount of hmGSH raised in the normal light intensity (white light) except for Ch, and the hmGSSG level was also upregulated in the CS genotype in the normal light intensity and Ch and Mir genotypes in the high light intensity. After cold acclimation, the significant decline in the hmGSSG/hmGSH ratio was noticed under normal light intensity in CD and Mir genotypes (Fig. 9C). Collectively, the pattern of the changes in hmGSH and hmGSSG was similar in the two sensitive and in the two tolerant genotypes, respectively.

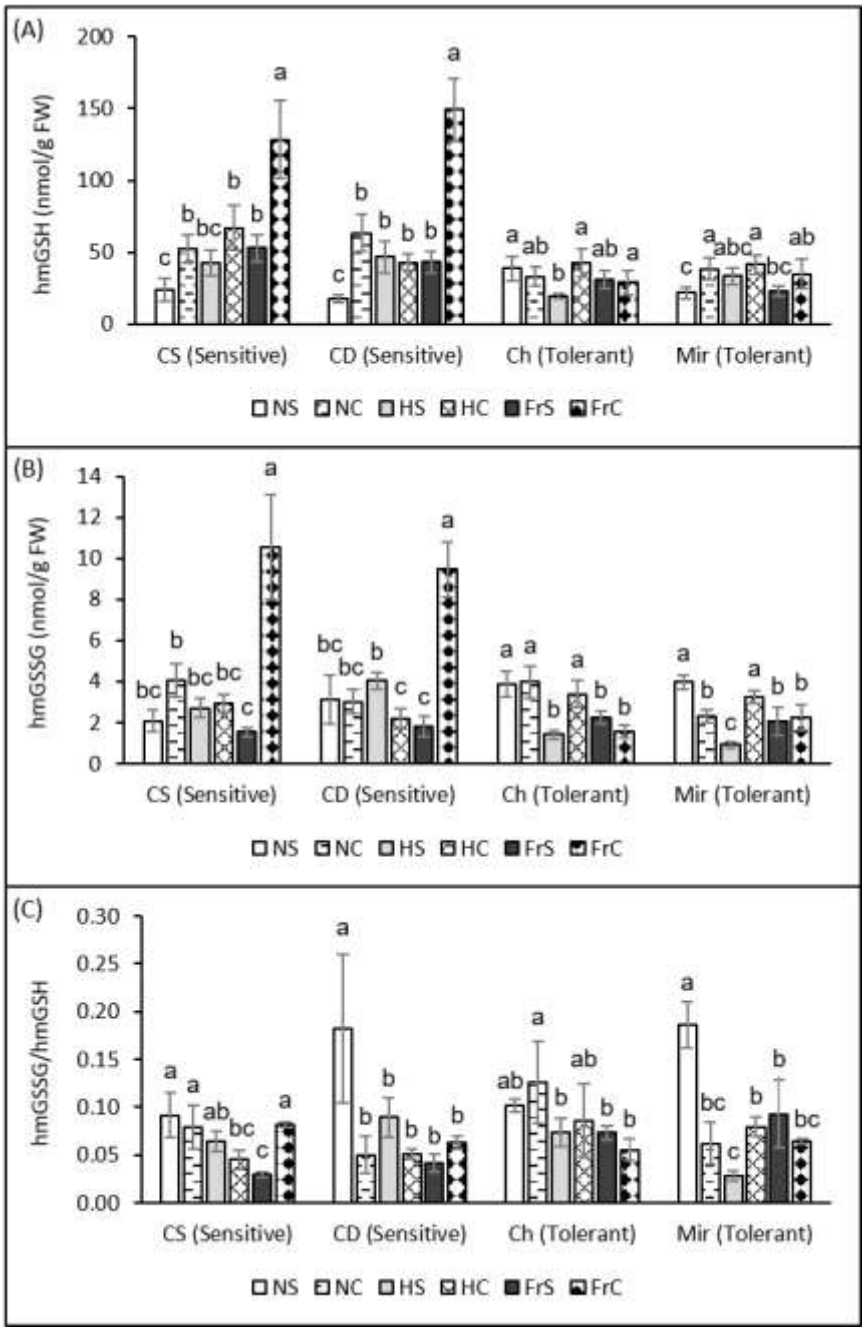

Fig. 9. Influence of the high light intensity and supplementary far-red light on redox state and amount of hydroxymethylglutathione during cold acclimation. The CS (Chinese Spring) and CD (Cappelle Desprez) are freezing-sensitive wheat genotypes, and Ch (Cheyenne) and Mir (Miranovskaya 808) are freezing-tolerant wheat genotypes. The seedlings were cultivated for two weeks at 20/17 °C, for one week at 5 °C, and as a recovery period, again at 20/17 °C for three weeks under three different light conditions. (A): hmGSH: hydroxymethylglutathione, (B): hmGSSG: hydroxymethylglutathione disulphide, (C): hmGSSG/hmGSH: ratio of the two forms. N: normal light intensity, H: high light intensity, FR: far-red light. In the case of each genotype, the different letters showed significant differences from each other at $p \leq 0.05$.

At 20/17 °C (normal growth temperature), the light intensity (high light) and spectrum (supplementary far-red light) had only a smaller influence on the cysteinylglycine (CysGly, degradation product of the GSH) and cystinylglycine (CySSGly) amounts. Whereas 5 °C (cold) induced 4- to -5-fold up-regulation in CysGly content under high light and supplementary far-red light as compared to normal light (Fig. 10A). In the CS genotype, the CySSGly content was substantially reduced by the cold treatment in all the light conditions (Fig. 10B). While other genotypes demonstrated weaker cold-induced variations in CySSGly levels. An obvious decline was found in CySSGly/CysGly ratio in all the studied wheat genotypes under all the light environments at 5 °C except for CD in normal light (Fig. 10C).

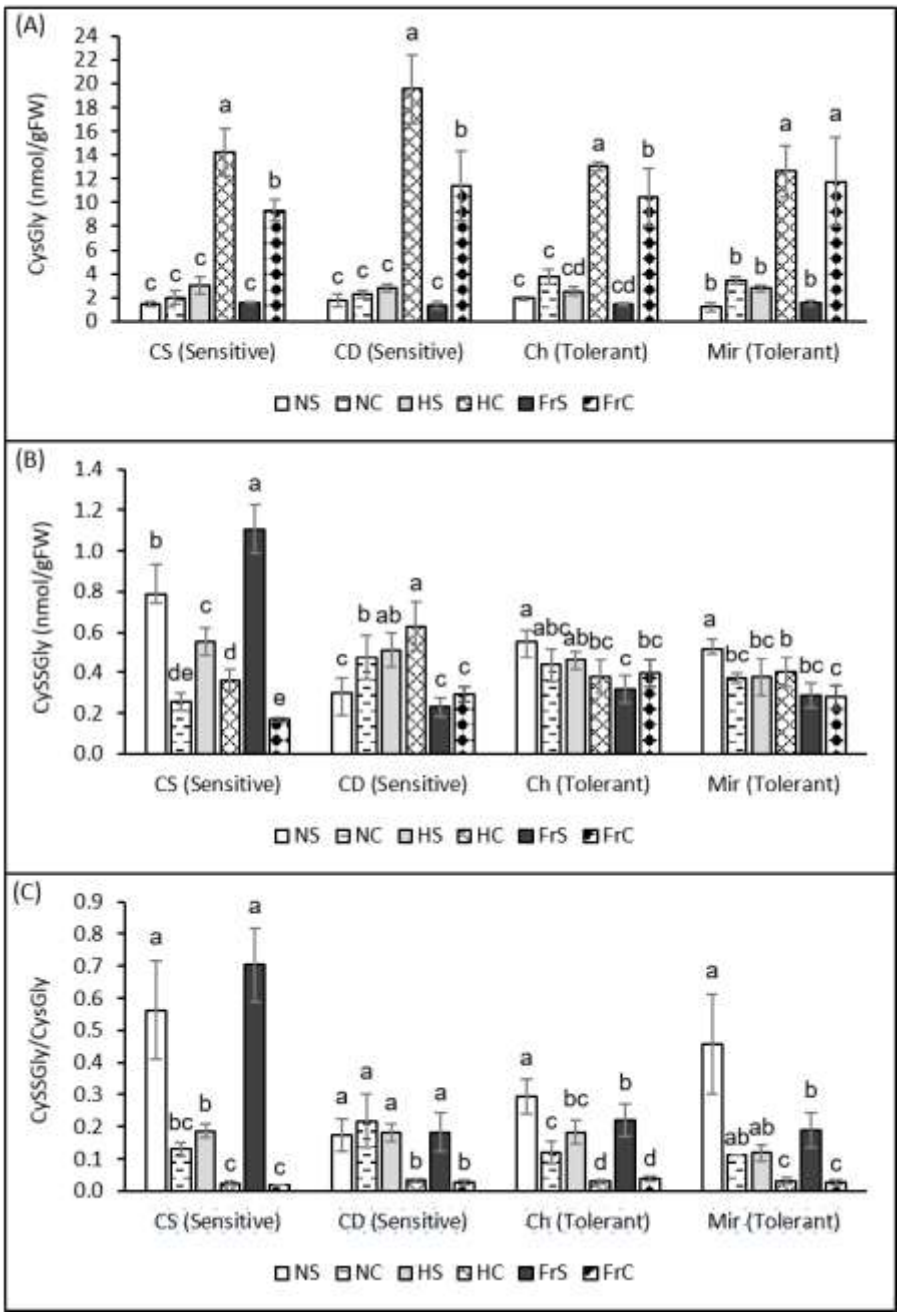

Fig. 10. Impact of the high light intensity and supplementary far-red light on redox state and amount of the cysteinylglycine during cold acclimation. The CS (Chinese Spring) and CD (Cappelle Desprez) are freezing-sensitive wheat genotypes, and Ch (Cheyenne) and Mir (Miranovskaya 808) are freezing-tolerant wheat genotypes. The seedlings were cultivated for two weeks at 20/17 °C, for one week at 5 °C, and as a recovery period, again at 20/17 °C for three weeks under three different light conditions. (A): CysGly: cyteinylglycine, (B): CySSGly: cystinylglycine, (C): CySSGly/CysGly: ratio of the two forms. N: normal light intensity, H: high light intensity, FR: far-red light. In the case of each genotype, the different letters showed significant differences from each other at $p \leq 0.05$.

5.1.4 Impact of light conditions on the activities of the ASA-GSH cycle-related enzymes during cold treatment

At normal growth temperature (20/17 °C), lower activities of the antioxidant enzymes were usually observed under high light conditions, while high light intensity did not influence them at low temperature (Fig. 11). At 20/17 °C, the high light intensity induced the considerable decline in the activities of DHAR, MDHAR, and GR with few exceptions, but their activities did not decrease further at 5 °C. This modification was weaker before the cold period in two sensitive wheat genotypes, whereas, their activities were inhibited by cold. Such variance between the wheat genotypes was not detected for APX in the case of high light intensity. The far-red light could not induce a pronounced effect in the enzyme activities compared to the normal light conditions. After cold application, the activities of all four enzymes of the ASA-GSH cycle showed a substantial reduction (50%) in all the tested wheat genotypes in all light regimes with few exceptions (Fig. 11).

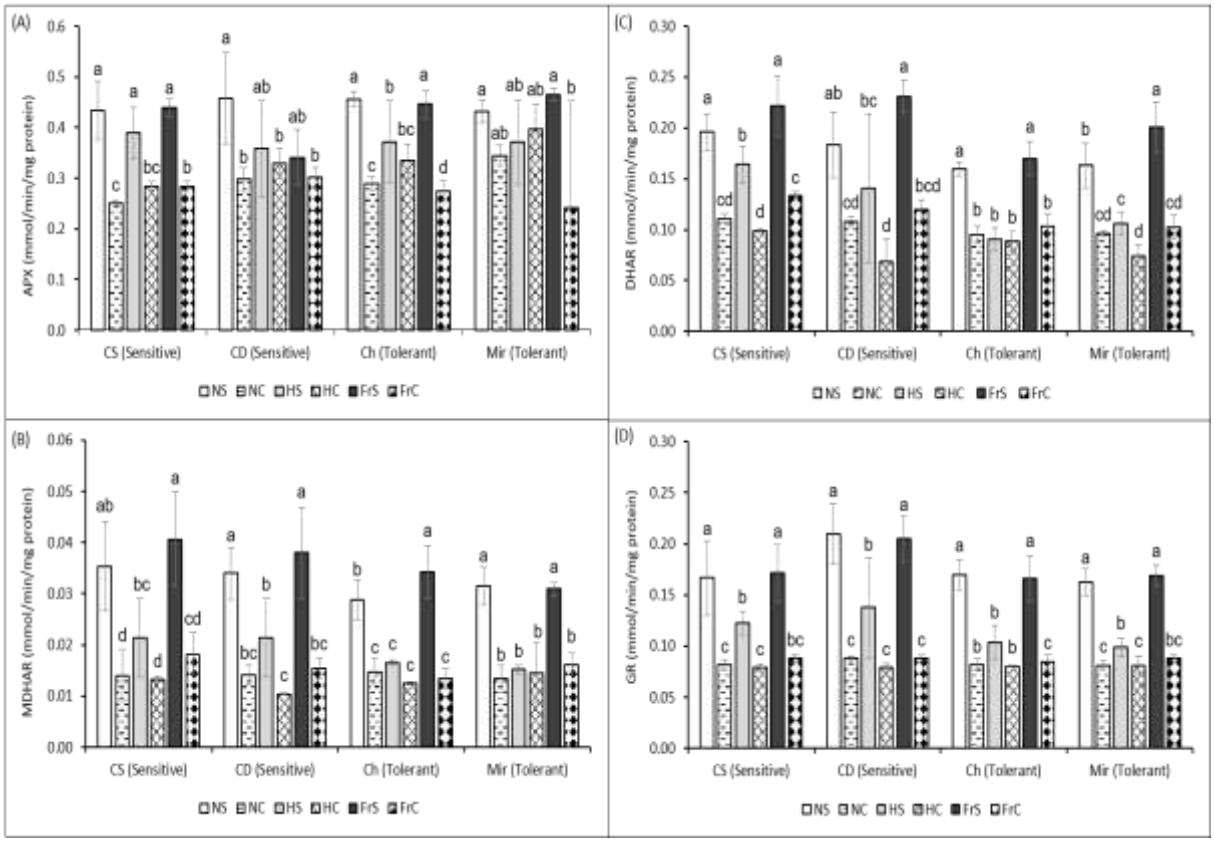

Fig. 11. Influence of the high light intensity and supplementary far-red light on the activity of the enzymes of the ascorbate-glutathione cycle during cold acclimation. The CS (Chinese Spring) and CD (Cappelle Desprez) are freezing-sensitive wheat genotypes, and Ch (Cheyenne) and Mir (Miranovskaya 808) are freezing-tolerant wheat genotypes. The seedlings were

cultivated for two weeks at 20/17 °C, for one week at 5 °C, and as a recovery period, again at 20/17 °C for three weeks under three different light conditions. (A): APX: ascorbate peroxidase, (B): MDHAR: monodehyroascorbate reductase, (C): DHAR: dehydroascorbate reductase, (D): GR: glutathione reductase. N: normal light intensity, H: high light intensity, FR: far-red light. In the case of each genotype, the different letters showed significant differences from each other at $p \leq 0.05$.

5.1.5 Influence of light conditions on transcript levels associated with glutathione and amino acid metabolism

According to hierarchical clustering, the glutathione- and amino acid metabolism-associated genes were considerably influenced by the light intensity in CS and Ch wheat genotypes before the cold environment, because the normal (white light) and high light intensity treatments were assembled separately (Fig. 12). But such difference was not found after the cold treatment. In contrast, in Ch and Mir, the far-red light could induce a pronounced effect on the transcript levels before the cold, but it had an impact after the cold treatment. The temperature had a pronounced effect on the transcript levels because the data obtained before the cold are on the left side of the heatmap, whereas recordings after the cold treatment are on its right side. In the case of genotype-specific effect, the values detected for the three light conditions (normal light, high light, and supplementary far-red light) were in the same cluster for Mir before the cold treatment and for CS and Ch genotypes after the cold.

Based on the expression changes of the investigated genes, the first main cluster was formed by *TaNR* and *TaAspTA*, where the enhancement of transcript levels was observed under cold in CS and Ch genotypes. The second cluster consisted of *TaCbf16*, *TatAPX*, *TacAPX*, *TaGS2*, and *TaGST*, in which the expression of most genes was decreased by cold. The genes in the third cluster showed higher expression levels under cold treatment in all the tested light regimens. Interestingly, numerous APX gene family members were clustered in different groups based on their expression patterns.

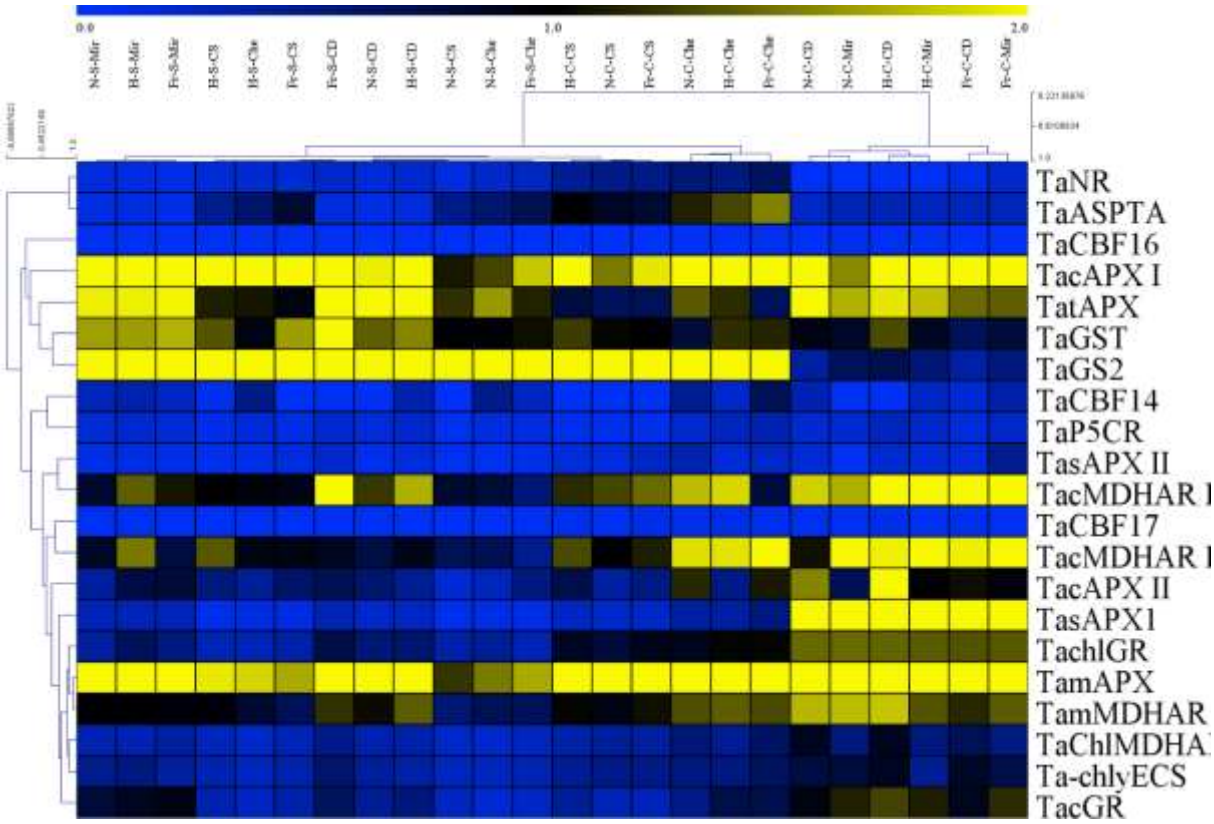

Fig. 12. Impact of the high light intensity and supplementary far-red light on the transcript levels of the genes associated with the metabolism of glutathione during cold acclimation. The CS (Chinese Spring) and CD (Cappelle Desprez) are freezing-sensitive wheat genotypes, and Ch (Cheyenne) and Mir (Miranovskaya 808) are freezing-tolerant wheat genotypes. The seedlings were cultivated for two weeks at 20/17 °C, for one week at 5 °C, and as a recovery period, again at 20/17 °C for three weeks under three different light conditions. N: normal light intensity, H: high light intensity, FR: far-red light.

5.2 Effect of ASA and H_2O_2 on the metabolism in wheat

5.2.1 ASA- and H_2O_2-induced alterations in growth indices

The untreated plants (without ASA or H_2O_2 application) displayed similar readings throughout the experiments, hence only the first-day recordings (before treatments) were shown. The wheat seedlings showed a significant increase in shoot length after a longer duration (7 days) of 5 mM and 20 mM ASA and 5 mM H_2O_2 additions relative to its value before the treatments (Fig. 13A). Most of the treatments induced the increment in the shoot fresh weight except for the 3-day 5 mM and 20 mM ASA and 20 mM H_2O_2 addition (Fig. 13B). Whereas the root length did not change after the application of the two compounds (ASA and H_2O_2) (Fig. 13C). Regarding root weight, a great increase was observed after 7 d 5 mM H_2O_2 addition (Fig. 13D).

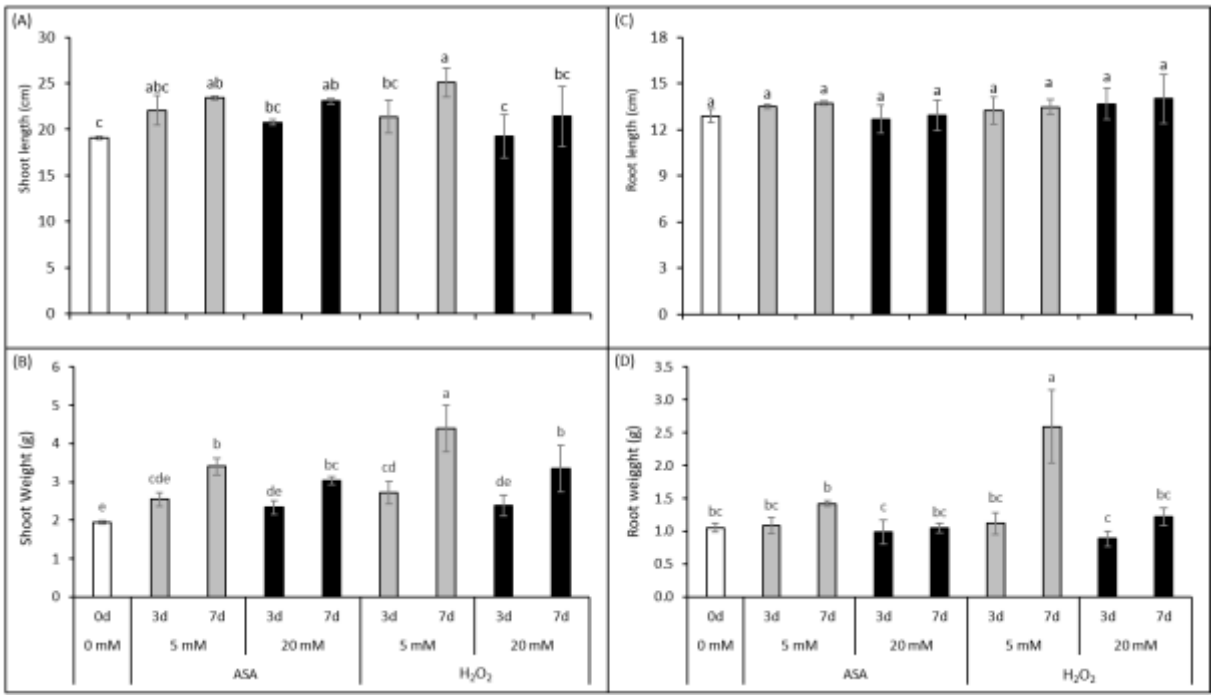

Fig. 13. Effect of the ascorbate (ASA) and hydrogen peroxide (H_2O_2) on the wheat growth indices. The different letters showed significant differences from each other at $p \leq 0.05$.

5.2.2 ASA- and H_2O_2-dependent changes in photosynthetic parameters

A 30-40% reduction was recorded for chlorophyll *a* contents following the treatments with a few exceptions (3-day 20 mM ASA and 5 mM H_2O_2). However, the chlorophyll *b* contents were not influenced by two compounds except for 20 mM H_2O_2 after 3 days (Table 1). ASA caused a significant reduction in the carotenoid content, but H_2O_2 did not have such effect except for the 7-day 5 mM H_2O_2 treatment (Table 1).

Prolonged ASA application resulted in a small, but noteworthy decline in F_v/F_m irrespective of its concentration (Table 1). Regarding H_2O_2, the only decline in F_v/F_m was recorded using its higher concentration (20 mM) for a longer duration (7 days).

Table 1. Influence of ascorbate (ASA) and hydrogen peroxide (H_2O_2) on the photosynthetic pigment contents, chlorophyll *a* fluorescence, and CO_2 assimilation attributes.

	ASA					H_2O_2			
	0 mM	5 mM		20 mM		5 mM		20 mM	
Parameters	**d0**	**d3**	**d7**	**d3**	**d7**	**d3**	**d7**	**d3**	**d7**
Pigment content of leaves									
Chl *a* ($mg\ g\ Fw^{-1}$)	1.55 $\pm0.27^{a}$	1.11 $\pm0.31^{bc}$	1.07 $\pm0.30^{bc}$	1.30 $\pm0.10^{ab}$	0.89 $\pm0.20^{c}$	1.28 $\pm0.12^{ab}$	1.06 $\pm0.11^{bc}$	0.96 $\pm0.03^{bc}$	1.14 $\pm0.11^{bc}$
Chl *b* ($mg\ g\ Fw^{-1}$)	0.37 $\pm0.04^{a}$	0.27 $\pm0.08^{a}$	0.34 $\pm0.14^{a}$	0.31 $\pm0.13^{a}$	0.251 $\pm0.05^{ab}$	0.26 $\pm0.05^{a}$	0.26 $\pm0.03^{a}$	0.11 $\pm0.00^{b}$	0.28 $\pm0.02^{a}$
Car ($mg\ g\ Fw^{-1}$)	0.43 $\pm0.06^{a}$	0.31 $\pm0.08^{bc}$	0.16 $\pm0.01^{d}$	0.34 $\pm0.00^{bc}$	0.22 $\pm0.03^{de}$	0.35 $\pm0.03^{abc}$	0.28 $\pm0.02^{cd}$	0.38 $\pm0.09^{ab}$	0.36 $\pm0.01^{abc}$
Photosynthetic parameters									
F_v/F_m	0.79 $\pm0.01^{a}$	0.79 $\pm0.01^{a}$	0.76 $\pm0.02^{bc}$	0.79 $\pm0.01^{a}$	0.75 $\pm0.02^{c}$	0.79 $\pm0.01^{a}$	0.78 $\pm0.01^{a}$	0.79 $\pm0.01^{a}$	0.77 $\pm0.02^{b}$
P_N ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	14.65 $\pm1.23^{a}$	12.28 $\pm0.43^{b}$	8.48 $\pm0.66^{d}$	11.4 $\pm1.1^{bc}$	7.98 $\pm0.46^{d}$	12.28 $\pm0.95^{b}$	10.35 $\pm1.23^{c}$	10.6 $\pm1.68^{c}$	8.35 $\pm0.53^{d}$
g_S ($mmol\ H_2O\ m^{-2}\ s^{-1}$)	132.6 $\pm5.5^{a}$	70.25 $\pm5.32^{c}$	68.67 $\pm16.86^{c}$	69.25 $\pm4.72^{c}$	51.5 $\pm5.45^{d}$	67.5 $\pm4.8^{c}$	87 $\pm9.49^{b}$	58 $\pm15.64^{cd}$	65.5 $\pm9.11^{c}$
C_i ($\mu mol\ CO_2\ mol\ air^{-1}$)	206.63 $\pm13.48^{a}$	122.75 $\pm10.31^{d}$	145 $\pm3.46^{c}$	87.75 $\pm13.28^{fg}$	156.25 $\pm25.85^{bc}$	100.5 $\pm14.39^{ef}$	138.5 $\pm7.72^{cd}$	78.5 $\pm7.68^{g}$	167.75 $\pm12.95^{b}$
E ($mol\ m^{-2}\ s^{-1}$)	1.78 $\pm0.07^{a}$	0.93 $\pm0.05^{c}$	0.78 $\pm0.13^{de}$	0.81 $\pm0.12^{cde}$	0.68 $\pm0.1^{ef}$	0.91 $\pm0.11^{cd}$	1.13 $\pm0.1^{b}$	0.55 $\pm0.13^{f}$	0.83 $\pm0.1^{cd}$

Chl *a*, Chl *b*: chlorophyll *a* and *b*; Car: carotenoids; F_v/F_m: maximal quantum efficiency of the PSII; P_N: net CO_2 assimilation rate; g_S: stomatal conductance; C_i: intracellular CO_2 concentration; E: transpiration rate. The different letters showed significant differences from each other at $p \leq 0.05$.

All the gas exchange attributes were determined before the application of the two compounds and after 3 and 7 days of the treatments. Relative to control, exogenous application of both compounds (ASA and H_2O_2) reduced the CO_2 assimilation rate (P_n) at both sampling time points (3rd and 7th days) (Table 1). Generally, the inhibition was amount and time-dependent: e.g. greater reduction was detected with the progression in the concentration and time of ASA or H_2O_2 application. Between the ASA and H_2O_2 applications, the only significant difference was noted when the compounds were supplied at 5 mM for a longer period (7 days). Namely, the ASA application caused a greater decline in P_N compared to the H_2O_2 application. The g_s and E were also inhibited by the exogenous application of both chemicals as compared to untreated plants (Table 1). Similarly, both ASA and H_2O_2 treatments decreased the intracellular CO_2 level, which was more severe after a shorter application (3 days) than a longer one (7 days).

5.2.3 Effect of ASA and H_2O_2 on oxidative stress indicators

Except for 20 mM ASA, all treatments reduced the level of endogenous H_2O_2, especially after longer application (Fig. 14A). Following 20 mM ASA application, the endogenous H_2O_2 level was higher after 3 days relative to the control, while after longer application (7 days), it was similar to it.

The lipid peroxidation (MDA) was elevated by all applied treatments (Fig. 14B). These variations were greater after 5 mM and 20 mM ASA application for 7 days and 20 mM H_2O_2 treatment for 3 days.

Although the application of both compounds did not affect electrolyte leakage after 3 days, they induced its increase ranging from 1.8- to 2.9-fold after 7 days (Fig. 14C). Taken altogether, it was noticed that the ASA application induced higher oxidative stress as compared to H_2O_2 application.

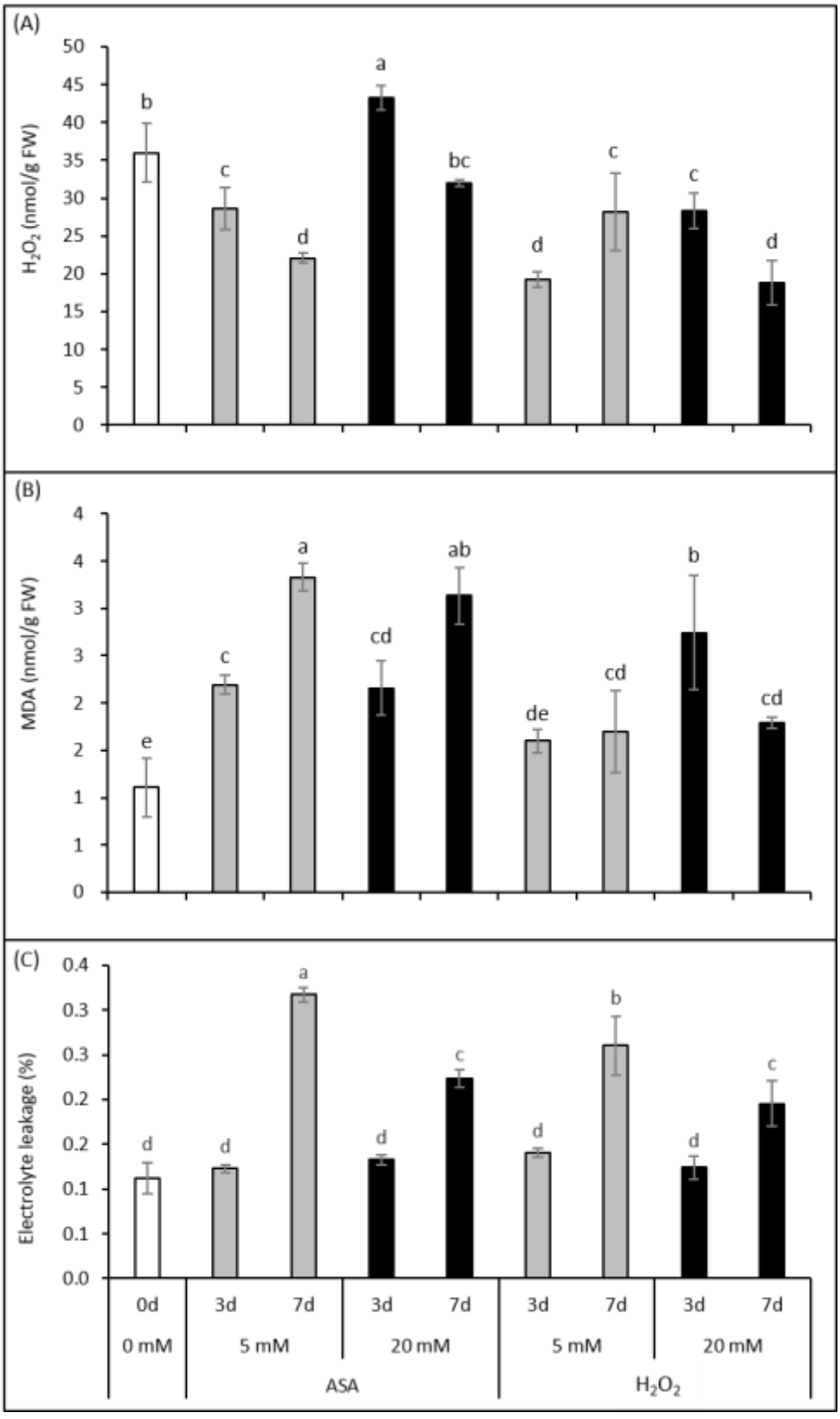

Fig. 14. Influence of ascorbate (ASA) and hydrogen peroxide (H_2O_2) on the oxidative stress indicators. (A): hydrogen peroxide (H_2O_2), (B): lipid peroxidation (MDA), (C): electrolyte leakage. The different letters showed significant differences from each other at $p \leq 0.05$.

5.2.4 Influence of ASA and H_2O_2 treatments on the amount of endogenous ASA and non-protein thiols

The amount of ASA and DHA was significantly enhanced by 7-day 20 mM ASA addition (Fig. 15A & B). A pronounced reduction in the DHA content was recorded after 7-day applications of 5 mM ASA and 20 mM H_2O_2 (Fig. 15B). The minimum ratio of DHA/ASA was noticed after 3-day 20 mM ASA and 7-day 20 mM H_2O_2 treatments (Fig. 15C).

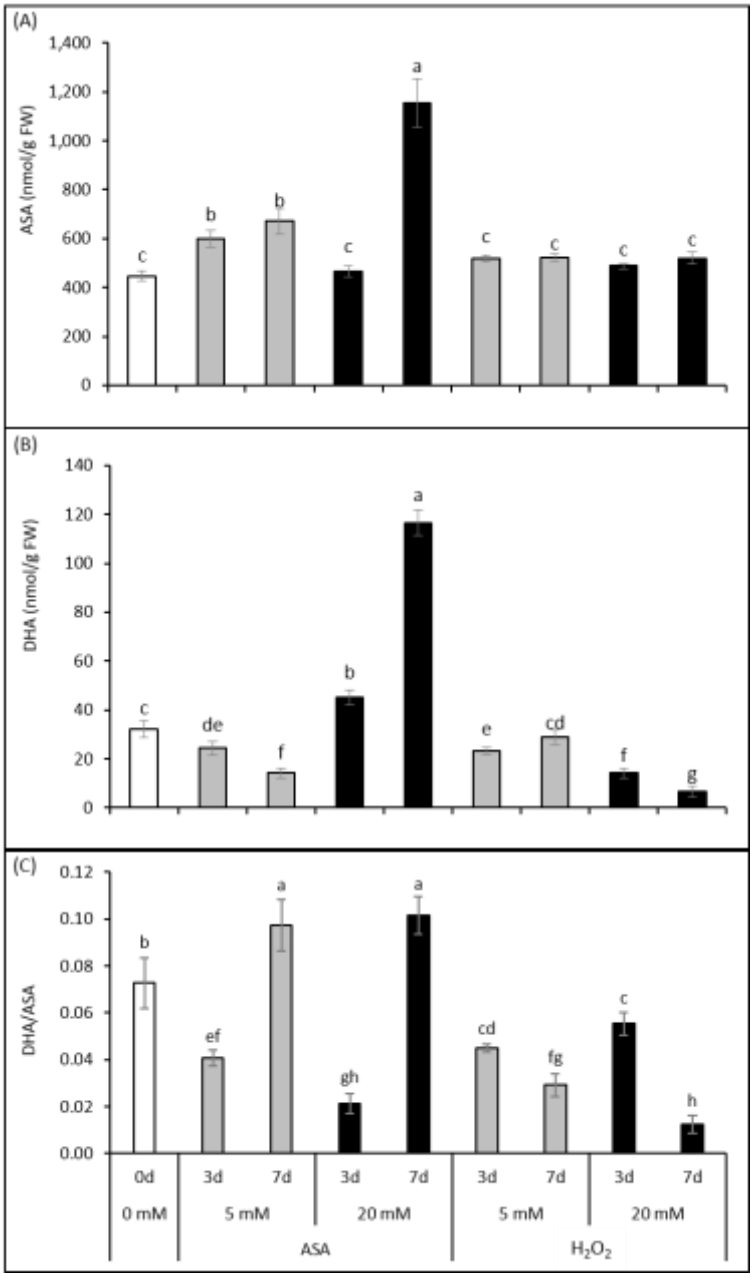

Fig. 15. Effect of ascorbate (ASA) and hydrogen peroxide (H_2O_2) on endogenous levels of ascorbic acid. (A): ascorbic acid contents (ASA), (B): dehydroascorbic acid contents (DHA), (C): DHA/ASA ratios. The different letters showed significant differences from each other at $p \leq 0.05$.

Compared to the untreated plants, a significant reduction in Cys (GSH precursor) amount was only seen following 7-day 20 mM ASA application (Fig. 16A). Following 5 mM ASA addition, the CySS content and the ratio of CySS/Cys were higher after 3 d and smaller after 7 d relative to control, whereas such changes were not observed after 5 mM H_2O_2 application (Fig. 16B&C). 20 mM ASA enhanced and 20 mM H_2O_2 declined these parameters throughout the experiment.

Both compounds substantially decreased the amount of GSH except for the 7-day addition of H_2O_2 (Fig. 16D). The tendencies of variations in GSSG amount and GSSG/GSH ratio were parallel to the changes in CySS and Cys/CySS (Fig. 16E&F). Overall, it was noticed that there was a large difference in the influences of 20 mM ASA and H_2O_2.

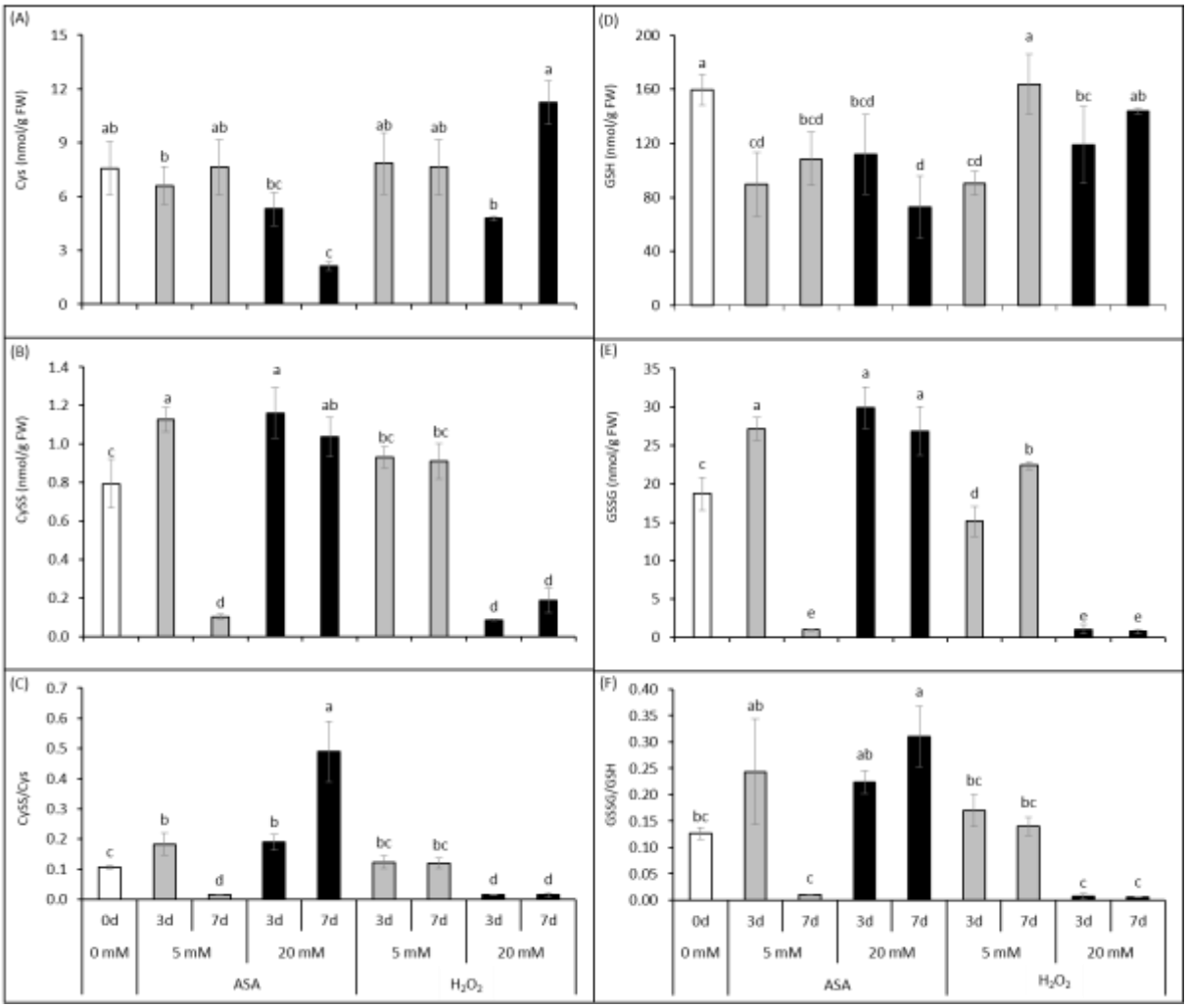

Fig. 16. Influence of the ascorbate (ASA) and hydrogen peroxide (H_2O_2) on the redox state and amount of the cysteine and glutathione. (A): Cys: cysteine (B): CySS: cystine, (C): ratio of the

two forms, (D): GSH: glutathione, (E): GSSG: glutathione disulphide, (F): ratio of the two forms. The different letters showed significant differences from each other at $p \leq 0.05$.

Among the treatments, the longer 20 mM ASA application reduced only significantly the hmGSH amount (Fig. 17A). Nonetheless, it was increased by 20 mM of H_2O_2 at both sampling points (3 and 7 days). The amount of hmGSSG was higher after 5 mM and 20 mM ASA applications under 3 and 7 days, respectively (Fig. 17B). Excitingly, longer application (7 days) of ASA with lower concentration (5 mM) or shorter treatment (3 days) with higher concentration (20 mM) considerably declined its level. Similarly, 20 mM H_2O_2 decreased the hmGSSG content significantly after 3 days. The ratio of hmGSSG/hmGSH was higher following 5 mM ASA addition for 3 days, while 5 mM and 20 mM H_2O_2 for 3 days, and 20 mM ASA- and H_2O_2-additions for 7 days decreased it (Fig. 17C).

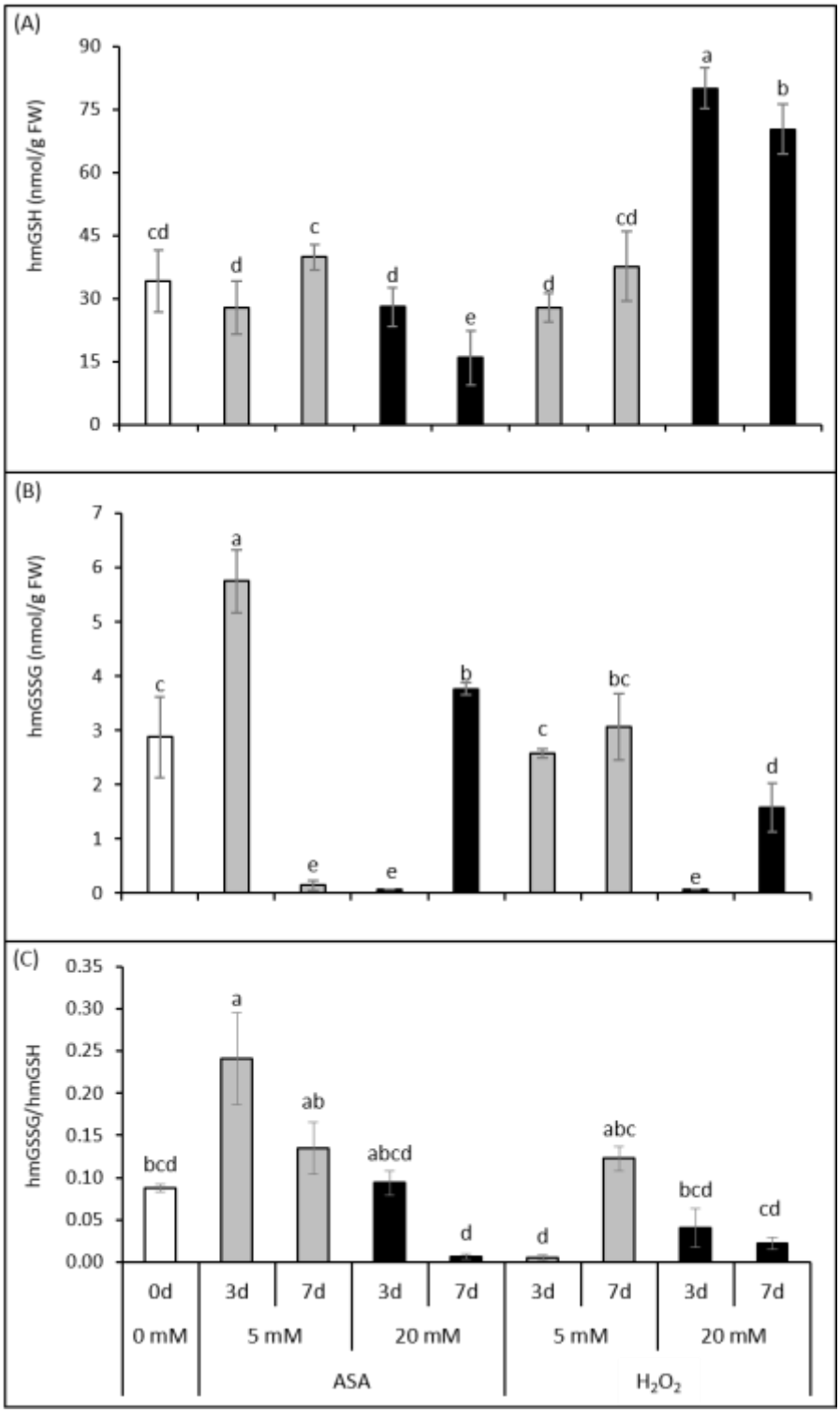

Fig. 17. Effect of ascorbate (ASA) and hydrogen peroxide (H_2O_2) on the redox state and amount of the hydroxymethylglutathione. (A): hmGSH: hydroxymethylglutathione, (B): hmGSSG: hydroxymethylglutathione disulphide, (C): hmGSSG/hmGSH: ratio of two forms. The different letters showed significant differences from each other at $p \leq 0.05$.

The $E_{DHA/ASA}$ value was increased by both concentrations of ASA after 7-day supplementation, but it was decreased by the other treatments except for the 5 mM H_2O_2 (7 days) application (Fig. 18A).

Following 5 mM ASA treatment, the $E_{CySS/Cys}$ value was higher after 3 d and smaller after 7 d relative to the control (Fig. 18B). The 20 mM H_2O_2 decreased it at either time point. The tendencies of the variations in the $E_{GSSG/GSH}$ value were parallel to those of the $E_{CySS/Cys}$ values (Fig. C). Compared to the control plants, 5 mM ASA (3 days) and 20 mM ASA (7 days) increased the $E_{hmGSSG/hmGSH}$ value significantly, however, it was reduced by the other treatments except for 5 mM H_2O_2 (3 d and 7 d) (Fig. 18D).

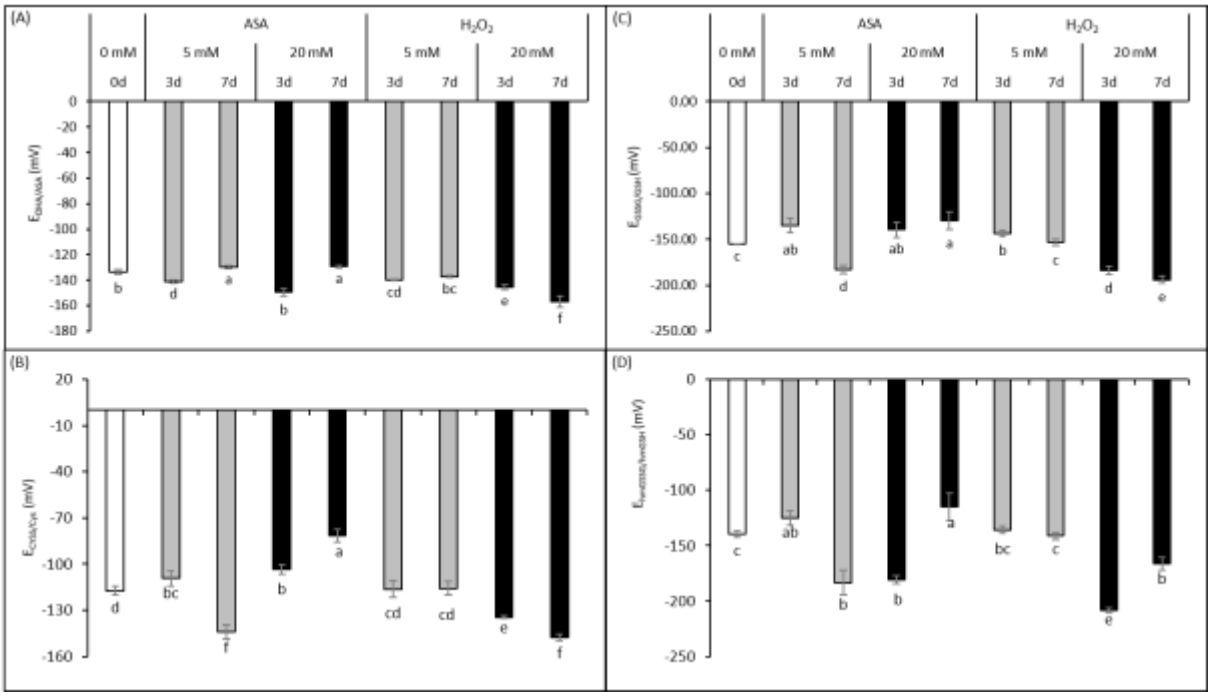

Fig. 18. Effect of ascorbate (ASA) and hydrogen peroxide (H_2O_2) on half-cell reduction potentials (E). (A): $E_{DHA/ASA}$: E-value of the dehydroascorbic acid/ascorbic acid redox couple, (B): $E_{CySS/Cys}$: E-value of the cystine/cysteine redox couple, (C): $E_{GSSG/GSH}$: E-value of the glutathione disulphide/glutathione redox couple, (D): $E_{hmGSSG/hmGSH}$: E-value of the hydroxymethylglutathione disulphide/ hydroxymethylglutathione redox couple. The different letters showed significant differences from each other at $p \leq 0.05$.

5.2.5 ASA- and H_2O_2-induced variations in the antioxidant enzymes

The activities of the studied antioxidant enzymes were augmented by most treatments except for GR and MDHAR (Fig. 19). Regarding APX, this change was considerably larger after using 20 mM ASA relative to the 20 mM H_2O_2 addition (Fig. 19A). The activity of MDHAR was only larger following the 3-day 5 mM application of ASA compared to the control (Fig. 19B). Interestingly, the activity of DHAR was up-regulated by all treatments except for the 7-day 20 mM ASA application (Fig. 19C). Likewise APX, the GR activity was greater following 20 mM treatment ASA than 20 mM H_2O_2 addition (Fig. 19D). The CAT and GST activities were enhanced by the addition of both compounds except for CAT activity following 7-day 5 mM H_2O_2 application and GST activity after 3-day 20 mM H_2O_2 treatment (Fig. 19E&F). The paramount changes in CAT and GST activities were observed following 20 mM and 5 mM H_2O_2 addition for 7 days, respectively.

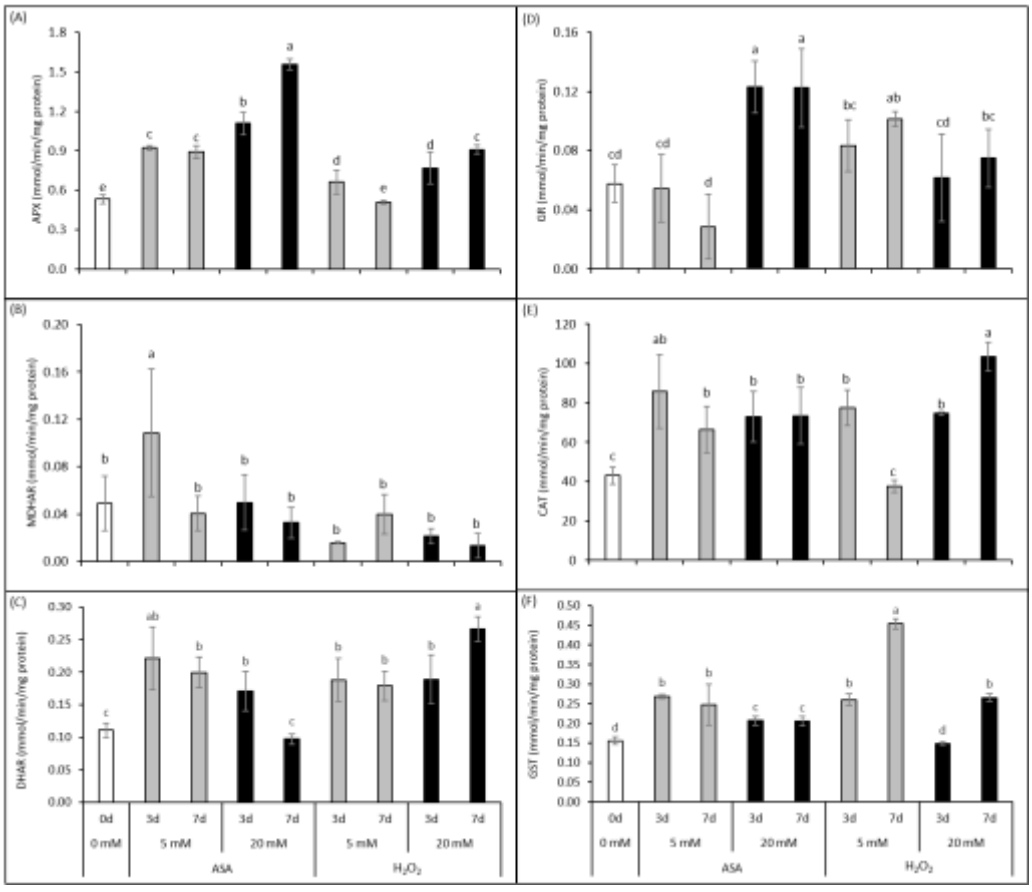

Fig. 19. Influence of the ascorbate (ASA) and hydrogen peroxide (H_2O_2) on the activities of antioxidant enzymes. (A): APX: ascorbate peroxidase, (B): MDHAR: monodehydroascorbate reductase, (C): DHAR: dehydroascorbate reductase, (D): GR: glutathione reductase, (E): CAT: catalase, (F): GST: glutathione S-transferase. The different letters showed significant differences from each other at $p \leq 0.05$.

5.2.6 Modifications in the metabolic profile of the wheat seedlings by ASA and H_2O_2

Altogether, 31 metabolites were determined in the wheat shoot samples and most of them were affected by the exogenous application of ASA and H_2O_2 (Fig. 20, Table S6A). According to hierarchical clustering, the metabolite profiles following all treatments were in a separate group from the control. The ASA- and H_2O_2-treated plants made separate groups except for the 7-day 5 mM ASA and H_2O_2 additions. The paramount difference was detected between the 7-day 20 mM ASA and H_2O_2 application, which were found on the two distinct borders of the heatmap.

Based on the resemblances of the redox compounds-inducted variations in the number of metabolites, there were three major groups. The first cluster is mainly comprised of organic acids and sugars, which displayed no or moderate modifications after the different treatments compared to the control. The second cluster contains sugars, organic acids, and amino acids whose levels exhibited great variations compared to the control and each other. The exogenous application of both compounds caused a reduction in the amounts of quinic acid and phosphoric acid. The third cluster mainly contained sugars and amino acids. The amount of sucrose, mannose, and galactose was only slightly affected by the treatment and they were present in greater amounts than the other compounds in all the measured samples. The level of the other compounds in this cluster was reduced by the longer treatments.

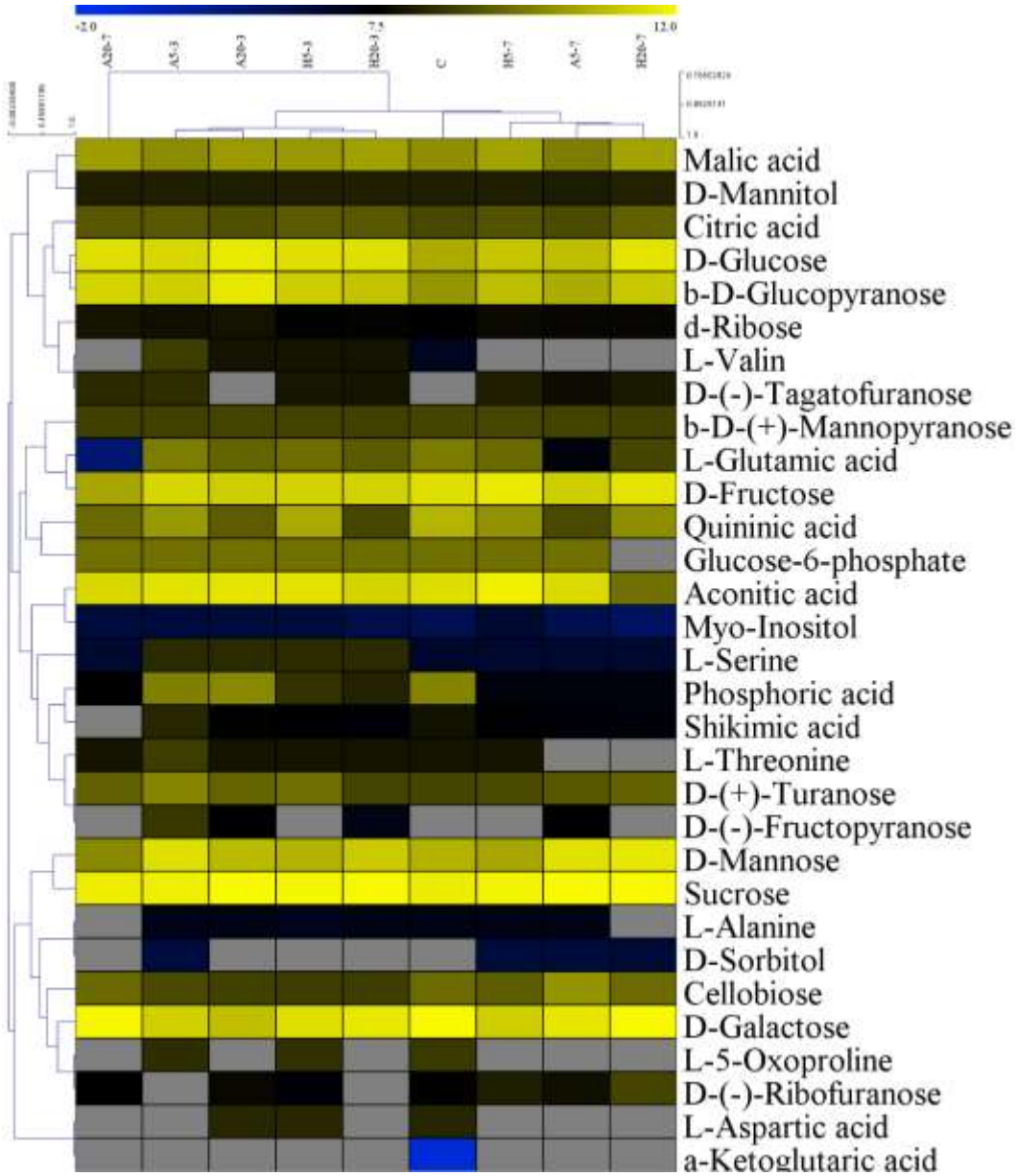

Fig. 20. Influence of the ascorbate (ASA) and hydrogen peroxide (H_2O_2) on the metabolite levels. The C, A5-3, A5-7, A20-3, A20-7, H5-3, H5-7, H20-3, and H20-7 treatments indicate the control, 5 mM ASA for 3 d, 5 mM ASA for 7 d, 20 mM ASA for 3 d, 20 mM ASA for 7 d, 5 mM H_2O_2 for 3 d, 5 mM H_2O_2 for 7 d, 20 mM H_2O_2 for 3 d and 20 mM H_2O_2 for 7 d treatments, respectively. ASA and H_2O_2 denote ascorbate and hydrogen peroxide, respectively. The grey boxes indicate where no amount was found. The data and their statistical analysis are shown in Table S6A.

5.2.7 ASA- and H_2O_2-dependent variations in free amino acid levels

The total protein amount had a similar reduction following all the applied treatments (Fig. 21A), nevertheless, the levels of the total free amino acid were differently influenced by the different

treatments (Fig. 21B). Great difference was found between the effect of the two compounds following 7 days, since 20 mM ASA decreased and 20 mM H_2O_2 greatly increased the amount of total free amino acid.

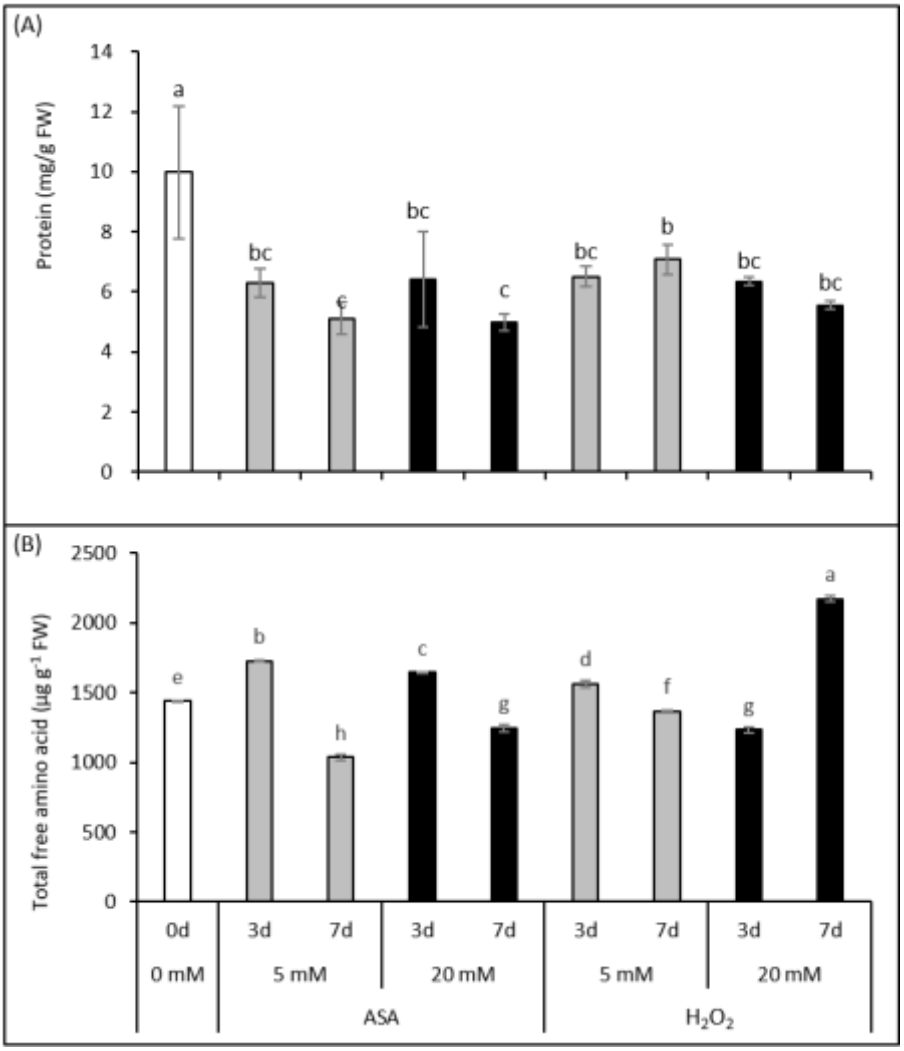

Fig. 21. Effect of the ascorbate (ASA) and hydrogen peroxide (H_2O_2) on protein and total free amino acid contents. (A): protein contents, (B): total free amino acid contents. The different letters showed significant differences from each other at $p \leq 0.05$.

Comparing the influences of various treatments on individual amino acid levels by hierarchical clustering, it is shown that the untreated and 5 mM H_2O_2-treated (at both sampling points) plants were clustered together. In the other main cluster, the 7-day 20 mM ASA application was separated into a sub-cluster from all other treatments. There was a large distance between the clusters of 7-day 20 mM ASA and H_2O_2 treatments (Fig. 22, Table S6B). Likewise, the total free amino acid amount and the levels of the individual amino acid were also lower after 20 mM ASA application than that of 20 mM H_2O_2 following 7 days.

Based on the clustering of redox treatment-dependent variations, the free amino acids made two groups. The first cluster contained 16 amino acids in which, the amino acid levels were parallel following most of the treatments except for 5- and 20-mM ASA application for 7 days. The Asn, Pro, 1mHis (1-methyl-L-histidine), His, Cysta (cysteamine), Val, 3mHis (3-methyl-L-histidine), Phe, Tyr, and Arg were placed in the second cluster in which great variations were observed in amino acid levels of individual groups; the most substantial divergence was perceived between the 5 mM H_2O_2 for 3 days and 20 mM ASA for 7 days treatments. Interestingly, the same amino acid family members were clustered in separate clusters based on the influence of the different treatments as revealed for the aspartate and glutamate family.

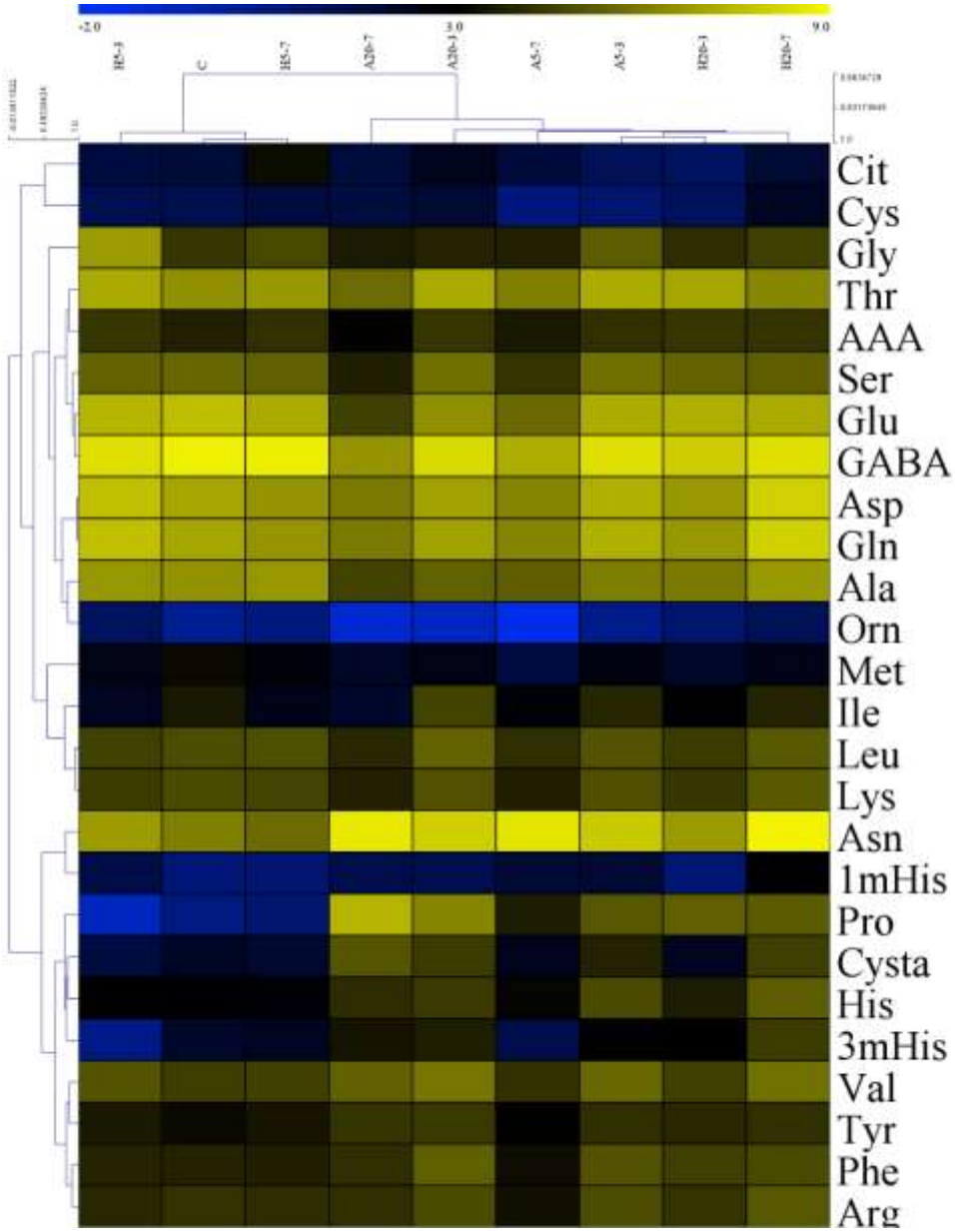

Fig. 22. Effect of the ascorbate (ASA) and hydrogen peroxide (H_2O_2) on the amino acid levels. The C, A5-3, A5-7, A20-3, A20-7, H5-3, H5-7, H20-3, and H20-7 treatments refer to control, 5 mM ASA for 3 d, 5 mM ASA for 7 d, 20 mM ASA for 3 d, 20 mM ASA for 7 d, 5 mM H_2O_2 for 3 d, 5 mM H_2O_2 for 7 d, 20 mM H_2O_2 for 3 d and 20 mM H_2O_2 for 7 d treatments, respectively. The ASA and H_2O_2 represent ascorbate and hydrogen peroxide, respectively. The data and their statistical analysis are shown in Table S6B.

5.2.8 ASA- and H_2O_2-dependent transcriptional modulation of glutathione and amino acid metabolism

The different cluster of the transcript levels for untreated plants highlights a pronounced influence of all the treatments (Fig. 23, Table S6C). This effect differed under ASA and H_2O_2 application as indicated by the separate clusters, except for 5 mM ASA and 20 mM H_2O_2 after 7 days. The main influence on the transcript levels was exhibited by the application of 3-day 5 mM H_2O_2 and 7-day 20 mM ASA treatments as indicated by the great decline and rise in the expression levels of genes, respectively.

Based on resemblances in the expression variations of the individual genes, two main clusters were formed (Fig. 23). In the first cluster, most of the genes were suppressed or not influenced by most of the treatments except *OrnATF*. This cluster is comprised of almost all the genes that regulate the metabolism of amino acids and those encoding the organellar enzymes of the antioxidant system or OPPP. In the second cluster, increased transcript levels were recorded in general. This cluster contained various genes encoding cytoplasmic enzymes of the antioxidant system.

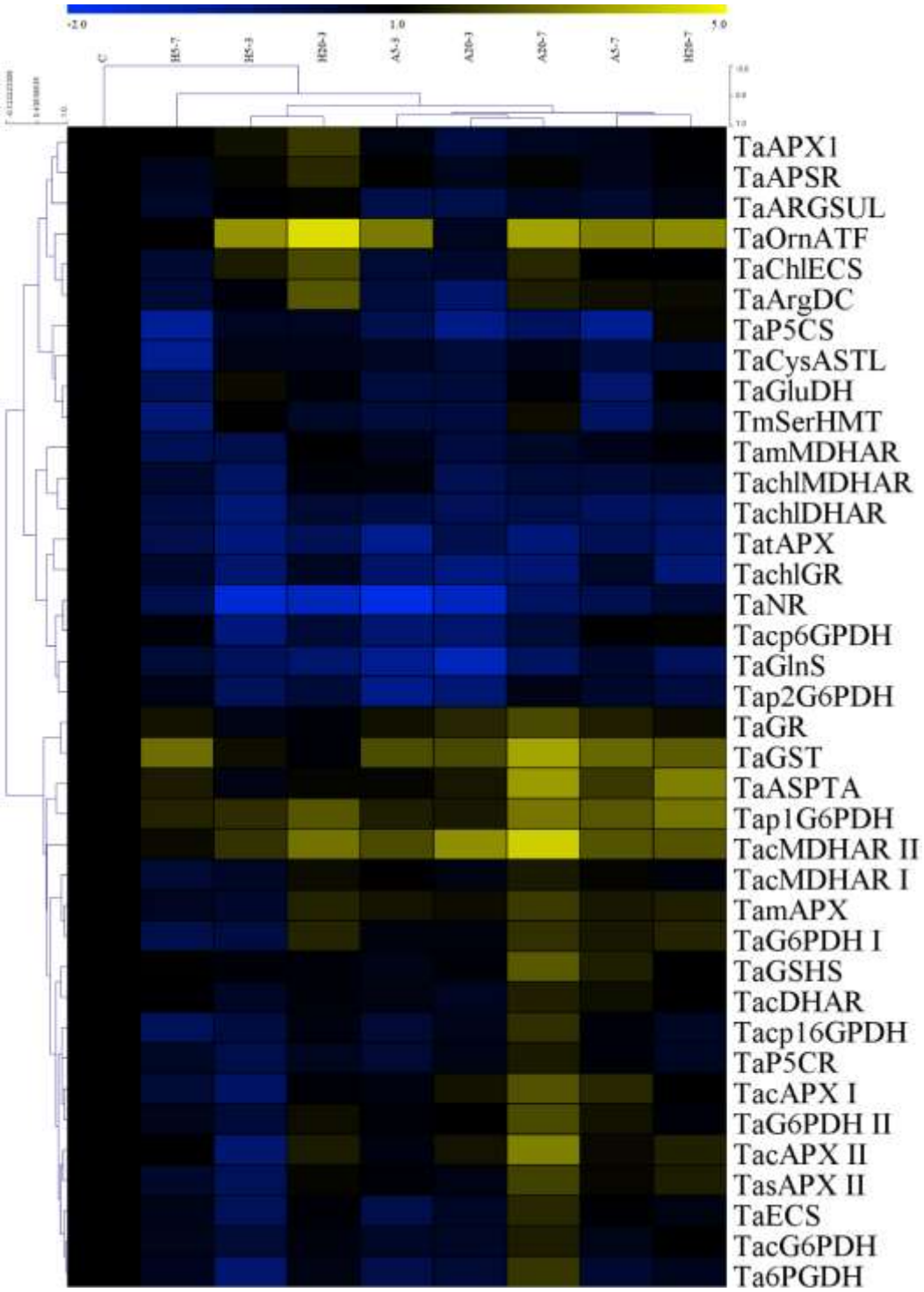

Fig. 23. Influence of the ascorbate (ASA) and hydrogen peroxide (H_2O_2) on expression levels of the genes related to the examined metabolites. The C, A5-3, A5-7, A20-3, A20-7, H5-3, H5-7, H20-3 and H20-7 treatments refer to control, 5mM ASA for 3 d, 5mM ASA for 7 d, 20mM ASA for 3 d, 20mM ASA for 7 d, 5mM H_2O_2 for 3 d, 5mM H_2O_2 for 7 d, 20mM H_2O_2 for 3 d and 20mM H_2O_2 for 7 d treatments, respectively. The ASA and H_2O_2 represent ascorbate and hydrogen peroxide, respectively. The data and their statistical analysis are shown in Table S6C.

6 Discussion

6.1 Effect of light conditions on the glutathione metabolism during cold acclimation

6.1.1 Light intensity-induced modifications in the glutathione metabolism and their role in the cold acclimation process

The light intensity had a considerable impact on glutathione metabolism, which is important in the regulation of the redox environment throughout the cold acclimatisation process, as demonstrated by significant increases in the amount of γEC (precursor of GSH) and CysGly (degradation product). The multivariate analysis of variance also presented the influence of the light intensity on the metabolism of glutathione (γEC, CysGly, hmGSH amounts, GR activity) (Table S5A). Since high light intensity considerably influenced the synthesis and the degradation of the glutathione, its levels displayed only slight up-regulation or no change. Conversely, this change was higher under normal light (white light) intensity-grown plants, except for the Ch genotype. These findings are corroborated by earlier results about the substantial increase in the levels of GSH after the cold acclimation period (first week) in wheat plants [277]. Together with changes in glutathione pool size, the alterations in its redox state are also very important in the acclimation process. A positive correlation (r: 0.62) was observed between the electrolyte leakage level (an indicator of freezing tolerance) and relative alteration in the redox state of the glutathione (GSSG/GSH ratio) after cold in the normal light (white light) intensity-grown plants (Table S4A). The high light enhanced this correlation (r: 0.98), suggesting the positive effect of light intensity on the redox-reliant acclimation processes (Table S4B). Furthermore, a parallel enhancement (0.5 to 0.9) was detected in the correlation between the freezing tolerance level and the variation in the GR activity controlling the redox state of glutathione. The redox state of other thiols involved in the glutathione metabolism may also influence the redox environment in the tissues. Fascinatingly, the cold-dependent modifications in the redox state of the γEC (GSH precursor) and CysGly (degradation product) were also allied with freezing tolerance levels as revealed by the correlation analysis (Table S4). Nevertheless, the CySS/Cys ratio exhibited a positive relationship with the level of freezing tolerance under normal light (white light) intensity, whereas the CySSGly/CysGly ratio showed a negative relationship. The trend of these correlations was different in the case of high-light conditions. The freezing tolerance-reliant, GSH-related valuable influence of the high light intensity on cold acclimation through its impact on the antioxidant potential is also shown by the constant activity of the ASA-GSH enzymes in freezing-tolerant wheat genotypes during the cold acclimation period. Contrarily, their activity was down-regulated in the sensitive wheat

genotypes. Even though, high light intensity adjusted the glutathione-associated redox system in a freezing tolerance-dependent manner and only the freezing-tolerant wheat genotypes grew based on shoot fresh weight recordings during the cold acclimation period. The impact of the light intensity on the antioxidant activities was also investigated in *Arabidopsis* and maize plants [255,278], in which the contribution of the high light intensity to the induction of the cold acclimation processes was observed [279]. In addition, the enhanced drought tolerance was also associated with the increased light intensity in wheat since it activated numerous protective and adaptive responses including higher production of proline and other amino acids [280].

Based on our correlation analysis, the different control of the individual members of a gene family by the light quantity could be proposed. However, the influence of the light intensity on cold-dependent/regulated modifications in glutathione metabolism could not be revealed usually at transcript levels, which suggests the post-transcriptional mediation of this mechanism. Hence, γEC amount exhibited a much higher cold-dependent up-regulation in high light in contrast to the normal one in all the examined wheat genotypes; a similar change was only observed for the γEC synthase gene in the CS and Mir wheat genotypes. Even with the up-regulation of the transcriptional levels of GR and APX coding genes in most of the cases under cold treatment, the activities of these enzymes were reduced or unchanged. Post-transcriptional regulation of γEC precursor (Glu) production is also likely since the cold-dependent inhibition of the transcriptional level of GS2 could not prevent the greater enhancement in γEC amount during the acclimation period. A remarkable influence of light intensity on glutathione metabolism-associated transcriptional levels was observed in earlier experiments in wheat as well [281]. The authors found a substantial decrease in the expression of genes encoding GS2, APX1, and GST enzymes. They were down-regulated by 60% in the high light intensity when compared with normal light (white light) intensity. They further observed a 86% down-regulation of the O-acetylserine (thiol) lyase (*ASTL*) gene following far-red light. In barley, the light intensity also influenced the cold acclimation-induced transcripts [282]. In *Arabidopsis*, the expression levels of the antioxidant enzyme coding transcripts (*DHAR*, *APX1*, and *GST*) were up-regulated under high light [283]. Thus, the earlier investigation highlighted the importance of light intensity-dependent regulation of transcripts of the antioxidant genes that also have crucial roles in the cold acclimation processes of plants [283].

The light intensity-dependent modulation of glutathione metabolism and the related redox environment was associated with freezing tolerance (characterized by electrolyte leakage

measurements) based on the correlation analysis. Similarly, high light intensity was able to improve freezing tolerance in six other wheat genotypes with different freezing tolerances [87]. Conversely, inadequate light in the acclimation period could increase the sensitivity of winter cereals to low temperatures [284]. Moreover, stunted Arabidopsis growth was observed under low light intensity during cold acclimation, but photoreceptor mutants and wild-type plants showed an inverse growth trend [285]. During cold acclimation processes, the critical role of light intensity was also verified in other plant species [279,286].

6.1.2 Far-red light-mediated alterations in glutathione metabolism and their association with cold acclimation

Besides light intensity, the spectrum could be also important in the control of glutathione metabolism. As shown by the multivariate analysis of variance, the far-red light greatly affected the glutathione metabolism (thiol amounts and ASA-GSH cycle-associated enzymes activities) (Table S5B). The influence of supplementary far-red light on cold-mediated alterations on GSSG/GSH ratio varied between freezing-susceptible and -tolerant wheat genotypes, but a similar variation was not detected for the redox state of its precursor (γEC) and the degradation product (CysGly). Interestingly, the positive relationship between freezing tolerance level and cold-mediated changes in the thiol/thiol disulphide ratios in normal light intensity turned into negative values in the supplementary far-red light regimen (Table S4C). Moreover, far-red light also influenced the correlation between the freezing tolerance level and cold-mediated alterations in ratios of GSSG/GSH and hmGSSG/hmGSH, since the relationship values varied from +0.62 and +0.34 (normal light intensity) to -0.82 and -0.87 (supplementary far-red light), respectively (Table S4A&C). This variation can be clarified by the cold-induced enhancement in the amount of (hm)GSSG and the ratio of (hm)GSSG/(hm)GSH in the two sensitive wheat genotypes, which was not observed in the two tolerant ones. As a result, the total (hm) GSH pool converted into more oxidised during cold, which can contribute to the lower level of freezing tolerance of CS and CD wheat genotypes. In this more oxidising redox environment the activity of proteins and the expression of many transcripts will be smaller [223,287]. Although a far-red light-dependent adjustment of the activities of antioxidant enzymes for the control of the redox environment was observed in salt-stressed tomato [184], a such spectral control of the activities was not observed in cold-treated wheat. In addition, the related transcript levels were also not influenced in the present experiments in wheat. Interestingly, the effect of far-red light on the expression of cold acclimation-associated and glutathione metabolism-related genes was verified in previous experiments in barley and wheat [281,282]. In another study, the involvement of far-red light in the regulation of oxidation-reduction

processes was also observed in *Eustoma grandiflorum* [288]. The authors further found that the far-red light supplementation up-regulated the expression of DEGs associated with GA and auxin biosynthesis, bHLH transcription factor, and internodal cell elongation of *Eustoma* plants.

Although the longer application of supplementary far-red light (14 d) before cold acclimation did not modify the growth indices and electrolyte leakage in this study, its short use had a positive influence on freezing tolerance level in earlier experiments as indicated by the membrane injury measurements [282,289]. In another experimental system, the use of lower red/far-red also resulted in enhanced freezing tolerance of the *Arabidopsis* plants when cultivated at 16 °C [290]. The activation of the CBF transcription factors was accountable for this advancement, which are responsible for the regulation of numerous signals together with those developing from the variations in the redox state [291]. The influence of far-red light on the cold-dependent modifications of the *TaCBF17* expression was also revealed in the current experimental system. Interestingly, there was a moderate positive relationship (r: 0.39) between *TaCBF17* expression and electrolyte leakage in white light, and a high negative one (-0.76) was detected in far-red light (Table S4A&C). This observation indicates that supplementary far-red light may induce the cold acclimation process through its effect on *TaCBF17*. In another study, it was revealed that PHYTOCHROME-INTERACTING TRANSCRIPTION 4 directly activated GA-INSENSITIVE 4, (*SlGAI4*), which acts as a positive modulator in the regulation of *CBF1* transcript level and low red/far-red-mediating cold tolerance in tomato plants during cold stress [292]. In *Setaria viridis*, *the* CO-like transcription factor (*BBX2*) was identified as responsive to chilling and low far-red light through coexpression network analysis [293].

6.2 Modification of redox environment by ASA and H_2O_2

6.2.1 Effect of ASA and H_2O_2 on photosynthesis, ROS production, and membrane stability

Photosynthesis is very sensitive to the alterations in the redox state of the leaf tissues [209]. Correspondingly, its modification by ASA and H_2O_2 reduced the CO_2 assimilation rate (Pn) of wheat seedlings in both time- and concentration-dependent manner. The inhibition of Pn is certainly related to the stomatal closure on the 3rd day of the treatments as shown by the decline of g_s. Additionally, stomatal closure also decreased the transpiration rate in wheat seedlings. The increment of C_i level following longer (7 days) applications (relative to 3 days of treatments) highlighted that some non-stomatal restriction can also be involved in the reduction of CO_2 assimilation. The chlorophyll *a* amount also declined following ASA and H_2O_2

treatments (7 days), which resulted in moderate, but substantial variations in F_v/F_m fluorescence attribute. In sum, the photosynthetic attributes, coupled with pigment contents show mild stress in wheat, which is about to get even worse if the addition of treatments (particularly ASA and 20 mM H_2O_2) would have been sustained for an extended period. The disturbed photosynthetic system may also be involved in the enhancement of lipid peroxidation which in turn resulted in larger electrolyte leakage (increased membrane injury/reduced membrane stability) following 7 days of ASA and H_2O_2 applications. Contrary to lipid peroxidation, the endogenous H_2O_2 levels did not change following most treatments, which might be due to the proficient initiation of the ASA-GSH cycle-associated enzymes for its scavenging. As detected in wheat plants, the ASA application (8 mM for 5/10 days) also enhanced lipid peroxidation in the *Arabidopsis* plants [105]. In parallel, its influence on lipid peroxidation was observed even after adding it in lower concentration for a short period (0.5 mM for 2 days) in tomato plants [210]. In contrast to wheat plants, the ASA caused even the increase in chlorophyll content and the reduction in membrane injury in tomato plants. These latter findings suggest the differential effects of ASA application on membrane stability and chlorophyll pigments in different plant species which is heavily dependent on the amount and duration of its application. Likewise, ASA, the effect of H_2O_2 application on the photosynthetic system also varied in wheat plants and other plant species as indicated by the non-significant effect of H_2O_2 application on the chlorophyll pigments and photosynthetic rate in cucumber plants [211]. Additionally, the 3-week-long treatment with 8 mM H_2O_2 did not affect the chlorophyll pigments and chlorophyll fluorescence parameters in *Ficus deltoidae* leaves [212]. Taken altogether, it is proposed that ASA and H_2O_2 had different influences on the growth and development of the plants based on the photosynthetic and ROS data.

6.2.2 Different modulation of redox state by ASA and H_2O_2

The current experiments revealed that the excessive presence of an oxidant or an antioxidant (reductant) could disturb the photosynthetic system, the proper function of which is essential for the maintenance of redox homeostasis. Likewise in wheat, the application of different reductants (ASA and GSH) and an oxidant (H_2O_2) resulted in increased and declined GSSG amount and GSSG/GSH ratio in *Arabidopsis*, respectively [267]. Interestingly, following a longer application (7 days) of ASA and H_2O_2 in higher concentration (20 mM), an overcompensation of the redox changes in contrasting directions was found. Thus, after ASA application, the GSH precursor (cysteine), GSH, and ascorbate pools converted into more oxidised form, and after H_2O_2 treatment they became more reduced relative to control as

indicated by their oxidised forms amounts, their redox states (oxidised/reduced ratios) and half-cell reduction potential data. In animal systems, a higher accumulation of ROS was also noted after treatment with reductants. This might be due to the deficiency of the efficient electron acceptor in the mitochondrial electron transport chain [294]. The more oxidizing redox environment following a 7-day 20 mM ASA application activated APX and GR enzymes to eliminate endogenous H_2O_2 levels. Transcriptional activation of the glutathione metabolism-associated enzymes was shown by similar alterations at the gene expression levels. The diverse redox modifications following the higher (20 mM) concentrations of both compounds (ASA and H_2O_2) were associated with the great inhibition of shoot and root growth based on the fresh weight data.

The current findings show the synchronized adjustment of the ASA-GSH cycle components, which was also observed in poplar plants by the enhancement of the endogenous ASA amount after GR overexpression [295]. In earlier studies, the efficient activation of the antioxidant enzymes such as SOD, CAT, and POD was seen after ASA application in wheat and other plant species [296–298]. The considerable influence of ASA application on the antioxidant system was also evidenced in an ASA-deficient *vtc-1 Arabidopsis* mutant, where MDAR and DHAR activities were reduced [299]. As noticed in the current experimental system, the exogenous H_2O_2 decreased the levels of endogenous ROS (superoxide radical and MDA) in other wheat genotypes, too [300,301]. In addition, mung bean plants showed increased GSH levels after H_2O_2 application [302]. These outcomes corroborate that reductants and oxidants (among them ASA and H_2O_2) substantially influence several components of the redox system, and have a significant role in the regulation of the redox environment to the changing environmental conditions.

6.2.3 ASA- and H_2O_2-induced changes at the metabolite level

The overcompensation of redox shifts after the application of ASA and H_2O_2 can originate from the effect of the ASA and H_2O_2 on NAD(P)H producing/consuming metabolic pathways, including glycolysis, saccharopine pathway and tricarboxylate cycle [25,303]. The unchanged glucose-6-P levels after ASA addition may limit its use for OPPP and/or glycolysis (Fig. 24). The decrease in α-aminoadipate and Lys levels by the ASA application may show the suppression of the saccharopine pathway. Consequently, the production of NAD(P)H in these pathways perhaps remained unchanged or became smaller. Its rise was not essential, since the exogenous application of ASA might guarantee sufficient reducing power and the less NADPH production was also enough for the reduction of its oxidised form. Thus, less $NADP^+$ was

formed during the DHA/MDHA reduction, which was not sufficient to accept all the electrons from the photosynthesis process, and ROS production was higher as mentioned earlier. After the application of H_2O_2, inhibition of glucose-6-P levels is an indication of the rise in the OPPP [NAD(P)H production] and/or glycolysis, which was noticed in various plant species under oxidative stress [208,223]. This adjustment lead to an increase in the levels of NAD(P)H, which could be involved in the rapid scavenging of H_2O_2 in the ASA-GSH cycle during which sufficient $NADP^+$ was accumulated for accepting electrons from the photosynthesis process, resulting in the reduction of the ROS production.

Treatment with ASA and H_2O_2 differently affected several metabolites of glycolysis and tricarboxylate cycle, which are starting points for the synthesis of amino acids. Consequently, the levels of most amino acids (except for Pro, Phe, Tyr, Val, Cys, Asn, Arg, oxoproline, and citrulline) declined under ASA application and increased or remained unchanged after H_2O_2 application (Fig. 24). Likewise wheat, certain metabolites levels in TCA cycle and glycolysis were also reduced in Arabidopsis plants under methyl viologen-induced oxidative stress [209]. Interestingly, the Pro presented a much larger rise, 123-fold and 27-fold when compared to other amino acid levels (7-fold proliferation), following ASA and H_2O_2 treatments, respectively (Fig. 24), which highlights its significance in the response to the redox changes. Alike wheat, the ASA application also increased the Pro level in the flax cultivars [22]. Its production is a NADPH-consuming process, while during its degradation NADPH is synthesized; henceforth, it is potentially involved in the adjustment of the cellular redox state [304]. The Glu serves as a precursor of Pro and GSH and has significant involvement in the interconnection of the Pro- and GSH-dependent redox adjustments. The larger rise in Pro level following ASA application was mainly accompanied by lower GSH and Glu content, and its slighter up-regulation after H_2O_2 addition was linked with unaffected Glu and GSH amounts (Fig. 24).

The redox response of the carbohydrate metabolism was shown by the diverse influences of ASA and H_2O_2 application on the levels of certain carbohydrates (sucrose, glucose-6-P, fructose, and mannose) (Fig. 24). Thus, being a precursor of ASA, the mannose amount was inhibited after ASA treatment and enhanced under H_2O_2 application. Although the organic acids of the citrate cycle (citrate and malate) have very significant roles in redox metabolism regulation [209,238], their levels remained unchanged after treatments by both chemicals except for the decrease in the alpha-ketoglutarate levels. This decline could arise from its larger use for the synthesis of the amino acids of the glutamate family [209]. After ASA addition, the great reduction in the Glu and alpha-ketoglutarate can be explained by the extremely large

increase in Pro accumulation (123-fold rise). Regarding H_2O_2 application, along with the smaller increase in Pro level (27-fold), the Arg and Gln levels became also greater which can be responsible for the constant Glu level.

The diverse influence of the ASA and H_2O_2 applications on ASA-GSH cycle-linked redox components and their associations with metabolite levels was also confirmed by the correlation analysis (Table S7). After the H_2O_2 application, the synchronised adjustment of the investigated components of the ASA-GSH cycle is indicated by their strong or very strong relationships except for ASA content. Nonetheless, such change was not noticed following ASA addition, because among the 36 comparisons only in the case of 10 were found strong or very strong correlations. Considering the metabolites presented in Fig. 24, many of them were inversely affected by ASA and H_2O_2 treatments as shown by the trends and levels of their correlations with the redox components (Table S7).

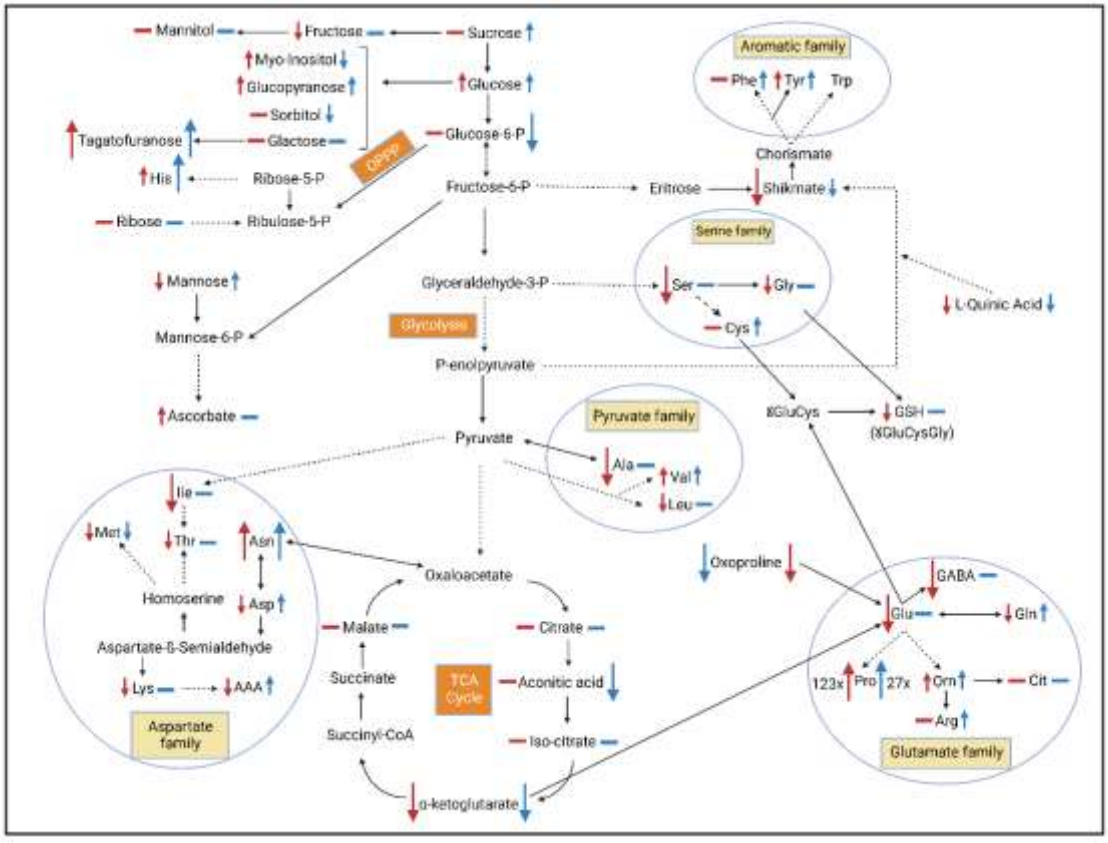

Fig. 24. The schematic representation of the metabolic changes involved in primary metabolic pathways of wheat seedlings exposed to a reductant (ASA) and an oxidant (H_2O_2). The red and blue arrows represent 7 d 20 mM ASA and H_2O_2 treatments, respectively. The horizontal lines refer to no change in the respective treatment. The relative variations in the amount of the various metabolites relative to control are indicated as follows: downregulation – small and

large downwards arrows for 0.7- to 0.35-fold and <0.35-fold changes, respectively; upregulation – small and large upwards arrows for 1.4- to 2.8-fold and >2.8-fold changes, respectively; no effect – horizontal lines for 0.7- to 1.4-fold changes. The highest ratio was around 7, but for proline, the increment was much higher as shown besides the arrows. The continuous lines display the direct metabolic association between two metabolites, whereas the dashed lines present various intermediate products between them. Along with the three-letter abbreviations of proteinogenic amino acids, the following ones were used: AAA: alfa-aminoadipic acid. Cit: citrulline, GABA: gamma-aminobutyric acid, ɣGluCys: gamma-glutamylcysteine, ɣGluCysGly: gamma-glutamylcysteinylglycine**,** GSH: glutathione, Orn: ornithine, OPPP: oxidative pentose-phosphate pathway, TCA: tricarboxylic acid. This figure was prepared in the Biorender software.

6.2.4 ASA- and H_2O_2-induced changes at the gene expression level

Likewise, metabolic profile, the expression levels of the evaluated genes connected with amino acid metabolism, OPPP, and antioxidant defense system was also differently regulated by shifting the cellular redox state to more reducing and more oxidizing directions following H_2O_2 and ASA treatments, respectively. Interestingly, the application of 5 mM H_2O_2 considerably decreased the expression levels of most genes after 3 days, whereas, a higher concentration (20 mM) of ASA for a longer duration (7 days) up-regulated their expressions relative to the control wheat seedlings, highlighting the existence of redox control at the transcriptional level. Regarding ASA application, the up-regulation of the genes encoding the enzymes associated with OPPP and the antioxidant defense system may be involved in the recovery of the redox environment. In the case of OPPP, various glucose-6-P dehydrogenase transcripts were released from their known reductive feedback decline [89]. An inverse change was expected following 20 mM H_2O_2 addition (7 days) for the modification of the redox state by the suppression of genes scavenging ROS. Surprisingly, such change was only observed under 5 mM H_2O_2 (3 days) addition. As perceived in wheat plants, many genes associated with the redox control of the metabolic pathways were also mediated by H_2O_2 application in *Arabidopsis* plants [305] and by methyl viologen in pepper plants [306]. A coordinated adjustment of the transcript levels of the evaluated genes related to the amino acid metabolism, antioxidant defense system, and OPPP was shown by the hierarchical clustering of genes, since the OPPP- and organellar redox system-associated genes were repressed or unchanged after most treatments, whereas the cytoplasmic genes were generally stimulated after ASA and H_2O_2 addition.

7 Summary

The sessile organisms, plants experience various abiotic stresses during their life span such as light (intensity and spectrum), extreme temperatures (low or high), exogenous application of the chemicals/compounds, water deficit conditions, soil salinity or heavy metals which ultimately result in ROS production, and in turn in disturbance of the redox homeostasis. Therefore, besides the study of the effect of light and cold, we have simulated the influence of the modified redox environment of the tissues by ASA and H_2O_2 treatments in order to see their effects on various physiological, biochemical, metabolic, and molecular mechanisms in wheat seedlings. For this purpose, two experimental systems were used:

During the study of the effect of light conditions on the response to cold treatment, two freezing-tolerant (Cheyenne – Ch, winter growth habit; Miranovskaya 808 – Mir, winter growth habit) and two freezing-sensitive (Cappelle Desprez – CD, winter growth habit; Chinese Spring – CS, spring growth habit) wheat (*Triticum aestivum* L.) genotypes were used. Three light treatments/conditions were as follows: white light; 250 µmol m^{-2} s^{-1} (denoted as normal light intensity; red/far-red ratio – 15:1; blue/red ratio – 1:2), white light; 500 µmol m^{-2} s^{-1} (denoted as high light intensity, blue/red ratio - 1:2; red/far-red ratio - 15:1) and far-red light; (blue/red ratio of 1:2, red/far-red ratio - 10:1) with 250 µmol m^{-2} s^{-1}. The subsequent cold treatment at 5 °C continued for a week and the recovery period lasted for 3 weeks at 20/17 °C. The sampling for physio-biochemical and molecular analysis (electrolyte leakage, enzyme activity, thiols, and gene expressions) was done before (14 days old plants) and after (21 days old plants) the cold treatment.

High light intensity and supplementary far-red light modified the redox environment through their influence on the level of the non-protein thiols and activities of ASA-GSH cycle-related enzymes in the shoot tissues of wheat. This led to an improved cold acclimation process as indicated by the increased freezing tolerance of leaf segments based on the reduced membrane damage. There was a positive correlation between freezing tolerance levels and the redox state of the GSH pool. The redox environment in the shoot tissues of freezing-sensitive genotypes became more oxidising compared to the tolerant ones under the modified light conditions, which can adversely affect the activities of the redox-sensitive proteins and the amount of the transcripts linked to the reduction of freezing-induced damage.

The influence of ASA and H_2O_2 on metabolism was studied in the wheat (*Triticum aestivum* L.) cultivar (Chinese Spring). The seedlings were treated with 0-, 5-, and 20-mM ASA (reductant) and H_2O_2 (oxidant). The sampling for physio-biochemical and molecular determinations (membrane injury, lipid peroxidation, hydrogen peroxide, photosynthetic parameters, enzyme activity, thiols, ascorbate and dehydro-ascorbate, metabolite profiling, amino acids, and gene expressions) was done following 0-, 3- and 7-day treatments in the middle of photoperiod.

Chemical modification of the redox state in the shoot tissues altered the metabolic processes. The longer (7 days) and higher concentrations (20 mM) of the reductant (ASA) and oxidant (H_2O_2) converted the redox environment of the wheat leaf tissues into the more oxidized direction and more reduced direction based on the size and redox status of the ascorbate and glutathione pools, respectively. These redox variations caused different modifications in the primary metabolic processes as shown by the free amino acid, carbohydrate, and organic acid levels. An adjustment exists, at least partly at the transcriptional level, and may be involved in the recovery of the redox state existing under control conditions.

The most important results obtained in the two experimental systems:

1) High light intensity decreased the electrolyte leakage (an indicator of the membrane damage) at the end of the cold treatment only in the two tolerant genotypes, but not in the two sensitive ones. This observation indicates that high light intensity activates the cold acclimation processes in a cold tolerance-dependent manner. Far-red light did not exhibit such an effect in the present experimental system.

2) Both high light intensity and supplementary far-red light intensities greatly enhanced the formation of ɣ-glutmylcysteine (intermedier product of GSH synthesis) and cysteinylglycine (GSH degradation product) during cold in all the genotypes independently of their freezing tolerance. This change resulted in a great reduction of the ɣESSE/ɣEC and CySSGly/CysGly ratios; therefore, the pool of both thiols became more reduced.

3) The cold-induced increase in GSH content was not or only slightly affected by the light intensity or supplementary far-red light. However, the GSSG content and consequently the GSSG/GSH ratio was greatly reduced before the cold treatment in supplementary far-red light in the two frost-sensitive genotypes, but not in the two tolerant wheat genotypes. The supplementary far-red light greatly induced the hmGSH and hmGSSG accumulation during the

cold treatment only in the two sensitive genotypes. These findings indicate the frost-tolerance-associated control of (hm)GSH metabolism in wheat.

4) At normal growth temperature, lower activities of the antioxidant enzymes were observed under high light intensity in all genotypes except for APX and DHAR in CS. A similar decrease only occurred under the other two light conditions following the cold treatment. These observations show the interactive effect of light and temperature on the antioxidant enzymes.

5) In contrast to the cold-induced decrease of the antioxidant enzyme activities in normal light and supplementary far-red light, the transcription of several of them even increased during cold treatment. Thus, their activities are not controlled at the transcriptional level.

6) ASA and H_2O_2 significantly reduced the studied photosynthetic parameters. The disturbed photosynthesis resulted in a similar increase in lipid peroxidation and electrolyte leakage (membrane damage) after both treatments.

7) Interestingly, the endogenous H_2O_2 level was greater after the exogenous application of ASA than following H_2O_2 treatment. Due to this difference, the redox state converted into more oxidized following ASA application and more reduced following H_2O_2 supplementation based on the ratios of oxidised and reduced ascorbate and glutathione.

8) The excessive ASA could repress, whereas H_2O_2 could stimulate the OPPP and glycolysis pathways producing reducing power as indicated by the unaffected and declined glucose 6-phosphate content, respectively. This difference may explain the greater H_2O_2 content after ASA treatment.

9) The proposed inhibition of the glycolysis and subsequently the citrate cycle by ASA and their induction by H_2O_2 may be responsible for the smaller and greater synthesis of amino acids from the intermediers of these pathways, respectively.

10) Following ASA addition, the more oxidising environment stimulated several transcripts related to the ASA-GSH cycle and the pentose phosphate pathway, which can contribute to the subsequent recovery of the optimal redox state. In contrast, after H_2O_2 treatment this effect was much weaker or did not exist.

8 Összefoglalás

A növények helyhez kötött élőlényként életük során különféle abiotikus stressz hatásoknak vannak kitéve, mint például a megváltozott fényviszonyok (intenzitás és spektrum), szélsőséges hőmérséklet (alacsony vagy magas), a mezőgazdaságban használt vegyszerek, vízhiány, a talaj nagy sótartalma vagy nehézfémek. Ezek ROS felhalmozódáshoz vezetnek, mely a redox homeosztázis egyensúlyát megzavarva oxidatív stresszt idéz elő. A fény és a hideg kezelések következményeinek vizsgálata mellett a különböző kedvezőtlen környezeti tényezők hatásait a szövetek redox környezetére ASA-val és H_2O_2-val történő kezeléssel szimuláltuk. Ezen kezelések befolyását a különböző élettani, biokémiai, metabolikus és molekuláris folyamatokra fiatal búzanövényekben tanulmányoztuk, két kísérleti rendszert használva:

A fényviszonyok hidegakklimációra gyakorolt hatásának vizsgálata során két fagytűrő (Cheyenne – Ch, őszi; Miranovskaya 808 – Mir, őszi) és két fagyérzékeny (Cappelle Desprez – CD, tavaszi; Chinese Spring – CS, tavaszi) búza (*Triticum aestivum* L) genotípust hasonlítottunk össze. A három fénykezelés a következő volt: fehér fény; 250 µmol m^{-2} s^{-1} (normál fényintenzitás; vörös/távoli vörös arány – 15:1; kék/vörös arány – 1:2), fehér fény; 500 µmol m^{-2} s^{-1} (nagy fényintenzitás, kék/vörös arány - 1:2; vörös/távoli vörös arány - 15:1) és távoli vörös fény; (250 µmol m^{-2} s^{-1}; kék/vörös arány 1:2, vörös/távoli vörös arány - 10:1). Az ezt követő hidegkezelés 5 °C-on egy hétig, a regenerációs időszak pedig 3 hétig tartott 20/17 °C-on. A biokémiai és a molekuláris analízisre (elektrolit-kiáramlás és enzimaktivitás mérések, tiol tartalom meghatározása és génexpressziós elemzések) a mintavételezés a hidegkezelés előtt (14 napos növények) és után (21 napos növények) történt.

A nagy fényintenzitás és a kiegészítő távoli vörös fény a nem-fehérje tiolok szintjére és az ASA-GSH ciklushoz kapcsolódó enzimek aktivitására gyakorolt hatásuk révén módosították a redox környezetet a búza hajtásszöveteiben. Ez a hideg akklimációs folyamat javulásához vezetett, amit a levélsejtek megnövekedett membránstabilitása jelez a csökkent ionkiáramlás alapján. Pozitív korrelációt találtunk a fagytűrés szintje és az összes glutationtartalom között. A fagyérzékeny genotípusok hajtásszöveteiben a redox környezet a módosított fényviszonyok mellett a toleránsakhoz képest oxidálóbbá vált, ami hátrányosan befolyásolhatja a redox-érzékeny fehérjék aktivitását és a fagytűrőképességgel összefüggő transzkriptumok mennyiségét.

Az ASA és a H_2O_2 metabolitprofilra gyakorolt hatását a Chinese Spring búzafajtában (*Triticum aestivum* L.) vizsgáltuk. A fiatal növényeket 0, 5 és 20 mM ASA-val (redukálószer) és H_2O_2-vel (oxidálószer) kezeltük. A biokémiai és a molekuláris meghatározásokra (elektrolit-kiáramlás, lipidperoxidáció és hidrogén-peroxid tartalom meghatározása, fotoszintetikus paraméterek és enzimaktivitás mérése, oxidált és redukált tiol, aszkorbát és dehidroaszkorbát tartalom valamint metabolitprofil meghatározása, aminosav és génexpressziós elemzések) a mintavételezés 0, 3 és 7 nappal a kezelés után történt a fotoperiódus közepén.

A redox környezet kémiai módosítása a hajtásszövetekben megváltoztatta az anyagcsere folyamatokat. A redukálószeres (ASA) és az oxidálószeres (H_2O_2) kezelés hosszabb időtartama (7 nap) és nagyobb mértéke (20 mM) a búzalevél szöveteinek redox környezetét az aszkorbát és a glutation redox állapota alapján az oxidáltabb, illetve a redukáltabb irányba tolta el. Ezek a redox változások különböző módosulásokat okoztak az elsődleges anyagcsere-folyamatokban, amit a szabad aminosavak, a szénhidrátok és szerves savak szintjének változása jelez. A szabályozás legalább részben transzkripciós szinten zajlik, és szerepet játszhat a környezeti hatásokra megváltozott redox környezet helyreállításában.

A két kísérleti rendszerben elért legfontosabb eredmények:

1) A nagy fényintenzitás csak a két fagytűrő genotípusban csökkentette az elektrolitkiáramlást (membránkárosodás jelzője) a hidegkezelés végén, a két érzékeny genotípusban viszont nem. Ez a megfigyelés arra utal, hogy a nagy fényintenzitás fagytűrés-függő módon aktiválja a hideg akklimatizációs folyamatot. A távoli vörös fény nem mutatott ilyen hatást a jelen kísérleti rendszerben.

2) Mind a nagy fényintenzitás, mind a kiegészítő távoli vörös fény nagymértékben megnövelte a gamma-glutamilcisztein (a GSH szintézis közbenső terméke) és a ciszteinil-glicin (GSH bomlástermék) képződését a hidegkezelés során minden genotípusban, függetlenül azok fagytűrő képességétől. Ez a változás a ɣESSE/ɣEC és a CySSGly/CysGly arányok nagymértékű csökkenését eredményezte, így mindkét tiol redukáltabbá vált.

3) A GSH tartalom hideg által kiváltott növekedését nem, vagy csak kis mértékben befolyásolta a nagy fényintenzitás vagy a kiegészítő távoli vörös fény. A GSSG tartalom és ennek következtében a GSSG/GSH arány azonban a hidegkezelés előtt nagymértékben csökkent a kiegészítő távoli vörös fényben a két fagyérzékeny genotípusban, de a két toleránsban nem volt ilyen változás. A távoli vörös fény csak a két érzékeny genotípusban indukálta nagymértékben

a hmGSH és a hmGSSG felhalmozódást a hidegkezelés során. Ezek az eredmények a (hm)GSH metabolizmus fagytűréssel összefüggő szabályozását jelzik búzában.

4) Normál növekedési hőmérsékleten az antioxidáns enzimek kisebb aktivitását figyeltük meg nagy fényintenzitás mellett minden genotípusban az APX és a DHAR kivételével a CS-ben. Hasonló csökkenés csak a hidegkezelést követően következett be a másik két fényviszonyon. Ezek a megfigyelések a fény és a hőmérséklet antioxidáns enzimekre gyakorolt interaktív hatását mutatják.

5) Ellentétben az antioxidáns enzimek aktivitásának hideg által kiváltott csökkenésével normál intenzitású fehér fényben és nagyobb arányú távoli vörös fényben, több enzim génjének a transzkripciója fokozódott a hidegkezelés során. Így aktivitásuk nem transzkripciós szinten szabályozott.

6) Az ASA és a H_2O_2 jelentősen csökkentette a vizsgált fotoszintetikus paramétereket. A fotoszintézis zavara a lipidperoxidáció és az elektrolit- kiáramlás (membránkárosodás) hasonló mértékű növekedését eredményezte mindkét kezelés után.

7) Érdekes módon az endogén H_2O_2 szint magasabb volt az ASA exogén alkalmazása után, mint H_2O_2 kezelést követően. Ennek a különbségnek köszönhetően, az oxidált és a redukált aszkorbát és glutation arányát figyelembe véve, a redox környezet az ASA alkalmazását követően oxidáltabbá, a H_2O_2-kezelés után pedig redukáltabbá vált.

8) Az ASA gátolhatja, míg a H_2O_2 indukálhatja a redukálóerőt (NADPH-t) biztosító oxidatív pentóz-foszfát útvonalat és a glikolízist , amit a változatlan, illetve a csökkent glükóz-6-foszfát tartalom jelez. Ez a különbség magyarázatot adhat az ASA-kezelés után megfigyelt nagyobb H_2O_2-tartalomra.

9) A glikolízis, ezáltal a citrátciklus ASA révén történő gátlása, illetve ezek H_2O_2 általi indukciója felelős lehet a két biokémiai folyamat köztes termékeiből történő kisebb, illetve nagyobb mértékű aminosav-szintéziséért.

10) Az ASA kezelés után az oxidálóbb környezet számos, az ASA-GSH ciklushoz és a pentóz-foszfát útvonalhoz kapcsolódó transzkriptumot stimulált, ami hozzájárulhat az optimális redox környezet későbbi helyreállításához. Ezzel szemben a H_2O_2-kezelés után ez a hatás sokkal gyengébb volt, vagy nem is létezett.

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10 List of publications

(MTMT identifier: 10082583)

10.1 Publications used in the thesis

1. **Asghar, M. A.,** Balogh, E., Ahres, M., Szalai, G., Gondor, O. K., Darkó, É., ... & Kocsy, G. (2022). Ascorbate and Hydrogen Peroxide Modify Metabolite Profile of Wheat Differently. *Journal of Plant Growth Regulation*, 1-16.

(**Q1, IF: 4.64**)

2. **Asghar, M. A.,** Balogh, E., Szalai, G., Galiba, G., & Kocsy, G. (2022). Differences in the light-dependent changes of the glutathione metabolism during cold acclimation in wheat varieties with different freezing tolerance. *Journal of Agronomy and Crop Science*, 208(1), 65-75.

(**D1, IF: 4.15**)

10.2 Other publications

10.2.1 Journal publications

1. **Asghar, M. A.,** Kulman, K., Szalai, G., Gondor, O. K., Mednyánszky, Z., Simon-Sarkadi, L., Gaudinova, A., Dobrev, P. I., , ... & Kocsy, G. (2023). Effect of ascorbate and hydrogen peroxide on hormone and metabolite levels during post-germination growth in wheat. *Physiologia Plantarum*. PPL.13887

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2. **Muhammad, A. A.,** JIANG, H. K., SHUI, Z. W., CAO, X. Y., HUANG, X. Y., Imran, S., ... & DU, J. B. (2021). Interactive effect of shade and PEG-induced osmotic stress on physiological responses of soybean seedlings. *Journal of Integrative Agriculture*, 20(9), 2382-2394.

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3. **Asghar, M. A.,** Du, J., Jiang, H., Li, Y., Sun, X., Shang, J., ... & Yang, W. (2020). Shade pretreatment enhanced drought resistance of soybean. *Environmental and Experimental Botany*, 171, 103952.

(**D1, IF: 4.02**)

4. **Asghar, M. A.,** Li, Y., Jiang, H., Sun, X., Ahmad, B., Imran, S., ... & Du, J. (2019). Crosstalk between abscisic acid and auxin under osmotic stress. *Agronomy Journal*, 111(5), 2157-2162.

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5. **Asghar, M. A.,** Mednyánszky, Z., Simon-Sarkadi, L., & Kocsy, G. (2021). Different induction of biogenic amine accumulation during cold acclimation in Triticeae genotypes with varying freezing tolerance. *Brazilian Journal of Botany*, 44, 11-15.

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6. **Asghar, M. A.,** Ahmad, B., Raza, A., Adil, B., Hassan Javed, H., Farooq, M. U., ... & Du, J. (2022). Shade and microbes enhance drought stress tolerance in plants by inducing phytohormones at molecular levels: a review. *Journal of Plant Ecology*, 15(6), 1107-1117.

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7. Raza, A., **Asghar, M. A.,**† Hussain, S., Bin, C., Shafiq, I., Ahmad, I., ... & Weiguo, L. (2021). Optimal NH4+/NO3− ratios enhance the shade tolerance of soybean seedlings under low light conditions. *Plant Biology*, 23(3), 464-472.

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8. Saleem, K., **Asghar, M. A.,**† M. H., Raza, A.,... & J. W.H. Yong. (2023). Biochar-mediated control of metabolites and other physiological responses in water-stressed Leptocohloa fusca. *Metabolites*. (Accepted).

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9. Raza, A., **Asghar, M. A.,**† Ahmad, B., Bin, C., Iftikhar Hussain, M., Li, W., ... & Weiguo, L. (2020). Agro-techniques for lodging stress management in maize-soybean intercropping system—a review. *Plants*, 9(11), 1592.

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10. Saleem, K., **Asghar, M. A.,**† Saleem, M. H., Raza, A., Kocsy, G., Iqbal, N., ... & Bhat, E. A. (2022). Chrysotile-asbestos-induced damage in Panicum virgatum and Phleum pretense species and its alleviation by organic-soil amendment. *Sustainability*, *14*(17), 10824.

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11. Kubar, M. S., Alshallash, K. S., **Asghar, M. A.,**† Feng, M., Raza, A., Wang, C., ... & Alshamrani, S. M. (2022). Improving Winter Wheat Photosynthesis, Nitrogen Use Efficiency, and Yield by Optimizing Nitrogen Fertilization. *Life*, 12(10), 1478.

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12. Lalay, G., Ullah, A., Iqbal, N., Raza, A., **Asghar, M. A.,*** & Ullah, S. (2022). The alleviation of drought-induced damage to growth and physio-biochemical parameters of

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13. Borbély, P., Gasperl, A., Pálmai, T., Ahres, M., **Asghar, M. A.,** Galiba, G., ... & Kocsy, G. (2022). Light Intensity-and Spectrum-Dependent Redox Regulation of Plant Metabolism. *Antioxidants*, 11(7), 1311.

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15. Hu, Y., Javed, H. H., **Asghar, M. A.,** Peng, X., Brestic, M., Skalický, M., ... & Wu, Y. C. (2022). Enhancement of lodging resistance and lignin content by application of organic carbon and silicon fertilization in Brassica napus L. *Frontiers in Plant Science*, 13, 217.

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16. Javed, H. H., Hu, Y., **Asghar, M. A.,** Brestic, M., Abbasi, M. A., Saleem, M. H., ... & Wu, Y. C. (2022). Effect of intermittent shade on nitrogen dynamics assessed by 15N trace isotopes, enzymatic activity and yield of Brassica napus L. *Frontiers in Plant Science*, 13.

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(**Q2, IF: 3.25**)

28. Hussain, M. I., Muscolo, A., Ahmed, M., **Asghar, M. A.,** & Al-Dakheel, A. J. (2020). Agro-morphological, yield and quality traits and interrelationship with yield stability in quinoa (Chenopodium quinoa Willd.) genotypes under saline marginal environment. *Plants*, 9(12), 1763.

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30. Kubar, M. S., Zhang, Q., Feng, M., Wang, C., Yang, W., Kubar, K. A., ... & **Asghar, M. A.** (2022). Growth, Yield and Photosynthetic Performance of Winter Wheat as Affected by Co-Application of Nitrogen Fertilizer and Organic Manures. *Life*, 12(7), 1000.

(**Q2, IF: 3.25**)

31. Ullah, A., Zeng, F., Noor, J., **Muhammad, A. A.,** Chai, X.,... & Zhang, Z. (2023). Application of GABA (γ-aminobutyric acid) to improve saline stress tolerance of chufa (*Cyperus esculentus* L. var. sativus Boeck) plants by regulating their antioxidant potential and nitrogen assimilation . *South African Journal of Botany*, SAJB4362.

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33. Hussain, S., Pang, T., Iqbal, N., Shafiq, I., Skalicky, M., Brestic, M., **Asghar, M. A.,**... & Yang, W. (2020). Acclimation strategy and plasticity of different soybean genotypes in intercropping. *Functional Plant Biology*, 47(7), 592-610.

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34. Ghafoor, A., Karim, H., **Asghar, M. A.,** Raza, A., Hussain, M. I., Javed, H. H., ... & Wu, Y. (2022). Carbohydrates accumulation, oil quality and yield of rapeseed genotypes at different nitrogen rates. *Plant Production Science*, 25(1), 50-69.

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35. Shafiq, I., Hussain, S., Raza, M. A., Iqbal, N., **ASGHAR, M. A.,** Ali, R. A. Z. A., ... & Feng, Y. A. N. G. (2021). Crop photosynthetic response to light quality and light intensity. *Journal of Integrative Agriculture*, 20(1), 4-23.

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(**Q1, IF: 1.42**)

37. Ishaaq, I., Farooq, M., Tahira, S., Maqbool, R., Barutçular, C., Yasir, M., **Asghar, M. A.,** ... & Rastogi, A. (2022). Exploration of Genetic Pattern of Phenological Traits in Wheat (Triticum aestivum L.) under Drought Stress. *Phyton-International Journal of Experimental Botany*, 91(12).

(**Q4, IF: 1.40**)

38. Ghafoor, A., Karim, H., **Asghar, M. A.,** Javed, H. H., Xiao, P., & Wu, Y. (2021). Effect of high-temperature, drought, and nutrient availability on morpho-physiological and molecular mechanisms of rapeseed-an overview. *Pakistan Journal of Botany*, 53(6), 2321-2330.

(**Q3, IF: 0.96**)

(**Commulative IF: 156.87**)

10.2.2 Book chapters and conference proceedings

1. Hussain, M. I., Abideen, Z., Danish, S., **Asghar, M. A.,** & Iqbal, K. (2021). Integrated weed management for sustainable agriculture. *Sustainable Agriculture Reviews*, 52, 367-393.

(**Book chapter**)

2. **Asghar, M. A.,** Balogh, E., Szalai, G., Gondor, O. K., ... & Kocsy, G. (2021). Redox-dependent adjustment of metabolites levels in wheat seedlings. *XII Congress of the Hungarian Society for Plant Biology, Hungary*.

(**Conference proceeding**)

3. **Asghar, M. A.,** Balogh, E., Szalai, G., Gondor, O. K.,... & Kocsy, G. (2022). Modification of metabolite profile by ascorbate and hydrogen peroxide in wheat. *7th International Plant Phenotyping Symposium, The Netherlands*.

(**Conference proceeding)**

11 Statement

As a supervisor of the Ph.D. work or/and the corresponding author of the following journal publications, I verify that all the results presented in this thesis and scientific publications were not used before to obtain any Ph.D. degree and will not be used in future as well:

Asghar, M. A., Balogh, E., Ahres, M., Szalai, G., Gondor, O. K., Darkó, É., ... & Kocsy, G. (2022). Ascorbate and hydrogen peroxide modify metabolite profile of wheat differently. *Journal of Plant Growth Regulation*, 1-16.

Asghar, M. A., Balogh, E., Szalai, G., Galiba, G., & Kocsy, G. (2022). Differences in the light-dependent changes of the glutathione metabolism during cold acclimation in wheat varieties with different freezing tolerance. *Journal of Agronomy and Crop Science*, 208(1), 65-75.

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12 Supplementary data

Figures

Fig. S1. The plants grown under the three various light conditions at the end of the recovery period. Left: high light intensity, middle: far-red light, right: normal light intensity.

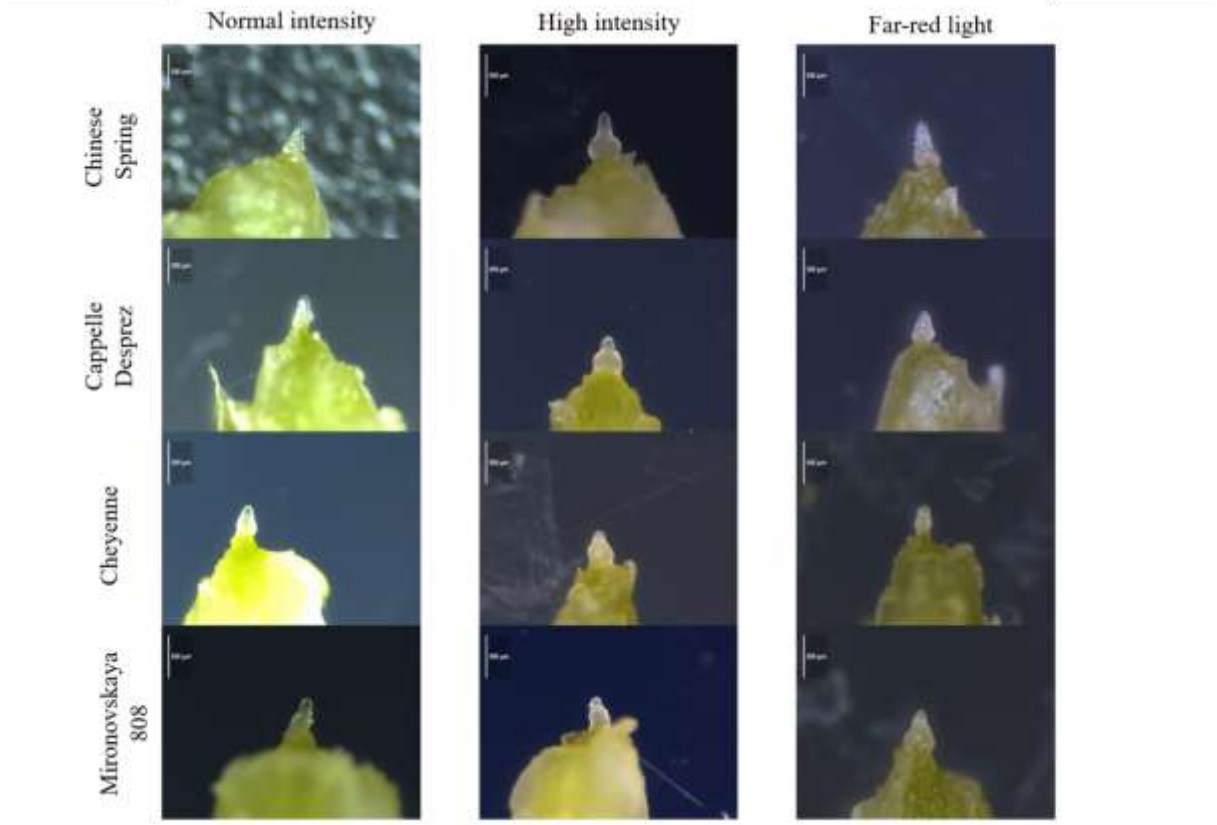


Fig. S2. The phenotype of shoot apices at the end of the recovery period.

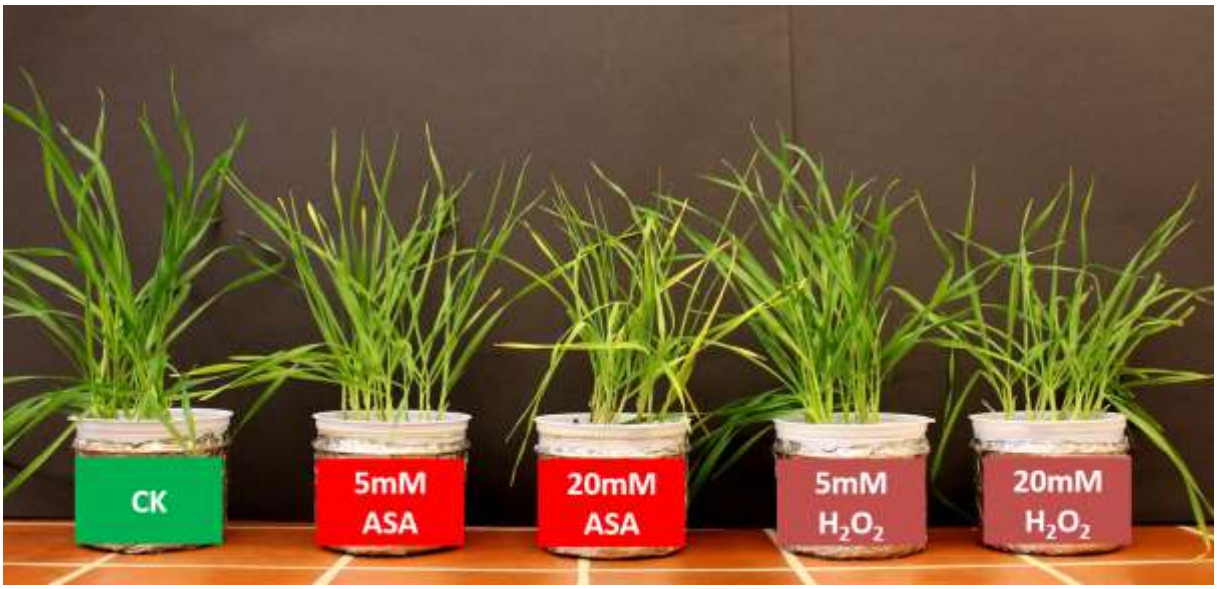


Fig. S3. The plants grown under different concentrations of ascorbate (ASA) and hydrogen peroxide (H_2O_2).

Tables

Table S1. Light conditions used in the first experiment (light and cold).

	Normal light intensity	High light intensity	Far-red light
PAR (umol/m^{-2}/s^{-1}	250	500	250
Blue/Red	1:2	1:2	1:2
Red/Far-red	15:1	15:1	10:1

Table S2. Primers used for the gene expression studies.

Gene name	Abbreviation	Forward primer	Reverse primer	Reference
Adenozine phosphosulfate reductase	*TaAPSR*	GCTCAAAGCCCCTCCATTAT	GACCGAGTGAACCACGAGTA	257
Aspartate transaminase	*TaASPTA*	TCGTCGCTGATGGTGGTG	GCTCCACGGTCCATTCG	12
Ascorbate peroxidase 1	*TaAPX1*	GCAGCTGCTGAAGGAGAAGTC	CAATTTCAAACCTCAAGCTACCAT	271
Arginine decarboxylase	*TaArgDC*	CAATGCCGTACCTGTCGTTC	AACTCCCACTCCTCGTCATC	12
Argininosuccinate lyase	*TaARGSUL*	GAACAGTTGGAGCGTGATGC	CAGTTCCAGCCAAAGCACAAG	12
C-repeat binding factor12	*TaCBF12*	GGCCGAAATGGACGCGGGCACGTAC	TGGGTCCACACCGTCCACGTACG	272
C-repeat binding factor14	*TaCBF14*	AACCGATGACGAGAAGGAAA	AACTCCGAGTAGCACGATCC	272
C-repeat binding factor15	*TaCBF15*	GTCGTCCATGGAAAATACCG	AATGTGTCCAGGTCCATTTCC	272
C-repeat binding factor16	*TaCBF16*	GCGGCATGCCTCCAACAGCGCAG	ACGTGCCCAGGTCCATCCCCG	272
C-repeat binding factor17	*TaCBF17*	ACTTCGAGTTCGACGTGTCCTGGGTG	ACTACTGACTCCAGAGCGCGGCGTC	272
chloroplast-Monodehydroascorbate reductase	*TachlMDHAR*	AGTATGAAGGAAGCTCCAGGAAAAT	AAACACCCTTCAATCGACTATCAGA	271
cytosol-Dehydroascorbate reductase	*TacDHAR*	GCCTGTGTATAACGGTGGTG	CGTGACAAAGGTGGAGAAGA	271
chloroplast-Dehydroascorbate reductase	*TachlDHAR*	AATTGGTCTGTCCCAGATGC	TCCAGCAATGACATCCTCTG	271
chloroplast-Glutathione reductase	*TachlGR*	TTGGGCTGTTGGAGATGTTA	CTCTGGTTTGGTCGGTTCAT	271
chloroplast-γ-Glutmylcysteine synthetase	*TaChlECS*	TGCCCACTTTGAACGATTGG	GTCCACCATCAGCCACCTTC	5
cytosol-Ascorbate peroxidase I	*TacAPX I*	CCTTCTTCAGTTGCCGACC	TCAGCAAAGAACGCATCCTC	271
cytosol-Ascorbate peroxidase II	*TacAPX II*	AGGAGAGGTCTGGCTTTGAG	GTCAGCAGGGTTTTGTCACTT	271
cytosol-Monodehydroascorbate reductase I	*TacMDHAR I*	CGATGAAGATCTACAACGACGAGA	CCGCCTCTTTCCCCTTGAT	271
cytosol-Monodehydroascorbate reductase II	*TacMDHAR II*	CGAGCAGGCAGTAAAGGCG	TCCCCGAACAGCACCGT	271
cytosol-Glucose-6-phosphate dehydrogenase	*TacG6PDH*	GAGCAAGAGGATGGGGTACG	TAACAGGCACTTGGTAGCGG	Own design
cytosolic/plastidic-1-6-Phosphogluconate dehydrogenase	*Tacp16GPDH*	ATTCAGAAGCCACGTGTCGT	CCACTCGTTTCCTCCGTCAA	Own design
cytosolic/plastidic-6-Phosphogluconate dehydrogenase	*Tacp6GPDH*	GGCAACGAGTGGTACCAGAA	TGATGTTGCTGTAGGCCTGG	Own design
Gamma-glutamilcystein synthetase	*TaECS*	TGCCCACTTTGAACGATTGG	GTCCACCATCAGCACCTTC	12
Glutathione reductase	*TaGR*	AACTGTAGACACAGACTCTTG	ATCAGATACACCGTATCACAC	12
Glutathione S transferase	*TaGST*	CAGGCAGTCAATCCTCGGAC	TGGAGAGGTTGGATGCTTGA	12
Glutathione synthetase	*TaGSHS*	TGGCCGTGACATATCCGATG	ATGACCTCCCCGTTTGTTCC	12
Glutamate dehydrogenase	*TaGluDH*	CGCTAAAGGTGGCATTGGG	TGTCTGTGCGTTGGTGCC	12
Glutamine synthetase	*TaGlns*	CATCATAAGACCTCCTATACG	ACCACCACTTCAAAGAAC	Own design
Glucose-6-phosphate dehydrogenase	*TaG6PDH I*	CGCTTCCTTCTCCGTGTGTA	TACACAAGTCACCCCACACG	Own design
Glucose-6-phosphate dehydrogenase	*TaG6PDH II*	CATACGCCTCCAGCCATCAG	TTGACATCTTGGTAACGCATCC	273
Mitochondria-Ascorbate peroxidase	*TamAPX*	CTACTGATAAGGCATTGTTGGAT	TGATCCGTGGTGTGAAGC	271
Mitochondria-Monodehydroascorbate reductase	*TamMDHAR*	GGACGACTACGAGGCGTTAT	AGGCACGTCTTTCTTGCTTT	271
Nitrate reductase	*TaNR*	CGAGATCCTCCCCATCAACG	CCGTCCAGAGTCACTTCCAC	12
O-acetylserine (thiol) lyase	*TaCysASTL*	CCTTGTTTCTGGCATCGGG	TTGTGTGGTCCAGGCTTTCC	12
Ornithine aminotransferase	*TaOrnATF*	GCACGGAGGCAAATGAGC	TCCGTCTGACCCGAAAGC	12
Phosphogluconate-dehydrogenase	*TaTA30797*	GCC GTG TCC ATG CCA GTG	TTA GCC TCA ACC ACC TGT GC	270 (Ref. gene)
Pyrroline-5-carboxylate reductase	*TaP5CR*	TGGTCCGGCCTACATTTTCT	TTTACCCGTCTCGCTAACCA	12
Pyrroline-5-carboxylate synthease	*TaP5CS*	CTCTGCGTCCCATTGCTG	CAAACCCCAGGCATAACCC	12
plastid-2-Glucose-6-phosphate dehydrogenase	*Tap1G6PDH*	CCTACGAGAGGCTGTTGCTC	AATACAACAGCCCAGCCCAG	Own design
plastid-1-Glucose-6-phosphate dehydrogenase	*Tap2G6PDH*	CGTCGGCTCATTTGGTTGTC	ACAAGAACACCGGCATCAGT	Own design
6-Phosphogluconate dehydrogenase	*Ta6PGDH*	TCTCTGTGGCTGCTCCTACC	TCCCTTCTCCGTGCTCTTGG	273
Serine hydroxymethyltransferase	*TmSerHMT*	TGCTGGTGTCATTCCGTCTC	TTATTTCTTTCACCCCCTTCCG	12
stroma-Ascorbate peroxidase II	*TasAPX II*	GGCGATCGGTGAGGTGC	TGGGGTGGCAGTGGGTT	271
thylakoid-bound-Ascorbate peroxidase	*TatAPX*	GCCGAAGCCCATGCTAAACT	ATCACTCGCTGGTGCTGGAT	271

Table S3. The freezing tolerance of the tetsted genotypes based on the re-growth level of the shoots after stress. The re-growth after freezing was investigated on a scale between 0 (no re-growth) and 5 (very good re-growth).

Genotype	**Sensitivity**	**-12 °C**	**-15 °C**
Chinese Spring	Sensitive	0.15 ± 0.07^{a}	0.09 ± 0.04^{a}
Cappelle Desprez	Sensitive	0.45 ± 0.23^{a}	0.36 ± 0.18^{a}
Cheyenne	Tolerant	3.44 ± 0.86^{b}	2.92 ± 0.72^{b}
Miranovszkaja 808	Tolerant	2.89 ± 0.94^{b}	2.65 ± 0.63^{b}

The values presented by different letters are significantly different at $p < 0.05$ level (ANOVA, LSD-test, 3 independent experiments, each with 3 parallels).

Table S4A. Correlation of the relative changes of the various parameters in plants grown under normal light conditions. The fold change in a certain parameter during cold was calculated and used for the analysis.

		Physiological parameters					Thiol contents															
		FT-(-12)	FT-(-15)	Shoot fresh weigh	Root fresh weight	Electrolyte leakage	Cys	CySS	CySSiCys	CysGly	CySSGly	CySSGlyiCysGly	γEC	γESSE	γESSEiγEC	hmGSH	hmGSSG	hmGSSGihmGSH	GSH	GSSG	GSSGiGSH
Physiological parameters	FT-(-12)		1.00	0.66	0.78	-0.26	0.78	-0.85	0.96	0.78	-0.14	-0.41	0.30	0.57	-0.17	-0.80	-0.08	0.34	-0.77	0.31	0.62
	FT-(-15)			0.68	0.81	-0.28	0.81	-0.84	0.97	0.82	-0.15	-0.42	0.34	0.53	-0.19	-0.78	-0.08	0.29	-0.74	0.25	0.57
	Shoot fresh weight				0.91	0.34	0.91	-0.17	0.84	0.79	-0.79	-0.94	0.88	-0.18	-0.85	-0.84	-0.72	0.36	-0.04	0.15	0.17
	Root fresh weight					-0.09	1.00	-0.41	0.91	0.99	-0.48	-0.72	0.82	-0.07	-0.67	-0.69	-0.37	0.04	-0.22	-0.13	0.06
	Electrolyte leakage						-0.09	0.60	-0.10	-0.30	-0.83	-0.63	0.32	-0.41	-0.57	-0.37	-0.89	0.66	0.53	0.52	0.12
Thiol contents	Cys							-0.41	0.91	0.99	-0.48	-0.72	0.82	-0.07	-0.67	-0.69	-0.37	0.04	-0.22	-0.13	0.06
	CySS								-0.68	-0.50	-0.37	-0.12	0.20	-0.88	-0.37	0.44	-0.41	-0.16	0.98	-0.26	-0.66
	CySSiCys									0.88	-0.37	-0.62	0.55	0.33	-0.43	-0.86	-0.30	0.33	-0.56	0.23	0.47
	CysGly										-0.28	-0.56	0.73	-0.01	-0.53	-0.56	-0.17	-0.13	-0.30	-0.26	0.00
	CySSGly											0.95	-0.77	0.48	0.91	0.65	0.99	-0.53	-0.42	-0.30	-0.04
	CySSGlyiCysGly												-0.87	0.36	0.93	0.78	0.91	-0.47	-0.22	-0.24	-0.10
	γEC													-0.61	-0.96	-0.49	-0.68	-0.02	0.37	-0.26	-0.32
	γESSE														0.66	-0.30	0.44	0.39	-0.95	0.57	0.84
	γESSEiγEC															0.52	0.85	-0.17	-0.50	0.09	0.26
	hmGSH																0.64	-0.75	0.41	-0.63	-0.67
	hmGSSG																	-0.61	-0.43	-0.40	-0.10
	hmGSSGihmGSH																		-0.27	0.97	0.82
	GSH																			-0.41	-0.77
	GSSG																				0.90
	GSSGiGSH																				

		Antioxidant enzyme activities				Transcript levels																	
		APX	GR	DHAR	MDHAR	*TaAPX I*	*TaAPX II*	*TasAPX II*	*TatAPX*	*TamAPX*	*TasAPX1*	*TachlGR*	*TacGR*	*pChlMDHA*	*pmMDHA*	*pcMDHAR*	*pcMDHAR*	*TaGST*	*TaNR*	*TaGS2*	*TaASPTA*	*TaP5CR*	*Ta-chlyECS*	*TaCBF14*	*TaCBF16*	*TaCBF17*
Antioxidant enzyme activities	APX		0.26	0.51	-0.02	-0.76	-0.42	-0.56	0.75	0.14	0.41	0.95	-0.06	-0.37	0.09	0.45	0.62	-0.90	-0.77	-0.70	0.93	-0.29	-0.49	-0.26	0.79	-0.50
	GR			-0.21	0.16	-0.71	-0.13	-0.05	0.30	-0.92	-0.75	0.23	-0.48	-0.78	0.44	0.60	0.60	0.15	0.42	0.50	0.24	-0.28	-0.55	-0.99	0.09	-0.06
	DHAR				0.70	0.09	0.50	0.33	0.86	0.47	0.36	0.24	0.83	0.53	0.60	0.64	0.66	-0.75	-0.64	-0.69	0.43	0.65	0.49	0.09	0.92	0.39
	MDHAR					0.70	0.09	0.50	0.33	0.86	0.47	0.36	0.24	0.83	0.53	0.60	0.64	0.66	-0.75	-0.64	-0.69	0.43	0.65	0.49	0.09	0.92
Transcript levels	*TacAPX1*						0.67	0.68	-0.38	0.44	0.08	-0.86	0.62	0.88	0.04	-0.33	-0.48	0.40	0.24	0.13	-0.79	0.68	0.89	0.64	-0.32	0.67
	TacAPXII							0.98	0.27	0.03	-0.34	-0.68	0.83	0.72	0.76	0.47	0.30	0.20	0.29	0.19	-0.52	0.97	0.90	0.00	0.21	0.99
	TasAPXII								0.13	-0.11	-0.49	-0.78	0.71	0.63	0.73	0.41	0.22	0.38	0.47	0.38	-0.65	0.91	0.85	-0.07	0.05	1.00
	TatAPX									0.05	0.08	0.52	0.48	0.04	0.70	0.87	0.93	-0.76	-0.52	-0.52	0.67	0.37	0.08	-0.39	0.97	0.19
	TamAPX										0.92	0.07	0.53	0.69	-0.35	-0.38	-0.32	-0.53	-0.74	-0.80	0.15	0.23	0.42	0.90	0.27	-0.08
	TasAPX1											0.41	0.22	0.36	-0.53	-0.41	-0.28	-0.68	-0.88	-0.89	0.45	-0.13	0.03	0.78	0.30	-0.45
	TachlGR												-0.34	-0.56	-0.16	0.22	0.42	-0.78	-0.63	-0.61	0.98	-0.57	-0.72	-0.24	0.56	-0.74
	TacGR													0.89	0.57	0.38	0.31	-0.31	-0.29	-0.39	-0.14	0.93	0.90	0.34	0.54	0.74
	TaChlMDHAR														0.20	-0.08	-0.17	-0.07	-0.19	-0.30	-0.41	0.81	0.94	0.68	0.15	0.65
	TamMDHAR															0.92	0.83	-0.08	0.18	0.15	-0.02	0.72	0.45	-0.57	0.56	0.76
	TacMDHARI																0.98	-0.35	-0.04	-0.04	0.35	0.46	0.12	-0.70	0.74	0.45
	TacMDHARII																	-0.50	-0.20	-0.18	0.54	0.32	-0.02	-0.69	0.82	0.27
	TaGST																		0.95	0.93	-0.88	0.00	0.12	-0.12	-0.87	0.32
	TaNR																			0.99	-0.77	0.07	0.08	-0.41	-0.69	0.41
	TaGS2																				-0.70	-0.03	-0.04	-0.48	-0.70	0.32
	TaASPTA																					-0.39	-0.56	-0.21	0.72	-0.60
	TaP5CR																						0.94	0.13	0.35	0.94
	Ta-chlyECS																							0.43	0.12	0.86
	TaCBF14																								-0.18	-0.07
	TaCBF16																									0.11
	TaCBF17																									

Table S4B. Correlation of the relative changes of the various parameters in plants grown under high light conditions. The fold change in a certain parameter during cold was calculated and used for the analysis.

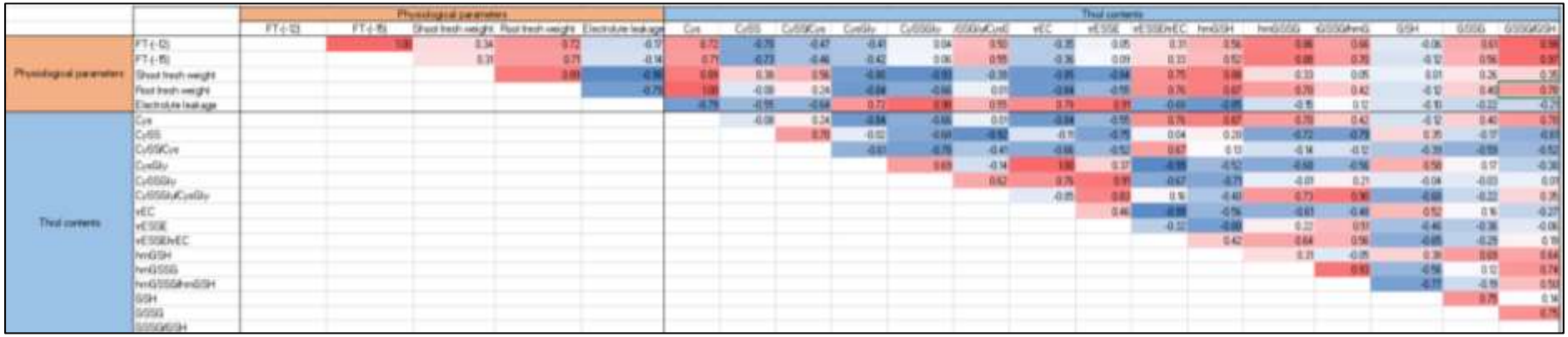


Table S4C. Correlation of the relative changes of the various parameters in plants grown under far-red light conditions. The fold change in a certain parameter during cold was calculated and used for the analysis.

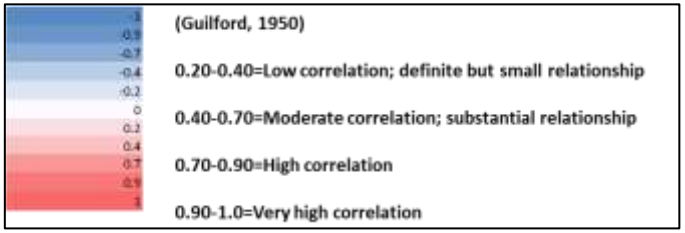


Table S5A. Statistical analysis of the general effect of genotype, light condition and cold for light intensity.

Multivariate Analysis of Variance							
Dependent Variable	Tests of Between-Subjects Effects						
	Genotype	Light Intensity	Cold Treatment	Genotype * Light Intensity	Genotype * Cold Treatment	Light Intensity * Cold Treatment	Genotype * Light Intensity * Cold Treatment
Shoot Fresh Weight	.000	.001	.000	.049	.000	.001	.049
Root fresh Weight	.000	.000	.001	.268	.015	.239	.407
Electrolyte Leakage	.003	.000	.000	.152	.438	.000	.004
Cys	.000	.069	.000	.009	.041	.675	.290
CySS	.000	.778	.001	.000	.131	.082	.510
CySS/Cys	.053	.066	.089	.000	.015	.739	.033
CysGly	.004	.000	.000	.000	.013	.000	.000
CySSGly	.188	.813	.000	.001	.000	.014	.032
CySSGly/CysGly	.029	.000	.000	.014	.000	.029	.000
vEC	.000	.000	.000	.001	.013	.000	.000
vESSE	.000	.001	.000	.004	.001	.000	.003
vESSE/vEC	.700	.000	.038	.259	.001	.000	.000
hmGSH	.001	.023	.000	.033	.067	.094	.000
hmGSSG	.111	.000	.036	.000	.000	.079	.000
hmGSSG/hmGSH	.118	.000	.001	.322	.001	.001	.001
GSH	.032	.702	.000	.169	.188	.000	.002
GSSG	.000	.024	.000	.001	.000	.000	.006
GSSG/GSH	.027	.038	.538	.091	.000	.705	.002
APX	.004	.337	.000	.362	.027	.000	.240
DHAR	.000	.146	.000	.000	.086	.225	.013
MDHAR	.037	.101	.000	.000	.112	.031	.004
GR	.033	.021	.000	.000	.068	.012	.001
TacAPX I	.000	.000	.005	.768	.000	.019	.504
TacAPX II	.000	.000	.000	.002	.000	.070	.000
TasAPX II	.191	.492	.001	.091	.046	.385	.086
TatAPX	.000	.887	.001	.409	.012	.759	.453
TamAPX	.000	.015	.000	.661	.001	.308	.159
TasAPX1	.000	.322	.000	.771	.000	.297	.787
TachlGR	.001	.564	.000	.903	.213	.929	.567
TacGR	.000	.048	.000	.807	.024	.443	.746
TaChlMDHAR	.000	.958	.000	.958	.010	.474	.827
TamMDHAR	.000	.214	.000	.009	.183	.062	.803
TacMDHAR I	.000	.008	.000	.005	.030	.055	.071
TacMDHAR II	.003	.006	.000	.459	.018	.382	.091
TaGST	.061	.038	.003	.645	.009	.421	.542
TaNR	.000	.758	.000	.271	.000	.956	.379
TaGS2	.017	.368	.000	.604	.001	.304	.583
TaASPTA	.000	.104	.000	.817	.000	.340	.745
TaP5CR	.000	.106	.003	.248	.161	.533	.541
Ta-chlyECS	.002	.813	.000	.251	.385	.199	.474
TaCBF14	.000	.841	.054	.299	.441	.034	.040
TaCBF16	.000	.002	.104	.000	.009	.209	.071
TaCBF17	.003	.005	.000	.573	.008	.135	.052

Significance levels in tables are designated as $p < 0.05$. Values were calculated from 3 independent biological replicates.

Table S5B. Statistical analysis of the general effect of genotype, light condition and cold for light quality.

Multivariate Analysis of Variance							
Dependent Variable	Tests of Between-Subjects Effects						
	Genotype	Light Quality	Cold Treatment	Genotype * Light Quality	Genotype * Cold Treatment	Light Quality * Cold Treatment	Genotype * Light Quality * Cold Treatment
Shoot Fresh Weight	.000	.017	.000	.234	.000	.018	.238
Root fresh Weight	.000	.000	.000	.203	.014	.331	.265
Electrolyte Leakage	.055	.009	.000	.301	.850	.005	.142
Cys	.000	.126	.000	.004	.000	.060	.063
CySS	.000	.051	.000	.000	.000	.006	.019
CySS/Cys	.027	.021	.014	.002	.009	.917	.015
CysGly	.055	.000	.000	.008	.054	.000	.013
CySSGly	.000	.001	.000	.000	.000	.001	.000
CySSGly/CysGly	.000	.000	.000	.000	.000	.000	.000
etc	.000	.000	.000	.000	.001	.000	.000
eESSE	.000	.000	.000	.004	.000	.000	.002
eESSE/eEC	.078	.000	.417	.174	.001	.000	.000
hmGSH	.000	.000	.000	.000	.000	.000	.000
hmGSSG	.000	.000	.000	.000	.000	.000	.000
hmGSSG/hmGSH	.048	.000	.004	.202	.001	.000	.000
GSH	.009	.000	.000	.213	.004	.000	.001
GSSG	.000	.000	.000	.002	.000	.000	.000
GSSG/GSH	.000	.025	.303	.002	.000	.389	.000
APX	.000	.054	.000	.000	.017	.000	.000
DHAR	.000	.000	.000	.000	.259	.342	.000
MDHAR	.000	.001	.000	.000	.411	.061	.000
GR	.000	.000	.000	.000	.028	.000	.000
TacAPX I	.000	.000	.858	.125	.000	.000	.000
TacAPX II	.000	.000	.000	.000	.000	.000	.000
TasAPX II	.789	.004	.000	.067	.092	.246	.003
TatAPX	.000	.051	.000	.523	.005	.870	.588
TamAPX	.000	.017	.000	.216	.015	.005	.001
TasAPX1	.000	.142	.000	.687	.000	.151	.709
TachlGR	.000	.832	.000	.966	.133	.976	.692
TacGR	.000	.058	.000	.884	.002	.301	.506
TaChlMDHAR	.000	.802	.000	.964	.019	.411	.609
TamMDHAR	.000	.049	.000	.090	.104	.074	.274
TacMDHAR I	.000	.000	.000	.000	.000	.229	.000
TacMDHAR II	.002	.018	.000	.339	.000	.118	.184
TaGST	.004	.018	.000	.539	.000	.001	.009
TaNR	.000	.029	.000	.668	.000	.161	.113
TaGS2	.002	.485	.000	.594	.000	.552	.843
TaASPTA	.000	.013	.000	.188	.000	.420	.018
TaP5CR	.000	.238	.000	.328	.001	.782	.165
Ta-chlyECS	.000	.564	.000	.686	.548	.426	.738
TaCBF14	.000	.826	.974	.224	.579	.000	.003
TaCBF16	.000	.001	.055	.000	.000	.396	.000
TaCBF17	.020	.004	.000	.248	.002	.187	.117

Significance levels in tables are designated as $p < 0.05$. Values were calculated from 3 independent biological replicates.

Table S6A. Influence of the ascorbate (ASA) and hydrogen peroxide (H_2O_2) on the metabolite levels.

	Malic acid	D-Mannitol	Citric acid	D-Glucose	b-D-Glucopyranose	d-Ribose	L-Valin	D-(-)-Tagatofuranose	b-D-(+)-Mannopyranose	L-Glutamic acid	D-Fructose	Quininic acid	Glucosephosphate	Aconitic acid	Myo-Inositol
C	1100.3 cd	273.35 ab	449.85 e	1582.8 e	1194.1 e	183.9 d	78.37 b	ND	438.45 ab	857.17 a	3049.55 b	1772 a	753.3 ab	2946 bcd	22.5 c
A5-3	1066.25 d	272.35 ab	563.78 bc	2744.125 c	2493.65 bc	223.5 ab	78.3 b	325.74 a	413.7 de	877.2 a	2681.75 c	1238.4 c	754.15 ab	3147.05 bc	35.17 bc
A5-7	873.22 e	259.98 c	475.18 de	2002.425 d	1615.45 de	200.2 cd	ND	213.50 d	431.02 abc	124.55 f	2393.75 d	460.05 f	752.25 ab	2862.15 cd	24.525 c
A20-3	1266.73 ab	264.95 bc	489.35 d	3434.37 a	3374 a	233.9 a	78.57 b	ND	434.77 ab	647.75 c	2401.3 d	592.05 e	753.55 ab	3227.75 b	37.6 b
A20-7	1322.65 ab	270.37 abc	550.57 bc	3011.45 b	2629.3 bb	235.7 a	78.45 b	312.10 a	407.55 e	9.71 g	1485.21 e	689.775 e	756.1 a	2775.45 d	36 b
H5-3	1242.1 bc	272.05 ab	577.12 b	3031.65 b	2371.43 bc	188.6 d	353.625 a	245.53 bcd	425.75 bcd	719.65 b	2572.15 cd	1531.2 b	750.85 ab	3137.7 bc	37.85 b
H5-7	1422.85 a	273.75 ab	532.65 c	2190.9 d	1977.15 cd	210.35 bc	ND	272.47 b	444.97 a	687 bc	3492.95 a	1157.71 cd	751.65 ab	3711.1 a	50.85 a
H20-3	1376 ab	273.35 ab	559.3 bc	3036.55 b	2092.1 bcd	190.45 d	ND	235.75 cd	413.75 de	574.9 d	2781.77 c	443.4 f	727.56 b	2624.8 d	26.62 c
H20-7	1425.1 a	277.85 a	618.3 a	3175.4 b	2262.7 bc	200.47 cd	ND	253.11 bc	415.8 cde	438.1 e	3192.2 b	1087.33 d	ND	752.1 e	14.4 d

	L-Serine	Phosphoric acid	Shikimic acid	.-Threonine	D-(+)-Turanose	D-Fructopyranose	D-Mannose	Sucrose	L-Alanine	D-Sorbitol	Cellobiose	D-Galactose	L-5-Oxoproline	D-(-)-Ribofuranose	L-Asparticacid
C	55.23 c	961.9 ab	230.28 b	236 c	441.82 e	ND	1760.7 bc	3558.65 d	103.95 a	ND	705.4 b	4372.5 b	380.02 a	189.46 de	295.15 c
A5-3	315.5 b	900.45 b	296.84 a	399 a	968.55 a	374.80 ab	2994.47 a	3658.76 d	99 a	35.56 b	466.65 cd	2532.5 de	327.1 c	ND	ND
A5-7	52.55 d	130.8 d	147.78 de	ND	555.97 cd	697.97 a	3103.2 a	5552.1 ab	101.45 a	37.56 b	1182.82 a	3296.9 c	ND	224.51 c	ND
A20-3	316.15 ab	1024.2 a	187.91 c	236.1 bc	638.4 bc	168.58 ab	1903.45 bc	4819.1 bc	102.15 a	ND	416.55 d	2026.75 e	ND	203.85 cd	305.9 a
A20-7	51.97 d	178.57 d	ND	236.32 b	617.02 c	ND	1032.75 d	3617.95 d	ND	ND	692.51 bc	4009.42 b	ND	176.42 e	0.00
H5-3	317.85 a	350.17 c	165.50 cd	236.1 bc	734.5 b	ND	1730.77 bc	3973.8 cd	85.25 b	ND	397.72 d	3022.06 cd	343.15 b	140.7 f	300.9 b
H5-7	53.26 d	129.55 d	157.76 de	235.95 c	471.75 de	ND	1498.33 cd	3857.7 cd	101.28 a	37.55 b	579.97 bcd	2485.35 de	ND	264.76 b	ND
H20-3	315.3 b	281.92 c	151.2 de	235.95 c	434.55 e	104.21 c	2324.47 b	4054.45 cd	103.66 a	ND	434.65 d	3337.25 c	ND	ND	ND
H20-7	51.55 d	124.95 d	132.9 e	ND	638.1 bc	ND	3341.32 a	6294.7 a	ND	42.33 a	702.67 b	5723.4 a	ND	435.26 a	ND

The C, A5-3, A5-7, A20-3, A20-7, H5-3, H5-7, H20-3 and H20-7 treatments represent the control, 5 mM ASA for 3 d, 5 mM ASA for 7 d, 20 mM ASA for 3 d, 20 mM ASA for 7 d, 5 mM H_2O_2 for 3 d, 5 mM H_2O_2 for 7 d, 20 mM H_2O_2 for 3 d and 20 mM H_2O_2 for 7 d, respectively. The ASA and H_2O_2 refer to ascorbate and hydrogen peroxide, respectively. The different letters showed significant difference from each other at $p \leq 0.05$.

Table S6B. Effects of ascorbate (ASA) and hydrogen peroxide (H_2O_2) on the amino acid levels.

	Asp	Thr	Ser	Asn	Glu	Gln	AAA	Pro	Gly	Ala	Cit	Val	Cys
C	165.51 e	89.54 d	45.81 c	68.87 g	198.79 a	131.11 d	14.06 b	1.35 e	20.58 de	94.68 c	3.69 b	23.04 e	2.72 cd
A5-3	222.79 b	147.34 a	52.98 a	236.02 e	148.06 c	153.86 c	18.41 a	35.21 c	38.21 b	65 d	2.42 b	46.47 c	1.79 ef
A5-7	113.33 g	70.34 e	19.41 d	365.51 c	47.43 e	75.14 f	12.49 b	13.10 d	14.1 fg	40.17 e	3.55 b	18.66 e	1.47 f
A20-3	180.12 cd	140.34 ab	52.85 a	266.53 d	91.6 d	123.04 d	20.11 a	75.4 b	14.73 fg	41.96 e	5.35 b	59.38 a	3.98 b
A20-7	188.98 c	48.26 f	13.72 e	390.63 b	23.85 f	61.86 g	8.54 c	166.72 a	12.83 g	24.81 f	3.53 b	41.50 c	3.27 bc
H5-3	172.14 de	139.99 ab	42.53 c	109.43 f	169.39 b	203.74 b	20.32 a	0.59 e	110 a	101.28 b	3.38 b	33.91 d	2.66 cd
H5-7	187.51 c	102.75 c	40.90 c	48.16 h	142.70 c	98.38 e	18.38 a	1.76 e	26.92 c	101.54 b	10.00 a	23.84 e	3.39 bc
H20-3	139.19 f	129.16 b	42.52 c	107.37 f	151.40 c	102.73 e	20.00 a	40.45 c	17.24 ef	63.24 d	2.16 b	23.84 e	2.29 e
H20-7	314.41 a	77.26 e	40.04 c	468.55 a	143.02 c	270.82 a	19.44 a	37.65 c	23.44 d	105.18 a	3.99 b	53.33 b	4.85 a
	Met	Cysta	Ile	Leu	Tyr	Phe	GABA	Orn	Lys	His	1mHis	3mHis	Arg
C	9.66 b	4.73 d	12.23 c	29.15 c	9.58 ef	14.52 f	438.18 a	1.086 d	28.62 bc	7.57 f	1.57 d	4.45 e	19.10 c
A5-3	6.46 b	14.44 c	15.12 b	32.41 c	17.83 bc	31.81 b	340.31 b	1.21 d	30.72 b	28.13 b	3.91 b	8.29 d	29.38 b
A5-7	3.35 d	5.37 d	6.95 d	17.94 e	8.46 f	10.11 f	150.01 e	0.32 e	13.70 e	8.77 f	3.89 b	2.81 f	10.85 e
A20-3	6.28 b	22.23 b	24.92 a	42.83 a	21.56 a	40.36 a	311.13 c	0.57 e	31.37 b	21.83 c	2.72 c	13.31 b	28.81 a
A20-7	4.66 c	34.69 a	4.38 e	15.51 e	19.91 ab	17.86 e	97.88 f	0.48 e	14.06 e	17.38 d	2.84 c	11.35 c	18.03 cd
H5-3	6.006 b	3.30 d	5.09 de	24.19 d	12.53 d	14.85 f	336.10 b	2.18 ab	22.23 d	7.94 f	3.04 c	1.23 g	16.01 d
H5-7	6.87 b	4.11 d	5.76 de	30.31 c	11.50 de	13.77 f	430.28 a	1.42 cd	25.49 c	7.37 f	1.73 d	4.95 e	17.72 cd
H20-3	4.45 c	5.04 d	7.57 d	22.07 d	16.21 c	23.26 d	248.64 d	1.79 bc	20.59 d	13.12 e	1.67 d	7.9 d	19.37 c
H20-7	5.86 a	23.45 b	14.77 b	36.05 b	18.48 bc	28.46 c	343.29 b	2.40 a	35.93 a	37.79 a	7.52 a	22.22 a	34.73 a

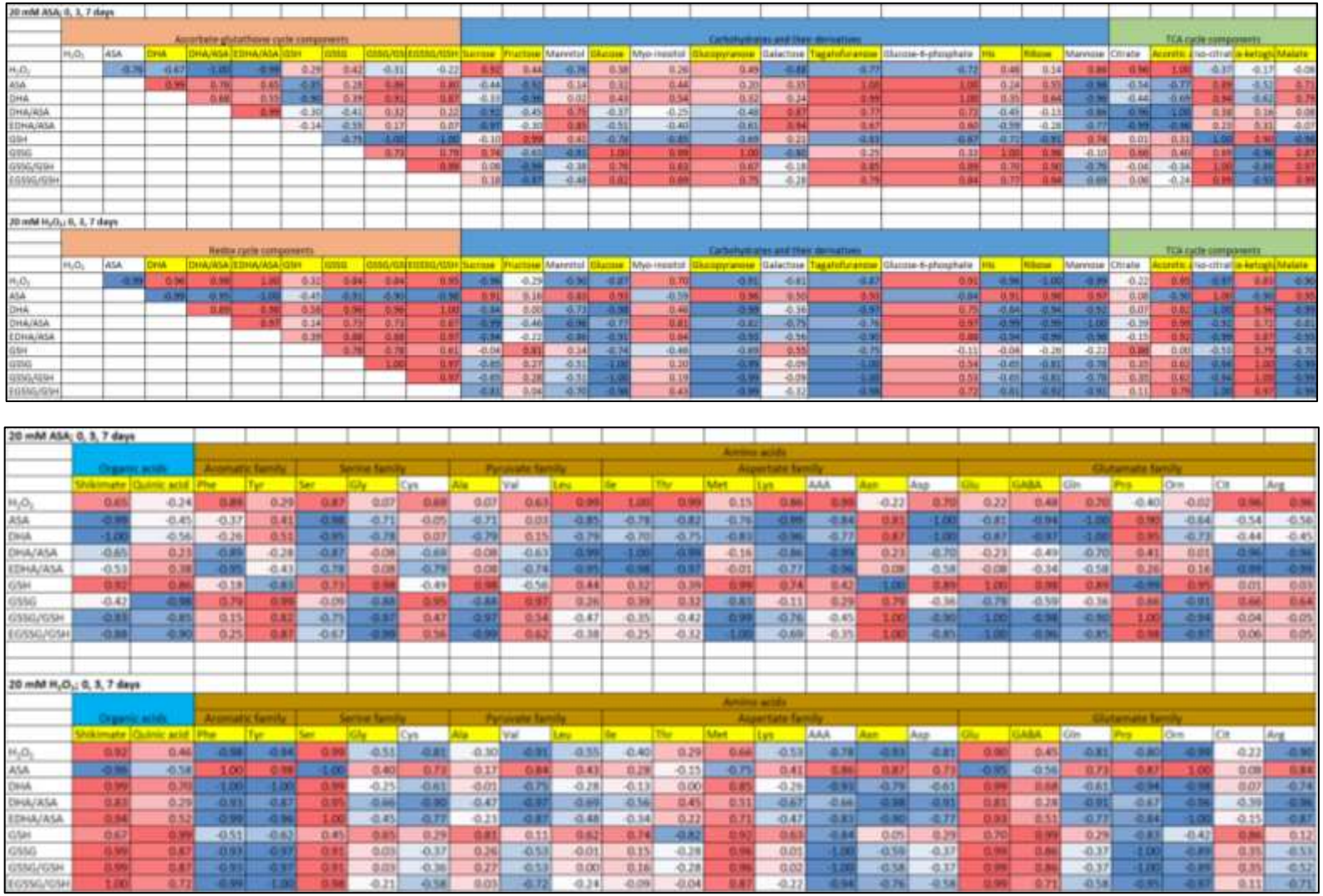
The C, A5-3, A5-7, A20-3, A20-7, H5-3, H5-7, H20-3 and H20-7 treatments represent the control, 5 mM ASA for 3 d, 5 mM ASA for 7 d, 20 mM ASA for 3 d, 20 mM ASA for 7 d, 5 mM H_2O_2 for 3 d, 5 mM H_2O_2 for 7 d, 20 mM H_2O_2 for 3 d and 20 mM H_2O_2 for 7 d, respectively. The ASA and H_2O_2 refer to ascorbate and hydrogen peroxide, respectively. The different letters showed significant difference from each other at $p \leq 0.05$.

Table S6C. Influence of the ascorbate (ASA) and hydrogen peroxide (H_2O_2) on the gene expression.

	TaGR	*TaNR*	*TaGST*	*TaGlnS*	*TaGSHS*	*TaP5CR*	*TaECS*	*TaASPTA*	*TaAPX1*	*TaChlECS*	*TaAPSR*	*TaARGSUL*	*TaCysASTL*	*TaGluDH*	*TaOrnATF*	*TaArgDC*	*TaP5CS*	*TmSerHMT*	*TacMDHAR I*
C	1 f	1 b	1 e	1 b	1 d	1 e	1 d	1 e	1 e	1 d	1 c	1 d	1 cd	1 c	1 d	1 e	1 c	1 cd	1 e
A5-3	2.45 bcd	0.30 c	4.73 cd	0.61 cd	1.66 cd	1.31 cde	1.08 d	2.15 de	1.75 bc	1.30 d	2.06 b	1.08 cd	1.49 abc	1.19 c	7.87 c	1.22 de	1.05 c	1.21 bcd	2 abcd
A5-7	2.85 bc	1.06 b	6.40 bc	1.37 a	2.84 b	1.82 b	1.91 b	3.83 c	1.63 bc	2.04 c	1.62 bc	1.35 bcd	1.15 bc	0.82 c	8.52 c	2.39 bc	0.61 d	0.89 d	2.15 abc
A20-3	3.04 b	0.41 c	4.49 d	0.43 d	1.82 cd	1.69 bc	1.46 c	2.60 cd	1.18 de	1.40 d	1.55 bc	1.08 cd	1.24 abc	1.24 c	1.54 d	0.84 e	0.66 d	1.18 bcd	1.73 bcde
A20-7	4.52 a	0.89 b	12.58 a	0.91 b	5.46 a	2.68 a	3.10 a	11.51 a	1.54 cd	3.09 b	2.09 b	1.46 bc	1.64 ab	1.87 b	12.34 b	2.79 b	0.93 cd	2.31 a	2.68 a
H5-3	1.68 e	0.35 c	2.36 e	0.93 b	1.84 cd	1.08 de	0.96 d	1.67 de	2.43 b	2.72 b	2.17 b	1.92 a	1.68 a	2.28 a	10.43 bc	1.82 cd	1.50 b	2.04 a	1.44 cde
H5-7	1.82 de	0.42 c	1.86 e	0.82 bc	1.86 cd	1.56 bc	1.86 b	2.15 de	3.78 a	4.56 a	3.16 a	2.10 a	1.64 ab	1.82 b	24.04 a	5.29 a	1.51 b	1.42 bc	1.29 de
H20-3	2.47 bcd	1.07 b	6.77 b	1.27 a	2.06 bc	1.43 bcd	1.63 bc	2.72 cd	1.99 b	1.35 d	1.58 bc	1.42 bcd	0.62 d	0.97 c	1.97 d	1.28 de	0.58 d	0.76 d	2.321 ab
H20-7	2.30 cde	1.35 a	5.59 bcd	0.96 b	2.02 bc	1.49 bc	1.70 bc	8.31 b	1.95 b	1.96 c	1.87 bc	1.72 ab	1.33 abc	1.97 b	9.47 c	2.28 bc	2.18 a	1.54 b	1.79 bcd
	TacMDHAR II	*TachlMDHAR*	*TamMDHAR*	*TacDHAR*	*TachlDHAR*	*TacAPX I*	*TacAPX II*	*TatAPX*	*TasAPX II*	*TamAPX*	*TachlGR*	*TaG6PDH-Other*	*TaG6PDH-CY*	*TaG6PDH-P1*	*TaG6PDH-CP1*	*TaG6PDH-CP*	*TaG6PDH-P2*	*TaG6PDH*	*Ta6PGDH*
C	1 e	1 c	1 c	1 d	1 a	1 e	1 cd	1 a	1 d	1 e	1 bc	1 d	1 d	1 d	1 cd	1 bc	1 bcd	1 e	1 d
A5-3	4.66 cd	1.78 ab	1.57 abc	1.72 bcd	1.13 a	1.76 cd	1.75 bcd	0.62 a	1.87 c	2.49 bcd	0.84 c	1.77 c	1.45 bcd	2.79 c	1.30 bcd	0.79 c	0.64 d	1.88 bcd	1.09 cd
A5-7	5.17 cd	1.22 abc	1.63 abc	2.38 ab	0.93 a	3.09 b	2.25 bc	1.01 a	2.18 bc	2.598 abcd	1.43 a	2.65 b	1.62 bc	5.37 b	1.82 b	1.91 a	1.38 ab	2.47 b	1.33 bcd
A20-3	9.98 b	1.02 bc	1.19 bc	1.52 cd	0.97 a	2.46 bc	2.50 b	1.06 a	1.70 cd	2.32 bcd	0.69 c	1.77 c	1.44 bcd	2.63 c	1.60 bcd	0.82 c	0.77 cd	2.02 bc	1.30 bcd
A20-7	20.38 a	1.23 abc	1.43 abc	2.88 a	1.09 a	4.96 a	8.55 a	0.74 a	4.21 a	3.90 a	0.79 c	3.49 a	2.77 a	7.51 a	3.43 a	1.26 b	1.67 a	4.63 a	3.75 a
H5-3	3.58 de	0.87 c	1.08 c	1.45 cd	0.77 a	0.88 e	0.79 d	0.72 a	0.98 d	1.38 de	0.80 c	1.152 cd	1.26 cd	3.36 c	1.15 bcd	0.72 c	0.95 bcd	1.23 de	0.83 d
H5-7	2.26 de	1.33 abc	1 c	1.92 bc	1.16 a	1.29 de	2.02 bcd	1.07 a	1.38 cd	1.51 cde	1.38 ab	1.07 cd	1.62 bc	2.94 c	0.95 d	1.83 a	1.70 a	1.60 cde	1.57 bc
H20-3	7.26 bc	1.82 a	1.96 a	1.82 bcd	1.31 a	1.88 cd	2.73 b	0.97 a	2.17 bc	2.98 ab	1.47 a	3.01 ab	1.78 bc	5.29 b	1.76 bc	1.28 b	1.26 ab	2.37 bc	1.71 b
H20-7	5.19 cd	1.36 abc	1.86 ab	1.98 bc	0.89 a	2 cd	2.88 b	0.83 a	2.74 b	2.83 abc	0.77 c	2.97 ab	1.99 b	7.30 a	1.46 bcd	2.10 a	1.18 bc	1.81 bcd	1.57 bc

The C, A5-3, A5-7, A20-3, A20-7, H5-3, H5-7, H20-3 and H20-7 treatments represent the control, 5 mM ASA for 3 d, 5 mM ASA for 7 d, 20 mM ASA for 3 d, 20 mM ASA for 7 d, 5 mM H_2O_2 for 3 d, 5 mM H_2O_2 for 7 d, 20 mM H_2O_2 for 3 d and 20 mM H_2O_2 for 7 d, respectively. The ASA and H_2O_2 refer to ascorbate and hydrogen peroxide, respectively. The different letters showed significant difference from each other at $p \leq 0.05$.

Table S7. Correlations between the components of the ascorbate-glutathione cycle and the amount of metabolites shown on Fig. 8. after 20 mM ascorbate (ASA) and H_2O_2 treatments.



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Thank you all for being there when the boat was rocking!!!

Muhammad Ahsan Asghar

14 Dedication

To My Parents (Haji Asghar Ali And Mrs. Parveen Asghar Ali)

for raising me to believe that anything is possible

And To My Wife (Mrs. Tayyaba Ahsan) And Daughter (Ms. Hoore Ain Ahsan)

for making everything possible!!!

"***Not all those who wander are lost***"

J.R.R. Tolkien