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Physiological, structural, and biochemical leaf traits of selected Poaceae species involved in oxidative stress protection and acclimation to different light conditions.

Ph.D. Thesis

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Prague, 2023

Student's declaration:

I hereby declare that I have written this thesis by myself, that I have listed all information sources and literature used, and that this thesis was submitted only once and has not been used to apply for another academic title.

Prohlášení Studenta:

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Abstract

This thesis examines physiological, structural, and biochemical leaf traits in grasses (Poaceae family) and how they contribute to oxidative stress protection. Light is a major factor contributing both to oxidative stress in plants and the induction of protective mechanisms at the leaf level. The agriculturally important species barley (*Hordeum vulgare*) was used to investigate responses to varying irradiance and atmospheric CO_2 levels. Barley was further used as a model organism to study the influence of different spectral qualities on oxidative protective mechanisms, particularly phenolic compound induction. This thesis also examines the protective leaf functional traits of wild grass species (*Nardus stricta, Calamagrostis villosa, Molinia caerulea,* and *Deschampsia cespitosa*) in a high-irradiance arctic-alpine tundra grassland.

Phenolic compounds are important protective secondary metabolites in plants that protect against oxidative stress from high irradiance. This thesis evaluates phenolic compounds and their contributions to plant stress tolerance and introduces a novel method of quantifying the histochemical detection of phenolics in leaf cross-sections. Key results indicate that light quantity and quality play major influential roles in the accumulation of phenolic compounds. High irradiance conditions promote high phenolic accumulation — although elevated CO_2 can induce a similar effect. When it comes to inducing phenolic compounds, blue light (400-500 nm) is an essential spectral component (in the absence of UV) for phenolic accumulation, especially protective di-hydroxylated flavonoids. Although often considered aggregately, individual phenolic compounds have varying responses to drivers of oxidative stress and offer varying levels of protection. Thus, addressing phenolic profiles rather than total phenolics can provide useful clues about grass stress tolerance and response mechanisms.

Additionally, plant functional traits, including biochemical profiles, physiology, and morphology, all contribute to the ability of a grass species to endure its environmental conditions. Differences between phenolic profiles and stomatal traits influencing water use efficiency are observed between barley genotypes; and differences in growth strategy, resource utilization, and stress indicators are observed between wild grass species. The adaptive significance of leaf functional traits in Poaceae, such as growth morphology, phenology of pigment accumulation, phenolic profile, and element utilization are discussed in the context of wild grasslands. In the absence of human intervention/grazing, tall grasses (especially with early phenological leaf-out and brad phenolic profiles) have an adaptive advantage that may allow them to spread aggressively (e.g., *C. villosa*). Conservative morphology (e.g., *N. stricta*) reduces the stress experienced by an individual, however, it can be a population-level disadvantage in an increasingly competitive environment. Localized changes can result in microhabitats that favor certain grasses (e.g., *D. cespitosa*) leading to expansion and decline in different areas.

Keywords:

Artic-alpine tundra; Barley; *Callamagrostis villosa; Deschampsia cespitosa;* Flavonoids; Grasslands; Histochemical detection, *Hordeum vulgare*; Irradiance; Leaf anatomy, Leaf functional traits; *Molinia caerulea; Nardus stricta;* Oxidative stress, Phenolic acids; Phenolic compounds; Photoprotection; Plant stress; Poaceae; Protective mechanisms; Secondary metabolites

Contents

Hunt et al., 2021a, Hunt et al., 2021b, Pech et al., 2022, Hunt et al., 2023

Abbreviations:

ABA: Abscisic acid
ABI5: ABA-Insensitive 5 gene
APXs: Ascorbate peroxidases
bHLH: Basic helix-loop-helix family of transcription factors
CAT: Catalase
C4H: Trans-cinnamate 4-hydroxylase gene
Chl: Chlorophyll
CHS: Chalcone synthase gene
CRY: Cryptochrome gene family (blue light receptors)
CUNI: Charles University in Prague
DFR: Dihydroflavonol 4-reductase gene
DPBA: Diphenylboric acid 2-aminoethyl ester
DPPH: 2,2-Diphenyl-1-picryl-hydrazil
EPF / EPFL: Epidermal patterning factor / Epidermal patterning factor-like
F3′H: Flavonoid 3′-hydroxylase gene
FACE: Free air CO_2 enrichment
GPx: Glutathione peroxidase
GR: Glutathione reductase
HPLC-HRMS: High performance liquid chromatography – high resolution mass spectrometry
H_2O_2: Hydrogen peroxide
HY5: Elongated Hypocotyl 5 gene
LEDs: Light emitting diodes
LHC: Light Harvesting Complex
MGDGs: monogalactosyl diacylglycerols
MYB: Myeloblastosis family of transcription factors
N: Nitrogen

NADPH: Nicotinamide adenine dinucleotide phosphate
O_2: Molecular oxygen
$^1O_2$: Singlet oxygen
$O_2^{\bullet -}$: Super oxide
O_3: Ozone
$OH^{\bullet}$: Hydroxyl radical
PAL: Phenylalanine ammonia lyase gene
PAR: Photosynthetically active radiation
PCA: Principal component analysis
PheCs/PhCs: Phenolic compounds
PID: protein kinase PINOID
PIN: PIN-FORMED protein family of auxin transporters
PSI and PSII: Photosystem I and Photosystem II
PTAL: Phenylalanine/tyrosine ammonia lyase gene
RDA: Redundancy analysis
RGB: Red Green Blue light
ROS: Reactive oxygen species
Rubisco: Ribulose bisphosphate carboxylase/oxygenase
SLA: Specific leaf area
SOD: Superoxide dismutase
UV: Ultra-violet
UVR8: UV-B resistance 8 (ultraviolet B receptor 8) gene
VOCs: Volatile organic compounds
WUE: Water use efficiency

1 Introduction

Poaceae, colloquially known as the grass family, are highly important both in terms of global economics and agriculture, as well as environmental conservation. While Poaceae has over 11,000 members, a handful of grass species (corn, wheat, rice, and barley) deliver the nutritional foundation for billions of people worldwide, as well providing forage for livestock, and supporting textile, biofuel, and brewing industries. Moreover, grasses play a key role in ecosystem succession: improving soil structure increasing nutrient cycling, sequestering carbon, and providing vital habitat (especially to invertebrates).

Poaceae can be challenging to study. From an agricultural perspective, grasses have complex genomes with large amounts of repetitive DNA and show a great amount of genetic diversity, making it difficult to study specific traits at the genetic level. However, the fact that barley has been cultivated for over 10,000 years has led to a wide diversity of barley cultivars. This genetic diversity provides a rich resource for breeding new varieties and makes it a promising model organism for eco-physiological investigations on the challenges posed by climate change.

From an ecological perspective, grasslands represent an important landscape type, accounting for 38 % of earth's surface — however, their reduced flowers can make parsing out individual species challenging, and eco-physiological instruments require some adaptation to be compatible with narrow leaf shapes. In the midst of climate change, research into plant stress, especially focused on Poaceae, is highly relevant.

Climate change is the most important issue of our time — concerns about which are particularly salient in the field of plant science and agricultural research. Disruptions in established precipitation and temperature patterns are a hallmark of climate change, as are increases in the frequency and intensity of severe climatic events, and uncertainty around crop yields. Food security is inherently tied to the ability of crop plants to survive and produce in their environments. Thus, the starting point of this Ph.D. work was examining barley leaves exposed to low, ambient, and elevated CO_2 in combination with low or high irradiance conditions, and then exposed to additional high temperature or drought stress. One aim of the first study of my Ph.D. was to develop a quantitative method for analyzing histochemical data. DPBA (diphenylboric acid 2-aminoethyl ester) staining was used on barley leaf cross-sections to show the localization of phenolic compounds accumulating in the leaves. Phenolic compounds act as antioxidants and protect plants from oxidative stress. The findings from this initial research project showed a very strong influence of light on phenolic compounds in terms of quantity, composition, and leaf tissue localization (**Hunt et al., 2021a**), while the influence of additional stressors (high temperature and drought) was difficult to untangle given our methodologies. Elevated CO_2 concentration also increased the accumulation of phenolic compounds, even in the absence of high light, supporting the theory that phenolic metabolism may act as a release valve to metabolically disperse excess carbon. This preliminary discovery led to a more careful examination of the role of light in determining leaf morphology and structural parameters, inducing biochemical protective mechanisms, and influencing overall physiology.

Climate change influences light conditions in a number of ways: alterations of the atmospheric composition change the amount of irradiation reaching the surface, which can be further influenced by temperature and changes in cloud cover. The second study of my Ph.D. compared high (light-saturating) irradiance conditions with low irradiance conditions, and their effect on protective phenolic profiles and stomatal behavior in barley. This stomatal research project combined classic eco-physiology with artificial intelligence (AI) to find and count stomatal complexes on barley leaves (**Hunt et al., 2021b**). These first two projects showed differences between oxidative stress-tolerant and oxidative stress-sensitive barley varieties, indicating that light serves as a useful variable for investigating overall stress response. Furthermore, the overall phenolic profile of the two barley varieties studied (Bojos and Barke) played a role in how receptive they were to environmental cues.

The influential role of light conditions barley continued in the third study of my Ph.D., but with a breakdown of the experimental light conditions into spectral components. The question to be answered was how specific spectral components of light (red versus green versus blue) might influence the phenolic profile of barley leaves in comparison to white (full spectrum) photosynthetically active radiation (PAR). From this work (**Pech et al., 2022**), it was confirmed that, in the absence of UV (ultra-violet), blue light had the greatest impact on phenolic compound accumulation – specifically di-hydroxylated flavonoids, which function more as antioxidants than as UV-screening compounds. This paper looked more carefully at gene expression and found enhanced expression of the F3′H (flavonoid 3-hydroxylase) gene is likely driving this increase in phenolic antioxidants.

The final work included in this thesis ventured out of the lab and into a natural ecosystem to observe how leaf functional traits and phenolic profiles might contribute to the changes in species composition. This project took place in the Krkonoše relict artic-alpine tundra grassland, where high elevation facilitates more incident PAR (photosynthetically active radiation) and UV exposure for plants. This inter-disciplinary project combined remote sensing data about changes in species composition over time, with ground-level eco-physiological investigations into the relative stress levels of the four main grasses in the area, showing that phenology of pigment accumulation as well as morphology and phenolic profiles play a role in the success of spreader species (**Hunt et al., 2023**). Because this study looked at four different species, we were able to evaluate different leaf functional traits, observe their potential advantages or disadvantages in the given environment, and relate this information to the changing species compositions observed in the area over the past few decades.

In summary, the articles contained in this Ph.D. thesis investigate the physiological, structural, and biochemical responses of Poaceae species to light conditions in the context of oxidative stress protection. A combination of methodological approaches provides an original integrated view on the structural-functional aspects of grass stress response. Furthermore, the contribution of individual phenolic profiles to stress protection in grasses is emphasized. This thesis aims to encourage researchers to look closer at what the phenolic profiles of grasses might convey, as the structural-functional intricacies are lost in many current studies available on the topic. I hope insights presented here will push forward research on the topic of stress response in grass species, encourage the consideration of functional traits in research aimed at grassland conservation, and highlight the fascinating role phenolic compounds play in oxidative stress protection.

Sketches of tundra grass species by the author, from left to right, Nardus stricta, Molinia caerulea, Deschampsia cespitosa, and Callamagrostis villosa.

2 Aims

The aims of this work were to expand the knowledge of protective mechanisms against oxidative stress in agricultural and wild members of the Poaceae family in the following ways:

1.) Examine the effect of light and CO_2 on leaf functional traits of barley leaves.
    a. Develop a novel quantitative method to analyze the accumulation of phenolic compounds in leaf cross-sections.
    b. Determine how light and CO_2 concentration interact to alter the accumulation and localization of phenolic compounds.
    c. Examine the differential effects of light and CO_2 on stomatal behavior and phenolic profiles in select barley cultivars.
2.) Investigate how spectral quality and intensity influence the profile and accumulation of phenolic compounds in barley.
    a. Ascertain which wavelengths (i.e., colors) of light are able to induce phenolic accumulation and at what intensities.
3.) Analyze how leaf functional traits, such as pigment accumulation, phenology, leaf shape, and element accumulation account for the expansion or retreat of wild grass species in an arctic–alpine tundra grassland.
    a. Identify leaf functional traits that could predict "spreader" species in high irradiation + high UV grassland ecosystems.
    b. Observe the role of phenolic compounds in plant success.
4.) To understand how different phenolic profiles confer stress protection in connection with other leaf functional traits.

3 Scientific Background

3.1 The double-edged sword of an oxygenated atmosphere: ROS and oxidative stress

The biochemical development of oxygenic photosynthesis by the ancestors of modern cyanobacteria is among the most important evolutionary steps influencing the trajectory of life on earth. Starting approximately 2.2 billion years ago, photosystem II's ability to oxidize water using energy from sunlight provided an abundant energy source for early cyanobacteria, producing O_2 as a waste product (Holland, 2020). This Great Oxidation Event, as its name suggests, radically increased the amount of oxygen present in the Earth's atmosphere over time, making O_2 available for aerobic respiration (an adaptation drastically more efficient at generating ATP than anaerobic fermentation and coincident with the advancement of complex eukaryotic life) (Raymond & Segrè, 2006). The reduction of oxygen provides one of the largest free energy releases per electron transfer (behind only fluorine and chlorine, which are far less abundant), thus, an oxygen-rich atmosphere provided a significant energy source to life on Earth (Catling et al., 2005). Furthermore, radicalization of O_2 in the upper atmosphere by UV radiation produced the ozone (O_3) layer, which filtered out much of the high energy UV-irradiation, allowing life on the Earth's surface to develop. The metabolic potential of O_2 came at a cost, however — the toxicity of ROS (reactive oxygen species) within cells.

ROS are defined as oxygen-containing molecules with a higher reactivity than molecular oxygen (O_2) (Waszczak et al., 2018). These include hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), the hydroxyl radical ($OH^\bullet$), as well as organic and inorganic peroxides (Mittler et al., 2022). ROS form easily from any misstep in electron transfer and are non-targeting and highly destructive inside the cells, oxidizing lipid membranes, damaging proteins, and altering DNA and RNA (Lane, 2002). ROS within the cell interact with phytohormones and epigenetic modifiers to influence development and stress response as signal stimuli, particularly influencing pathways involved in stomatal behavior, pathogen defense, and programmed cell death (Huang et al., 2019). The overproduction and accumulation of ROS results in a state of oxidative stress, leading to significant damage of cellular components and ultimately cell (and possibly organismal) death. The effect of unchecked oxygen radicals is perhaps one of the oldest stresses known to life on Earth (Dowling & Simmons, 2009). However, harsh environmental conditions, including temperature extremes, drought, metal toxicity, and pollutants have all been shown to induce ROS generation (Huang et al., 2019). Aerobic life forms had to develop novel methods to maintain safe levels of ROS, including enzymatic and non-enzymatic antioxidant systems.

3.2 Functional significances of ROS

For a long time, ROS were considered just a toxic by-product of aerobic metabolism (Das & Roychoudhury, 2014). Over the past few decades, evidence for ROS serving an essential role as signaling molecules has emerged (Dröge, 2002). It seems that aerobic organisms have evolved to not only survive the presence of ROS, but to make advantageous use of them. ROS signaling facilitates numerous processes in plants, including cell cycling (Fehér et al., 2008), hormone signaling (Devireddy et al., 2021), pollen tube and root hair formation (Mangano et al., 2016), and cell wall development (Kärkönen & Kuchitsu, 2015). The function of ROS as signaling molecules relates to predictable site- and time-specific modulations (Mittler, 2017). Stress can disturb the balance between ROS generation and detoxification, resulting in an uncontrolled ROS formation and the damaging effects of oxidative stress. ROS can also be synthesized de novo via NADPH oxidases and peroxidases on the plasma membrane — effectively increasing ROS as a means to trigger stress-response pathways (Demidchik, 2010). Accumulation of ROS in plants can be summarized into three categories (Vaahtera et al., 2014): (i.) Accumulation of background ROS due to inefficient ROS-scavenging; (ii) Metabolic imbalances as a result of stress that result in elevated ROS levels; (iii) Active ROS production via enzymes (i.e., oxidative bursts).

The accumulation of ROS affects the redox state of enzymes, receptors, and small molecules involved in stress-response signal transduction pathways, ultimately altering gene expression and influencing plant response to stress (Mittler et al., 2022). Cellular proteins vary in their sensitivity to ROS, however, misfolded proteins are more often the target of oxidative damage and subsequent proteolytic degradation (Davies, 1987). Paradoxically, the production of ROS can prevent cells from succumbing to oxidative damage. However, the initiation of ROS perception as well as complete signaling pathways remain unclear.

3.2.1 Common ROS in plants and their distinctions

As the term "reactive" suggests, these molecules are short-lived, and tend to be compartmentalized. Individual ROS have distinct lifetimes, reactivity, and roles in redox pathways. In general, radical forms of ROS are more reactive than non-radical forms (Janků et al., 2019).

The most potent and simultaneously the shortest-lived ROS is the hydroxyl radical ($OH^{\bullet}$). It is produced in the cell wall and plasma membrane, and is generated by a range of superoxid dismutases (SODs), NADPH oxidases, peroxidases, and transition metal catalysts (Richards et al., 2015). $OH^{\bullet}$ is essential for cell growth as its radical nature loosens the polysaccharides within the cell wall matrix (Schopfer, 2001). It also facilitates seed germination, stomatal closure, and immune responses (Richards et al., 2015).

Singlet oxygen (${}^1O_2$) is mostly produced in the thylakoid membranes of mesophyll cells and represents the lowest excited electron state of molecular oxygen. It forms as a side-product of triplet state molecular oxygen (${}^3O_2$) generated in the oxidation of H_2O in PSII (photosystem

II). $^1O_2$ can diffuse out of the chloroplast, relaying information to the nucleus and influencing nuclear gene expression (Dmitrieva et al., 2020).

Superoxide (O_2^-) is generated in the mitochondria, as well as in the chloroplasts by a one-electron reduction of O_2 at PSI (photosystem I) (Waszczak et al., 2018). It is then dismutated to H_2O_2 in the stroma spontaneously or by SODs. O_2^- can also be released in an oxidative burst in response to wounding (Prasad et al., 2017). O_2^- may initiate reaction cascades generating H_2O_2 for signal transduction.

Hydrogen peroxide (H_2O_2) is the least reactive ROS. It is produced by leaky electron transport chains in the chloroplasts and mitochondria, by plasma membrane NADPH oxidases, peroxisomal oxidases, and via the dismutation of O_2^- by SOD. H_2O_2 functions well as a signaling molecule as it can diffuse across lipid membranes and through aquaporins. It is degraded through the action of numerous enzymatic antioxidants. H_2O_2 signaling is mediated through proteins with redox sensitive moieties, such as metal centers or cysteine thiols, whose oxidation controls their activity (Antunes & Brito, 2017). Apoplastic H_2O_2 aids in polymer cross-linking, lignification, and cell expansion (Smirnoff & Arnaud, 2019). Excess H_2O_2 triggers chloroplast and peroxisome autophagy and programmed cell death in plants (Gadjev et al., 2008).

Certain regions, such as the stroma and cytosol, are highly buffered against ROS accumulation, while signal transduction is more likely to occur in areas, such as the thylakoid lumen or apoplast (Foyer & Noctor, 2016). ROS are likely sensed directly at the intersection of the apoplast and plasma membrane after activation of NADPH oxidase, which in turn initiates intercellular signaling resulting in changes in gene expression (Tripathy & Oelmüller, 2012). It is yet unknown how ROS are sensed specifically (if by some unidentified receptors), however, certain compounds, such as transcription factors may be sensitive to overall cellular redox, and ROS may directly disrupt phosphatase activity (Tripathy & Oelmüller, 2012).

Although redox regulation is relevant in other biological systems, plants present a unique situation as they rely on photosynthesis and thus expose themselves to a continuous light-dependent generation of both O_2 and ROS. Moreover, as sessile organisms, they have limited control over their ambient light and temperature environments, which intrinsically drive their metabolism. Most environmental stress manifests as oxidative stress within cells. A basal level of ROS is essential to cellular homeostasis (Mittler, 2017), and is maintained by a balance between the rate of ROS production and the rate of ROS scavenging by antioxidant compounds and enzymes. Cellular homeostasis can be disrupted by various distinct stressors: high and low temperatures, high-intensity light (especially UV radiation), drought, pollution, pathogens, and chemical pesticides/herbicides (Inzé & Montagu, 1995; Xie et al., 2019). This is a major issue in agriculture as crop productivity depends on the ability of plants to tolerate their environmental conditions. The issue becomes more pressing each year as anthropogenic climate change ushers in unprecedented instability to annual temperature and precipitation norms.

3.3 Major sites of ROS production in plants

The main sites of ROS production in plants are the chloroplasts and peroxisomes in the presence of light, and the mitochondria independent of light conditions (Choudhury et al., 2013), although they are also found in the cytosol and apoplast (Tripathy & Oelmüller, 2012). Amine oxidase in cells walls and NADPH oxidase in the plasma membrane produce ROS in response to stress (Tripathy & Oelmüller, 2012).

3.3.1 Chloroplasts

Chloroplasts are the organelles responsible for the conversion of PAR from electromagnetic energy to chemical energy. Only a minor portion of incident photons drive the chemical reactions necessary for photosynthesis, while the rest represents excess energy that must be safely dissipated to avoid ROS formation. Incident photons do not strike the reaction center directly but are funneled through an antenna complex of light-absorbing pigments, known as the light-harvesting complexes (LHC), to reach the reaction center. LHCs are embedded in the thylakoid membrane and serve as essential cofactors for PSI and II reaction centers (Fahnenstich et al., 2008). Excess incident irradiation is partially dissipated by a group of carotenoids, another type of pigment molecule found in the LHCs, which participate in the xanthophyll cycle – violaxanthin, antheraxanthin and zeaxanthin. Under high irradiation, the carotenoid violaxanthin is rapidly converted to zeaxanthin via antheraxanthin, and the process is reversed under low irradiation conditions. This allows for dissipation of excess photons as heat via zeaxanthin, and increases photon capture by violaxanthin under light-limiting conditions (Demmig-Adams & Adams, 1996). Regardless, ROS production is inevitable in chloroplasts.

When PAR strikes the chloroplast, pigment molecules in the LHC pass along the excited state until it reaches a particular chlorophyll molecule in the reaction center, where a charge separation occurs. Upon excitation, the electron in the reaction center of chlorophyll moves to a higher energy state, making it a strong reducing agent, which can then pass an electron to an electron acceptor (Wayne, 2009). The chemical energy is then conserved in $NADPH_2$ and ATP. Both PSI and PSII produce ROS. An excess of excitation energy in PSII leads to a triplet state excited chlorophyll molecule, which can transfer its electron to O_2, generating singlet oxygen $^1O_2$. The $^1O_2$ production within the thylakoid membranes is mainly responsible for the photodamage experienced by lipids and proteins (Triantaphylidès & Havaux, 2009). Furthermore, electron leakage on the acceptor side of PSII leads to the production of O_2^-, which then undergoes dismutation to produce H_2O_2 ; H_2O_2 is reduced by a non-heme iron producing $OH^\bullet$ (Pospíšil, 2016). In PSI, production of O_2^- and H_2O_2 occurs as a result of PSII-derived electrons being passed to O_2 via PSI electron transport machinery. Two enzymatic antioxidants act to detoxify these species: superoxide dismutase (SOD) catalyzes dismutation of O_2^- to O_2 and H_2O_2 and ascorbate peroxidases (APXs) detoxify H_2O_2 via the ascorbate-glutathione cycle (Li & Kim, 2022). See Figure 1 for an overview of ROS production in the chloroplasts.

Following its acquisition by early eukaryotes, the chloroplast genome was significantly reduced, meaning that basic processes, such as assembly of protein complexes, require coordination with the nucleus (Rousseau-Gueutin et al., 2018). This communication between the nucleus and the plastids occurs as part of plastid formation (biogenic signaling) or as a way of responding to oxidative stress experienced within the plastid (operational signaling) (Chan et al., 2016). Chloroplast-associated operational signaling may contribute significantly to overall cellular homeostasis and stress response (Li & Kim, 2022). Chloroplasts are further involved with stress-related molecules, including reactive nitrogen and sulfur species, volatile compounds, precursors of the phytohormones salicylic, jasmonic, and abscisic acids, as well as secondary metabolites (more on this in Section 4).

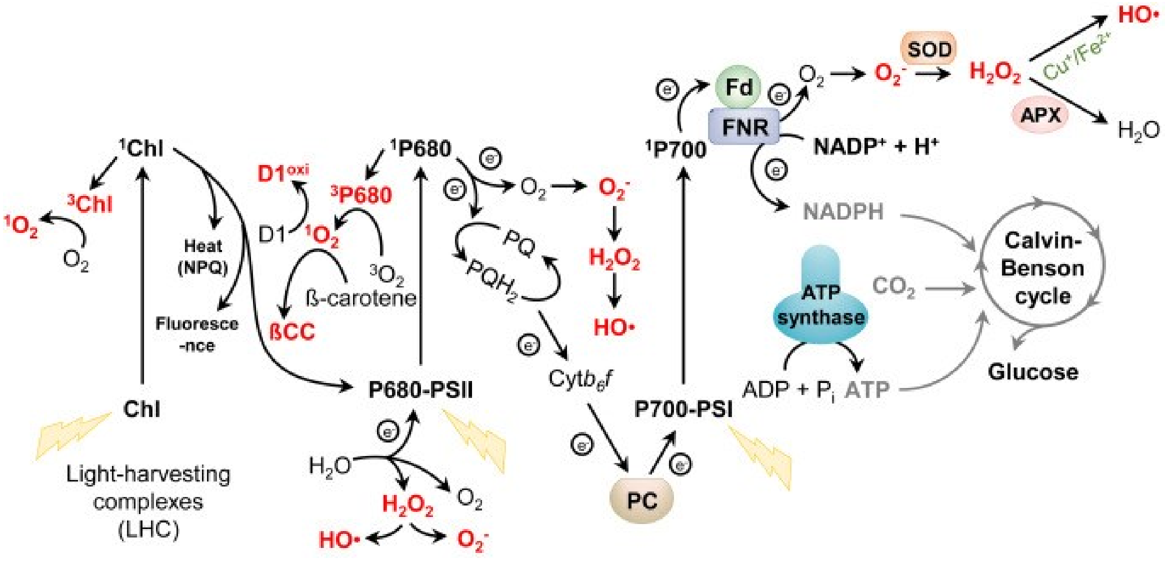

Figure 1 (from Li and Kim et al 2022): production of ROS in the chloroplasts. Photon striking a chlorophyll (Chl) in the light harvesting complex (LHC) generates an excited state chlorophyll (^{1}Chl). The excess energy can be dissipated as heat in a process of non-photochemical quenching (NPQ), or re-emitted as fluorescence. ^{1}Chl can also decay into triplet state chlorophyll (^{3}Chl), which can transfer energy to molecular oxygen (O_2) producing singlet oxygen $^1O_2$. An excited state at the photosystem II (PSII) reaction center Chl (P680) can also form $^1O_2$ if the electron transport chain (ETC) is over-reduced. H_2O_2 is produced by two oxidations of H_2O on the donor side of PSII, and superoxide O_2- can be formed by a one-electron reduction on the acceptor side of PSII. When the ETC is over-reduced, the PSI electron acceptor ferredoxin (Fd)-NADP$^+$ oxidoreductase (FNR) transfers an electron to O_2, generating O_2^- (rather than NADP$^+$, to produce NADPH and drive ATP synthesis). O_2^- is changed into H_2O_2 by superoxide dismutase (SOD) a H_2O_2 is scavenged by ascorbate peroxidases (APXs) in the ascorbate-glutathione cycle. The interaction of H_2O_2 with a reduced transition metal ion (such as Fe^{2+} or Cu^+) generates hydroxyl radical $OH^{\bullet}$ via the Fenton reaction.

3.3.2 Peroxisomes

Peroxisomes are a single-membrane bound organelle housing diverse metabolic reactions. They are a potent source of H_2O_2, $O_2^{\bullet-}$, and $OH^{\bullet}$, which are produced during processes such as photorespiration, fatty acid β-oxidation, and nucleic acid and polyamine catabolism (Sandalio et al., 2013). Photorespiration is the main source of intracellular H_2O_2 (Foyer & Noctor, 2009). It occurs when O_2 rather than CO_2 binds with the active site on the enzyme rubisco (ribulose bisphosphate carboxylase/oxygenase), forming 2-phosphoglycolate, which must be converted into 3-phosphoglycerate. This synthesis of 2-phosphoglycolate is accompanied by stoichiometric H_2O_2 production by peroxisomal O_2-dependant glycolate oxidase (Sørhagen et al., 2013). Peroxisomes have a high concentration of H_2O_2-generating oxidases, as well as detoxifying enzymes, such as catalases (Bonekamp et al., 2009). The key role of peroxisomes in oxidative metabolism is highlighted by estimates that they are responsible for 20 % of cellular oxygen consumption and 35 % of H_2O_2 production (Boveris et al., 1972).

3.3.3 Mitochondria

Finally, mitochondria (the primary ROS generator in animal cells) vary in their contribution to ROS in plants. It is the main producer of ROS in non-green tissues or under dark conditions (Navrot et al., 2007). Curiously, in animal cells at least, exposure to blue light can induce production of mitochondrial ROS (King et al., 2004). Respiration occurs through a process of electron flow culminating in charge acceptance by O_2. Under normal circumstances, $O_2^{\bullet-}$ is produced as a byproduct of the respiratory chain, however decreased respiratory rates generate a highly reduced mitochondrial electron transport chain and ultimately, an increase in ROS production (Janků et al., 2019). SOD, present in the mitochondrial matrix, disproportionations $O_2^{\bullet-}$ into H_2O_2 and O_2 (Morgan et al., 2008).

Although not always the main producers of ROS, mitochondria were found to be the main site of oxidative damage in drought-stressed wheat plants, with 9-28 fold more oxidatively modified proteins compared to the chloroplasts or peroxisomes (Bartoli et al., 2004).

3.4 Phenolic Compounds

Plants synthesize an enormous number of chemical compounds — over 200,000 individually identified and isolated entities (Lattanzio, 2013). They are classically separated into primary and secondary metabolites. The former contains compounds considered essential to basic plant growth and development connected with carbon metabolism (sugars, fatty acids, amino acids, nucleotides), while the latter contains compounds that allow the plant to be competitive in their environments (Teoh, 2015). However, this distinction is weak as even basic survival would not be possible without secondary metabolites in most environments. Phenolic compounds are the largest group of secondary metabolites (8,000 naturally occurring compounds), ranging from simple structures containing a single aromatic ring with one or more hydroxyl groups, to structurally complex polymers. Despite being "secondary metabolites," products of the phenylpropanoid pathway account for about 40 % of organic carbon on Earth (de Vries et al., 2021). Phenolic compounds serve diverse roles in plants, ranging from structural support, defense against biotic stress, attenuation of UV irradiation, and scavenging of ROS (Dixon & Paiva, 1995). The ability to synthesize phenolic compounds via the phenylpropanoid pathway has been considered basal to terrestrial plant evolution (Emiliani et al., 2009). Phenolic compounds have a weak yellow autofluorescence, which can be enhanced histochemically via treatment with DPBA (see Figure 2).

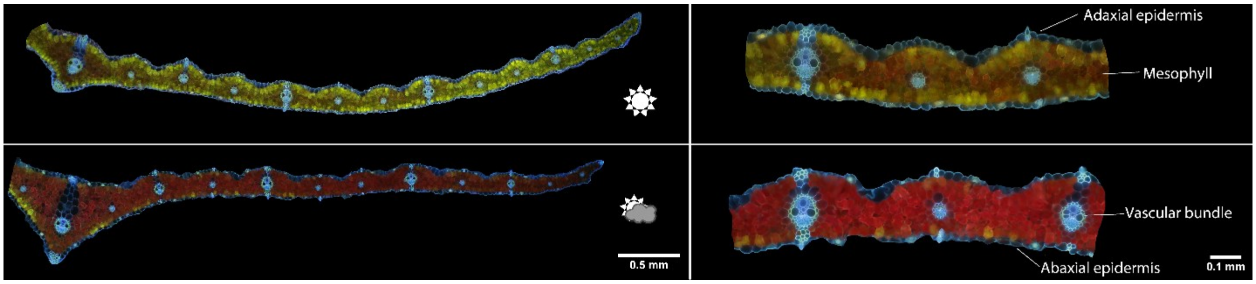

Figure 2: Cross-sections of barley (Hordeum vulgare) leaves treated with DPBA to enhance the fluorescence of phenolic compounds (yellow). Chlorophyll fluorescence (red) and cell wall fluorescence (blue) are also visible. The top leaf cross section (detail on right) comes from a leaf grown in high light (1500 µmol $m^{-2}s^{-1}$ PAR with 4 W m^{-2} UV-A maxima) and has a greater phenolic presence, especially deeper in the mesophyll, compared to the bottom leaf cross section, which was grown in low light conditions (400 µmol $m^{-2}s^{-1}$ PAR with 0.75 W m^{-2} UV-A maxima) and shows predominantly chlorophyll fluorescence, with a slight phenolic presence near to leaf surfaces.

The phenylpropanoid pathway generates diverse and multifunctional compounds. Lignin, for example, is a complex polymer made up of phenolic subunits, which endows rigidity to cells walls, and enables long-distance transport of water and minerals thanks to its hydrophobic properties (Liu et al., 2018). Without lignin, the diversity of modern plants as we know it would not be possible. A useful characteristic of the phenolic ring structure is its ability to absorb UV radiation (Aleixandre-Tudo et al., 2018).

In terms of environmental stress response, research has been focused on phenolic acids and flavonoids. Phenolic acids and flavonoids (including anthocyanins) are major non-enzymatic antioxidants, alongside ascorbic acid, tocopherols, glutathione, and carotenoids (Ashraf et al., 2019). Non-enzymatic antioxidants work in tandem with enzymatic antioxidants — SOD, CAT (catalase), GPx (glutathione peroxidase), and GR (glutathione reductase), to maintain sub-toxic levels of ROS. Antioxidative activity is determined by a compound’s reactivity as a hydrogen or electron donating agent and the stability of the subsequent antioxidant-derived radical, as well as its reactivity with other antioxidants (Rice-Evans et al., 1996).

3.4.1 Structural-functional properties of phenolic compounds

3.4.1.1 Flavonoids

Phenolic compounds radiate out from a few basic forms, broadly categorized as simple phenols (one phenol group) and polyphenols (two or more phenol groups). Flavonoids, the largest class of secondary metabolites, are polyphenols with at least two phenol subunits. They are characterized by 15 carbon atoms arranged as two aromatic rings (A and B), which may be connected by a 3-carbon bridge ($C_6C_3C_6$ carbon skeleton). Modification of this basic structure by various enzymes results in subclasses, such as flavonols, anthocyanins, anthocyanidins, flavanones, isoflavones, and flavones (Santos et al., 2017), see Figure 3. Polyphenols with three or more phenol subunits are classified as tannins.

The basic structure of a flavonoid is alglycone (not conjugated with a sugar moiety), however, many flavonoids occur conjugated with sugars as glycosides or as methylated derivatives (Kumar & Pandey, 2013). The 7-OH and 3-OH groups in flavones and flavonols, respectively, are most typically glycosylated (S. Kumar & Pandey, 2013). Glycosylated flavonoids are more soluble in the cytosol, allowing them to be transported to cellular compartments, such as the central vacuole, or be secreted to the plasma membrane or cell wall (J. Zhao & Dixon, 2010). Glycosylated flavonoids have reduced antioxidant activity compared to their corresponding aglycones (Rice-Evans et al., 1996). Flavonoids can also be found in the nucleus, chloroplast, and mitochondria (Pucker & Selmar, 2022). Some enzymes involved in flavonoid biosynthesis co-localize with the tonoplast (Marinova et al., 2007) and nucleus (Saslowsky et al., 2005), highlighting the differential targeting involved with flavonoid metabolism. Nucleus-localized flavonoids are hypothesized to protect against DNA damage (Melidou et al., 2005), while chloroplast and mitochondrial flavonoids prevent and scavenge ROS (Agati et al., 2012).

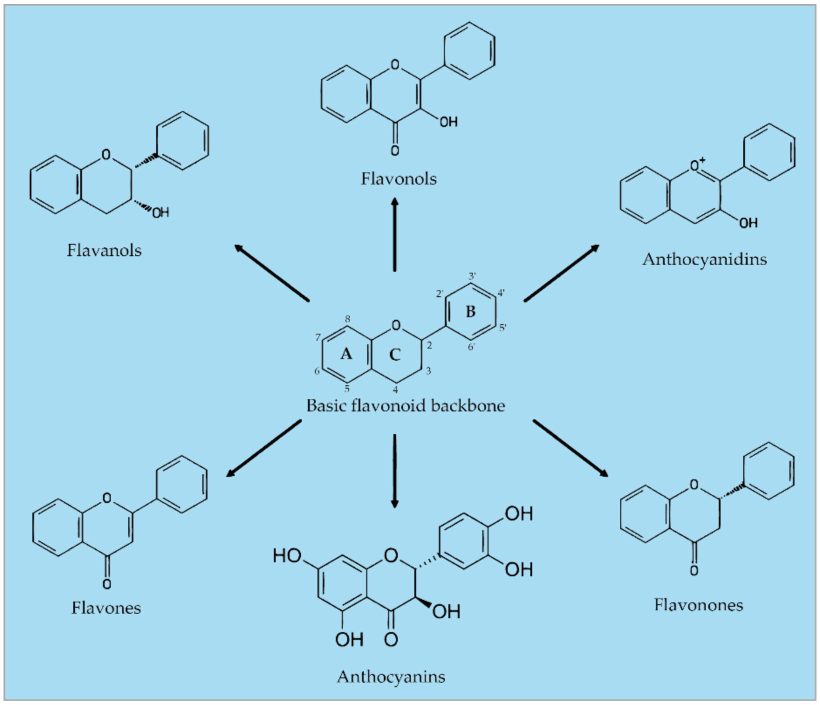

Figure 3: Structure of flavonoids, demonstrating the basic flavonoid backbone and variations resulting in respective subclasses of flavonoids. Aromatic rings A and B, with a 3-carbon bridge forming ring C. Modifications at each ring produce changes in bioactivity,

Different properties of flavonoids are influenced by hydroxylation patterns on their A, B, and C rings, with certain positions enhancing bioactivity. Antioxidative ability is most associated with the hydroxylation of the B ring (Spiegel, Andruniów, et al., 2020). For example, among three flavonols which share the same A and C ring configuration but vary in B ring hydroxylation — myricetin (3′,4′5′-OH), quercetin (3′4′-OH) and kaempferol (4′-OH) — tri-hydroxylated myricetin has the highest radical scavenging activity, followed by the di-hydroxylated quercetin and then the mono-hydroxylated kaempferol. Radical scavenging ability is thus increased with B ring hydroxyl groups. Di-hydroxylation at the 3′ and 4′ positions of the B ring is most common in flavones and flavonols (Rice-Evans et al., 1996). Anthocyanins (the glycoside conjugates of anthocyanidins) are pigment molecules whose colors darken from red to magenta to blue with increasing hydroxylation of the B ring (Tanaka & Ohmiya, 2008). Structural modifications on the other rings are under investigation as well. One study found that a hydroxyl group at the C5 position on the A ring (as on the flavonol quercetin) enhances membrane binding (Sinha et al., 2014). Another found that the enolic 3-hydroxy group on the C ring had a mesomeric pro- or anti-oxidative effect (Chobot et al., 2013).

3.4.1.2 Phenolic Acids

The phenolic acids consist of a benzene ring bearing a carboxyl and one or more hydroxyl groups. Phenolic acids are categorized as either hydroxycinnamic acid or hydroxybenzoic acid derivatives based on the length of the chain bearing the carboxyl group (See Figure 4). Hydroxycinnamic and hydroxybenzoic acid derivatives possess C_6C_3 and C_6C_1 carbon skeletons, respectively. The most common hydroxycinnamic acids are ferulic, caffeic, *p*-coumaric, and sinapic acids and the most common hydroxybenzoic acids are *p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids (Kumar & Goel, 2019). Hydroxycinnamic acids are abundant in most plants, presenting as esters with quinic acid or glucose: esterification generally enhances lipophilicity and antioxidant properties (Chalas et al., 2001). Between the two subgroups, the CH=CH-COOH structure of hydroxycinnamic acids generally affords them a greater antioxidant efficiency compared to the COOH group in hydroxybenzoic acids (Rice-Evans et al., 1996). A recent investigation into the structural features influencing antioxidant activity found phenolic acids with two or more hydroxyl groups in *ortho* or *para* position to one another has the highest antioxidant properties — greater than those with two hydroxyl group in *meta* position to each other, or to monohydroxylated compounds (Spiegel et al., 2020).

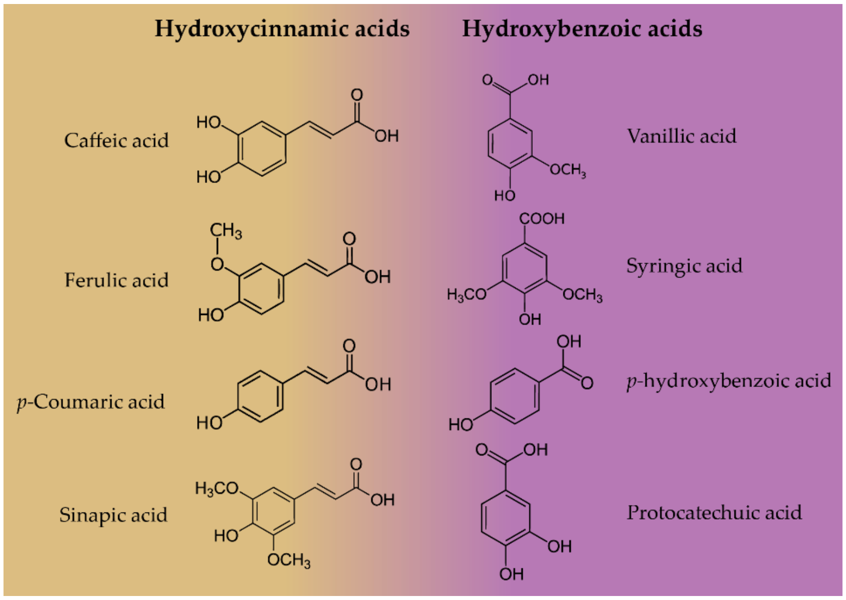

Figure 4: Structure of common phenolic acids, showing hydroxycinnamic acids on the left and hydroxybenzoic acids on the right. Colors correspond to the visual abstract for Hunt et al., 2021b (see page 28).

3.4.2 Phenolics in Poaceae

The enzyme PAL (phenylalanine ammonia lyase) is the first step in the phenylpropanoid pathway for synthesizing polyphenols, catalyzing the conversion of phenylalanine into cinnamate. Cinnamate is the precursor to B ring-deoxy-flavonoids. PAL occurs widely in cyanobacteria, plants and fungi, while Poaceae also possesses a bifunctional PTAL (phenylalanine/tyrosine ammonia lyase) (Barros & Dixon, 2020). The next step in the phenylpropanoid pathways is the hydroxylation of the aromatic ring by C4H (*trans*-cinnamate 4-hydroxylase), forming coumarate. Coumarate can also be formed directly from tyrosine through the action of PTAL, and is a precursor for coumarins, anthocyanins and isoflavones. This enables monocots of the Poaceae family to produce free phenolic acids more efficiently than most dicots.

Grass PTALs are thought to have emerged in the Paleocene during an ancient genome duplication event separating the order Poales from other monocots (Jiao et al., 2014). This would have coincided with a period of increased temperature and humidity known as the Pleocene-Eocene Thermal Maximum, resulting in increased fungal (Smith et al., 2020) and insect (Currano et al., 2008) diversification and placing new pressures on plant defense mechanisms. The possibility of using phenylalanine or tyrosine as a substrate for phenylpropanoid biosynthesis likely provided an adaptive advantage to grass cell walls, promoting diverse lignin compositions, cell-wall bound hydroxycinnamates, and presence of the flavonoid tricin (Barros & Dixon, 2020). The accumulation of these compounds would have increased grass structural support and aided ancient grasses in resisting pathogens and herbivores, in addition to the native UV shielding properties of phenolics.

Phenolic compounds are abundant in Poaceae and can be useful in determining interactions between grass species/genotypes and their environment. Some of the most common phenolic compounds identified in Poaceae include the hydroxycinnamic acids — chlorogenic acid, ferulic acid, caffeic acid, sinapic acid, and *p*-coumaric acid, and the hydroxycinnamic acids — gallic acid, vanillic acid, *p*-hydroxybenzoic acid, syringic acid and salicylic acid (Gebashe et al., 2020). When looking at eleven phenolic compounds across grass genera, ferulic acid, followed by *p*-coumaric acid, were found to be the most useful due to their high concentrations (Míka et al., 2005). Cell-wall bound ferulic acid in Poaceae contributes to the heightened blue-green autofluorescence in grass leaves compared to dicot plants — ferulic acid fluorescence is stronger than even chlorophyll fluorescence in grasses, while the opposite is true of dicots (Lichtenthaler & Schweiger, 1998). *P*-coumaric and caffeic acids also occur bound to grass cell walls, and hydroxycinnamic acids occur in vacuoles as well, however, these contribute relatively little to the characteristic blue-green fluorescence of Poaceae. As far as flavonoids, common examples found in barley and other grasses include flavones (apigenin, luteolin, isovitexin, homoorientin, and saponarin), flavonols (kaempferol and quercetin), and anthocyanins (cyanidin, cyanidin 3-glucoside, delphinidin, pelargonidin, pelargonidin glycosides, and petunidin 3-glucoside)(Hunt et al., 2021, 2023; Idehen et al., 2017). The principal flavonoid constituent found in barley is saponarin (apigenin-6-C-glucosyl-7-O-glucoside) (Seikel & Geissman, 1957). While saponarin occurs constitutively, high irradiance can positively affect ratios of lutonarin relative to saponarin in young barley leaves (Holub et al., 2019).

3.5 Irradiance effects on plants

Light is perhaps the most influential environmental factor for plants, intrinsically linked to metabolism, development, and stress response. Visible light is made up of a spectrum of electromagnetic radiation, with low energy red light (700 nm wavelength) on one side and high energy blue light (400 nm wavelength) on the other. The spectrum of visible light closely corresponds to the spectrum of PAR. Plants experience a range of light intensities and spectral properties as a result of atmospheric conditions and relative positions of foliage within a canopy. Different spectra of light influence plant physiology in various ways, and environmental information can be obtained by the spectral composition of light perceived by plants. For example, a below-canopy shade position can be detected by a high ratio of far-red to red light, indicating red light has mostly been absorbed by chlorophyll in other plants (Smith et al., 2010). As it pertains to ROS production, red light (620-750 nm) contains the energy to transition an electron to the first excited state, while blue light energy contains excess energy, exciting an electron to a higher excited state, which then loses energy to arrive back at the first excited state (Cardona et al., 2012). Red and blue light also influence stomatal function, with high fluence red light driving stomatal opening in relation to CO_2 demands, and low fluence blue light initiating stomatal opening at dawn (Matthews et al., 2020). Green light is mostly reflected or transmitted, accounting for the green appearance of leaves, however, the scattering of green light inside the leaf means it is able to stimulate photosynthesis at locations deeper in the mesophyll than blue or red photons normally reach (Smith et al., 2017). Rapidly changing light conditions (e.g., as a result of solar angle, cloud cover, or sun flecks produced by shifting canopies) can cause temporary stress and reduce carbon gain as photosynthesis rates become decoupled from actual conditions (Slattery et al., 2018).

3.5.1 UV radiation

Plants require sunlight for photosynthesis; however, this unavoidably exposes them to damage by UV irradiation. UV radiation accounts for about 7-10 % of the sun's electromagnetic output and is comprised of UV-A (315-400 nm), UV-B (280-315 nm), and UV-C (100-280 nm). Only wavelengths greater than 290 nm reach the Earth's surface: UV-C is absorbed by atmospheric gases. UV-B is also mostly absorbed by the ozone layer, however UV-A and a small amount of UV-B (95 % and 5 %, respectively) still affect organisms on the Earth's surface (Frohnmeyer & Staiger, 2003). This remaining UV radiation can still damage proteins and DNA — causing pyrimidine dimers, cyclobutene pyrimidine dimers, and pyrimidine-pyrimidone 6-4 photoproducts (Kemp & Sancar, 2012). These distortions of DNA structure throttle gene transcription and DNA replication at the damaged point; Interestingly, this can be reversed through a process called photoreactivation, where visible light is utilized to split the bond forming the cyclobutene ring (Cooper, 2000).

The photosynthetic apparatus is another sensitive target for UV radiation. Even very low fluence UV-B causes degradation of the D1-D2 protein heterodimer of the PSII reaction center; the process is accelerated in the presence of visible light (Booij-James et al., 2000). Some view this degradation process as more of a targeted repair cycle — "a feature, rather than a bug" of UV exposure (Jansen et al., 1996). As with ROS, it is dose-dependent and the line between improving acclimation and causing damage can become blurry. In fact, "damage" resulting in reduced metabolic efficiency and plant productivity can manifest as an increase in UV-tolerance. UV-B does not directly influence PSI, however, it can inactivate the oxygen evolving complex (OEC), thereby reducing electron flow to PSI and preventing photoinhibition in cold-stress (Zhang et al., 2016).

Plants exhibit variation in UV-B sensitivity and morphological adaptations to UV irradiance load along ecological gradients (Sullivan et al., 1992). Areas of low latitude or high elevation generally have a higher UV radiation load. Certain effects of UV radiation are highlighted in conditions of low PAR but high UV-B, such as decreased photosynthetic pigments, disruptions in thylakoid structure, reduced Rubisco activity, and increased stomatal diffusion (Jansen et al., 1998). Even at low levels, UV radiation stimulates the synthesis of phenolic compounds (Interdonato et al., 2011; Wang et al., 2022).

3.6 Leaf Functional Traits

Investigations into functional traits aim to recognize the ecological and evolutionary underpinnings of why plants "behave" as they do: why they grow in some places and not others, and how they interact with their biotic and abiotic environment (Reich, 2014). Leaf functional traits refer to key chemical, structural, and physiological leaf characteristics that determine plant productivity and competitive interactions within a given environment, as well as variations in species composition across environmental gradients (Rawat et al., 2021). In order for a trait to be truly functional at the individual level, it must contribute to one of the three components of plant fitness —growth, reproduction, and survival (Violle et al., 2007). Functional traits can be observed at the organ, individual, population, community, or ecosystem level.

An ecological community may be characterized by a lack/excess of the necessities of plant life or by competition for resources, resulting in selection for individual traits that can survive in that community, and influencing population levels and community dynamics. Examples of individual leaf functional traits include plant height, specific leaf area, leaf nitrogen concentration, chlorophyll/pigment concentrations, phenology, and morphology. Each functional trait represents a physiological or morphological trade-off the plant is making. For an overview of common plant functional traits and their significances, see Figure 5.

The theory of the "plant economic spectrum" provides a framework for evaluating species according to their trade-offs — strategies of rapid acquisition or resource conservation (Freschet et al., 2010). In general, resource-limiting environments favor slower growth rates, long leaf lifespans, low SLA (specific leaf area), and high levels of carbon-based secondary metabolites (e.g., phenolics, terpenoids, alkaloids). Conversely, in environments where water, light, or nutrients are available, the selective pressure of competition favors short lifespans, high photosynthetic rates, high SLA and leaf nitrogen content, and low investment in secondary metabolites (Jardine et al., 2020). Additional factors add selective pressure to fine-tune these strategies. For example, fast resource acquisition is only advantageous when matched by fast resource processing, and a balance between carbon, nutrients, and water — otherwise, it can lead to expensive uptake and a need for storage, ultimately wasting resources for the plant (Reich, 2014). Functional traits help explain the distributions of plant species and communities across gradients of light, water, and nutrient availability. The coexistence of multiple species within a community is fostered by the ability of plants to exploit the spatial and temporal variation in resources, i.e., microenvironments or niches. Greater plant community diversity in turn promotes higher diversity in insect populations, which continues to promote diversity at higher trophic levels.

Analysis of leaf functional traits may also highlight specific adaptations that benefit a given plant in its environment by comparing stress levels among plants exposed to the same conditions. Investigating sites with known constraints, such as low nutrient availability or high UV exposure, can reveal which leaf characteristics are preferentially selected by that environmental stressor. Locally adapted genotypes show a minimized range of potential leaf traits (Read et al., 2014). Analysis of functional traits has been used to forecast ecosystem responses to drought (Yan et al., 2019), biological invasions (Richardson et al., 1990), grazing (Jäschke et al., 2020), as well as contributions to climate regulation (Hanisch et al., 2020).

Moreover, remote sensing can be used to predict grassland functional trait and species diversity, aiding in grassland conservation efforts (Zhao et al., 2021).

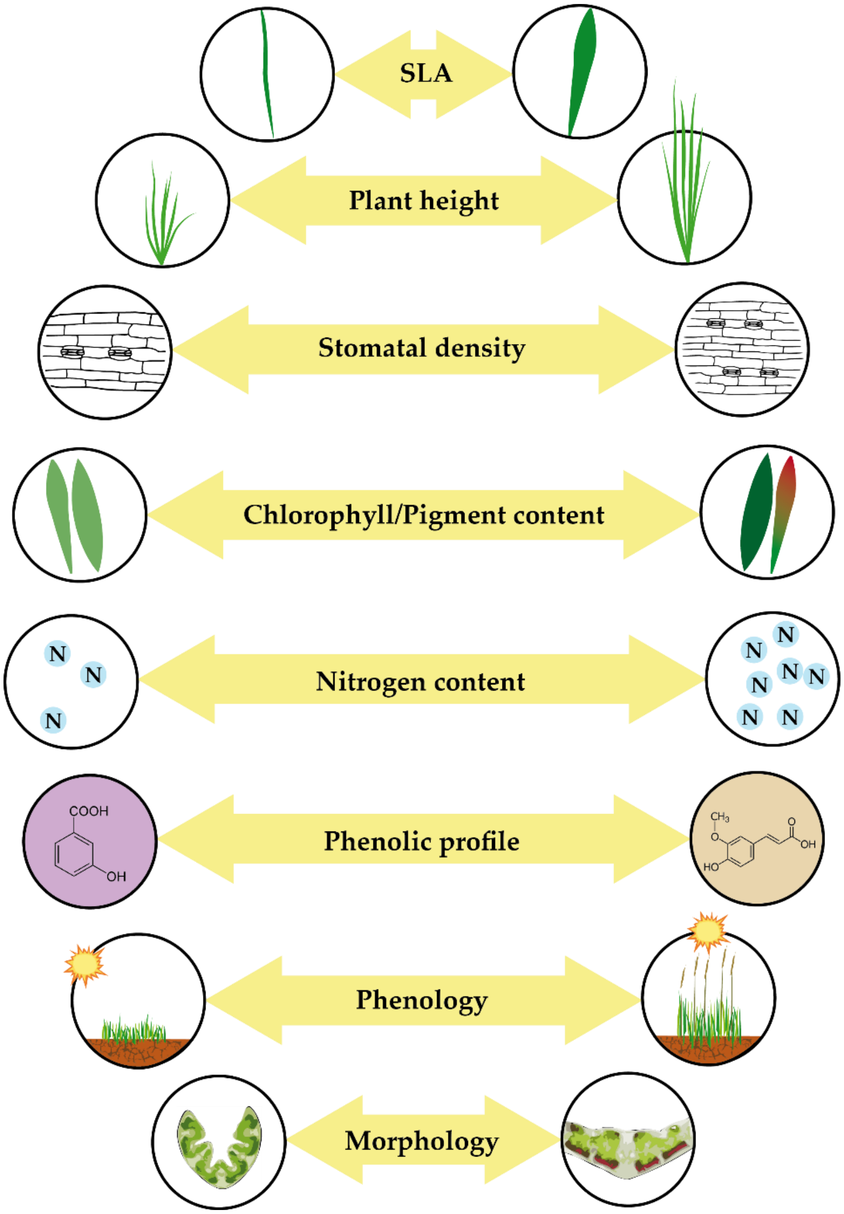

*Figure 5: Examples of commonly investigated leaf functional traits. From top to bottom: **SLA** (specific leaf area) is the ratio of leaf area to leaf dry mass. Low SLA is found in plants that allocate carbon resources to produce thicker or more structurally reinforced leaves, while high SLA is found in plants that prioritize leaf expansion, and more rapidly produce leaves with a larger surface area relative to biomass. **Plant height** determines the canopy structure in a community and influences competition for light. Plant height can be altered in response to environmental stress and long-term grazing. **Stomatal density** is influenced by genotype, light conditions, and atmospheric CO_2 levels. Low stomatal density may reduce transpirational water loss, however high stomatal density allows for a more calibrated response to environmental conditions. **Chlorophyll content** functions as a proxy for leaf photosynthetic capacity, and changes in chlorophyll content may reflect stress or nutrient deficiencies (such as N, or Mg). **Pigment content**, such as carotenoid and anthocyanin content, is indicative of a leaf's capacity to endure certain types of environmental stress. Carotenoids function to dissipate excess light energy while anthocyanins provide a range of protective functions, notably scavenging ROS, and absorbing UV radiation. **Nitrogen content** is essential for producing chlorophyll, thylakoid proteins, and enzymes (including Rubisco) and so there is a close relationship between nitrogen content and photosynthesis. **Phenolic profile** is an emerging area of interest, however, ratios of certain phenolic compounds may influence how tolerant leaves are to particular stressors, such as high PAR, UV radiation, temperature extremes, herbivory, and pathogens. **Phenology** refers to the timing of seasonal events, such as leaf expansion, pigment accumulation, flowering, and seed set. The phenology of a plant influences its ability to attract pollinators, compete with neighbouring species for resource acquisition, and exploit temporal windows to establish a population in an area. Phenology also has implications for remote sensing techniques used to classify species throughout the growing season the spectra of a given species may change as it moves through its life stages. **Morphology** plays a role in how well-suited a species is to its environment, and changes in morphology can occur as a result of specific stressors influencing the phenotypic plasticity of a species.*

3.7 Poaceae

While arguably the most successful plant family, Poaceae are thought to be rather young on the evolutionary timescale. Poaceae are a subgroup of monocots, meaning they have only one embryonic leaf. Grasses are distinct from other monocots in their rapid embryonic development compared to seed maturation, and their unique single-ovule fruit: the caryopsis or grain (Kellogg, 2000). The fossil pollen record suggests that Poaceae arose between 55-60 million years ago, i.e., after the extinction of the dinosaurs which ended the Cretaceous period (Kellogg, 2001); However, silica phytoliths found in dinosaur coprolites now suggest grasses may have evolved earlier (Prasad et al., 2005). The expansion of grasses created ecosystems and precipitated novel floral and faunal ecosystems (Jacobs et al., 2000). Grasslands and savannas set the stage for bipedalism to evolve and hominin species to diversify (Bonnefille, 2010). While there is evidence that humans collected and ate grains over 100,000 years ago (Mercader, 2009), the organized cultivation of grasses began around 10,000 BC, starting off the agricultural revolution (Braidwood, 1960). Currently, approximately 38 % of the plant's terrestrial surface is dominated by grasses, including croplands, pastures for grazing, and wild grasslands (FAOSTAT, 2023). Poaceae have had a major impact on both global ecology and human progress, and impact global carbon, silica, and nitrogen cycles (Linder et al., 2018).

Grass-dominated ecosystems contribute 33 % of global primary productivity, which is incredible given that they account for only 3 % of plant species (Beer et al., 2010). Still, with 11,000 member species, Poaceae are the fifth largest angiosperm family (Campbell, 2023). The success of grasses as colonizers has been explained as "Viking syndrome" — a combination of efficient dispersal (30 % of all wind-pollinated species are Poaceae), fast population expansion, resilience to disturbance, phenotypic plasticity, and ecosystem transforming growth habits (Linder et al., 2018) According to Linder et al. in the aforementioned study, some of the traits most contributive to the success of Poaceae include: i) Their unique meristem position at the base of the shoot during their vegetative stage, enabling them to tolerate defoliation from herbivores, fire, or drought; ii) Exploitation of several dispersal mechanisms including by wind and by animals (on their coats or after ingestion); iii) Their short generation times, enabling them to utilize minor spatio-temporal opportunities to establish a population and respond rapidly to selective pressure; iv) The prevalence of polyploidy providing genetic variance and increasing phenotypic plasticity. In some cases, such as the tropical alpine region of the Andes, grasslands are able to match productivity of forests in terms of net primary productivity (Oliveras et al., 2014). Grasses have evolved functional traits allowing them to cope with disturbances related to extreme climates, specific soils, fire regimes, and ever-presence herbivory. In fact, traditional land management practices (such as grazing, harvesting hay, and cutting down trees for wood) likely mimicked the natural disturbance regimes (e.g. fires) Poaceae adapted to exploit, facilitating the spread of semi-natural grasslands in Europe during the Middle Ages (Petermann & Buzhdygan, 2021). However, in the past couple centuries, the conversion of natural ecosystems into agricultural lands has been a major driver of greenhouse gas emissions, as well as biodiversity and sequestered-carbon loss. Moreover, cropland per capita for producing food has steadily fallen as the global population continues to rise (FAOSTAT, 2023). Improving agricultural productivity is a necessity, not only to produce enough food to sustain humanity, but also to preserve dwindling natural grass communities.

4 Summary of Published Papers

4.1 **Hunt et al., 2021a**: Light and CO_2 Modulate the Accumulation and Localization of Phenolic Compounds in Barley Leaves

Lena Hunt, Karel Klem, Zuzana Lhotáková, Stanislav Vosolsobě, Michal Oravec, Otmar Urban, Vladimír Špunda, and Jana Albrechtová

Antioxidants **2021**, *10*, 385. (IF_{2021}: 7.675)

This paper describes a novel method for evaluating the histochemical localization of phenolic compounds in barley leaf cross-sections. The detection method itself is not new, DPBA was discovered by the 1950s by Richard Neu to significantly enhance the yellow and green autofluorescence of phenolic compounds (Neu, 1957). Previously, however, it had been used only to demonstrate the presence or absence of phenolic compounds in a sample of plant tissue, (e.g., Hutzler et al., 1998; Wuyts et al., 2006). In this paper, cross-sections of barley leaves are measured for pixel intensity at 5 vertical intersections, generating quantitative data about the gradient of phenolic accumulation within the leaf. This data was used to show different patterns of accumulation, as well as differences in overall intensity. In addition to image analysis, an optical sensor was used to measure levels of chlorophyll and UV-screening compounds, and a profile of specific phenolic compounds was determined using targeted high performance liquid chromatography-high performance mass spectroscopy (HPLC-HRMS).

These methods were deployed on barley leaves subject to high or low light conditions in combination with low (200 ppm), ambient (400 ppm), or elevated (700 ppm) CO_2 conditions. As expected, high light enhanced the accumulation of phenolic compounds throughout the mesophyll, especially near the leaf epidermal surfaces. Plants grown in low light had lower accumulations, except along the lower mesophyll. This pattern was true in low and ambient CO_2, however, elevated CO_2 drastically reduced the differences between high and low light, and showed an overall greater phenolic accumulation. Accumulation patterns correlated with optical sensor findings. Two barley varieties were used, one known to be tolerant to oxidative stress (Bojos) and one known to be sensitive (Barke). HPLC-HRMS revealed that these varieties accumulated similar levels of flavonoids, but differed in their phenolic acid profiles: Bojos had mainly hydroxycinnamic acids and Barke had mainly hydroxybenzoic acids. Thus, we suggest that hydroxycinnamic acids may contribute more oxidative stress tolerance than hydroxybenzoic acids.

The barley plants for this project were grown at the Global Change Research Institute, Czech Academy of Sciences in Brno (CzechGlobe). Sectioning and staining were carried out in our lab by Drahomíra Bartáková, Zuzana Lhotáková, Eva Neuwirthová, and myself. I captured the microscope images with Miroslav Barták and developed the image analysis method. Optical sensor data and HPLC-HRMS analysis were performed by Karel Klem, Michal Oravec, and Otmar Urban from CzechGlobe. Stanislav Vosolsobě from our home Department of

Experimental Plant Biology consulted on statistics. As the lead author on this study, I wrote the initial text of the manuscript under supervision of Jana Albrechtová with contributions from Karel Klem. Zuzana Lhotáková, Otmar Urban, Vladimír Špunda reviewed the manuscript before submission. This study was funded by the Czech Science Foundation (GAČR 18-23702S).

Graphical Abstract: Light and CO_2 Modulate the Accumulation and Localization of Phenolic Compounds in Barley Leaves

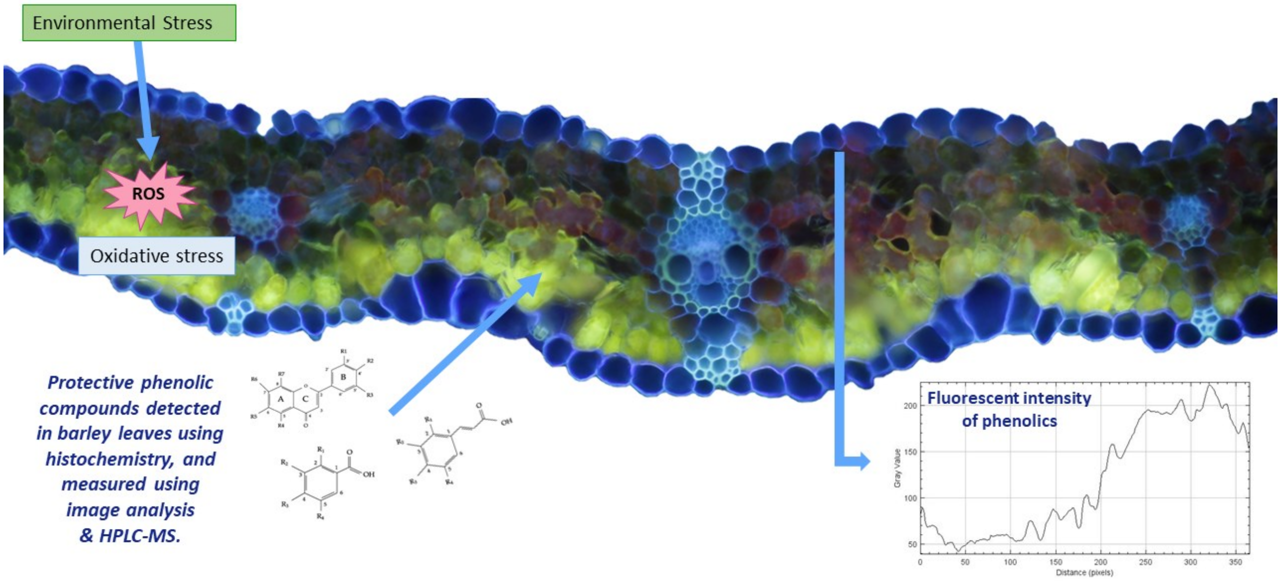

*Figure 6: Graphical abstract for Hunt et al., 2021a. The image shows a section of a barley (*Hordeum vulgare*) leaf cross-section. Yellow fluorescence indicating the accumulation of phenolic compounds in response to environmental stress is measured in terms of fluorescent intensity through the mesophyll, demonstrating the spatial distribution of phenolic compounds in plants grown in various light conditions.*

4.2 **Hunt et al., 2021b**: Barley Genotypes Vary in Stomatal Responsiveness to Light and CO_2 Conditions

Lena Hunt, Michal Fuksa, Karel Klem, Zuzana Lhotáková, Michal Oravec, Otmar Urban, and Jana Albrechtová

Plants **2021**, *10*, 2533. (IF_{2021}: 4.658)

The novelty of this paper is the use of artificial intelligence — a convolution neural network — to count stomata from micrographs of epidermal imprints. We wanted to find a faster way to process large quantities of micrographs without counting individual stomatal complexes. This paper used the same experimental material as the previous paper — barley varieties tolerant or sensitive to oxidative stress (Bojos and Barke, respectively) grown in low or high light, and in low, ambient, or elevated CO_2 conditions. We examined stomatal density, stomatal conductance, photosynthesis, and transpiration, as well as levels of saccharides - pentoses, hexoses, disaccharides - and abscisic acid (ABA).

We found that stomatal density was greater in low CO_2, but did not differ between ambient and elevated CO_2; High light also increased stomatal density in all cases. There were pronounced differences between the two barley varieties. Barke (sensitive) had an overall higher stomatal density, but lower levels of stomatal conductance, and thus showed better water use efficiency. Moreover, Barke showed more variability between treatments in terms of stomatal density, sugar accumulation, and ABA levels, indicating that it was more responsive to its environmental conditions. While not explicitly tested, one hypothesis raised in the discussion to account for the differences between Barke and Bojos could be their phenolic profiles (tested in the previous paper). While a high accumulation of hydroxycinnamic acids improves oxidative stress tolerance by scavenging ROS, it may simultaneously dull ROS signaling, making Bojos less sensitive to environmental cues.

The initial idea to use a convolution neural network was conceived by myself and Michal Fuksa, and we collaborated on it together. Michal Fuksa coded the program and I consulted with ensuring proper identification of cells and stereoscopy principals. Stomatal imprints were prepared by students supervised by Zuzana Lhotáková and Jana Albrechtová. Stomatal conductance and metabolite data was collected by our collaborators at CzechGlobe, Karel Klem and Michal Oravec. As the lead author on this study, I wrote the initial text of the manuscript. Karel Klem, Zuzana Lhotáková, Otmar Urban, and Jana Albrechtová contributed through reviewing and editing the manuscript to prepare it for submission. This study was funded by the Czech Science Foundation (GAČR 18-23702S).

Graphical Abstract: Barley Genotypes Vary in Stomatal Responsiveness to Light and CO$_2$ Conditions

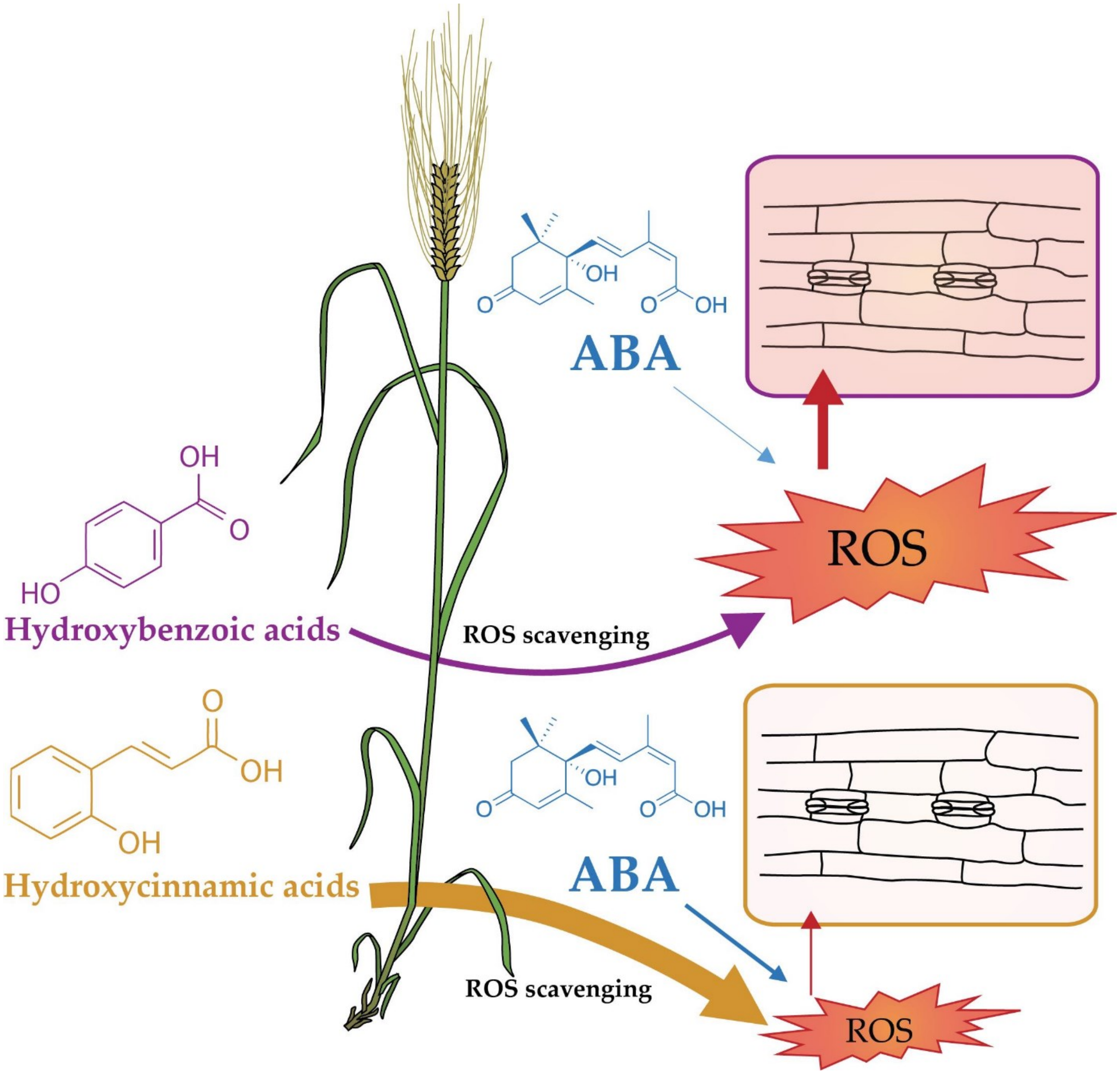

Figure 7: Graphical abstract for Hunt et al., 2021b showing how the accumulation of predominantly hydroxybenzoic or hydroxycinnamic acids effects the ROS scavenging capacity of the plant. Less ABA is required to induce stomatal closure when ROS accumulate.

4.3 **Pech et al., 2022**: Regulation of Phenolic Compound Production by Light Varying in Spectral Quality and Total Irradiance

Radomír Pech, Adriana Volná, Lena Hunt, Martin Bartas, Jiří Červeň, Petr Pečinka, Vladimír Špunda, and Jakub Nezval

International Journal of Molecular Sciences **2022**, *23*, 6533 (IF$_{2022}$: 6.208)

This paper highlights the importance of blue light in the production of phenolic compounds. While it is well established that high PAR and UV irradiance can enhance the accumulation of phenolic compounds, the goal of this experiment was to see how the main spectral components of PAR contribute to phenolic production. Barley plants were cultivated in low, medium, or high intensity light consisting of either isolated red (R), green (G), or blue (B) light, or white (RGB) light. It was found that light intensity increased total phenolics, but that this effect was strongest under light conditions with a B spectral component (i.e., blue and white light). Light also affected the types of phenolics accumulated by barely, with high intensity light increasing isovitexin derivatives (mono-hydroxylated), but blue light specifically increasing homoorientin derivatives (di-hydroxylated). The significance of this is that the di-hydroxylated homoorientin derivative have higher ROS scavenging capacity and offer greater protection against oxidative stress. This was reflected in the enhanced antioxidative capacity for leaves cultivated with a blue spectral component. Furthermore, expression analysis of genes related to enzymatic antioxidants (SOD and APX) and phenolic compound biosynthesis (PAL, CHS, F3’H) were found to be proportional to blue light irradiance.

This article was written in the same research consortium as the previous two barley papers. The study was conceived by Vladimír Špunda, and Jakub Nezval. HPLC analysis, UV-A shielding and antioxidant capacity were contributed by Radomír Pech. Adriana Volná provided transcriptome analysis and qPCR. Martin Bartas, Jiří Červeň, and Petr Pečinka supervised interpretation and processing of bioinformatic data. Radomír Pech, Adriana Volná and Jakub Nezval prepared the initial text of the manuscript. Although I did not take part in data collection for this article, I contributed through writing and interpretation additions to the introduction and discussion sections, and in overall review and editing the manuscript for publication in collaboration with Vladimír Špunda and Jakub Nezval. This research was funded by the Czech Science Foundation GAČR 21-18532S.

4.4 **Hunt et al., 2023**: Leaf Functional Traits in Relation to Species Composition in an Arctic–Alpine Tundra Grassland

Lena Hunt, Zuzana Lhotáková, Eva Neuwirthová, Karel Klem, Michal Oravec, Lucie Kupková, Lucie Červená, Howard E. Epstein, Petya Campbell, and Jana Albrechtová

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This article was the result of a multidisciplinary collaboration between plant scientists, ecologists, and remote sensing specialists to investigate changes in species composition in the endangered relict artic-alpine tundra grassland of the Krkonoše National Park. The Krkonoše tundra is a prime example of a semi-wild grassland subject to changing climate and anthropogenic disturbance. While *Nardus stricta* is the dominant grass species, there has been a visible increase in the abundance of *Calamagrostis villosa* over the past decade. Our remote sensing collaborators were able to classify the major plant species from aerial photographs (orthophotos) and confirm the spread of *C. villosa*, and the retreat of *N. stricta* between 2012 and 2018. *Deschampsia cespitosa* was found to increase in one area and decrease in another, while *Molinia caerulea* remained the same in that time.

We set out to investigate what leaf functional traits could explain these changes in grass species abundance in an environment characterized by high irradiance, low temperatures, and a short growing season. We looked at physical traits, such as specific leaf area and leaf morphology; biochemical traits, such as pigment accumulation, phenolic profiles and how they change over the growing season; physiological traits, such as chlorophyll fluorescence indicators of photosynthetic functioning and overall stress; and element analysis. We found that *N. stricta* is morphologically well-suited to the high-irradiance, low-nutrient environment and showed the lowest stress levels of all species. *D. cespitosa* showed the highest stress levels but was found to be expanding in the area more disturbed by human activity — a result possibly driven by the creation of micro niches suitable for its higher Mg and pH demands. While both *C. villosa* and *M. caerulea* are known to be rapid spreaders in other grasslands, only *C. villosa* was found to be expanding in the Krkonoše tundra grassland. The success of *C. villosa* over *M. caerulea* could be attributed to its rapid accumulation of pigments early in the growing season, and its diverse phenolic profile lending it greater protection against oxidative stress.

This project was conceived by Zuzana Lhotáková, Jana Albrechtová, and myself from our home Department of Experimental Plant Biology and Lucie Kupková and Lucie Červená from the department of Geography, CUNI. Fieldwork and sample collection was carried out by Zuzana Lhotáková, Lucie Červená, Eva Neuwirthová, and myself with assistance from master student Petr M. Mamula. HPLC-HRMS analysis was performed by Karel Klem and Michal Oravec. I performed the histochemical staining and microscopy and prepared images and figures for publication with assistance from Miroslav Barták. Eva Neuwirthová contributed statistical analysis. As the lead author, I prepared the initial manuscript. Zuzana Lhotáková,

Lucie Červená, Howard E. Epstein, and Petya Campbell made additions to the text. The manuscript was finalized for submission by myself, Lucie Kupková, and Jana Albrechtová. This research was funded mainly by the Ministry of Education, Youth and Sports of the Czech Republic, scheme INTER-EXCELLENCE, INTERACTION, grant number LTAUSA18154, and the Czech Science Foundation (GAČR grant number 21-18532S).

Graphical Abstract: Leaf Functional Traits in Relation to Species Composition in an Arctic–Alpine Tundra Grassland

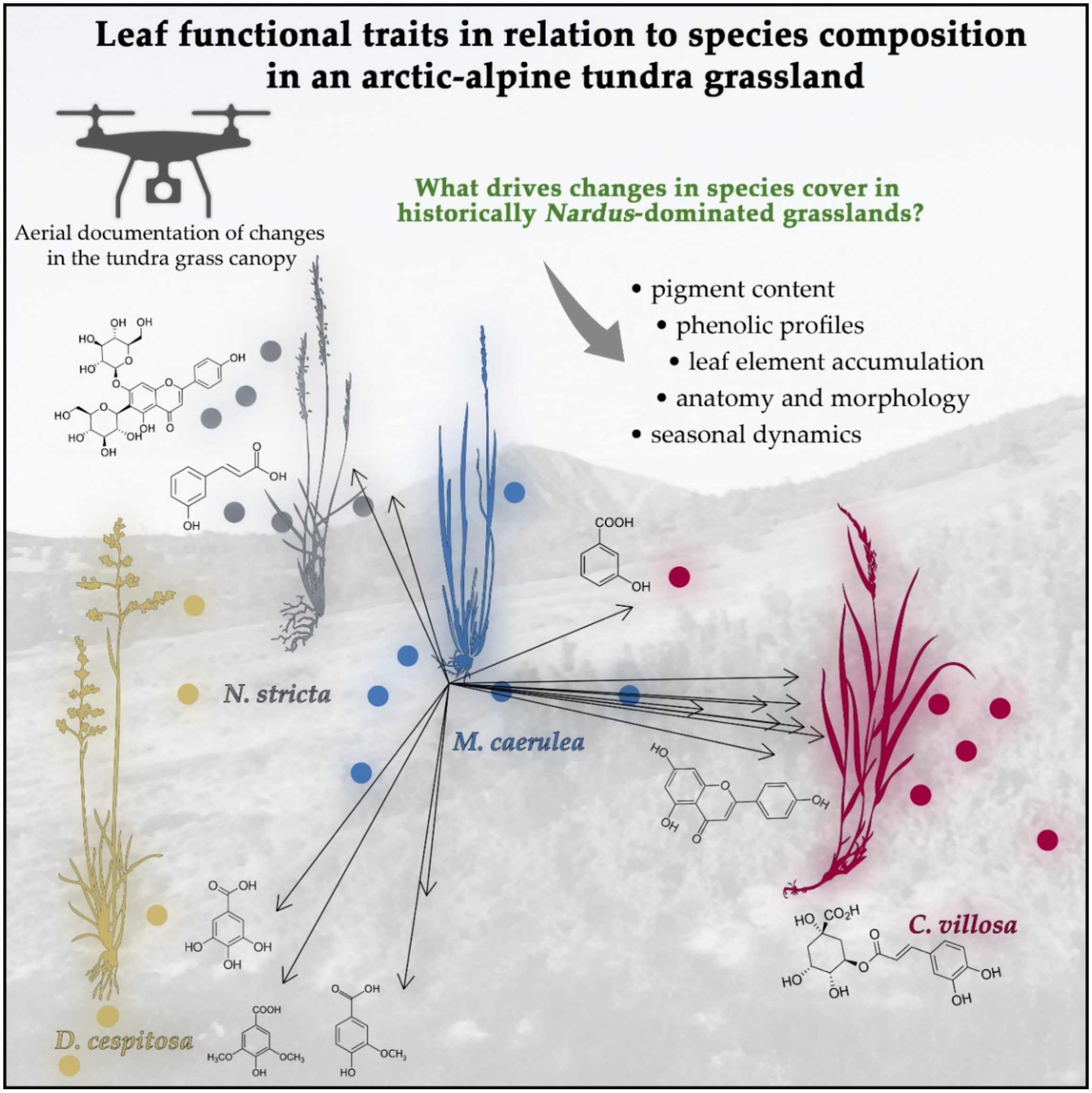

Figure 8: Graphical abstract for Hunt et al., 2023, showing the four main grasses of the study (Nardus stricta, Molinia caerulea, Deschampsia cespitosa, and Calamagrostis villosa) and a principal component analysis (PCA) of the specific phenolic compounds each species accumulated. A drone to symbolize remote sensing is present in the upper left corner. The background is an original photo of the field site by the author with Sněžka (The highest peak of the Czech Republic) visible in the background.

5 Overview of Main Findings

The four articles in my thesis focus on leaf traits in Poaceae as they relate to oxidative stress protection.

In the first study, (**Hunt et al., 2021a**), a relationship was demonstrated between the light and CO_2 conditions and the accumulation of phenolic compounds in leaf mesophyll tissue. While high light and elevated CO_2 both increased overall leaf phenolic accumulation, the individual phenolic profiles were specific to the two genotypes investigated, Barke and Bojos. Oxidative stress tolerant Bojos accumulated more hydroxycinnamic acids while oxidative stress sensitive Barke accumulated more hydroxybenzoic acids.

The following study (**Hunt et al., 2021b**) looked at the same two barley genotypes with respect to their stomatal behavior. In addition to the expected findings of increased stomatal density with high light and decreased stomatal density with elevated CO_2, the relative changes in stomatal density between experimental treatments varied considerably by genotype. Barke, the oxidative stress tolerant genotype, was more sensitive to light and CO_2 and ABA, resulting in overall higher water use efficiency (WUE).

In the third study, (**Pech et al., 2022**), the effects of both light intensity and spectral quality on the accumulation of phenolics and antioxidative enzymes in barley (Bojos genotype) were investigated. The results of this study confirmed that overall light intensity influenced phenolic accumulation but showed the importance of blue light specifically in these systems. Barley plants exposed to medium or high irradiance with a blue spectral component had more phenolic compounds and antioxidative enzymes than those exposed to the same irradiance of only red or green light. While some phenolic compounds were present in all samples, others required a certain intensity of blue light to be induced at all. Thus, the connection between light spectral quality and differential phenolic accumulation was demonstrated.

Finally, the last study, (**Hunt et al., 2023**), examined four wild grass species in an oxidative stress prone tundra grassland environment. Data from this study suggests that morphological adaptations can measurably reduce stress (as in the case of dominant grass *N. stricta*), however, in the absence of grazing/mowing, tall grass species (*C. villosa* and *D. cespitosa)* have an advantage when it comes to expanding territory. Success in the tundra grassland is not only a matter of growth form, but of rapid accumulation of pigments to aid non-photochemical quenching and scavenge ROS, as well as a diverse phenolic profile to combat environmental stress and protect photosynthetic structures.

6 Discussion

6.2 Differential roles and responses of major phenolic groups

Phenolic compounds are a large family, with under-studied complexity. Much of the current literature available emphasizes the relationship between total phenolics and antioxidative capacity of a sample. I hope this thesis can encourage researchers to look closer at what the phenolic profiles of grasses might convey, as the structural-functional intricacies are lost in many studies that look only at "total phenolics". All four articles presented in this thesis demonstrate that individual phenolic profiles confer differential stress protection in grasses. Particularly, in **Hunt et al., 2021a**, the tolerant and sensitive genotypes both increase their total phenolics in response to high light and elevated CO_2, and in **Hunt et al., 2023**, all species accumulate a similar level of total phenolics. However, outcomes are clearly different among cultivars and species. Sensitive Barke develops necrotic spots under oxidative stress conditions (Wu & von Tiedemann, 2004) and reduces photosynthesis in response to UV (Klem et al., 2012). And in the tundra grassland, species accumulating a narrow and more weakly antioxidative phenolic profile were outperformed by *C. villosa*, which invested in a wide range of flavonoids, hydroxycinnamic and hydroxybenzoic acids. I believe it is useful to look at these families of phenolic compounds individually for clues on how they may each aid plants in stress tolerance.

6.2.1 Response to irradiance

6.2.1.1 Irradiance preferentially enhances flavonoid accumulation

PAR and UV irradiance induce different phenolic profiles on barley. One of the pioneers in the field of phenolic research in plants, Massimiliano Tattini, observed that glandular trichomes of *Phillyrea latifolia* synthesized exclusively hydroxycinnamic acids in shade, but flavonoids in full sunlight (Tattini et al., 2000). Further investigation on the effects of light on hydroxycinnamic acids versus flavonoids showed that, although hydroxycinnamic acids are better at shielding UV irradiance, it is flavonoids that are preferentially accumulated in response to high natural irradiance. This increase in flavonoid biosynthesis was associated with higher scavenging rates of O_2^- (Tattini et al., 2004).

We confirmed this finding in **Hunt et al., 2021a**, demonstrating an increase in mesophyll-localized flavonoids, versus epidermal-localized hydroxycinnamic acids. In a recent study, the combination of PAR and UV increased common barley flavones (saponarin, homoorientin, and isovitexin) but decreased certain hydroxybenzoic acids (vanillic and syringic). By contrast, hydroxycinnamic acids were positively regulated by PAR alone and not UV (Klem et al., 2022). Thus, flavonoids accumulation is the most important phenolic defense against UV radiation and associated oxidative stress. Different classes of flavonoids (flavonols, flavones, anthocyanins) are regulated by biosynthesis genes controlled by the UV photoreceptor UVR8, and all contribute to UV screening although they vary in UV absorption spectra (Ferreyra et al., 2021).

Not all flavonoids are equally protective in high irradiance conditions. UV radiation is known to increase the ratio of di-hydroxy : mono-hydroxy B ring substituted flavonoids (i.e., flavonoids with higher ROS scavenging capacity are preferentially accumulated). This is observed as an increase in luteolin-to-apigenin / quercetin-to-kaempferol / homoorientin-to-isovitexin ratios, depending on the predominant flavonoids in a given species. Our findings in **Pech et al., 2022** demonstrated this phenomenon also with blue light in the absence of UV radiation. Both UVR8 and CRY photoreceptors (responsive to UV and blue light, respectively) control F3'H (responsible for catalyzing the addition of hydroxyl groups to the flavonoid skeleton) and CHS (responsible for initial steps of flavonoid synthesis) via downstream signaling pathways (Nascimento & Tattini, 2022; Vrábl et al., 2023). However, as with many factors, leaf age plays a role in how leaves react to spectral components — previous studies indicate that young leaves accumulate flavonoids in response to high PAR, while older leaves respond more to UV (Klem et al., 2012). Post-harvest UV exposure is also currently being investigated as a means to prolong storage half-life and increase the nutritional quality of fruits and vegetables by increasing flavonoid content (Zhang & Jiang, 2019). Still much remains to be discovered about the specific induction of various flavonoids and their individual roles in oxidative stress reduction.

6.2.1.2 Flavonoids influence phytohormones in response to irradiance

Another perspective on the preferential accumulation of flavonoids under high irradiance arises from their ability to modulate phytohormone signaling pathways. The ROS scavenging activity of flavonoids impacts both auxin and ABA signaling (Nascimento & Tattini, 2022). Auxin regulates plant architecture, and disruptions as a result of flavonoid accumulation likely contribute to the stress-induced morphology changes, such as overall dwarfing, reduction in stem and petiole lengths, and smaller thicker leaves (reduced SLA) (Qian et al., 2021). This occurs as flavonoids act on serine-threonine PINOID (PID) kinases that regulate localization of PIN proteins (auxin efflux facilitators) and additionally reduce auxin catabolism (Brunetti et al., 2018). Quercetin (a di-hydroxy flavonoid) in particular is synthesized in response to excess light from bryophytes to angiosperms (Brunetti et al., 2018). Among the thousands of possible flavonoids, quercetin shows a superior ability to scavenge ROS, effect phytohormone signaling, and screen UV-B radiation, making this flavonoid an important regulator of plant-environmental interactions (Brunetti et al., 2018).

ABA is the phytohormone most associated with stress response and is a key regulator of stomatal closure. The regulatory functions of ABA in integrating light signaling pathways has also recently been gaining attention. ABA was shown to positively affect accumulation of anthocyanins in *Vitis vinifera* L. (Olivares et al., 2017). ABA promotes the binding of HY5 gene (Elongated Hypocotyl 5) to ABI5 gene (ABA-Insensitive 5, a gene involved in light-induced morphogenic response). HY5 gene is downstream from UVR8 (as with many key phenolic biosynthesis genes) and phytochrome A photoreceptors. Additionally, HY5 activates MYB12 and MYB111 gene expression, involved in flavonol biosynthesis (Brunetti et al., 2019). Thus, plants integrate light and ABA signaling when it comes to phenolic accumulation. Recent evidence shows that the presence of ABA in cell cultures can enhance the production of phenolic compounds in callus cells of *V. vinifera*, with dose-dependent and light-dependent results: 200 µM ABA in light promoted piceid biosynthesis, while the same concentration in dark promoted resveratrol, and 50 µM ABA in light promoted anthocyanins (Andi et al., 2021)

6.2.1.3 Irradiance downregulates hydroxybenzoic acids

It has been established that hydroxybenzoic acids are generally less protective against oxidative stress induced by high irradiance compared to hydroxycinnamic acids (Cuvelier et al., 1992, results supported by **Hunt et al., 2021a**). However, recent evidence suggests that light may actually downregulate hydroxybenzoic acid biosynthesis. This was observed for vanillic and sinapic acids in barley (Klem et al., 2022). Research on callus cultures of *Camellia sinensis* grown in light versus dark conditions show a 2.4 fold decrease in hydroxybenzoic acid derivatives in light conditions (Ossipov et al., 2022). This decrease in hydroxybenzoic acid derivatives was also observed in by Wang et al., (also in *C. sinensis*) with the given explanation that hydroxybenzoic acids and flavonoids compete for precursor material (Wang et al., 2012). Ossipov et al., (2022) goes further to explain changes in carbon flux distributions through shikimate pathway. Shikimate kinase controls the allocation of carbon and his highly dependent on ATP; in the dark, the lower availability of ATP and NADPH/NADP+ reducing potential lowers the activity of shikimate kinase, redirecting carbon from flavonoid synthesis to the less costly gallic acid synthesis pathway. Another factor may be the dark-grown calluses having more poorly differentiated cells. In planta, young buds and leaves of *C. sinensis* have high expression of genes involved with hydroxybenzoic acid biosynthesis, and low flavonoid content — a pattern which is reversed with growth and development of the plant (Ossipov et al., 2022).

6.3 Functions of hydroxybenzoic acids beyond irradiance

With the epithet of "oxidative stress sensitive" in the articles presented in this thesis (**Hunt et al., 2021a and b**), it may be surprising to the reader that Barke is one of the most commonly planted barley cultivars in Europe — and a descendant of the Moravian landrace used in the first Pilsner beer (Junkersfeld, 2018). The preferential accumulation of hydroxybenzoic acids in Barke is unusual, as hydroxybenzoic acids generally occur in lower quantities compared to hydroxycinnamic acids (Manach et al., 2004). Observational evidence in literature discussing barley malting varieties praises Barke for its resistance to common fungal maladies (leaf rust, leaf scald, net blotch, and mildew) (Oliver, 2011), which may be a more pressing factor in barley cultivation in Northern and Central Europe than high irradiance. Recent multi-omic analysis examining wild barley populations found differential gene expression for genes relating to phenylpropanoid biosynthesis and plant-pathogen interactions. Wild barley native to drought-prone areas were found to have reduced phenylpropanoid biosynthesis and increased sugar metabolism, while those native to moister areas rich in fungal pathogens had more phenolics (Cai et al., 2021). The functional role of phenolics, especially hydroxybenzoic acids, is related to their chemical structure.

A 2011 study reported that antibacterial activity correlated with a decreasing the number of hydroxyl groups or substitution of hydroxyl groups with methoxy in hydroxybenzoic acids, with no or minor effects on hydroxycinnamic acids (Sánchez-Maldonado et al., 2011). There are some inconsistencies, however, this trend has generally held up in recent analysis (Liu et al., 2020). In addition, the carboxylic acid groups were found not to affect antioxidant or antimicrobial activity, meaning it can be react with other biomaterial monomers without loss of function (Liu et al., 2020). Another recent study on this topic reports that antiradical (quenching) activity of hydroxybenzoic acids is at least partially pH-dependent, but does confirm the low antioxidant activity of compounds with hydroxyl groups in the *meta* positions (di-hydroxybenzoic acid, α-resorcylic acid, and γ-resorcylic) and higher activity for gallic acid with three hydroxyl groups, two in ortho position to the third hydroxyl group (Kalinowska et al., 2021).

Hydroxybenzoic acids, such as gallic and protocatechuic acids, are also precursors to tannins. The antibacterial function of tannins is reportedly a function of the number of hydroxyl groups and the release of H_2O_2 to combat infection. However, this antipathogenic effect has confused the literature about their antioxidative properties as their oxidation actually produces ROS (albeit the least damaging and most useful as a signaling molecule) (Tong et al., 2022). It must be noted, however, that condensed tannins were not detected in either barley variety using the vanillin HCl histochemical assay (**Hunt et al., 2021)**. This could be result of leaf age, as tannin content increases with leaf maturity and decrease upon leaf senescence. The leaves sampled for histochemical analyses were sampled from the youngest developed leaves, potentially too early to detect tannins, although data on the course of tannin accumulation in Poaceae leaves remains scant.

6.3 Influences of other environmental stress factors on phenolics

6.3.1 Elevated carbon

The role of elevated CO_2 was explored in **Hunt et al., 2021a** and **2021b**. A clear increase in phenolic accumulation in the leaves of barley cultivars grown in elevated CO_2 was observed in **Hunt et al., 2021a**. This effect has also been confirmed in wheat (*Triticum aestivum*) cultivars grown in FACE (free air CO_2 enrichment) experiments (Blandino et al., 2020). Additionally, elevated CO_2 was found to alter herbivore resistance mechanisms away from silicon accumulation in favor of phenolic accumulation (Johnson et al., 2022). These results support an early hypothesis that phenolic compounds can act as a sink for "excess carbon". The RDA (Redundancy analysis) from **Hunt et al., 2021a** suggests that CO_2 was associated with the accumulation of two phenolic acids: sinapic acid, and 3-hydroxybenzoic acid. FACE (free air CO_2 enrichment) experiments on wheat also showed an increase in soluble sinapic acid with elevated CO_2. Curiously, a report on phenolics in rice grains found that sinapic acid and hydroxybenzoic acid showed the greatest reductions in response to elevated CO_2 (Goufo et al., 2014). As far as I know, a study has not yet addressed if the phenolic profiles of different plant organs differentially respond to the same environmental cue (i.e., an increase in sinapic acid in leaves but a decrease in grains). However, it seems plausible as an increase in foliar phenolics with elevated CO_2 has been documented in other grain crops — maize (*Zea mays*), rice (*Oryza sativa*) (Li et al., 2008), wheat (Blandino et al., 2020), and barley (**Hunt et al., 2021a**), and reductions in grain phenolics in elevated CO_2 have been documented in rice (Goufo et al., 2014) and rye grass (*Lolium rigidum* Gaude) (Fernando et al., 2019). A study on buckwheat (*Fagopyrum esculentum* Moench) indicated that the concentrations of phenolic metabolites are independently determined in flowers, leaves, and grains, and that leaves had higher concentrations of phenolics than grains (Vollmannová et al., 2021). Elevated CO_2 predictions raise some important concerns about the future nutritional quality of food. Grains remain a food staple around the world, and yet reductions in phenolics and grain protein content under future atmospheric conditions may become a challenge (especially for items like bread flour, which requires quite high — 11.5% - protein content) (Blandino et al., 2020).

6.3.2 Nutrient deficiency

The "excess carbon" hypothesis of phenolic accumulation explaining the expected increase in foliar phenolics in elevated CO_2 conditions is based on the potential fates of phenylalanine. Phenylalanine could be converted into proteins, however if carbon resources outpace nitrogen (N) availability, phenylalanine will be diverted to pathways generating phenolic compounds instead (Lambers, 1993). Genetic evidence confirms that the PAL enzyme and other phenylpropanoid-pathway genes are more expressed in N depleted conditions (Olsen et al., 2008). Recent experiments confirm that barley plants grown in high N availability conditions do, in fact, accumulate fewer phenolics than those grown in conditions with lower nitrogen availability (Klem et al., 2022). In that study, high PAR and UV in the presence of adequate N resulted in an increased accumulation of amino acids. This "non-necessary" accumulation of phenolics explains the similarity between phenolic accumulation patterns for all barley plants grown in elevated CO_2 in **Hunt et al., (2021a)**, compared to the distinct accumulation patterns between light treatments observed at ambient or low CO_2.

A similar nutrient-deficiency enhancement of phenolic metabolism has also been observed in wheat cultivars in response to phosphorus (P) deficiency. Decreasing P availability led to enhanced total phenolic and lignin accumulation, as well as a concurrent reduction in oxidative damage and chloroplast H_2O_2 production (Pontigo et al., 2018). This effect was modulated by different cultivars' relative P-uptake efficiencies.

Foliar anthocyanin (a sub-category of flavonoids) accumulation is a side effect of both N and P deficiencies, albeit with slightly different phenotypic presentations. Studies on Arabidopsis mutants with deficiencies flavone and anthocyanin biosynthesis genes (MYB75 and DFR — dihydroflavonol 4-reductase) suggest that anthocyanins contribute more to plant survival under N starvation than flavones (Liang & He, 2018). Like all flavonoids, anthocyanins contribute to UV-shielding and ROS scavenging, however anthocyanins are also functionally well-suited to reversibly store carbon during periods of reduced growth due to nutrient stress as they can accommodate many sugar moieties (Jezek et al., 2023). The role of anthocyanins as sugar-sinks could help to explain the abaxial localization of anthocyanins observed in some grasses (e.g., *C. villosa* in **Hunt et al., 2023**). The abaxial localization doesn't make sense from a light-attenuation stand-point, and the abaxial mesophyll is unlikely to require more ROS-scavenging than the more photosynthetically active adaxial (upper) leaf side. In the case of tundra grasses, abaxial anthocyanins may also play a role in cold acclimation/tolerance (see next section).

6.3.3 Temperature

Temperature is another factor affecting the phenolic profile of barley leaves. A recent paper on phenolic compounds in barley leaves found that light is the main driver of accumulation, but results are modulated by temperature conditions. While high irradiance predictably increased flavonoids, high temperature increased flavonoids acylated by sinapic acid and low temperature increased those acylated by ferulic acid (Vrábl et al., 2023). This is likely a factor of the different oxidative loads experienced by plants in high versus low temperature environments. Sinapic acid shows higher ROS scavenging than ferulic acid in DPPH$^{\bullet}$ (2,2-Diphenyl-1-picryl-hydrazil) radical scavenging assays (Kikuzaki et al., 2002), and thus may be more beneficial to plants in high temperatures.

The effect of low temperatures on phenolics is less studied, however, cold stress is a major limiting factor for overall plant productivity. The Vrábl et al. (2023) study noted that for high irradiance treated barley plants, the epidermal UV-A shielding index was lower in plants cultivated in low temperatures compared to normal or high temperature conditions. This finding is in contrast to the finding that low temperatures did increase total phenolics (although localization plays a role in shielding, the increase in total phenolics was not concentrated near the adaxial epidermis). A concomitant increase in sugars was also observed in barley plants grown in combination of high irradiance and low temperature (Vrábl et al., 2023), indicating a potential connection between accumulation of sugars as growth rates slow in low temperatures, reducing alternative sinks, reducing N uptake, and consequently upregulating phenolic biosynthesis. Cold stress is known to regulate bHLH and MYB families of transcription factors regulating flavonoid (especially anthocyanin) and terpenoid biosynthesis (He et al., 2022), (although correlations between sugar content and expression of CHS and F3'H genes were not found in the He study).

6.4 Spectral influences on secondary metabolism

The third study in the present Thesis (**Pech et al., 2022)** highlights the importance of a blue spectral component for the upregulation of total phenolics, and the requirement of a certain intensity of blue light to induce biosynthesis of select phenolic compounds (e.g., feruloylquinic acid) in barley leaves. A recent study on basil (*Ocimum basilicum)* also looks at how light spectra influence secondary metabolites, however, this study utilized three spectral combinations rather than individual red, blue, and green LEDs (light emitting diodes) (Kivimäenpä et al., 2022). In that study, the three light conditions (A, B, and C) consisted of increasing proportions of UV-A, blue, and green-yellow wavelengths and decreasing proportions of red to far-red wavelengths from A to C. What is interesting about this experiment was that the highest accumulation of phenolics was *not* found in the spectral conditions with the highest proportion of blue light. Treatment A, with the highest levels of blue and UV-A spectral components, had the lowest total phenolics, although they were preferentially localized to the epidermis and upper-mesophyll (i.e., strategically protective against high energy wavelengths) (Kivimäenpä et al., 2022). Treatment C, which had the lowest amount of blue light, (although the highest ratio of blue:green wavelengths and the lowest ratio of red:far-red wavelengths) accumulated the highest amount of phenolic compounds (Kivimäenpä et al., 2022). Unfortunately, the study did not report specific phenolic compounds, and whether significant differences occurred between treatments. Still, this study implicates complexity in the ratios of spectral components reaching plants, beyond the presence/absence of specific wavelengths. While our research in **Pech et al., 2022** does hypothesize a "minimum intensity" requirement from blue light to induce significant upregulation of PAL, CHS, and F3'H over blue-exclusion treatments, this area of research remains ripe with questions for how specific light ratios influence plant metabolism and localization of secondary metabolites.

6.4.1 Influence of far-red light

Interestingly, the low red:far-red spectral conditions favoring phenolic accumulation in the above-mentioned basil study were the opposite of those favoring terpenoid and volatile organic compounds (VOC) accumulation, indicating a light-induced shift in secondary metabolism (Kivimäenpä et al., 2022). Far-red light is a useful signal, as the photosynthetically active red and blue spectral components are absorbed as light travels through a canopy, leaving understory plants with a lower ratio of red:far-red light. Far-red light upregulates genes associated with morphology favoring light capture, such as plant height and leaf area (Tan et al., 2022). Previous research has also shown that lowered ratios of red:far-red light reduce VOC emissions in barley — potentially allocating carbon resources to elongate stems as a shade-avoidance mechanism while downregulating secondary metabolism (Kegge et al., 2015). While a low red:far-red ratio is not usually considered stressful the same way, high-intensity light is, plants cultivated under a low red:far-red ratio can still accumulate ROS as a result of long-term low availability of reductants and assimilates in the chloroplasts. These conditions were observed to trigger an increase of the antioxidant glutathione in wheat chloroplasts (Gasperl et al., 2022). Research looking at the effects of far-red light on barley (e.g., Klem et al., 2019) does not report the upregulation of phenolics observed in basil mentioned above — instead, phenolic accumulation is influenced by blue and UV spectral components.

Ratios of red:far-red light do seem to have a significant effect on phytohormones, including gibberellins, ABA, auxin, ethylene, and cytokinins, although connections between light signals and hormone pathways are still under investigation (Lau & Deng, 2010). One recent study showed that supplementation with far-red light enhanced cold tolerance in barley by modulating the phytohormones ABA and auxin (Ahres et al., 2021). This occurs as the red:far-red ratio is naturally reduced in autumn and winter in the Northern Hemisphere. Thus, ratios of red:far-red light contribute not only to shade avoidance, but also cold tolerance. The same study showed downregulation of PAL as a result of far-red light, further indicating that phenolics are unlikely to accumulate in barley in response to far-red light (Ahres et al., 2021). A subsequent experiment revealed that supplementing white light with both far-red and blue spectral components increased monogalactosyl diacylglycerols (MGDGs) — lipid component of chloroplast membranes enhancing membrane stability and photosynthetic performance under cold-stress conditions (Ahres et al., 2023).

6.5 Stomata

6.5.1 Phenolic compounds in guard cells

Grasses have distinct stomatal complexes from dicots. The grass stomatal apparatus is made up of four cells: two dumbbell-shaped guard cells, each flanked by a subsidiary cell. Grass stomata develop in parallel rows, embedded in the parallel rows of epidermal pavement cells. In **Hunt et al., 2021a**, we demonstrate the accumulation of phenolic compounds in barley guard cells, but not in the surrounding subsidiary or pavement cells (see Figure 2f,g). Phenolic presence only in stomatal cells was also observed for Arabidopsis (Watkins et al., 2014). However, we received pushback from reviewers for this because the "common wisdom" is that phenolic compounds occur in the epidermis. As mentioned in the discussion of **Hunt et al., 2021a**, this could also be an effect of leaf age. Pavement cell localization was detected in some individuals after an additional two weeks of stress exposure; however, the effect was not universal and a detailed description of the course of localization over leaf age has not yet been conducted. (See supplementary Figure 2 of **Hunt et al., 2021a** for paradermal images of barley leaves demonstrating a lack of phenolic compounds in pavement cells.) In addition to our histochemical detection, the presence of flavonoids, hydroxycinnamic acids and hydroxybenzoic acids within guard cells has been confirmed by metabolic analysis (Misra et al., 2015).

The influence of phenolics on ABA-induced stomatal closure has received scattered interest in the past few decades. One of the earliest papers on the subject (Purohit et al., 1991) noted that flavonoids, hydroxybenzoic acids (and hydroxycinnamic acids with a double bond in the side chain) had the ability to reverse ABA-induced stomatal closure. A 2014 paper by Watkins et al., showed a correlation between reduced stomatal closure in response to ABA and decreased ROS levels as a result of phenolic accumulation. The implication being that the ROS scavenging activity of phenolic compounds may lessen the efficiency of ABA signal transduction influencing stomatal behavior (Watkins et al., 2014). Recent research has highlighted the relationship between phenolics and ABA in drought tolerance — a study on sea buckthorn (*Hippophae rhamnoides* L.) showed a reversible increase in flavone and ABA content under drought stress with effects on stomatal conductance (Gao et al., 2021). This supports a hypothesis laid out in **Hunt et al. (2021b)**, that phenolic profiles may be partially responsible for the observed differences in stomatal sensitivity to environmental conditions between Barke and Bojos cultivars. However, an article from this year on stomatal response of drought-tolerant barley cultivars found different regulation strategies and responses to ABA and ROS, and different dependencies on antioxidant systems (Lv et al., 2023). This finding emphasizes the complexity of the issue, and the limitations of any single model of stomatal response mechanisms.

6.5.2 Recent advances in the study of grass stomata

Regulation of stomatal density in Arabidopsis, as well as crop grasses, is regulated by Epidermal Patterning Factor (EPF) and Epidermal Patterning Factor-like (EPFL) signaling peptides. Manipulation of EPFs and EPFLs to reduce stomatal density has produced more drought-tolerant rice (Caine et al., 2019) and barley mutants (Hughes et al., 2017) by increasing relative WUE. However, in our study (**Hunt et al., 2021b)**, we found the cultivar with higher stomatal density (Barke) to also have a higher WUE, demonstrating that water use is more than an issue of stomatal density alone. In fact, a recent study on the model grass *Brachypodium distachyon* suggests a positive relationship between stomatal density and rapid stomatal response. This may be a result of proximity effects prompting stomata closure in a coordinated manner (Nunes et al., 2022). Another recent study on grass stomata showed that operational stomatal aperture was more influential on stomatal conductance than stomatal density (Israel et al., 2022). Lower WUE was found for grasses with slower stomatal movements and wider stomatal apertures, leading to wasteful transpiration, especially during light transitions. Rapid stomatal response is key, especially in changing light environments: models suggest as much as a 20% increase in WUE if stomatal conductance could instantaneously match changes in light intensity (Lawson & Blatt, 2014).

In the study **Hunt et al. (2021b)** we used a convolution neural network as a time-saving way to calculate stomatal density. Although one neural network-based program for counting stomata existed at the time (Fetter et al., 2019), we found that results were unreliable on our grass samples. This year, a new tool for counting stomata has been introduced (Sai et al., 2023), which has been trained on both monocot (barley) and dicot (Arabidopsis) epidermal samples. Furthermore, automated measurements of stomatal width, length, and pore aperture have until now only catered to dicot stomata — Sai's StomaAI offers a reliable automated way to measure these parameters in grasses for the first time (Sai et al., 2023). In our study **Hunt et al. (2021b), we** used epidermal imprints, a reliable and durable method for capturing stomatal density. However, another recent publication provides a method for isolating intact epidermal samples from grasses, which are still physiologically sensitive to the effects of potassium chloride, light, and ABA; the process involves keeping the leaf in a buffer solution while stripping away all non-epidermal parts (Brett & Ng, 2022). Assuming that stomatally-localized phenolics remain undisturbed, this could provide a promising way to evaluate the direct effects of phenolic compounds on stomatal reactivity. Additionally, single-nucleus RNA sequencing has now been carried out on grass stomatal complex cells for the first time (Méteignier, 2022). Transcription results suggest that guard cells are less photosynthetically active than previously thought, and rely on imported glucose to regulate turgor, and that guard cells rather than subsidiary cells are the targets of ABA fluxes (Méteignier, 2022). This is exciting progress in the field and will hopefully make research into stomatal parameters of climate-resilient crops more accessible.

6.6 Grass leaf functional traits

As mentioned in the introduction, Poaceae is a family of plants with strong colonizing abilities, and invasive proclivities. They are well suited to exploit environments marked by disturbance, and may even contribute to continual disturbance (i.e., through morphologies that encourage fire spread, allelochemicals that discourage competing plants, and rapid growth forms that establish populations in a short amount of time). The functional traits of Poaceae become even more interesting when observed in competition with other members of the family. The findings of our study **Hunt et al. (2023)** observe an interplay between leaf biochemical traits, phenology, and morphology driving relative changes in grass abundance in the Krkonoše artic-alpine tundra grassland. The four studied species (*N. stricta, C. villosa, D. cespitosa,* and *M. caerulea*) fall in various places on the plant economic spectrum, and their relative success is a matter not only of the environmental stress present, but of the anthropogenic changes in the area (rising temperatures, sulfur deposition as a result of pollution, fertilization/changes in soil nutrient content, and cessation of grazing and mowing practices).

6.6.1 Biochemical traits

One notable finding in **Hunt et al., 2023** was that total phenolics did not vary significantly between the four grass species. It was not until the results of HPLC-HRMS were revealed that the various strategies in phenolic accumulation could be observed. *C. villosa*, the species demonstrating the most aggressive population spread, was found to accumulate a wide range of phenolic compounds including flavonoids, hydroxycinnamic acids, and hydroxybenzoic acids, as well as a visible layer of anthocyanin accumulation on its abaxial leaf side (**Hunt et al., 2023**). A recent study looking at phenolic profiles of plants in Patagonia rangelands found the types of phenolics accumulated by a species depended on intrinsic species attributes. Fast growing perennial grasses were found to have lower concentrations of phenolics compared to other non-grass species, such as shrubs (Saraví Cisneros et al., 2022). While this study targets a very different ecosystem than the Krkonoše grassland, the observed prioritization of growth over phenolic accumulation is mirrored in our findings (**Hunt et al., 2023)**: *C. villosa* shows early leaf expansion and photosynthetic pigment accumulation, while phenolic accumulation does not occur until later in the season. Although phenolic accumulation is not prioritized early in the growing season, phenolic acids in leaves influence plant-plant interactions after the leaves decompose. Leaching of phenolics can result in the allelopathic inhibition of co-occurring species. Litter leachate from *C. villosa* was found to inhibit the germination of Norway spruce (*Pinus abies)* in the Hrubý Jeseník mountains of Czech Republic , while other herb leachate samples had no effect (Zeidler, 2023). This indicates that at least some of the secondary metabolites accumulated by *C. villosa* have allelochemical effects, and its continued spread may have wider implications for the community dynamics, i.e., maintaining the boundaries between forest- and grassland-type landscapes and selecting woody species (germination of non-native *Pinus mugo* was not affected) (Zeidler, 2023). This inhibition may be further heightened by the soil acidification experienced by the region during the 1980s (Reininger et al., 2011).

For commercial grass species, such as barley, increased temperatures associated with climate change will likely increase total leaf phenolic content, increasing antioxidant capacity to deal with the heat stress (Martínez-Subirà et al., 2021). Wild grasses in the artic-alpine tundra, by contrast, may see a reduction in phenolic accumulation with warming temperatures. The tundra grassland is characterized by a combination of low temperature, high UV radiation, and low nutrient content. Alleviation of low temperature stress was found to may shunt more carbon toward growth pathways while simultaneously reducing total phenolics for alpine shrubs (Zhou et al., 2022). The most effective protective mechanisms for plant species are dependent on environmental constraints and thus, vary between agricultural grass crops planted in monocultures and wild grasses in (semi-)natural environments. High temperature may prioritize epidermal and epidermal adjacent phenolic accumulation, rather than the more or less homogenous distribution observed in our study (**Hunt et al., 202**3), although this pattern may also be the result of nutrient poor soil and thus irreversible by temperature alone.

6.6.2 Structural-Morphological traits

Grass morphology plays a role in how plants utilize or conserve resources and respond to stress. Water and nitrogen are two major resources influencing plant growth. As with woody plants, recent research confirms that grass height negatively correlates with xylem-level drought resistance (Griffin-Nolan et al., 2023). However, in the same study it was observed that increasing tiller density positively correlated with drought resistance in grass leaves. Drought tolerance in grasses can also be increased through adjustments in leaf functional traits such as SLA, leaf dry matter content, and leaf carbon/nitrogen/phosphorus contents (Yan et al., 2019), although this is limiting for plants growing in nutrient-poor environments. A survey of European Poaceae species found that leaf traits, especially high SLA and low leaf dry matter content were most associated with rapid uptake of NH_4^+ and NO_3^-, and overall more exploitative growth habits (Grassein et al., 2015). *N. stricta* was one of the species included in the survey, demonstrating the proposed correlation between a conservative growth form (low tissue turn-over, an adaptation to nutrient poor soil) and the lowest observed NH_4^+ uptake (Grassein et al., 2015). Interspecific differences in nitrogen uptake (NH_4^+ versus NO_3^-) has been proposed as one mechanism by which species can coexist in a community. Another recent study showed that leaf traits, such as leaf dry matter content and leaf nitrogen content, may correlate with plants species useful for reducing excess environmental nitrogen that might otherwise result in downstream eutrophication (Hanisch et al., 2020).

Environmental filtering results in some functional traits being highly represented in specialist species of certain environments. Adaptive functional traits in *Nardus*-dominated grassland species — short height, low SLA, slow growth, temporally short flowering window, and late seed shedding — were also found to be common in alpine species. By contrast, functional traits common in meadow species included higher SLA, taller canopies, earlier and longer periods of flowering, and rapid clonal lateral spread (Ladouceur et al., 2019). Based on these observations, *C. villosa* better fits the description of a meadow species. However, for many species, suitable ranges are shifting with climate change — moving up in latitude or altitude for temperate species (Couet et al., 2022). This shift in range is happening more rapidly for species with short lifespans and fast regeneration abilities, likely favoring meadow-specialists over alpine-specialists, and resulting in the spread of *C. villosa* observed in the Krkonoše grassland (**Hunt et al., 2023**).

6.6.3 Phenological traits

One of the "Viking syndrome" traits expounded by Linder et al. (2018) is Poaceae's ability to exploit even minor spatial-temporal windows of opportunity to establish a population and begin spreading. This is reflected in our findings in **Hunt et al. (2023)** that part of the success of *C. villosa* may be due to its early leaf expansion and accumulation of chlorophyll and carotenoid pigments compared to the other species (especially the otherwise morphologically similar *M. caerulea*). Phenology plays an additionally important role for grasses in areas used for grazing livestock. While both *C. villosa* and *N. stricta* are avoided by grazing animals, early leaves *of D. cespitosa* were recently found to be a "more important than expected" fodder source for livestock (Kotowski et al., 2023). The leaf expansion and abundance of neighboring fodder species could be quite influential in whether *D. cespitosa* has the opportunity to expand or is limited by severe grazing early in its growing season. Interestingly, grazing in alpine grasslands can change the area from a carbon source to a carbon sink, although the opposite trend is seen in temperate grasslands (Zhang et al., 2023).

Climate change complicates previously stable weather cycles and increases instances of ecosystem disturbance. Environmental cues regulating phenological traits, such as leaf expansion and flowering times can become de-coupled under changing climatic conditions, potentially shifting phenological niches in favor of invasive species (Archer et al., 2023). A study exploring the restoration of vegetative communities disrupted by severe wind storms in Italy suggests that the resilience of future plant communities to expected disturbances should be considered (Giupponi et al., 2023). Major disturbance events create opportunities which can be used to encourage colonization by plants more tolerant to environmental disturbance, encourage biodiversity, and continue to thrive under future climate conditions. The effects of climate change are already altering the phenology of alpine grasslands in Tibet, promoting earlier and faster growth. However, this has not resulted in an overall biomass increase, i.e. increased carbon sequestration, as water-stress shortens their growing season in autumn (H. Wang et al., 2020).

6.6.4 Effects of plant traits on the ecosystem level

As we move deeper into the Anthropocene, plants are facing new challenges. Subalpine montane communities in the Czech republic have lost plant diversity since the 1970s, owing in part to cessation of grazing and increased atmospheric pollution (Klinkovská et al., 2023). Conservation efforts have slowed this trend, however, the issue merits continued attention. An emerging area of research is the ability of plant communities to survive and even reverse the effect of anthropogenic activities, such as soil acidification: Recent research in montane forests of the Czech Republic compares the ability of beech versus spruce forests to replenish soil nutrients through litter decomposition and to increase exchangeable base cations lost during soil acidification. Beech forests were found to perform better at mitigating the hostile effects of soil acidification (Thai et al., 2023). A similar study looking at how grasslands of varying compositions contribute to ecosystem function and repair could be valuable.

While drought is a very real consideration for agricultural lands (Trnka et al., 2016), natural grasslands contain comparatively higher soil moisture content than other landscape types (e.g., managed grasslands, meadows, beech and conifer forests) (Šípek et al., 2020), likely as a result of lower transpiration demands. A few key functional traits were found to affect water cycling and erosion, including root depth, leaf area, canopy structure, percentage fine roots, stem dry matter content and root nitrogen content (Hanisch et al., 2020). The effects of elevated CO_2 reducing stomatal density and increasing stomatal resistance will likely positively affect water balance in montane grasslands, although this is frequently neglected in literature (Vremec et al., 2023). The physiological effects of elevated CO_2 must be considered when creating and analyzing soil water fluxes and changes to watersheds under future CO_2 conditions.

The works contained in this thesis have focused exclusively on leaf traits, however, interactions between plant below-ground biomass, soil characteristics, and rhizome-associated microorganisms all contribute significantly to the overall picture of plant physiology and climate change. Recent studies suggest that changes in plant community compositions will influence soil microbial communities, decomposition processes, and carbon/nutrient cycling in tundra ecosystems (Koranda et al., 2023). Root functional traits, such as root depth and percentage fine roots, positively affect soil structure, reduce erosion, and contribute to below-ground carbon storage (Hanisch et al., 2020). Thus, I hope to expand my research outlook in the future to accommodate the effects of below-ground processes in grasses.

7 Conclusions

This thesis examined the physiological, structural, and biochemical responses of Poaceae species to light conditions in the context of oxidative stress protection, with an emphasis on the role of phenolic compounds:

- While both high light and elevated CO_2 positively affect phenolic accumulation, the phenolic profiles of barley were found to be genotype specific — at least in terms of phenolic acids.
- Phenolic profiles are often overlooked in favor of total phenolics; however, phenolic compounds are not equally protective against oxidative stress, and may provide other protective advantages in grasses.
- The biosynthesis of different phenolic groups was found to be differentially dependent on light intensity and spectral quality. Blue light in particular is necessary for the induction of certain oxidative-stress protective di-hydroxylated flavonoids and antioxidative enzymes.
- Analysis of the accumulation of specific phenolic compounds may provide valuable insight into stress tolerance mechanisms of plants — including stomatal regulation.

Phenolic profile is one important functional trait in grasses, however, stress tolerance results from the integration of multiple traits, including SLA, plant height, leaf dry matter content, stomatal density/responsiveness, leaf nitrogen content, pigment accumulation, as well as their seasonal dynamics.

Grass functional traits represent a spectrum of resource utilization strategies with adaptive advantages unique to certain environments, however, anthropogenic disturbance may be altering the adaptive advantages of certain leaf functional traits.

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Sketch of Hordeum vulgare *by the author.*

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8 Supplement

The published articles included in this thesis (**Hunt et al., 2021a, Hunt et al., 2021b, Pech et al., 2022, Hunt et al., 2023**) can be found in the following section as supplements. They are presented in chronological order by publication date.