

University of Zagreb

FACULTY OF AGRICULTURE

Jurica Duvnjak

INFLUENCE OF DROUGHT STRESS DURING ANTHESIS ON AGRONOMIC AND MORPHOLOGICAL TRAITS OF WINTER WHEAT AND ITS PHYSIOLOGICAL AND MOLECULAR RESPONSE

DOCTORAL THESIS



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ABSTRACT:

Wheat (*Triticum aestivum* L.) is the most important cereal crop in the world. It contributes as a major source of protein and calories in the daily human diet. Wheat production is severely threatened by unfavourable climate changes resulting in drought stress. Drought can significantly reduce wheat growth and development as well as final grain yield. Therefore, achieving a high grain yield of wheat is a challenge for breeders. In order to explore the effects of drought on different agro-morphological and physiological traits of six wheat varieties, experiment was set up during two growing seasons (2021/2022 and 2022/2023) in field conditions, where it was not possible to isolate one stress. Therefore, corresponding experiment was set up also in a greenhouse where drought stress was simulated at two intensities (mild and severe) by withholding watering for 45 and 65% of the volumetric soil moisture content (VSMC) for 14 days at anthesis stage. The objectives of this research were to determine the agro-morphological and physiological response of winter wheat varieties to two intensities of drought stress at the anthesis stage, and to define the expression level of genes related to drought resistance of wheat varieties.

In the field conditions, different amounts of rainfall and average temperatures during the two-year field experiment were recorded. In the first growing season a dry period (from January till April) was recorded, but however good grain yield was achieved. Further, grain yield was significantly positively correlated with 1000 kernel weight and maximum quantum yield of primary photochemistry (TR₀/ABS) at the second measurement point which coincidence with tillering stage. Only in the highest-yielding variety Bubnjar, the TR₀/ABS value was at the same significance between the second and third measurement points (tillering and stem elongation stage). In contrast to first growing season, there was more rainfall in the second growing season (especially through April and May), which led to occurrence of more diseases resulting in reduced grain yield. The three highest-yielding varieties escaped yellow rust pressure at the stem elongation stage more efficiently, and showed the lowest increase in TR₀/ABS at this stage. In both growing seasons, variety Bubnjar had the highest grain yield and the lowest protein content.

In a greenhouse, on 14th day after drought stress, morphological parameters were measured and flag leaves were collected to identify physio-biochemical and molecular changes. Severe drought stress led to abscisic acid (ABA) accumulation in the flag leaves and resulted in a significant decrease of chlorophyll a content in the drought sensitive variety, indicating a severe loss of photosynthetic reaction centres. The content of carotenoids, which could play an important role in resistance to drought stress, tended to increase in drought tolerant variety. Further, a significant decrease in catalase (CAT) activity was observed at mild drought stress, compared to control, in drought sensitive variety, and at severe drought stress, in all medium sensitive varieties. Drought tolerant and medium tolerant varieties responded to both drought stresses with a decrease in total glutathione (tGSH) content to enhance their defence system. The results of gene expression analysis showed that severe drought increased the levels of DHN5 and WZY2 genes in drought tolerant variety, whose grain weight, area, and length did not significantly change in maturity. On the other hand, drought sensitive and medium sensitive varieties showed the greatest decrease in grain yield-related traits. Both drought stress conditions induced variety-specific responses that depended on the severity of the drought stress. The identification of different mechanisms under drought can contribute to the selection of drought tolerant varieties.

Keywords: drought stress; grain yield; hormonal response; molecular response; physiology; photosynthesis; quality traits; winter wheat

EXTEND ABSTRACT IN CROATIAN

Utjecaj sušnog stresa tijekom klasanja na agronomska i morfološka svojstva ozime pšenice te njezin fiziološki i molekularni odgovor

Pšenica (Triticum aestivum L.) je najvažnija, vrlo hranjiva, široko uzgajana i najviše konzumirana žitarica u svijetu. Pridonosi kao glavni izvor proteina i kalorija u svakodnevnoj ljudskoj ishrani. Povećana potražnja za hranom zbog rasta populacije čini važnost proizvodnje pšenice još većom. Ima višestruku upotrebu u različitim industrijama, poput mlinarske i prehrambene. Također ima veliki utjecaj u industriji stočne hrane i stočarstvu. S napretkom načina života dolazi do sve većeg iskorištavanja prirodnih resursa, a negativne posljedice klimatskih promjena povećavaju učestalost raznih vrsta abiotičkog stresa što u konačnici rezultira smanjenjem produktivnosti pšenice. Suša je postala jedan od najznačajnijih abiotičkih stresova koji značajno utječe na cjelokupnu proizvodnju pšenice na globalnoj razini. Glavni uzroci sušnog stresa su nedovoljne količine oborina ili njihov nepovoljan raspored tijekom vegetacije, povećana razina CO2 u atmosferi, porast atmosferske temperature te vrući i suhi vjetrovi. Suša je u biljkama uzrok morfoloških, fizioloških i biokemijskih promjena kao što su smanjenje sadržaja klorofila i fotosintetskih aktivnosti u biljnim tkivima, smanjenje nakupljanja škroba što u konačnici smanjuje urod zrna. Sušni stres se može pojaviti tijekom svih faza rasta i razvoja pšenice, no najkritičniji je onaj koji se javlja tijekom reproduktivne faze rasta jer dovodi do značajnog smanjenja uroda zrna i postao je važan ograničavajući faktor za sigurnost hrane na globalnoj razini.

Kako bi se istražio utjecaj sušnog stresa na agronomska i morfološka svojstva ozime pšenice te njezin fiziološki i molekularni odgovor, šest sorti ozime pšenice (Bubnjar, Anđelka, Pepeljuga, Rujana, Fifi i Silvija) izloženo je sušnom stresu u stakleniku, a ujedno pokus je postavljen i u poljskim pokusima kroz dvije vegetacijske godine (2021/2022 i 2022/2023) na Poljoprivrednom institutu Osijek (45°27' N, 18°48' E). Tijekom obje vegetacijske godine primijenjene su standardne agrotehničke mjere. Mjerila su se morfološka svojstva (visina stabljike i biljke) te parametri fotosinteze. Zabilježen je datum klasanja, a nakon žetve izmjerena su agronomska svojstva i kvaliteta zrna. Budući da je u poljskim uvjetima prisutan cijeli niz stresnih čimbenika pokus je postavljen i u stakleniku gdje su simulirana dva intenziteta sušnog stresa smanjenjem zalijevanja za 45 i 65% volumetrijskog sadržaja vlage u tlu tijekom 14 dana počevši od faze klasanja. Nakon dvotjednog simuliranja sušnog stresa izmjereni su morfološki parametri (visina stabljike i bilike, duljina i širina lista zastavičara, broj listova po biljci i broj plodnih izdanaka) i uzorkovani su listovi zastavičari za daljnje fiziološke, hormonske i molekularne analize. Nakon zriobe utvrđen je broj klasića po klasu, broj zrna po klasu, masa 1000 zrna i morfološka svojstva zrna (duljina, širina, površina i kružnost)

Uspoređujući vremenske uvjete u dvogodišnjim poljskim pokusima, prvu vegetacijsku godinu karakterizirala je pojava sušnog perioda od siječnja do travnja, ali su unatoč tome ostvareni visoki urodi zrna. Za pretpostaviti je da su bilike pšenice vierojatno apsorbirale vodu iz rezervi tla akumuliranih u razdoblju od listopada do prosinca 2021. Posebno bi ovo moglo biti od značaja za sortu Bubnjar, koja je ranije bila deklarirana kao otporna na sušu, a kod koje je ujedno zabilježen i najveći urod zrna. Nadalje, urod zrna bio je u značajnoj pozitivnoj korelaciji s masom 1000 zrna i maksimalnim kvantitativnim prinosom fotosustava II (TR₀/ABS) u drugom mierenju (faza busanja). Samo je sorta Bubnjar pokazala vrijednosti TR₀/ABS na istoj značajnoj razini između druge i treće točke mjerenja (faze busanja i vlatanja). Drugu vegetacijsku godinu karakterizirala je veća količina oborina tijekom travnja i svibnja što se u konačnici negativno odrazilo na urod zrna. Rezultat smanjenja uroda zrna je bio jači intenzitet bolesti (pojava žute hrđe). Najmanje smanjenje uroda zrna zabilježeno je kod sorte Bubnjar koja je ujedno bila najrodnija i u drugoj vegetacijskoj godini, a s najnižim sadržajem proteina. Sorta Bubnjar je ujedno i jedna od najviših i najkasnijih sorti koja je učinkovitije izbjegla pritisak žute hrđe u fazi vlatanja s najvećim porastom indeksa učinkovitosti na bazi apsorpcije (PI_{ABS}) u zadnjem sedmom mjerenju (faza zriobe) u odnosu

na šesto (faza cvatnje). Na taj način dulje je održana vitalnost lista zastavičara što se u konačnici pozitivno odrazilo na urod zrna.

Smatra se da listovi svojim oblikom, veličinom, intenzitetom voštane prevlake i brzinom kojom ulaze u senescenciju mogu pridonijeti tolerantnosti na sušni stres. U ovom istraživanju kod većine ispitivanih sorti nisu zabilježene značajne promjene u veličini lista zastavičara (duljina i širina) u uvjetima oba sušna stresa u odnosu na kontrolu, ali je relativni sadržaj vode u listu zastavičaru značajno smanjen kod većine ispitivanih sorti, što ukazuje na gubitak vode u uvjetima jačeg sušnog stresa. Značajno smanjenje visine biljke uslijed jačeg sušnog stresa zabilježeno je kod dvije sorte (Bubnjar i Rujana). Nadalje, tolerantne sorte tijekom sušnog stresa održavaju nižu visinu biljke kako bi se smanjila potreba za vlagom i spriječio gubitak vlage zbog transpiracije. Stoga se čini da sorta Bubnjar reagira na jači sušni stres značajnim smanjenjem visine stabljike i cijele biljke. Također, jači sušni stres rezultirao je povećanim nakupljanjem apscizinske kiseline (ABA) u listu zastavičaru kod svih ispitivanih sorti, što nije bio slučaj za salicilnu kiselinu (SA) kod koje je zabilježen blagi porast i neznačajna promjena u odnosu na kontrolu. Nakon zrelosti, Bubnjar i Pepeljuga (tolerantna i srednje tolerantna sorta), nisu značajno smanjile broj zrna po klasu i masu 1000 zrna u oba sušna stresa, što ih definira tolerantnijima na sušni stres od ostalih. S druge strane, Rujana, Fifi, a posebno Silvija imale su najveće smanjenje svojstava vezanih uz urod zrna, te se smatraju osjetljivijima na sušni stres.

Pšenica je razvila različite mehanizme tolerantnosti kako bi preživjela u uvjetima sušnog stresa. Prema prijašnjim istraživanjima, nakupljanje prolina, proizvodnja raznih enzima poput, askorbat peroksidaza (APX), katalaza (CAT), osmotske prilagodbe i nakupljanje ABA u biljnom tkivu rezultiralo je jačom otpornosti na sušni stres. U ovom istraživanju uslijed jačeg sušnog stresa zabilježeno je značajno smanjenje sadržaja klorofila u sorti osjetljivoj na sušni stres, što ukazuje na snažan gubitak fotosintetskih reakcijskih centara. Nasuprot tome, kod Bubnjara, sorte deklarirane kao tolerantne na sušni stres sadržaj karotenoida se značajno povećao u uvjetima blažeg sušnog stresa. Nadalje, kod sorte osjetljive na sušni stres (Silvija) zabilježeno je značajno smanjenje CAT u oba sušna stresa, u usporedbi s kontrolom, te kod srednje osjetljivih sorti (Rujana i Fifi) pri jačem sušnom stresu. Također, oba intenziteta sušnog stresa rezultirala su padom ukupne koncentracije glutationa kod tolerantnih i srednje tolerantnih sorti, a za pretpostaviti je da su na ovaj način uključile svoj obrambeni sustav. Dehidroaskorbat reduktaza (DHAR), monodehidroaskorbat reduktaza (MDHAR) i glutation reduktaza (GR) bili su ključni enzimi uključeni u askorbat-glutation ciklus zajedno s CAT, sudjelujući u detoksikaciji proizvedenih reaktivnih kisikovih jedinki (ROS) pri jačem sušnom stresu. Rezultati analize ekspresije gena pokazali su da je jači sušni povećao razine gena DHN5 i WZY2 u tolerantnoj sorti na sušni stres, čija se masa, širina i duljina zrna nisu značajno mijenjale pri blažem sušnom stresu u usporedbi s kontrolom. Također, vidlijvo je da je ekspresija *DHN5* bila u značajnoj pozitivnoj korelaciji s duljinom zrna i sadržajem prolina pri blažem sušnom stresu.

Ukratko, oba sušna stresa uzrokovala su odgovore specifične za sortu, koji su ovisili o jačini sušnog stresa. Razumijevanje povezanosti aktivnosti antioksidativnih enzima i ekspresije gena s genetskim varijacijama u tolerantnosti na sušu važno je za daljnju identifikaciju čimbenika koji kontroliraju antioksidacijsku obranu. Odgovor obrambenih sustava u tolerantnim sortama pokazao je da proučavani geni i enzimi imaju značajnu ulogu u obrambenim reakcijama na sušu. To je vrlo značajno jer je najbolja opcija za proizvodnju usjeva i poboljšanje stabilnosti uroda zrna u uvjetima sušnog stresa razvoj sorti otpornih na sušni stres.

Ključne riječi: sušni stres; urod zrna; hormonski odgovor; molekularni odgovor; fiziologija; fotosinteza; parametri kvalitete; ozima pšenica

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List of abbreviations:

- 1. Total glutathione (tGSH)
- 2. Reactive oxygen species (ROS)
- 3. Deoxyribonucleic acid (DNA)
- 4. Ribonucleic acid (RNA)
- 5. Superoxide dismutase (SOD)
- 6. Catalase (CAT)
- 7. Ascorbate peroxidase (APX)
- 8. Monodehydroascorbate reductase (MDHAR)
- 9. Dehydroascorbate reductase (DHAR)
- 10. Glutathione reductase (GR)
- 11. Abscisic acid (ABA)
- 12. Salicylic acid (SA)
- 13. European Union (EU)
- 14. Genetics (G)
- 15. Environment (E)
- 16. Relative water content (RWC)
- 17. Carbon dioxide (CO₂)
- 18. Hydroxyl radical (OH)
- 19. Superoxide anion (O₂)
- 20. Hydrogen peroxide (H₂O₂)
- 21. Singlet oxygen (¹O₂)
- 22. Glutathione peroxidase (GPX)
- 23. Polyphenol oxidase (PPO)
- 24. Glutathione (GSH)
- 25. Dlutathione disulfide (GSSG)
- 26. Pyrroline-5-carboxylate synthetase (P5CS)
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- 32. Single nucleotide polymorphism (SNP)
- 33. Volumetric soil moisture content (VSMC)
- 34. Performance index on absorption basis (PI_{ABS})
- 35. Grain stage (GS)
- 36. Principal component analysis (PCA)
- 37. Chlorophyll (Chl)
- 38. Malondialdehyde (MDA)

List of appendices:

Appendices 1. Scientific paper: Duvnjak J., Lončarić A., Brkljačić L., Šamec D., Šarčević H., Salopek-Sondi, B., Španić V. (2023). Morpho-physiological and hormonal response of winter wheat varieties to drought stress at stem elongation and anthesis stages. Plants 12(3): 418. doi: 10.3390/plants12030418

Appendices 2. Scientific paper: Duvnjak J., Katanić Z., Šarčević H., Španić V. (2024). Analysis of the photosynthetic parameters, grain yield, and quality of different winter wheat varieties over a two-year period. Agronomy 14: 478. doi: 10.3390/agronomy14030478

Appendices 3. Scientific paper: Duvnjak J., Sarcevic H., Vukovic R., Spanic V. (2024). Effects of drought at anthesis on flag leaf physiology and gene expression in diverse wheat (*Triticum aestivum* L.) genotypes. Agronomy 14(7): 1522–1547. doi: 10.3390/agronomy14071522

Scientific papers	Base	Category	Quartile	Impact factor
 Duvnjak, J., Lončarić, A., Brkljačić, L., Šamec, D., Šarčević, H., Salopek-Sondi, B., Španić, V. (2023) Morpho- Physiological and Hormonal Response of Winter Wheat Varieties to Drought Stress at Stem Elongation and Anthesis Stages. Plants 2(3): 418. 	WoS	A1	Q1	4.4
2. Duvnjak, J ., Katanić. Z., Šarčević, H., Španić, V. (2024) Analysis of the Photosynthetic Parameters, Grain Yield, and Quality of Different Winter Wheat Varieties over a Two-Year Period. Agronomy 14(3): 478.	WoS	A1	Q1	3.7
3. Duvnjak, J ., Šarčević, H., Vuković, R., Španić, V. (2024) Effects of Drought at Anthesis on Flag Leaf Physiology and Gene Expression in Diverse Wheat (<i>Triticum aestivum</i> L.) Genotypes. Agronomy 14(7): 1522.	WoS	A1	Q1	3.7
Explanation of the connection between recearch hypotheses and published				

Explanation of the connection between research hypotheses and published research papers

Research hypotheses	Explanation of research hypotheses
H1. Morphological and	In Scientific Paper number 1, the morphological,
physiological response to	physiological and hormonal response of winter wheat
drought stress will involve	varieties to two drought stress conditions simulated
different mechanisms in	by reducing water content by 45 and 65% of the
different wheat varieties and	volumetric soil water content (VSMC) was analyzed.
will depend on the intensity of	The results confirmed the assumed research
stress	hypothesis, that is, during the research under the
	influence of different drought stress conditions,
	recorded differences in the tested traits were
	observed in all tested varieties. In addition, severe
	drought stress resulted in a significant decrease or

increase in the tested traits in all tested varieties
compared to mild drought stress.
In Scientific Paper number 2, the photosynthetic
efficiency was analyzed using the maximum
quantitative yield of primary photochemistry
(TR_{\circ}/ABS) and performance index on absorption
hasis (PL). Significant differences in the analyzed
traite were found between the tested verifies were
traits were found between the tested varieties, years
and different measurement points. In the first growing
season, only the variety Bubnjar maintained
TR_O/ABS and PI_{ABS} at the same significant level
during the second and third measurements points
(tillering and stem elongation phases), while in the
other varieties recorded increase in the mentioned
parameters during the transition from tillering to stem
elongation stage, when mild drought stress
prevailed. It follows that the variety Bubnjar had a
lower photosynthetic efficiency than the other tested
varieties in the mentioned period and achieved a
more productive tillering, which eventually led to a
high grain yield and 1000 kernel weight, which were
significantly positively correlated with the TR ₀ /ABS at
tillering stage.
In Scientific Paper number 3, the influence of mild
and severe drought on the physiological traits and
gene expression in the flag leaf of the tested varieties
was analyzed. The carried out analyzes showed that
the plants used different mechanisms of the
antioxidant system, that is, in drought sensitive
varieties sensitive a significant decrease in catalase
(CAT) was recorded under mild drought stress, while
in varieties declared as tolerant to mild and severe
drought stress responded by reducing the
concentration of total glutathione to strengthen their
defense system, which allows them to tolerate

	drought. The obtained results indicate that in addition
	to CAT, the enzymes of the ascorbate-glutathione
	(AsA-GSH) cycle (glutathione reductase,
	monodehydroascorbate reductase and
	dehydroascorbate reductase) are also important
	components of the antioxidant defense system due
	to the severe drought stress.
110 Lovel of expression of	In Scientific Departmenter 2, the effects of mild and
H2. Level of expression of	In Scientific Paper number 3, the effects of mild and
the gene encoding the DREB	severe drought on physiological traits and gene
transcription factor will be	expression (DHN5, WZY2, P5CS, DREB1 and
more strongly associated with	DREB2) in the flag leaf of the tested varieties were
the resistance of varieties to	analyzed. After the analysis, it was found that severe
drought stress compared to	drought stress led to increased accumulation of
expression level of other	carotenoids and higher expression of the DHN5 and
analysed genes	WZY2 genes in drought tolerant variety. The above
	results do not confirm the assumed research
	hypothesis, that is a significant increase in the
	expression of the DREB1 gene was found only in the
	variety Rujana at mild drought stress, while a
	significant increase in the expression of the DREB2
	gene was found in Rujana at both drought stress and
	a decrease in Bubnjar at a mild drought stress.

1. INTRODUCTION

Wheat (Triticum aestivum L.) is one of the most important grain cereal used for human nutrition, and ranks second in total cereal production worldwide. It is also the main food for more than one third of the world's population, providing approximately 20% of protein and daily calories (Shiferaw et al., 2013). This cereal has significantly contributed to human civilization, and is cultivated in a wide region between 30° and 60° north and 27° and 40° south latitudes (Tadesse et al., 2019). At the same time, the wide adaptation and cultivation of wheat across all continents lead to the harvest of wheat in each month of the year at least in one region of the world (Tadesse et al., 2019). However, most of the global harvest takes place between April and September in the temperate zone of the northern hemisphere. There is continued urgency to enhance wheat productivity to ensure global food security given continued global population growth (Erenstein et al. 2022). A sustainable increase in wheat production is a prerequisite for meeting the current and future world food needs. However, the current climate change has increased the frequency of biotic and abiotic stresses that can adversely affect wheat productivity. High or low temperatures, salinity, drought or flooding, nutrient deficiency or metal toxicity are some of the main abiotic stresses occurring during the growing season of wheat. One of the most devastating abiotic stress for wheat production is the lack of water resulting in drought, which is the main limiting factor for wheat production worldwide in the last few decades. In plants, drought can cause different morphological, biochemical, physiological, and molecular changes. In addition, drought can occur at any stage of wheat growth and development, and grain yield may be affected to some degree regardless of the developmental stage at which drought stress occurs. It is believed that the highest grain yield losses will occur when drought occurs during the reproductive and grain-filling stages (Cappelli and Cini, 2021).

Wheat grain yield losses during reproductive stage may be the result of adverse effects of drought stress on morphological and physiological traits. Drought affects the morphology of certain plant organs and their physiological characteristics, which results in changes in the anatomy of certain plant tissues (Bhusal et al., 2019; Wasaya et al., 2021). The chlorophyll content in the flag leaves influences their photosynthetic activity and consequently the grain yield potential of wheat. For example, under drought stress the reduced chlorophyll content in the leaf led to leaf necrosis or reduced photosynthesis (Yang et al., 2001). Li et al. (2012) also reported that severe drought stress reduced chlorophyll content in wheat flag leaves. For this reason, the maintenance of an efficient photosynthetic apparatus is considered crucial under stress conditions, because the longer the plants have

photosynthetic activity, the longer assimilates can be translocated into the grain (Muhammad et al., 2021).

Under drought conditions, reactive oxygen species (ROS) are produced, which in higher concentrations can cause deoxyribonucleic (DNA) and ribonucleic (RNA) acids damage, and protein degradation. To remove ROS, plants developed an antioxidant defence system that includes non-enzymatic antioxidants and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and enzymes of the ascorbateglutathione system (ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR)). In addition to the antioxidant system, hormones are among the most important signalling molecules that have a role in the plant's response to drought. Among the most important are abscisic acid (ABA) and salicylic acid (SA), which control the growth rate under unfavourable conditions by inhibiting plant growth, protein synthesis, and ion transport. These two hormones have a role as chemical messengers in response to drought stress that lead to the activation of diverse plant physiological processes, including accumulation of osmolyte, stomatal closure, and root growth stimulation to avoid water loss (Sharma et al., 2019). ABA is plant hormone governing multiple biological processes in plants under drought conditions (Kishor et al., 2022). For example, ABA induced stomatal closure during drought, which resulted in the prevention of intra-cellular water loss (Saradadevi et al., 2017). In addition, more efficient protection against drought can be achieved by excessive production of SA through enhanced activity of enzymes of the SA biosynthetic pathway (Khan et al., 2015). Furthermore, some genes have been shown to have a function in drought tolerance, although many of them are of low effect. Identification of genes controlling physiological changes can lead to rapid genetic improvement of wheat tolerance to drought.

Taking account all facts about drought as a major problem in sustainable wheat production there is a need for development of new wheat varieties with better drought tolerance where physiological and molecular pathway may can have significant role. This represents a major challenge for wheat breeders, but also is a justification for setting up research that will reveal the impact of drought on the changes in agronomical and morphological traits of wheat caused by physiological and molecular response. Such research will provide information on the impact of drought on wheat production and help breeders to identify more drought tolerant varieties.

1.1. Hypotheses and research goals

1.1.1. Hypotheses:

1. Morphological and physiological response to drought stress will involve different mechanisms in different wheat varieties and will depend on the intensity of stress.

2. Level of expression of the gene encoding the DREB transcription factor will be more strongly associated with the resistance of varieties to drought stress compared to expression level of other analysed genes.

1.1.2. Objectives:

1. To determine the morphological and physiological response of winter wheat varieties to two intensities of drought stress at the anthesis stage.

2. To determine the expression level of genes related to the drought resistance of wheat varieties.

2. REVIEW OF RELEVANT LITERATURE

2.1. Wheat

2.1.1. Wheat origin

Wheat is a crop that is the foundation of human civilization. Between eight and ten thousand years ago in the earliest permanent agricultural settlements of the Fertile Crescent, farmers cultivated bread grain from emmer grass (Smith, 2003). Wheat comes from the Levant region in the Middle East and the Ethiopian highlands, and was grown in ancient Greece, Persia, Egypt and Europe, from where it was brought to China, India, Australia and America. Some wild relatives of wheat are still cultivated in Lebanon, Syria, northern Israel, Iraq and eastern Turkey. In terms of cultivation, there are wild, primitive and cultivated species. Wild species can be recognized by strong tillering, spike brittleness, where small grain is tightly wrapped in glumes. As a result of breeding and the influence of nature, cultivated wheats were created, which are characterized by a firm spike with the grain that is released from the glumes during threshing (Španić, 2016).

Wheat has large genetic diversity, which is manifested through different subspecies with different numbers of chromosomes. It belongs to the *Poaceae* family and the genus *Triticum*. The species of the genus *Triticum* are divided into three groups: diploids (2n = 2x = 14, AA), tetraploids (2n = 4x = 28, BBAA), and hexaploids (2n = 6x = 42, BBAADD) (Tadesse et al., 2019). The most important wheat species cultivated today are bread wheat (*Triticum aestivum*) and durum wheat (*T. turgidum*) (Španić, 2023). A third species, eincorn (*T. monococcum*), has great historical but currently very small agricultural importance (Feuillet et al., 2008). Bread or common wheat is allohexaploid with 21 pairs of chromosomes, which contributes 95% to wheat production. After the Green Revolution from the 1960s, wheat production tripled occupying large areas (Shiferaw et al., 2013). Europe also increased the production of quality bread wheat in order to reduce wheat imports, while simultaneously increasing grain yields through the introduction of semi-dwarf wheat varieties (Cornucopiaalchemy, 2023).

2.1.2. Wheat in the world

Ensuring global food security, while preserving the environment and sustainable biodiversity, is scientific challenge facing humanity (Cassman, 2012). According to previous data obtained by Food and Agriculture Organization of the United Nations, food production is projected to increase by about 70% between 2007 and 2050 in order to provide enough

food for a population estimated to grow to more than nine billion (FAO, 2022). The three main cereals in the world are wheat, rice and maize, with wheat being most important for food production having the widest adaptation of all cereals (Briggle and Curtis, 1987). Work on wheat breeding began at the beginning of the 19th century, which led to a significant increase in grain yield and quality (Španić, 2016). After an extremely difficult period in India (1942-1943), when a great famine took nearly three million human lives, priority was given to agriculture through reforms to increase irrigated land, and increased investment in science. Furthermore, the Green Revolution (1960-1980) led to the development of semi-dwarf and high-yielding varieties, which significantly contributed to the reduction of hunger and poverty in the countries of India and Pakistan (Renkow and Byerlee, 2010). During this period the grain yield of wheat increased from 1 or 2 t ha⁻¹ to 5 t ha⁻¹ in India (Španić, 2016). Growth in wheat grain yields continued in Mexico and Pakistan, resulting in increase of wheat production worldwide.

According to available data, the largest producers of wheat in the world are China and the European Union (EU) (FAOSTAT, 2023). In 2021/2022, 808 million tons of wheat were produced worldwide with an average grain yield of 3.7 t ha⁻¹, of which 282 million tons were produced in the EU with an average grain yield of 4.5 t ha⁻¹ (FAOSTAT, 2023), which corresponds to about 35% of the total world production. The amount of produced wheat provides more than 20% of the world's food supply (Kettlewell et al., 2023). Although the area sown with wheat at the world level decreased by 1% in the period from 2016 to 2018, compared to the period from 1992 to 1994, wheat production increased by 36.3% as a result of the increase in grain yield by 37.8% (Erenstein et al., 2022). Due to the disruption of the agricultural market and the large amount of wheat exported from Russia in 2023, compared to 2022, wheat prices on the world stock exchanges fell. The negative trend continued in 2024.

2.1.2. Wheat in Croatia

According to the sown fields in Croatia, wheat is at second place, being sown on 170 000 hectares in 2022/2023, with an average grain yield of 4.8 t ha⁻¹ (CBS, 2022). If this compared with the total used agricultural area (land and gardens) in Croatia, which is about 898 000 ha (CBS, 2022), wheat in Croatia is represented by 20% in the sowing structure. Wheat production is twice as high as domestic consumption, that is, in Croatia there is self-sufficiency in own production, which is extremely desirable and important. In the last ten years, the average grain yield ranged from 4.2 to 6.7 t ha⁻¹, on produced areas from 116 000 ha (2017) to 204 000 ha (2013). The lowest average harvest in the last ten years was achieved in 2014, and the highest in 2021 (CBS, 2022). If the period of the last ten years is

compared with the previous multi-year period, it can be concluded that the average wheat production has increased despite changes in many climatic factors, of which the lack of precipitation is the most significant. Although the area of Croatia is characterized by great variability in the amount of precipitation during the wheat growing season, the annual frequency of dry periods has increased in the last few years (Marinović et al. 2021).

As a result of unfavourable climate change, wheat production in Croatia during 2022/2023 crop season was decreased than in the previous year (Španić, 2023). Wheat producers believe that they do not need to invest too much in production (fertilization, disease and pest protection) because the wheat prices are too low to justify inputs in production, which often results in lack of application of sufficient protection against diseases, pests or weeds, and ultimately leading to a decrease in grain yield (Španić, 2023). In same book, author reported that the use of fertilizers is not in accordance with the recommendations of the agronomical profession, mainly due to the high prices of mineral fertilizers and protective agents. The lower average grain yield in Croatia is mainly the result of poor agricultural policy and inadequate implementation of agro-technical measures.

2.2. Drought stress

Abiotic stress poses a significant limitation for plant growth and food production in many regions of the world and is expected to intensify. Drought stress is one of the most important abiotic stresses faced by wheat producers (Mansour et al., 2020). Wheat crops require 300-500 mm of water during the growing season, which is much more compared to other crops such as maize (Poudel et al., 2020). Globally, only 346 895 ha are irrigated, and the rest of the area depends entirely on natural rainfall (Nyaupane et al., 2024). In developing countries, the major cause of lower wheat production is the lack of irrigation compared to developed countries (Shiferaw et al., 2013). Drought was the cause of reduction of 50-60% of grain yield (Zhao et al., 2020a). If drought stress continues, there will be an additional decrease in wheat production, which will have a negative impact on the world's food security (Mansour et al., 2020).

Drought stress has a complex effect on morphology, physiology and biochemistry of wheat plants resulting in growth retardation, reduction in grain yield and ultimately reduction in productivity. According to previous research, the major causes of drought stress were climate-related changes such as changes in the amount of precipitation, increased levels of CO₂ in the atmosphere, an increase in atmospheric temperature, and hot or dry winds (Arbona et al., 2013; Dai, 2013; Nezhadahmadi et al., 2013; Hossain et al., 2016). Furthermore, recent study in Nepal found that the annual average temperature increased

by 0.05 °C, between 2000 and 2015, while at the same time the annual precipitation decreased by 16.09 mm per year (Paudel et al., 2020).

Plants can experience a lack of water in the soil, even when there is sufficient moisture in the soil as a result of various edaphic factors (salinity, low soil temperatures, and floods) that interfere with the absorption of water by the roots. This condition is a physiological drought (Lisar et al., 2012; Arbona et al., 2013). Besides, the final effect of drought on plants will depend on soil type and environmental effects (Semenov et al., 2014), varietal tolerance (Wu et al. 2014), and production technology (Haque et al., 2012). With increasing frequency of drought, wheat is likely to be grown more in rain-fed areas in the future (Ortiz et al. 2008). The negative effects of drought can be large as it can damage wheat in critical growth and development stages, such as germination and emergence, tillering and booting, flowering and grain filling (Akram, 2011; Wang et al., 2015; Sarto et al., 2017), with the flowering and grain filling stages being the most drought sensitive (Yang et al., 2006) causing grain yield losses of up to 69% (Khan et al., 2023).

2.2.1. Drought effects on agro-morphological traits of wheat

Effects of drought stress on plants depend on its duration and intensity (Ercoli et al., 2008). Drought stress especially affects the grain yield and quality of wheat (Javadinejad et al., 2021). Ma et al. (2014) reported that severe drought stress caused a greater reduction in grain yield than milder drought stress. In contrast, mild drought stress at the grain filling stage can promote the remobilization of carbon assimilates in the grain, accelerate grain filling and ultimately improve grain yield of wheat (Yang et al., 2001). In the same research, authors reported that during grain filling stage, drought significantly reduced 1000 kernel weight which is a key component of grain yield. The speed and duration of grain filling determine grain weight (Islam et al., 2021). Grain filling stage usually begins with endosperm cell division followed by an increase in cell volume through assimilate accumulation that comes from two primary sources (mobilization of reserves in the stem and photosynthesis) (Ehdaie et al., 2006). Since photosynthesis decreases under drought conditions, grain filling mainly depends on the remobilized reserves from the stem (Hossain et al., 2011). Stored stem reserves, mainly water-soluble carbohydrates (glucose, fructose, and sucrose) accumulate in the stem during the stem elongation stage until the beginning of grain filling stage, that can be remobilized in the later stages of grain filling (Wardlaw and Willenbrink, 2000). Thus, every additional millimetre of stored reserves remobilized during grain filling can result in increased grain yield during drought stress (Kirkegaard et al., 2007).

Mehraban et al. (2018) reported that before flowering drought stress reduced the number of grains per unit area, while after flowering affected the number of grains per spike and 1000 kernel weight (Knezevic et al., 2012). Also, during flowering stage, the occurrence of drought affected the reduction of the total number of grains per spike and the number of grains per spikelet (Sangtarash, 2010). It is known that drought can cause grain yield loss because of pollen sterility and ABA accumulation in spikes in drought sensitive wheat varieties (Ji et al., 2010). Drought stress also negatively affects grain filling (Rajala et al., 2009), leading to shrivelled wheat grains (Mitchell et al., 2013). Increased senescence of leaves after flowering was increased causing a drastic loss of grain yield of wheat (Ji et al., 2010). Overall, during reproductive stage, drought stress resulted in a reduction in the number of potential grains in the wheat spike (Dong et al., 2017), while for wheat grain size, drought stress was the most harmful during and immediately after the flowering stage (Sangtarash, 2010).

2.2.2. Drought effects on wheat quality

Wheat production is very important in the context of increasing demands for food enhanced with nutrients due to population growth (Hussain and Jatoi, 2021). Wheat grain contains 1.8% fiber, 9.4% protein, 69% carbohydrates, and 2.5% fat (Ahmad et al., 2022). Among all grains, wheat has the best gluten that results in good bread quality. Wheat grain quality is defined by the concentration and composition of starch and protein, grain hardness, and other physical and chemical parameters (Jernigan et al., 2017, Španić, 2023). The food industry uses wheat flour as a basic raw material for the production of many other products, such as bread, noodles, pasta, pastries, cookies, and couscous. However, grain quality is influenced by genetics (G), environment (E), and their interactions (G × E) (Jernigan et al., 2017). Among environmental factors, water availability has a major influence on grain protein. Drought during the reproductive stages of growth usually increases grain protein concentration (Xu et al., 2005). Consequently, the protein content of flour increases significantly (Ozturk and Aydin, 2004), mainly due to higher rates of nitrogen accumulation and lower rates of carbohydrate accumulation. Drought stress before flowering stage, and from flowering to grain filling stage has a serious impact on the quality of final grain in terms of protein content and bread-making efficiency (Yang et al., 2011; Zörb et al., 2017). A number of researchers have shown that protein content and sedimentation value, parameters related to gluten quality, increase in water-deficient environments (Saint Pierre et al., 2008a; Flagella et al., 2010,). The increase in grain protein is probably a reaction to a decrease in grain yield caused by drought.

Although researchers have documented the effects of drought stress on protein content and composition, glutenins swelling, and dough mixing traits, there is still a lack of information on how and to what extent drought stress impacts rheological parameters and bread baking quality. One reason for this is that researchers have mainly conducted drought experiments in greenhouses or plant growth chambers, limiting the quantities of ground material and flour available for the trials.

2.3. Morphological, physiological and hormonal responses of wheat to drought stress

2.3.1. Morphological response of wheat to drought stress

Under drought, various morphological changes occur in plants. The root system of plants is crucial for adapting to different types of abiotic stresses, especially drought, which adversely affects root depth (Wasaya et al., 2021). Morphological traits of the root can play an important role in the signal generation process, because stomatal closure during water shortage is a response to signals generated and transmitted by the root itself. Under drought stress, roots keep growing in search for water, while the growth of aerial parts like stems and leaves may be limited (Ahmad et al., 2018). Plants with longer root systems use water from deeper soil layers and thus contribute to a reduction of drought effects (Charney, 1975).

Drought also reduces plant height as a result of the reduction in photosynthetic efficiency and nutrient translocation during drought stress (Sarto et al., 2017). The reduction in plant height depends on the drought tolerance of the variety and the intensity of the drought stress. Plants with better drought tolerance tend to maintain a lower plant height to prevent moisture loss and reduce the need for moisture (Su et al., 2019). The number of leaves and the size of individual leaves also decrease under drought. Leaf expansion usually depends on turgor pressure, and under drought stress there is a decrease in turgor pressure and slower photosynthetic activity, which results in limited leaf expansion (Nonami, 1998). Photosynthetic capacity of the plant is under influence the size of leaf area and therefore may influence overall plant productivity (Ramya et al., 2016). Furthermore, drought stress reduces nitrogen uptake in plants which causes re-mobilization of nitrogen from leaves and stems into grain causing early leaf senescence (Hajibarat and Saidi, 2022).

Drought stress in the flowering stage reduces the viability of pollen, makes pollen sterile, which leads to spikelet sterility, that is unsuccessful fertilization (Varga et al., 2015; Rawtiya and Kazaly, 2021). Consequently, due to spikelet sterility, the number of grains

and their weight per spike decreased (Fahad et al., 2017; Sarto et al., 2017). Spike length was also found to decrease under drought (Mohammed and Kadhem, 2017). There are data previously obtained for grain weight per spike under drought that decreased by 15% (Poudel et al., 2020). Further, drought reduced nutrient uptake and photosynthesis, and accelerated early senescence and maturity resulting in a significant reduction of 1000 kernel weight (Rawtiya and Kazaly, 2021). Drought can reduce during phenological phases of wheat (Ahmad et al., 2022). To escape drought, wheat plants undergo some phenological phases faster (Chowdhury et al., 2021).

2.3.2. Physiological response of wheat to drought stress

Drought stress has a serious effect on grain yield through disruption of various physiological processes such as assimilation of nutrients, mobilization and accumulation of stable reserves, gametogenesis, fertilization, embryogenesis, endosperm development, and grain growth (Cakir, 2004). Furthermore, drought reduces relative water content (RWC) and chlorophyll content in leaves (Ahmad et al., 2022). Maghsoudi et al. (2016) reported that drought reduced RWC in leaves of different wheat cultivars by 33%. It also provoked plant growth retardation through reduction of cell extensibility and slowed down embryogenesis (Sharma et al., 2022).

To reduce water loss through transpiration plants close stomata (Pirasteh-Anosheh et al., 2016). On the other hand, the closure of the stomata leads to a reduction in the supply of CO₂ for photosynthesis and to a lower photosynthetic rate (Ahmad et al., 2018). Furthermore, plants have developed numerous mechanisms to control reactive oxygen species (ROS), which accumulate under drought conditions and have a toxic effect in plant cells (Hasanuzzaman et al., 2020). ROS found in the cell as radicals are hydroxyl radical (OH) and superoxide anion (O_2), while hydrogen peroxide (H_2O_2), and singlet oxygen (1O_2) are found in molecular form. Although radicals are considered as by-products and harmful to living cells, they play an important role in physiology, for example in the tolerance to abiotic and biotic stress (Liu and He, 2016). ROS have a double role: excessive formation causes oxidative damage, while low ROS accumulation acts as a signal to induce a response to abiotic stress according to the adaptation process. Strong ROS accumulation leads to oxidative damage to the membrane and oxidative destruction of the cell (Mittler, 2002), but also to various changes in the protein composition of the plant cell (Liu et al., 2015). Thus, in order to control the negative effect of ROS, plant cells have developed a series of detoxification mechanisms (Choudhury et al., 2017). One of the most important defence mechanisms against drought is the antioxidant system, which detoxifies

prooxidants such as ROS and lipid peroxyl radicals, and therefore plays an important role in plant growth and development by modulating various processes.

Antioxidant can be considered as any organic substance capable of neutralizing the negative effect of oxidation (Gupta, 2015). ROS directly or indirectly reacts with antioxidants, whose activity is directed towards scavenging of ROS (Kozlov et al., 2024). The antioxidant system of plant cells consists of non-enzymatic (ascorbate, reduced glutathione, tocopherol, carotenoids, flavonoids, alkaloids, and non-proteinogenic amino acids) and enzymatic antioxidants (superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), polyphenol oxidase (PPO)) (Hasanuzzaman et al., 2020). Drought tolerant plants tend to reduce water content, amino acid stability, and photosynthetic activity. On the other hand, they increase the proline and chlorophyll content and induce enzymatic and non-enzymatic components of antioxidant activities.

2.3.2.1. Changes of enzymatic and non-enzymatic system during drought stress

In order to maintain a balance between ROS scavenging and production, plants developed non-enzymatic and enzymatic antioxidant defence. Glutathione (GSH) is essential in maintaining the stability of the redox state in plant cells and is one of the most abundant and essential non-enzymatic antioxidants (Noctor and Foyer, 1998). It has been reported that GSH had the most important role in the antioxidant response to drought (Vuković et al., 2022). GSH is usually present in reduced form, and acts as a ROS scavenger, where it is oxidized into glutathione disulfide (GSSG) (Koek et al., 2011). Furthermore, as mentioned earlier, the non-enzymatic antioxidant defence system includes proline, ascorbate, tocopherols, phenolic compounds, and carotenoids. The increased accumulation of proline is one of the common phenomena in most cereals under drought (Marcińska et al., 2013). Drought stress resulted in increased proline content in wheat with reduced RWC and dry matter production (Tatar and Gevrek, 2008). Accumulated proline is osmotically active having an important role in membrane stability (Bandurska et al., 2008), and triggering many mechanisms that help adaptation to drought (Marcińska et al., 2013). In plants, there are two pathways for proline biosynthesis, the preferred one involving the conversion of glutamate to proline in two sequential reactions catalysed by enzymes $\Delta 1$ pyrroline-5-carboxylate synthetase (P5CS) and Δ 1-pyrroline-5-carboxylate reductase (P5CR) (Meena et al., 2019).

The ascorbate-glutathione (AsA-GSH) pathway comprises ascorbate (AsA) and glutathione (GSH), which serves to maintain cellular homeostasis and remove ROS

(Hasanuzzaman et al., 2020). The AsA-GSH cycle is essential in ROS detoxification and interacts with other defence systems to reduce oxidative damage caused by abiotic stress. Together with the two powerful antioxidants (GSH and AsA) four enzymes (ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR)) are involved in this cycle (Pandey et al., 2015; Hasanuzzaman et al., 2019).

Plants enhance the activity of peroxidases and glutathione to reduce oxidative damage caused by drought stress (Ahmad et al., 2018). Nikolaeva et al. (2010) reported that APX activity in wheat varieties varied depending on the development stage and the duration of the drought stress. Also, researchers recorded a higher activity of SOD, POD, and CAT in drought tolerant varieties, compared to drought sensitive varieties (Hasheminasab et al., 2012). SOD is one of the most important enzymes of the antioxidant system that catalyses the dismutation of the superoxide radical into molecular oxygen and hydrogen peroxide in the presence of a metal ion (Španić, 2016). The glutathione system enables the reduction of peroxidases and thus protects cell proteins and membranes from oxidation. The GR enzyme plays an important role under drought as an important part of the AsA-GSH cycle, protecting chloroplast from oxidative stress by maintaining a high GSH/GSSG ratio in the plant cell (Wang and Frei, 2011). Carotenoids are important nonenzymatic antioxidant molecules that remove free radicals, thereby protecting the components of the photosystem, while flavonoids have the potential to neutralize free radicals, thereby reducing cellular damage because of lipid peroxidation (Hasanuzzaman et al., 2020).

2.3.2.2. Changes in photosynthetic activity during drought stress

Photosynthesis is a universal process in the plant kingdom that takes place in various green organs, such as young stems (Nilsen, 1995), leaves (Smith et al., 1997), green fruits (Cipollini and Levey, 1991) and spikes before maturity (Kriedemann, 1966). Overall, photosynthesis is one of the most important processes during the growth and development of wheat plants, and therefore in the formation of grain yield. During the grain filling stage, photosynthetic efficiency gradually declines. It is characterized by the cessation of leaf growth, accelerated leaf aging and improper operation of photosynthetic compounds (Wahid et al., 2007). Since the photosynthesis of the flag leaf in wheat plays the greatest role in the assimilation of nutrients for grain filling, it is desirable that it remains photosynthetically active as long as possible.

Any stress affecting photosynthesis can make the efficiency of the maximum quantitative yield of photosystem II (PSII) very sensitive, making chlorophyll fluorescence (ChIF) usable as a rapid indicator for stress (Jansen et al., 2014). One of the most commonly used parameters derived from ChIF induction measurements that estimates PSII is the maximum quantum yield of primary photochemistry (TRo/ABS or Fv/Fm ratio) (Španić et al., 2023).

Photosynthesis is particularly sensitive to abiotic stress. Drought stress can limit the efficiency of photosynthesis because of damage to the thylakoid membrane and reduced chlorophyll *a* content (Tanveer et al., 2019). High temperatures that increase evapotranspiration often complement drought stress, causing reduced photosynthesis and ultimately reduced grain yield (Flexas et al., 2004; Mir et al., 2012). According to previous research, drought resulted in a decrease in the rate of photosynthesis (Dawood et al., 2019) by changing the internal structure of chloroplasts and mitochondria as well as the content of minerals and chlorophyll (Huseynova et al., 2016). Furthermore, in drought conditions, wheat varieties that maintain high TRo/ABS are considered drought tolerant and show effective protection of PSII activity (Zlatev, 2009). Also, there were significant changes recorded in the amount of chlorophyll *a* content in flag leaves. Drought sensitive varieties had greater losses in chlorophyll content, while tolerant varieties retained elevated amounts of chlorophyll (Khayatnezhad, 2011). Nikolaeva et al. (2010) reported that a decrease in chlorophyll content of 13-15% was recorded in the leaves of wheat varieties because of limited water supply.

Drought stress during flowering resulted in a significant drop in the chlorophyll content in the flag leaf and traits associated with a decrease in grain yield, such as the duration of grain filling, and number and 1000 kernel weight. (Talukder et al., 2014). The positive correlation between chlorophyll content and wheat grain yield was also reported in other studies (Talebi, 2011; Kumari et al., 2013). Stay green trait represents longer retention of green colour in photosynthetic tissues indicates delayed senescence of leaves, and this is another important indicator of tolerance to drought stress (Lopes and Reynolds, 2010). The colour of leaves or other tissues can be visually evaluated in that manner (Campos et al., 2004), but confusing results can occur if the studied varieties differ in the length of vegetation (Sanchez-Bragado et al., 2016). In breeding for drought tolerance stay green trait is desirable because it is associated with increased chlorophyll content.

2.4. Hormonal response of wheat to drought stress

Plant hormones are small molecules that have different roles in the regulation of growth, development, and reactions of plants to stress. Abiotic stresses, such as drought, have negative effects on plant growth, development, and survival. Adaptation and tolerance to abiotic stresses require sophisticated mechanisms, whereas plant hormones have significant role (Jiang and Asami, 2018). Many hormones such as auxin, gibberellin, cytokinin, abscisic acid, ethylene, brassinosteroids, jasmonic acid, salicylic acid, and strigolactones are known as plant hormones. Hormones act at the site of their biosynthesis or further away (Peleg et al., 2011; Rasool, 2022). Hormonal biosynthesis, distribution, and patterns of their signal transduction significantly change under stress conditions (Eyidogan et al., 2012).

One of the most important signalling phytohormones under drought are abscisic acid (ABA) and salicylic acid (SA). Reduction in available soil water triggers ABA hormone signalling (Sah et al., 2016) and reduces plant development (Tuteja, 2007). Under drought, wheat varieties differ in their ability to produce ABA, but also the sensitivity of varieties to ABA is different (Saradadevi et al., 2017). In that case, ABA controls plant growth by modifying development of leaves and roots of plants (Reddy et al., 2014; Farooq et al., 2014). Therefore, ABA signalling has a role both as a developmental signal transducer and as a stress regulator (Shinozaki et al., 2015). ABA signalling that occurs during drought stimulates excessive ROS production and causes an increase in ROS scavenging mechanisms, including increased CAT activity (Guajardo et al., 2016).

According to previous research carried under drought, ABA was accumulated in the apoplast of the leaf inducing the closure of the stomata, which prevented the loss of water inside the plants' cells (Saradadevi et al., 2017). Also, it is known that the accumulation of ABA results in a decrease in the influx of CO₂, which limits photosynthesis (Flexas et al., 2004). Overall, ABA had a positive effect on grain yield by affecting the redistribution of carbohydrates from the stem to the grain (Travaglia et al., 2007).

SA is another phytohormone and growth regulator that has a vital role in plant growth and development, and induction of flowering and respiration in many plants (Kang et al., 2012; Kumar, 2014). It regulates vital physiological processes of plants, such as water uptake and ion transport, transpiration, and photosynthesis (Klessig et al., 2018). According to previous research, SA, an endogenous signalling hormone, was produced in a very small amount. It activated several physiological and biochemical processes in response to biotic and abiotic stresses (Hafez, 2016). It was also involved in the regulation of the response to drought by strengthening the antioxidant capacity in plants (Saruhan, 2013).

2.5. Molecular response of wheat to drought stress

There are many important physiological traits that can mitigate the effects of drought stress in wheat. Genes controlling these physiological traits are very important because they are useful source for genetic improvement of drought tolerance. There are many molecular markers for important genes having a role in drought tolerance, such as *DREB* (Rasheed et al., 2016). Two most characterized transcription factors families involved in plant abiotic stress are *WRKY* and *AP2/EREBP* of the *DREB* group that regulates developmental, physiological, and metabolic processes (Javed et al., 2022). The transcription factor *WRKY* plays a key role in the control of numerous developmental processes in plants (Yu et al., 2023). According to a recent research, a total of 124 *WRKY* genes, including 294 homogeneous copies, have been identified in wheat (Ye et al., 2021). However, relatively few *WRKY* members involved in drought stress have been examined (Ge et al., 2024). Gao et al. (2018) reported that overexpression of *WRKY2* increased the wheat tolerance to drought and heat stress. Furthermore, it was confirmed that two wheat genes (*WRKY2* and *WRKY19*) had a role in modulating the response to drought stress (Niu et al., 2012).

DREB transcription factors have been reported to enhance drought tolerance in transgenic wheat (Saint Pierre et al., 2012). According to recent research, it was estimated that DREB1 was affected by low temperatures and DREB2 by drought and salt stresses (Khan et al., 2019). But, according to the research of Rustamova et al. (2021) carried under drought, the transcriptional level of DREB1 expression increased, especially in drought tolerant varieties. In another research, drought tolerant variety had higher P5CS gene expression, compared to sensitive variety (Wang et al., 2018; Nasirzadeh et al., 2021). This coincides with a recent study which showed that under drought wheat seedlings increased P5CS expression and proline accumulation, which were positively correlated (Vuković et al., 2022). Furthermore, overexpression of the P5CS gene in transgenic wheat with increased drought tolerance was also reported (Vendruscolo et al., 2007). The reasons for the increased level of P5CS gene expression under drought was the result of ABA accumulation (Bandurska et al., 2017).

Dehydrins are another group of stress proteins involved in the formation of plants' protective reactions to drought and osmotic stress. Although the function of dehydrins has not been clearly understood, many studies have demonstrated that dehydrins have
important roles in tolerance to abiotic stress including drought (Liu et al., 2015; Bao et al., 2017). Increasing dehydrin accumulation intensified stress tolerance of the plants (Porcel at al., 2005). Another dehydrin gene, WZY2, was identified as a drought stress responsive gene (Zhu et al., 2014). Further, Liu et al. (2020) reported that TabHLH49 positively regulated expression of WZY2 gene and improved drought tolerance of wheat. Also, overexpression of *DHN5* gene increased tolerance to osmotic stress in Arabidopsis plants due to regulation of proline metabolism and antioxidant response (Saibi et al., 2015). In wheat, drought stress highly induced the expression of genes encoding dehydrins (*DHN5* and *WZY2*) (Vuković et al., 2022). Similar findings were recorded in barley, showing that the relative expression of few *DHN* genes was greatly increased in drought tolerant varieties (Guo et al., 2009).

2.6. Progress in the production of drought tolerant varieties in wheat

Climate change will have a major impact on increasing drought consequence in the agriculture and will pose a risk to food security. Drought tolerance of variety is a very complex trait that is approached from several aspects. Careful consideration should be given to the growth stage at which varieties are tested under drought and methods can be specified for the development stage, for example, for germination and seedling, for tillering when lateral shoots are formed, or for the grain filling stage in which drought stress occurs most often in our region. Also, when testing varieties in the field, drought is often influenced by other factors such as the occurrence of diseases, pests, weeds etc. Therefore, it is preferable to conduct the tests in controlled conditions (plant growth chambers or greenhouses) so that only provoked stress would be present. However, there is a need to test wheat varieties both in controlled and field experiments in order to select promising varieties for targeted traits (Sallam et al., 2016).

The real challenge is the identification and selection of drought tolerant varieties. One of the first steps for crossbreeding is to select parental components based on their drought tolerance in combination with other agro-morphological traits. After selection, the varieties are crossed to incorporate as many genes for drought tolerance as possible into the offspring. Measurements of drought tolerance in the field are often influenced by various environmental effects, and for this very reason multi-year testing at multiple locations are necessary. Thus, the effectiveness of selection is affected by the variety x environment (VxE) interaction, especially if the environments are contrasting. Drought tolerance is usually a trait with low heritability. To overcome low heritability, breeders have integrated

DNA molecular markers into their programs with good results in increased drought tolerance (Arifuzzaman et al., 2014). Recently, advances in DNA sequencing have enabled new techniques for genotyping by producing high-density single nucleotide polymorphism (SNP) markers (Eltaher et al., 2018). Furthermore, new technologies such as genomic selection and gene editing can be used to improve drought tolerance in wheat (Singh and Singh, 2015). Identification of genes encoding enzymatic activities under drought stress in wheat is very important in breeding programs. Also, genetically modified plants with overexpression of the MDHAR and DHAR genes had a higher tolerance to abiotic stress (Wang and Frei, 2011).

3. RESULTS AND DISCUSSION

3.1. Review of published qualification papers

3.1.1. Morpho-physiological and hormonal response of winter wheat varieties to drought stress at stem elongation and anthesis stages

Drought stress can significantly reduce wheat growth and development as well as grain yield. This study investigated morpho-physiological and hormonal (abscisic (ABA) and salicylic (SA) acids) responses of six winter wheat varieties during stem elongation and anthesis stage as well grain yield-related traits were measured after harvest. To examine drought response, plants were exposed to moderate non-lethal drought stress by withholding watering for 45 and 65% of the volumetric soil moisture content (VSMC) for 14 days at separate experiments for each of those two growth stages. During the stem elongation phase, ABA was increased, confirming the stress status of plants, and SA showed a tendency to increase, suggesting their role as stress hormones in the regulation of stress response, such as the increase in the number of leaves and tillers in drought stress conditions, and further keeping turgor pressure and osmotic adjustment in leaves. At the anthesis stage, heavier drought stress resulted in ABA accumulation in flag leaves that generated an integrated response of maturation, where ABA was not positively correlated with any of investigated traits. After harvest, the variety Bubnjar, followed by Pepeljuga and Anđelka, did not significantly decrease the number of grains per ear and 1000 kernel weight (except Andelka) in drought treatments, thus, declaring them more tolerant to drought. On the other hand, Rujana, Fifi, and particularly Silvija experienced the highest reduction in grain yield-related traits, considering them drought-sensitive varieties.

Keywords: abscisic acid; drought stress; salicylic acid; winter wheat

3.1.2. Analysis of the photosynthetic parameters, grain yield, and quality of different winter wheat varieties over a two-year period

Due to increasingly frequent unfavourable climate changes, achieving a high grain yield of wheat is a challenge for breeders. The relationships between wheat productivity and photosynthesis traits are not very well understood during the growing season. This study investigated the effect of chlorophyll a fluorescence parameter (maximum quantum yield of primary photochemistry (TR₀/ABS) and performance index on absorption basis (Pl_{ABS})) on grain yield and the yield-related and technological guality traits of six wheat varieties over two growing seasons. In the first growing season (2021/2022), grain yield was significantly positively correlated with 1000 kernel weight and TR₀/ABS at the second measurement point (growth stage 25 (GS 25)). Only the highest-yielding variety Bubnjar (104.0 dt ha⁻¹) showed values of TR_0/ABS at the same significance level between the second and third measurement points. Due to elevated virus and disease infections in the second growing season (2022/2023), the grain yield of the investigated varieties decreased between 37.9% (Bubnjar) and 67.6% (Anđelka) relative to the first growing season. The three highest-yielding varieties (Bubnjar, Rujana, and Silvija) in 2022/2023 were the tallest, were later in maturity, escaped yellow rust pressure at the stem elongation stage more efficiently, and also showed the lowest increase in TR₀/ABS at this stage (fourth measurement point at GS 47, compared to the third at GS 32). In addition, the highestyielding variety Bubnjar showed the highest increase in PIABS at the last measurement (seventh) at GS 71 compared to the sixth (GS 69), thus maintaining the vitality of flag leaves at the grain-filling stage, while the other varieties showed a very small increase or even a significant decrease. Therefore, plant photosynthetic activity over the entire growing season contributes to crop productivity.

Keywords: grain yield; photosynthesis; quality traits; wheat

3.1.3. Effects of drought at anthesis on flag leaf physiology and gene expression in diverse wheat (*Triticum aestivum* L.) genotypes

The current study aimed to quantify the effects of two drought intensities achieved by deprivation of watering for 45 and 65% of the volumetric soil moisture content (VSMC) for 14 days after wheat anthesis to identify physio-biochemical and molecular changes associated with drought tolerance in six genotypes with different drought tolerance. Drought at 65% of VSMC induced a significant decrease in the chlorophyll a content in the droughtsensitive genotype, which indicated a strong loss of photosynthetic reaction centres. Further, in the drought-tolerant genotype, the content of carotenoids, which could play a vital role in resisting water shortage stress, tended to increase. The increased production of malondialdehyde showed that the antioxidant system in the drought-sensitive genotypes was not properly activated. A significant decrease in catalase (CAT; EC 1.11.1.6) was observed at a 45% reduction in VSMC, compared to the control, in the drought-sensitive genotype, and at a reduction in VSMC of 65%, in all medium sensitive genotypes. Further, the drought-tolerant and -medium tolerant genotypes responded to drought with a decline in total glutathione concentrations with the intention to reinforce their defence system. Thereby, dehydroascorbate reductase (DHAR; EC 1.8.5.1), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), and glutathione reductase (GR; EC 1.6.4.2) were critical enzymes involved in the ascorbate-glutathione cycle together with CAT, showing their main role in the detoxification of ROS produced with the reduction in VSMC by 65%. The results of gene expression analysis showed that severe drought increased the levels of the DHN5 and WZY2 genes (that were significantly positively correlated) in the drought-tolerant genotype, whose grain weight, area, and length did not change in maturity. Also, it was seen that DHN5 expression showed a significant positive correlation with grain length and proline content at a 45% reduction in VSMC. The identification of different mechanisms under drought can contribute to the selection of drought-tolerant genotypes.

Keywords: drought; molecular response; physiology; wheat

3.2. Unified discussion

Climate change scenarios predict increasing irregular rainfall, which will lead to drought stress at some point in the wheat growth cycle. It is known that drought stress in wheat impairs plant growth, development, and grain productivity. Since drought stress severely limits the productivity of wheat worldwide, the research work began with the preparation and construction of experiments. The experiment was set up under field conditions over two growing seasons, but as field experiments involve a number of stresses, the experiment was also set up under controlled conditions in the greenhouse. To investigate the impacts on morphological, physiological, developmental and molecular adaptations, an experiment was conducted with two different intensities of drought stress in winter wheat at the anthesis stage.

3.2.1. Morphological response of wheat to drought stress

Morphological traits (such as plant height and number of tillers) are considered potential indicators for indirect selection of drought tolerant wheat varieties (Liu et al., 2015). Singh et al. (1973) reported that leaf development in wheat was more susceptible to water stress than root development. In Article 1 (3.1.1), the research results showed the effects of drought stress on different morphological traits of wheat. After simulating two intensities of drought in duration of 14 days after anthesis, the following parameters were measured: number of leaves per plant, fertile tillers per plant, flag leaf width and length, relative water content (RWC) of flag leaf, stem height, and plant height. In this Article, a significant increase in the number of leaves per plant was observed in varieties Andelka and Silvija under both drought intensities, compared to the control. When counting the number of fertile tillers after drought stress at the anthesis stage (Article 1), it was found that only Andelka had a significant increase in the number of fertile tillers per plant. The shape and size of leaves can also contribute to drought tolerance (Rijal et al., 2021). In addition, the number of leaves per plant, leaf size, and leaf longevity can be reduced under drought stress (Shao et al., 2008). In Article 1 (3.1.1), flag leaf size (width and length) did not significantly change or there was slight tendency of increase under drought. In two varieties (Rujana and Bubnjar) a significant decrease in plant height was observed due to severe drought stress. This is consistent with the research of Qaseem et al. (2019) which found the plant height of wheat decreased due to drought. This can be explained by the fact that cell elongation was disrupted by drought, which affected the growth and overall height. In Article 1 (3.1.1), a drought induced reduction in plant height of 10% on average was observed in all varieties.

This is consistent with the research of Caverzan et al. (2016) who reported that the drought induced plant height reduction at the grain filling by 7%.

According to research of Nayyar et al. (2004) drought tolerant plants maintained a lower plant height and a lower plant area index in order to reduce moisture demand and avoid moisture loss through transpiration. In Article 1 (3.1.1), the variety Bubnjar responded to drought stress with significantly shorter stems and decreased plant height, suggesting that Bubnjar is more tolerant to drought than the other tested varieties.

Further, leaf water potential is considered a reliable parameter for quantifying the response of plants to drought stress, but Sinclair and Ludlow, (1985) suggested that RWC is a better indicator of water status than water potential. In addition, RWC has been utilized for the selection of drought tolerant varieties in wheat (Bayoumi et al. 2008). In Article 1 (3.1.1), RWC was significantly reduced in the flag leaves of all varieties except Silvija and Fifi, indicating higher water loss under drought. Similarly, Karimpour, (2019) reported that tested varieties under drought conditions had a significantly lower RWC in flag leaves. Also, a decrease in RWC in plants under drought stress may be related to a reduction in plant vigour and has been observed in many plants (Liu et al., 2002).

3.2.2. Hormonal response of wheat to drought stress

In Article 1 (3.1.1), the aim was to determine the concentration of stress hormones (ABA and SA) and to investigate the mechanisms underlying the correlation between two endogenous levels of ABA and SA at the anthesis stage under two intensities of drought stress. All varieties showed a tendency for an ABA increase under mild drought stress, with a significant increase in severe drought (except in Pepeljuga). Pepeljuga was the only variety in which ABA concentration did not change significantly between drought and control treatments, but it was also the only variety that showed increased SA concentration under mild drought stress. Stress hormones such as ABA and SA are among the most important signalling molecules that regulate adaptive responses to various abiotic stresses. In addition, a reduction in available water triggers the signalling of the hormone ABA and impacts plant growth (Tuteja, 2007; Sah et al., 2016). The application of exogenous ABA under water stress accelerates the accumulation of osmolytes and improves the water status of plants, resulting in higher grain weight in drought sensitive wheat varieties (Nayyar et al., 2004). Also, ABA controls plant growth by refining root development and modifying leaf elongation and expansion during drought conditions (Farooq et al. 2014). In addition to ABA level, ABA sensitivity to drought is also an important trait for plant survival under stress conditions. Experiments with Arabidopsis and wheat showed that plants with a high drought tolerance had a significantly higher ABA sensitivity than drought sensitive varieties (Kurahashi et al., 2009; Li et al., 2018). As mentioned in Article 1 (3.1.1), ABA under drought stress showed significant changes under severe drought, and accordingly, our data suggested that ABA is a good stress marker in all wheat varieties under drought.

SA, as a phytohormone, is a promising compound for increasing plant tolerance to drought and can influence many aspects of physiological and biochemical processes. Also, SA has great potential to improve the photosynthesis rate and chlorophyll content in wheat. The role of SA may be even more controversial than that of ABA under abiotic stress, as some researchers have reported that the application of SA has a beneficial effect in protecting plants from oxidative damage caused by drought stress (Wang et al., 2019; Sankari et al., 2019). Article 1 (3.1.1) showed that all varieties exhibited a slight increase or no significant change in SA concentration under both drought conditions at the anthesis stage. In addition, SA is a phenolic acid with proven antioxidant activity and its presence in the plant may also be positive as a ROS scavenger. Similarly, SA maintained the integrity of the cell membrane and enhanced ROS scavenger activity, such as CAT (Khalvandi et al., 2021).

3.2.3. Analysis of grain yield data under drought stress

According to previous research, drought stress during grain filling was the most damaging to yield, as grain development was impaired by unbalanced levels of growth hormones (Abid et al., 2017). Grain yield in wheat is a polygenic trait that is influenced by interactions between environment and variety throughout all stages of plant growth. Also, grain yield is the ultimate product of photosynthesis and is closely linked to physiological processes. Grain yield is usually divided into three components: number of spikes per area, grain number per spike, and 1000 kernel weight (Simmonds et al., 2014). Furthermore, previous research reported that the period of seven to ten days before anthesis and five days after anthesis was the most critical period for the reproductive development (Sato et al., 2002). Drought stress at and shortly after the anthesis stage was most detrimental to wheat grain size (Sangtarash et al., 2010), which coincides with the period of drought stress applied in Article 1 (3.1.1).

In Article 1 (3.1.1), as mentioned above, drought stress was simulated at the anthesis stage for two weeks, after which the effects of drought stress on the number of spikelets per spike, grain number per spike, and 1000 kernel weight were analysed. All varieties showed a tendency toward reduced grain number per spike and 1000 kernel weight under drought stress, although the changes were not statistically significant in all varieties and

drought intensities. One of the varieties in which the number of grains per spike did not change significantly, compared to control, was Bubnjar, which was previously declared as a drought tolerant variety. Also, 1000 kernel weight was significantly reduced in four out of six tested varieties, but a slight, non-significant reduction was observed in all varieties under severe drought stress, compared to the control. This partially aligns with the research (Ji et al., 2010), which reported that under drought stress, average kernel weight was significantly reduced in all tested varieties compared to the controls, or that drought during the anthesis stage mainly caused a reduction in grain size (Ji et al., 2010). During reproductive development, drought stress reduced grain number in the spike of wheat (Dong et al., 2017), with premature flower death resulting in a reduced number of potential grains in the spike (Dolferus et al., 2011). The occurrence of drought during anthesis affected the number of grains per spikelet and the total number of grains per spike (Sangtarash et al., 2010), which was also demonstrated in Article 1 (3.1.1). Further, Khalili et al. (2013) reported that increased drought intensity significantly reduced grain yield and harvest index of maize.

In Article 2 (3.1.2) part of the analysed data included the grain yield data during two growing seasons. The accumulated total rainfall in the first growing season (2021/2022) was 440.8 mm and 564.2 mm in the second growing season (2022/2023). The first growing season had a few dry months (from January to April), but despite this, good grain yields were recorded. Wheat plants were probably able to absorb water from the soil reserves accumulated in the period from October to December of the first growing season. In a recent study, it was reported that an adequate level of soil water storage in the early stage of plant growth can ensure the promotion of tillering in winter wheat and eventually increase the effective number of tillers to increase grain yield (Li Chao et al., 2017). This could be particularly true for the variety Bubnjar, which was previously declared as drought tolerant, as well as for Rujana. These two varieties showed higher grain yields than the other four varieties in the first growing season. Therefore, the observed differences in grain yield between the six varieties could to some extent be the result of their different tolerance to drought.

In contrast to the first season, a larger amount of rainfall was recorded in the second growing season, especially through April and May in 2023. Although rainfall increased, grain yield decreased drastically in all varieties, compared to the grain yield measured in the first growing season, mainly due to a strong increase in leaf and spike diseases combined with a high incidence of aphids in the autumn, which are vectors of barley yellow dwarf virus that weakened the plants already in the autumn. In the second growing season, disease development was also favoured by large amounts of rainfall through April and May

in 2023. In addition, high infestation pressure from yellow or stripe rust (*Puccinia striiformis*) was observed from April to May.

Article 2 (3.1.2), showed that the highest yielding varieties were the latest in maturity, as the heading date was significantly positively correlated with plant height, which in turn was positively correlated with grain yield. Heading date is of crucial importance as this is the stage before flowering, which can be delayed and result in accelerated reproductive development, resulting in lower grain filling. Španić et al. (2016) reported a negative correlation between plant height and grain yield in very tall varieties used in the past. In contrast, in Article 2 (3.1.2) plant height and grain yield were significantly positively correlated, and it was observed that the two highest yielding varieties were also the tallest, which is consistent with the research of Mahdy et al. (2022). Furthermore, the correlation matrix showed that grain yield was significantly positively correlated with 1000 kernel weight and plant height, which is in accordance with previous studies (Sharma et al., 2008: Mohammadi et al., 2012).

3.2.4. Grain quality under drought stress

Maintaining the grain quality of wheat under adverse climate change conditions is essential for human nutrition, as well as for functional properties for the end consumer and commodity value. In Article 2 (3.1.2), four quality parameters (protein content, sedimentation value, wet gluten content, and Hagberg falling number) were analysed after harvest. Grain protein is one of the most important parameters of wheat, while sedimentation value is a composite assessment of both the quantity and quality of wheat protein. This means that a high sedimentation value indicates high protein content and high protein quality in the wheat flour. The wet gluten content is very important for baking quality. Elasticity is a key property of gluten that provides structural strength to dough. High-quality dough must be sufficiently stretchy so that baked goods have the right volume and texture. Further, the Hagberg falling number is used to measure the α -amylase activity in wheat flour to determine the suitability of the wheat for bread making. Higher values are of course desirable and indicate higher quality. The lowest protein content was obtained in Bubnjar which was expected as this variety was the highest yielding and it is well known that higher grain yields are associated with lower protein concentration (Jablonskyte-Rašče et al., 2013). It is difficult to achieve high grain yield and high protein content at the same time as there is a negative correlation between these two traits (Saint Pierre et al., 2008b; Bogard et al., 2012; Geyer et al., 2022). Further, in Article 2 (3.1.2), protein content and wet gluten content were significantly positively correlated, which is in accordance with the previous

research of Kaushik et al. (2015), as the gluten protein complex is derived from the storage proteins of wheat grain.

In Article 2 (3.1.2), it was found that the sedimentation value was significantly positively correlated with the Hagberg falling number. In the study by Laidig et al. (2017), strong correlations were found between protein content, sedimentation value and bread loaf volume.

3.2.5. Analysis of the photosynthetic parameters under drought stress

In Article 2 (3.1.2), the effects of different weather conditions on the main photosynthetic parameters and the effects of photosynthesis on grain yield and quality of winter wheat were investigated. Maximum quantum yield of primary photochemistry (TRo/ABS) and performance index (PI_{ABS}) parameters showed differences in the flag leaves during seven measurement points in the two growing seasons.

In the first growing season (2021/2022), PCA showed that grain yield was significantly positively correlated with 1000 kernel weight, which is consistent with previous studies (Sharma et al., 2008; Mohammadi et al., 2012; Nofouzi et al., 2018). In addition, grain yield was significantly positively correlated with TRo/ABS at the second measurement, which coincides with the tillering stage. It is important to emphasise that at this stage, plants begin to form lateral tillers, which are very important for wheat productivity (Tilley et al., 2019). It is also important to note that the tillers that are first formed on the plant always have an advantage in growth and development over those formed later (Tilley et al., 2019). According to previous research, improved photosynthesis, even at the level of a single leaf, can increase plant yields (Makino, 2011).

Previously, it was found that more drought tolerant wheat varieties can preserve water content in the photosynthetic tissues, represented in our case by the leaves, where water losses through evapotranspiration may be less pronounced (Izanloo et al., 2008). Bubnjar, previously described as the drought tolerant variety, is also the only variety where TR_O/ABS and PI_{ABS} remained at the same level during the second and third measurements (tillering and stem elongation stage). We can assume that Bubnjar had lower photosynthetic activity than the other varieties during this phase, which allowed more productive tillering. This means that all wheat varieties except Bubnjar accelerated photosynthesis during tillering and probably went through this stage faster than Bubnjar.

According to research of Tilley et al. (2019) tillers with a larger leaf area will produce more and heavier kernels. Accordingly, Bubnjar had the highest 1000 kernel weight in Article 2 (3.1.2). The 1000 kernel weight correlated strongly with the values of TR_0 /ABS at the last (7th) measurement, which coincided with the grain filling stage. Zhang et al. (2021) suggested that increased photosynthesis of flag leaves improves 1000 kernel weight and thus contributes to high grain yield.

In contrast to the first growing season, there was a severe disease infestation in the second growing season (2022/2023), which severely damaged wheat leaves. Accordingly, high infestation pressure from yellow rust was observed from April 2023. It was found that yellow rust can lead to a reduction in grain yield of more than 25% (Wellings, 2011). The presence of any type of stress can inactivate or damage photosystem II (PSII), leading to a decrease in TR₀/ABS (Spanic et al., 2021). In addition, drought stress during the flowering stage can have a negative effect on the net photosynthetic rate and will reduce the photosynthetic time and considerably increase the senescence of the flag leaves (Wu et al., 2014). At the fourth measurement (stem elongation stage where severe yellow rust infestation occurred), TRo/ABS was significantly negatively correlated with the grain yield, suggesting that lower TR₀/ABS at this stage resulted in higher grain yields. It is likely that the varieties that maintained TR₀/ABS at lower rates during this period conserved energy for the generative stage of development.

In addition, TR_o/ABS at the fourth measurement showed a negative correlation with heading dates, meaning that later maturing varieties may have escaped virus/disease pressure more efficiently than varieties that were earlier in maturity. Further, the varieties with the highest grain yield (Bubnjar and Rujana) showed an increase in Pl_{ABS} between the sixth and seventh measurements which coincided with the anthesis and grain development stages. Photosynthetic efficiency is especially important during flowering and early grain filling stages as its reduction at these stages can lead to spikelet sterility and lower grain yield (Spanic et al., 2021). Moreover, it has been reported that 70% of grain yield is produced by photosynthesis in the leaf and spike tissues after the heading stage (Mu et al., 2010).

In the Article 2 (3.1.2), biotic stress as a result of virus and disease attacks occurred much earlier than the heading stage began. Varieties with better photosynthetic efficiency were better able to store energy for future growth and development.

3.2.5.1. Pigments involved in photosynthesis during drought stress

Photosynthates and assimilates, organic compounds produced during photosynthesis and nutrient assimilation, must be transported to places of use or storage. That means they are transported to the developing grain from the flag leaf and from the reserves before anthesis in tissues such as the stem and the spike. The typical source organ is the leaf which produces more photosynthates than it needs for its own growth and metabolism. Further, the chlorophyll content in the flag leaf of plants is highest at the beginning of the flowering phase (Simova-Stoilova et al., 2009). According to the research of Fotovat et al. (2007), drought stress in wheat significantly reduced leaf chlorophyll content, but Ahmad et al. (2018) reported that variations in chlorophyll content were not only caused by drought stress but also existed between varieties. Article 3 (3.1.3) showed that Chl a, Chl b and Chl a+b tended to increase in the flag leaves of most wheat varieties under mild drought stress, while some varieties showed no significant changes. In contrast, under severe drought stress, all wheat varieties showed the same significant levels of chlorophylls as under control conditions, except for Silvija (previously declared as the most sensitive variety), which showed a significantly reduced Chl a content. According to previous research, drought reduced chlorophyll content and photosynthesis in the leaf of wheat (Prasad et al., 2011), i.e. the content of Chl a, Chl b, total Chl, and carotenoids (Chakraborty and Pradhan, 2012). Only Bubnjar (variety declared as drought tolerant) showed a significantly increased content of carotenoids even under mild drought conditions. Carotenoids protect photosystems by reacting with lipid peroxidation products and scavenging singlet oxygen (Anjum et al., 2017). It has been reported that carotenoids together with ascorbate, glutathione (GSH), and α -tocopherol, are good indicators of drought tolerance (Ahmed et al., 2020). The loss of chlorophyll content may be the first sign of photosynthesis inactivation. In Article 3 (3.1.3) various metabolic processes were impaired under severe drought, resulting in a significant reduction of chlorophyll content in drought sensitive variety (Silvija). The varieties that showed increased chlorophyll content under drought could lead to the conclusion that they have switched on the ROS scavenging system to some extent.

3.2.6. Physiological response of wheat to drought stress

The physiological response of wheat to drought stress depended on the variety and severity of drought stress, with more pronounced effects in severe drought stress. The antioxidant defence system ensures a balance between the production and degradation of ROS in plant cells (Dumanović et al., 2021). Increased ROS levels at the cellular level

influenced protein degradation, inhibition of enzymes, oxidative damage to DNA and RNA, and lipid peroxidation in membranes, leading to cell death (Checa et al., 2020). According to previous research, the main ROS detoxification process was under the influence of enzymes, including APX, GR, DHAR and MDHAR, which are involved in AsA-GSH metabolism (Foyer and Noctor, 2011). In Article 3 (3.1.3), the antioxidant status in wheat flag leaves was determined by measuring the glutathione content (tGSH and GSSG) and the activities of antioxidant enzymes and enzymes of AsA-GSH pathway (CAT, APX, GST, MDHAR, DHAR, and GR). The changes in enzyme activities were dependent on the variety and the intensity of drought stress. According to Devi et al., (2012) a variety was probably drought tolerant if the activity of the specified enzyme was upregulated in certain tissues under drought.

In Article 3 (3.1.3), a significant reduction in CAT activity was observed in drought sensitive and medium sensitive varieties, especially under severe drought stress. Similar results were obtained by Chakraborty and Pradhan (2012), who reported that CAT and SOD activity decreased during drought periods in more drought sensitive wheat varieties. Under stress conditions, a strong increase in CAT activity in leaves can protect chloroplasts, which are the main generators and targets of ROS (Sofo et al., 2005). Thus, the stability of CAT activity in leaves is probably responsible for the elimination of photorespiratory hydrogen peroxide (H_2O_2).

The activity of the enzymes of the AsA-GSH scavenging pathway (APX and GR) was increased under drought (Khalegi et al., 2019). APX is an integral component of the AsA-GSH cycle, which scavenges superoxide radicals and H₂O₂ in the chloroplast under drought conditions (Keles and Oncel, 2002). According to D'Arcy-Lameta et al. (2006), transcript levels of cytosolic and peroxisomal APX genes were increased in a drought sensitive variety. Similarly, in Article 3 (3.1.3), Silvija increased APX activity in flag leaves in an attempt to detoxify ROS during severe drought. Also, when wheat plants were exposed to mild drought, leaf APX activity increased, while prolonged water deficit decreased APX activity due to increased production of malondialdehyde (MDA) (Nikolaeva et al. 2010). In addition, drought sensitive and medium sensitive varieties showed significantly reduced GR activity during severe drought stress, which did not contribute to the detoxification of ROS. Due to the reduced GR activity in these varieties, GSH could not be sufficiently recycled, as shown by the significant increase in tGSH content in drought sensitive and medium sensitive varieties under both drought intensities. In contrast, the drought tolerant variety (Bubnjar) showed no significant differences in tGSH content under control and both drought intensities, which minimised the formation of ROS. It was previously observed that varieties

with different drought tolerance showed a decrease in tGSH and a higher GSH/GSSG ratio after one month of drought (Loggini et al., 1999).

However, in Article 3 (3.1.3) it was found that an increase of tGSH content was only observed in drought sensitive and medium sensitive varieties. In sensitive and medium sensitive varieties, tGSH content significantly increased later than in tolerant and medium tolerant varieties, in which antioxidant function may have been enhanced earlier to resist drought. It can be assumed that drought sensitive and medium sensitive varieties were still unable to defend themselves against oxidative stress after two weeks of drought, and therefore tGSH content remained elevated.

Previous study on the role of GST in drought stress is relatively inconsistent (Vuković et al., 2022). Our research (Article 3) also failed to provide a clear picture of the role of GST in the drought tolerance of wheat varieties. However, one research found that an increase of GST could reduce the accumulation of H_2O_2 and MDA and contribute to the maintenance of the GSH/GSSG ratio under salt stress (Rajput et al., 2021). Also, Galle (2011) reported that GST activity was induced by osmotic stress in moderately drought tolerant wheat varieties.

In Article 3 (3.1.3), the drought tolerant and medium tolerant varieties showed no significant changes in MDHAR activity under both drought treatments, compared to the control, while the drought sensitive and medium sensitive varieties showed a tendency for significant MDHAR reduction. Similarly, the research of Shokat et al. (2020) showed that increased MDHAR activity in the leaves of wheat under drought was a predictive biomarker for increased grain number. In addition, increased ascorbate levels in transgenic rice plants were previously found as a result of increased MDHAR and DHAR activities (Kim et al., 2022). DHAR is involved in catalysing the reduction of DHAR using reduced GSH, resulting in ascorbic acid and GSSG, thus maintaining an ascorbate redox state (Rajput et al., 2021). In Article 3 (3.1.3), drought tolerant and medium tolerant varieties showed no significant changes in DHAR between any of the two drought treatments and control.

MDA is a naturally occurring product of lipid peroxidation (Zhao et al., 2020b), and the MDA content in plants is often used as a parameter to evaluate the damage to plant cells due to stress (Porcel et al., 2004). According to research of Wang et al. (2022), MDA content increased rapidly under drought, with significantly higher levels observed in the drought sensitive variety, compared to the drought tolerant variety. Similarly, in Article 3 (3.1.3), drought sensitive and medium sensitive varieties showed a significant increase in MDA levels under severe drought stress, in contrast to drought tolerant and medium tolerant varieties. Similar results were obtained by Sultan et al. (2012), who reported that drought tolerant varieties had significantly increased proline and RWC content, while MDA content decreased under drought. Moreover, plants with lower MDA content under drought are generally considered to be more drought tolerant (Ma et al., 2015). Accordingly, in Article 3 (3.1.3), MDA content was reduced or significantly unchanged under both drought conditions in drought tolerant and medium tolerant varieties, suggesting a stronger antioxidant response in these varieties.

Proline content has also been recognized as a valuable indicator of drought tolerance and could be used as a selection criterion in creating drought tolerant varieties (Mwadzingeni et al., 2016). Previous studies indicated that proline is among the major biochemicals that accumulate significantly in plants when exposed to different types of stress (Hong-Bo et al., 2006; Khamssi, 2014). Increased proline accumulation was found in drought tolerant varieties under drought, compared to drought sensitive varieties (Maghsoudi et al., 2018). In contrast, some researches showed that proline is associated with drought sensitivity (Chun et al., 2018; Zhang et al., 2022). In Article 3 (3.1.3), all varieties showed significantly increased proline content in severe drought and four varieties in mild drought. Similarly, Johari-Pireivatlou (2018) reported that the accumulation of proline in wheat was increased by drought stress. According to Khaleghi et al. (2019), the increased proline content in plants under severe drought conditions could play an important role in recovery after stress.

3.2.7. Gene expression in wheat under drought stress

Plants use different adaptation and acclimatization strategies under drought stress, ranging from changes in morphological or physiological traits or changes in genes' expression in which a large number of transcription factors are induced. Therefore, in Article 3 (3.1.3), the expression patterns of the drought-responsive gene *P5CS*, genes encoding dehydrins (*DHN5* and *WZY2*), and genes encoding transcription factors (*DREB1* and *DREB2*) were analysed to determine the expression level associated with drought tolerance of wheat varieties. The increase in relative expression of the analysed stress-responsive genes *WZY2* and *DHN5* was upregulated under drought conditions. According to previous researches, *DHN* genes are important in regulation of abiotic stress tolerance (Xie et al., 2012; Liu et al., 2015; Bao et al., 2017). *TaDHN* genes responded strongly to stress conditions such as drought (Hao et al., 2022). Accordingly, Article 3 (3.1.3) showed that the most drought tolerant variety (Bubnjar) strongly overexpressed *DHN5* and *WZY2* genes under severe drought, compared to all other varieties. Dehydrin gene *WZY2*, whose relative expression was increased in most plants under abiotic stresses such as drought,

was identified as a drought stress-responsive gene (Huang FaPing et al., 2009; Zhu et al., 2012), which improves drought stress tolerance in wheat (Vuković et al., 2022).

In Article 3 (3.1.3), the expression of the *P5CS* gene had a tendency to increase under severe drought in all tested varieties, but significantly in Silvija, Fifi, and Anđelka. Further, only Rujana showed a significant increase in *P5CS* under mild drought conditions, together with an increase in proline content. In addition, *P5CS* was found to have a significant positive correlation with proline content under mild drought conditions. Similarly, it was previously reported that increased P5CS enzyme activity strongly correlated with proline accumulation (Maghsoudi et al., 2018), ultimately leading to increased stress tolerance (Vendruscolo et al., 2007).

Genes from the DREB family are involved in the response of plants to dehydration and may play a crucial role in the response to abiotic stress (Hou et al., 2023). According to Liu et al., (1998), two major subgroups of the DREB subfamily include the DREB1 and DREB2 genes. The role of DREB1 transcription factors is evident from their up-regulation in response to drought stress (Shen et al., 2003). In contrast, Yousfi et al. (2016) reported that drought sensitive varieties increased the expression of DREB1A, compared to drought tolerant varieties, suggesting that the expression of this gene is not related to drought tolerance. Similar findings were also observed in Article 3 (3.1.3), where Rujana was the only variety in which DREB1 expression increased significantly under mild drought. Due to the significant expression of DREB1 in only one variety, it is not possible to draw a reasonable conclusion about the role of this gene, although DREB1 showed a negative correlation with grain width and area under severe drought stress. According to Yousfi et al., (2016), the expression of DREB2B under drought conditions was similar to or lower than in control plants, suggesting that this gene does not play a role in drought tolerance. Reduction of some expressed genes may simply reflect impairment in the normal metabolic function of a plant. Also, in Article 3 (3.1.3), only the drought tolerant variety (Bubnjar) showed significantly reduced DREB2 expression under mild drought. Accordingly, the expression of DREB2 could lead to the conclusion that the expression levels could serve as an indicator of the degree of stress in relation to plant metabolism.

4. CONCULUSIONS

The results of this doctoral thesis provide scientific evidence of the negative effects of drought stress on wheat production and how drought stress affects different wheat varieties. The aim of the work was to determine the morphological and physiological response of winter wheat varieties at two intensities of drought stress at the anthesis stage, and to determine the level of expression of genes related to drought tolerance in winter wheat varieties. The following conclusions were formulated on this basis:

1. Hypothesis 1 states that morphological and physiological response to drought stress will involve different mechanisms in different wheat varieties and will depend on the intensity of stress. The research carried out confirm the above hypothesis. It was observed that morphological and physiological changes during drought stress were observed depending on the varieties and stress intensity. For example, correlation analysis and principal component analysis (PCA) showed that varieties declared as drought tolerant and medium tolerant, showed the least changes in morpho-physiological traits under drought stress conditions.

- The expression of the antioxidant enzymes ascorbate peroxidase (APX) and glutathione S-transferase (GST) under drought was variety-specific and depended on the intensity of drought stress. In addition to catalase (CAT), the enzymes of the ascorbate glutathione (AsA-GSH) cycle (glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR)) also function as important components of the antioxidative defence system under severe drought.
- All tested varieties showed a tendency to reduce the number of grains per ear as well as 1000 kernel weight under both drought stress conditions, although the changes were not statistically significant in all cases. For example, the varieties declared as drought tolerant and medium tolerant did not significantly change the number of grains per ear compared to the control plants.
- Wheat varieties differed in their ability to produce abscisic acid (ABA) under both drought stresses, whereas drought tolerance is variety specific.

2. Hypothesis 2 states that the level of expression of the gene encoding the transcription factor *DREB* will be more strongly associated with the tolerance of varieties to drought stress, compared to the level of expression of other analysed genes. In contrast, this doctoral thesis concluded that the expression levels of the genes *DHN*5 and *WZY2* under drought stress were more strongly associated with the resistance of varieties to drought

than the gene encoding the *DREB* transcription factor. For example, the relative expression of the *DHN5* and *WZY2* genes under severe drought was higher in the drought tolerant variety. These two genes also showed a significant positive correlation under severe drought, while no correlation was observed between them under controlled conditions and mild drought stress. This shows that the expression of the analysed genes depends on the variety and the intensity of drought stress.

3. In general, drought tolerant and medium tolerant wheat varieties were better able to acclimatize to drought and induce antioxidant systems earlier than drought sensitive varieties. Understanding these responses of different wheat to drought stress can help breeders to develop genetically improved drought tolerant varieties.

5. LITERATURE

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6. CURRICULUM VITAE

Jurica Duvnjak was born on September 6 1994 in Našice, Croatia. He successfully graduated from the secondary school of civil engineering in Osijek in 2013. In the same year, he began his studies at the Faculty of Agrobiotechnical Sciences Osijek at the University of Josip Juraj Strossmayer in Osijek. In 2016, he finished studies and obtained the title of Bachelor of Plant Production. He enrolled postgraduate studies in Plant Production in 2016, and finished in 2019 thus obtaining the title of Master of Engineering in Agronomy. After completing his studies, he worked for a short time at Croatia Osiguranje and Timac Agro as a sales consultant, and at Jerković d.o.o. as head of agricultural production. Since 2021, he is employed at the Agricultural Institute Osijek as an assistant (PhD student) as part of a project of the Croatian Science Foundation. He started his PhD study in 2021 at Faculty of Agriculture of University of Zagreb. In 2021/2022, he completed two doctoral trainings at the Ruder Bošković Institute in duration of ten days, where he investigated hormonal response of wheat to drought stress. In 2023/2024, he was a fellow at the Leibniz Institute of Plant Genetics and Crop Plant Research in Gatersleben (Germany) for six months where he investigated metabolic response of seedlings under drought. During his academic career, he has participated in twelve international and national conferences with posters or oral presentations, and improved his skills in various workshops and courses. He is the author or co-author of ten scientific articles and one chapter in a book.

List of publications:

Scientific papers in journals:

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7. APPENDICES

Article 1. Duvnjak J., Lončarić A., Brkljačić L., Šamec D., Šarčević H., Salopek-Sondi, B., Španić V. (2023). Morpho-physiological and hormonal response of winter wheat varieties to drought stress at stem elongation and anthesis stages. Plants 12(3): 418. doi:10.3390/plants12030418

Article 2. Duvnjak J., Katanić Z., Šarčević H., Španić V. (2024). Analysis of the photosynthetic parameters, grain yield, and quality of different winter wheat varieties over a two-year period. Agronomy 14: 478. doi:10.3390/agronomy14030478

Article 3. Duvnjak J., Sarcevic H., Vukovic R., Spanic V. (2024). Effects of drought at anthesis on flag leaf physiology and gene expression in diverse wheat (*Triticum aestivum* L.) genotypes. Agronomy 14(7): 1522–1547. doi:10.3390/agronomy14071522





Article Morpho-Physiological and Hormonal Response of Winter Wheat Varieties to Drought Stress at Stem Elongation and Anthesis Stages

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Abstract: Drought stress can significantly reduce wheat growth and development as well as grain yield. This study investigated morpho-physiological and hormonal (abscisic (ABA) and salicylic (SA) acids) responses of six winter wheat varieties during stem elongation and anthesis stage as well grain yield-related traits were measured after harvest. To examine drought response, plants were exposed to moderate non-lethal drought stress by withholding watering for 45 and 65% of the volumetric soil moisture content (VSMC) for 14 days at separate experiments for each of those two growth stages. During the stem elongation phase, ABA was increased, confirming the stress status of plants, and SA showed a tendency to increase, suggesting their role as stress hormones in the regulation of stress response, such as the increase in the number of leaves and tillers in drought stress conditions, and further keeping turgor pressure and osmotic adjustment in leaves. At the anthesis stage, heavier drought stress resulted in ABA accumulation in flag leaves that generated an integrated response of maturation, where ABA was not positively correlated with any of investigated traits. After harvest, the variety Bubnjar, followed by Pepeljuga and Anđelka, did not significantly decrease the number of grains per ear and 1000 kernel weight (except Anđelka) in drought treatments, thus, declaring them more tolerant to drought. On the other hand, Rujana, Fifi, and particularly Silvija experienced the highest reduction in grain yield-related traits, considering them drought-sensitive varieties.

Keywords: abscisic acid; drought stress; salicylic acid; winter wheat

1. Introduction

Wheat (*Triticum aestivum* L.) is one of three major cereal crops providing daily calories and protein intake with annual global production of 780 million tons [1]. It is expected that demand for wheat will increase by up to 60% by 2050, whereas wheat production might be decreased by 29% due to climate change imposed by environmental stress [2]. This may lead to an uncertain future for world resources due to an increase in global average surface temperature [3]. Thus, drought is an important and challenging issue in wheat research because it has become one of the major problems worldwide as a result of climate change [4] that negatively affects wheat growth, development, and grain yield [5].

Wheat plants may be more susceptible to drought at critical growth stages such as germination and seedling stages [6], tillering and stem elongation stages [7], and anthesis and grain filling stages [8]. According to previous research by Sarto et al. [8], droughts with



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Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). different intensities that occurred during different growth stages of crops differently influenced grain yield. During the germination stage, drought stress resulted in the reduction of germination rate and percentage, leading to prolonged germination time [9,10]. As drought stress levels increased, there was a significant decrease in the root and shoot fresh weight of wheat seedlings [11]. Furthermore, leaf wilting reflected the effect of drought stress on plant leaves during drought treatment [12]. Moreover, plant growth was hampered due to the turgor loss of plant cells [13]. Previously, drought impact on the plant density in the initial phase, on the tiller number per plant in the tillering phase, and on the plant height in the stem elongation phase was reported [8]. As the cell elongation was disrupted, wheat's growth and height were also reduced [14]. Drought can shorten the stem elongation phase and consequently reduce the dry weight of ears and the number of fertile florets at anthesis resulting in lower grain yields [15]. Although drought impeded wheat performance at all growth stages, it was more critical during flowering and grain-filling stages resulting in substantial grain yield losses [16]. Moreover, drought stress influenced the fullness of wheat grains during grain filling [17]. It was reported that drought in the pre-anthesis stages decreased grain number per unit area, while drought in the post-anthesis stage affected the grain weight [18].

Aside from morphological adjustments, wheat plants can adapt themselves to drought conditions by activation of different molecular, biochemical or physiological processes [19]. In that case, plants take a step at the cell level against drought stress through the development of a mechanism that maintains the osmotic adjustments at the tissue level [20]. In general, drought-tolerant plants accumulate soluble sugars, proline content, amino acids, chlorophyll content, and enzymatic and non-enzymatic antioxidant activities [21]. It was previously concluded that more drought-tolerant wheat varieties could conserve water content in photosynthetic tissue and are less affected by evapotranspiration water losses [22]. Further, phytohormones play a significant role in response to abiotic stress, besides their physiological functions and involvement in the regulation of most developmental functions in plants [23]. One of the most important signaling phytohormones under drought stress is abscisic acid (ABA) [24]. According to previous research, ABA was accumulated in the leaf apoplast and induced stomatal closure under drought stress, whereas stomatal closure prevented intracellular water loss [25]. Consequently, the photosynthesis of plants was also impaired by drought, with decreased carbon assimilation [26,27]. Further, ABA helped seeds overcome stress conditions and germinate only when conditions were suitable for germination [28]. Along with ABA, salicylic acid (SA) also has a major role in modulating the plant response to drought with diverse roles in physiological processes, including germination, flowering, photosynthesis, modulation of stomatal opening and closing, and thermotolerance [29,30]. It also regulates the deterioration of reactive oxygen species (ROS) and the function of the antioxidative system [31] and induces genes responsible for encoding chaperones, heat shock proteins, and secondary metabolites [32]. Protection against drought could be accomplished through the overproduction of SA via the enhanced activity of SA biosynthetic pathway enzymes [33]. In addition, signaling cross-talk of phytohormones, such as the interaction between SA and ABA, has been recorded under both normal and stressed conditions [34].

Our previous study reported the significant negative impact of osmotic stress caused by polyethylene glycol (PEG) treatments on germination and seedlings growth of six winter wheat varieties: Silvija, Rujana, Bubnjar, Fifi, Anđelka, and Pepeljuga [10]. The objectives of the present study were (i) to investigate the effects of drought application on morpho-physiological traits in six bread wheat varieties during stem elongation and anthesis stages, (ii) to investigate the impacts of two different intensities of droughts on winter wheat by studying the occurrence timing of droughts relative to the growth stage of winter wheat, (iii) to compare the performances of elite wheat varieties under drought in terms of ABA and SA concentrations, and (iv) to identify the superior wheat varieties that can be used in breeding for drought suffered environments. We hypothesize that morphophysiological and hormonal stage-specific traits may be potential targets for the future selection of drought-tolerant wheat varieties and that drought-tolerant varieties in early developmental stages will manifest drought tolerance in the latter developmental stages.

2. Results

2.1. Stem Elongation Phase

During the stem elongation stage, the number of tillers per plant was significantly reduced in Rujana by 50.0% and Anđelka by 63.6% at a 45% reduction of volumetric soil moisture content (VSMC), compared to the control (Figure 1A). At 65% reduction of VSMC, compared to the control, Silvija and Anđelka significantly reduced the number of tillers by 60.0 and 28.6%, respectively, while Fifi increased it by 33.3%. Varieties Rujana, Fifi, and Anđelka significantly increased the number of tillers at a 65% reduction of VSMC, compared to a 45% reduction of VSMC.



Figure 1. Number of tillers (**A**) and leaves (**B**) per plant, leaf length (**C**) and width (**D**), and relative water content (**E**) during the stem elongation stage in six winter wheat varieties. Data are average values of six biological replicates \pm SD. Each biological replicate consisted of one plant. Different lowercase letters represent significantly different values (p < 0.05) within one variety under three treatments.

The number of leaves per plant was considerably reduced at 45% and 65% reduction of VSMC, compared to the control, in Anđelka, while Fifi had a significantly higher number of leaves per plant at 65% reduction of VSMC, compared to the control (Figure 1B).

Variety Silvija significantly increased leaf width at a 45% reduction of VSMC by 11.6%, compared to the control, while Rujana and Anđelka significantly reduced it by 13.6 and 9.5%, respectively, at a 65% reduction of VSMC. A significant reduction of leaf width was

observed in the variety Silvia at a 65% reduction of VSMC, compared to a 45% reduction of VSMC (Figure 1C). At a 45% reduction of VSMC, a significant reduction of leaf length was observed in Rujana, Silvija, and Pepeljuga, compared to the control (Figure 1D). The leaf length was significantly reduced in all varieties at a 65% reduction of VSMC compared to the control. The greatest reduction was noticed in Rujana (29.0%) and Silvija (24.7%).

Variety Fifi significantly reduced relative water content (RWC) by 8.5% at a 45% reduction of VSMC, compared to the control, while Anđelka significantly reduced it by 10.9% at a 65% reduction of VSMC, compared to the control (Figure 1E).

All varieties showed an increasing trend of ABA concentration in leaves with increasing drought stress, but statistically significant changes, compared to the control, were observed only at 65% reduction of VSMC in varieties Silvija (46.5%), Rujana (41.7%), Pepeljuga (35.7%), Bubnjar (27.1%), and Fifi (24.3%) (Figure 2A).



Figure 2. Concentration of abscisic acid (**A**) and salicylic acid (**B**) in leaves during the stem elongation stage of six winter wheat varieties. Data are average values of three biological replicates \pm SD. Each biological replicate consisted of one plant. Different lowercase letters represent significantly different values (p < 0.05) within one variety under three treatments.

Bubnjar and Fifi significantly increased SA concentration in leaves at a 65% reduction of VSMC by 38.4 and 59.7%, respectively, compared to the control, while Pepeljuga significantly increased it by 65.4% at a 45% reduction of VSMC (Figure 2B). Signs of the withering of bottom leaves were observed at a 65% reduction of VSMC in some varieties (Figure 3).

2.2. Anthesis Stage

Only variety Anđelka significantly increased the number of leaves and fertile tillers per plant by 62.5 and 62.5%, and by 25.0 and 19.6%, respectively, at 45 and 65% reduction of VSMC, compared to the control, while Silvija significantly increased only the number of leaves per plant by 26.8 and 31.0% at the two drought intensities (Figure 4A,B).



Figure 3. Withering of the bottom leaves at 65% reduction of volumetric soil moisture content (VSMC) in variety Silvija two weeks after drought treatments (pot with control plants, pot with plants at 45% reduction of VSMC, and pot with plants at 65% reduction of VSMC).



Figure 4. Number of leaves (**A**) and fertile tillers (**B**) per plant, leaf width (**C**) and length (**D**), and relative water content (**E**) during the anthesis stage of six winter wheat varieties. Data are average values of six biological replicates \pm SD. Each biological replicate consisted of one plant. Different lowercase letters represent significantly different values (p < 0.05) within one variety under three treatments.

Variety Silvija significantly increased the leaf width by 12.8%, at a 45% reduction of VSMC, compared to the control, while Bubnjar significantly increased it by 13.1% at a 65% reduction of VSMC (Figure 4C).

The leaf length was significantly increased by 33.3, 24.4, and 24.1% in Silvija, Bubnjar, and Fifi, respectively, at a 65% reduction of VSMC, compared to the control (Figure 4D). Significant reductions of RWC by 52.9, 35.2, 27.5, and 17.1% were observed in Rujana, Anđelka, Bubnjar, and Pepeljuga, respectively, at 65% reduction of VSMC, compared to the control, while for Anđelka the reduction of RWC was also observed at 45% reduction of VSMC (Figure 4E).

The number of spikelets per ear was significantly reduced by 12.1, 11.6, 11.1, and 10.3% in Bubnjar, Silvija, Rujana, and Fifi, respectively, and at a 45% reduction of VSMC, compared to the control, while varieties Silvija, Rujana, Pepeljuga, and Bubnjar significantly reduced it by 25.0, 16.6, 11.6, and 10.6%, respectively, at 65% reduction of VSMC, compared to the control (Figure 5A). In the control plants, the non-significant higher stem height was recorded for all varieties, compared to drought treatments, by 45 and 65% reduction of VSMC, but only varieties Fifi and Bubnjar significantly reduced it at 65% reduction of VSMC, compared to control plants, by 6.3 and 18.7%, respectively (Figure 5B). There was no significant change in plant height between plants in control and a 45% reduction of VSMC. A significant reduction of plant height was recorded at 65% reduction of VSMC, compared to control plants, for Rujana and Bubnjar by 10.5 and 16.0% (Figure 5C).



Figure 5. Number of spikelets per ear (**A**), stem height (**B**), and plant height (**C**). Data are average values of six biological replicates \pm SD. Each biological replicate consisted of one plant. Different lowercase letters represent significantly different values (p < 0.05) within one variety under three treatments.

Significant increase in the concentration of ABA occurred in flag leaves of Fifi, Anđelka, Bubnjar, Silvija, and Rujana at 65% reduction of VSMC, compared to control plants, by 67.7, 52.4, 45.2, 43.0, and 38.1%, respectively (Figure 6A). Only Pepeljuga significantly increased SA in flag leaves at a 45% reduction of VSMC, compared to the control, by 48.1% (Figure 6B).



Figure 6. Concentration of abscisic acid (**A**) and salicylic (**B**) acid in flag leaves during the anthesis stage of six winter wheat varieties. Data are average values of three biological replicates \pm SD. Each biological replicate consisted of one plant. Different lowercase letters represent significantly different values (p < 0.05) within one variety under three treatments.

2.3. Grain Yield-Related Measurements after Harvest

A reduction of VSMC by 45% did not cause significant changes in the number of grains per ear in comparison to the control (Figure 7A). The number of grains per ear was significantly reduced by 33.2, 32.9, and 29.9% in Fifi, Silvija, and Rujana, respectively, at 65% reduction of VSMC, compared to the control, while no significant reduction was recorded in other varieties. Only Anđelka significantly reduced 1000 kernel weight at 45% reduction of VSMC, compared to the control, while significant reduction by 20.7, 18.7, 13.8, and 9.1% was observed in Fifi, Silvija, Anđelka, and Rujana, respectively, at 65% reduction of VSMC, compared to the control (Figure 7B).

2.4. Correlation and Principal Component Analysis

Correlation analysis at the stem elongation stage (Table S1), anthesis stage (Table S2), and after harvest (Table S3) were performed to show relationships among traits. Further, to visualize the relationships between morpho-physiological traits, plant stress hormones, and the level of drought tolerance of six winter wheat varieties, PCA analysis was conducted for two developmental stages, stem elongation (Figure 8A) and anthesis stage (Figure 8B) as well as grain yield-related data obtained after harvest (Figure 9).



Figure 7. Number of grains per ear (**A**) and 1000 kernel weight (**B**) after harvest of six winter wheat varieties. Data are average values of six biological replicates \pm SD. Each biological replicate consisted of one plant. Different lowercase letters represent significantly different values (p < 0.05) within one variety under three treatments.

At the stem elongation stage, it was shown that leaf length was in significant positive correlation with leaf width, as well as the number of leaves per plant with the number of tillers per plant. ABA and SA were in significant negative correlation with leaf length, as well as ABA with leaf width. In addition, ABA and SA were significantly positively correlated (Table S1). The PCA biplot showed that at the stem elongation stage, 41.17% of the total variability was explained by the first principal component (PC1) and 27.11% by the second principal component (PC2) (Figure 8A). The first two principal components (PCs) together explained 68.28% of the total variability. As can be seen from the biplot, morphophysiological traits were grouped on the left side, while stress hormones were grouped on the right side of the PCA plot, indicating a negative correlation between morphophysiological traits and stress hormone concentrations. At the same time, the positioning of wheat varieties in the control conditions and two treatments showed that the controls of all varieties were grouped closely on the left side of the PCA plot, while the treatments (T1 and T2) were shifted toward the right side of the PCA plot. The shift was larger the more drastic changes the varieties exhibited under stress conditions. Furthermore, the shifts to the right were consistent with the severity of the stress (the T2 treatments were positioned further to the right compared to the T1 treatments for all varieties). Accordingly, the variety Silvija experienced the most drastic changes under drought, while Bubnjar experienced the less drastic changes, indicating that Silvija was the most sensitive and Bubnjar the most tolerant variety at the stem elongation stage to the applied drought treatments.



Figure 8. Principal component analysis (PCA) showing the relationship of morpho-physiological traits and stress hormones at (**A**) stem elongation stage and (**B**) anthesis stage (Rujana, Silvija, Fifi, Anđelka, Bubnjar, and Pepeljuga) under two drought regimes (T1 = VSMC-45% and T2 = VSMC-65%) and control (C). PCA was performed on the correlation matrix of average values of morpho-physiological attributes (number of leaves, leaf length, leaf width, number of tillers, RWC, number of spikelets per ear, stem height, plant height), and concentrations of stress hormones (abscisic acid and salicylic acid).

At the anthesis stage, a significant positive correlation was observed between the number of leaves per plant and the number of fertile tillers per plant, the number of leaves per plant and the leaf width, the number of spikelets per ear and RWC, as well as between stem and plant height. The number of leaves per plant and leaf length, the number of fertile ears per plant and RWC, the number of fertile tillers per plant and leaf length, as well as the number of spikelets per ear and leaf width, were negatively correlated. A significant

negative correlation was also observed between ABA and RWC, as well as between SA and the number of spikelets per ear. On the other hand, SA was in a significant positive correlation with leaf width (Table S2). PCA showed that PC1 accounted for 33.23% and PC2 for 25.28% of the total variability (Figure 8B), explaining together 58.51% of the total variability. As can be seen from the biplot, traits such as RWC, leaf length, and spikelets per ear were positioned opposite to stress hormones, stem and plant height, tillers per plant, leaves per plant, and leaf width. Varieties were positioned according to certain changes exhibited under stress treatments. Overall, Bubnjar, Pepeljuga, and Anđelka seem to undergo less drastic changes under stress compared to their controls, while Rujana, Fifi, and particularly Silvija experienced more drastic changes under stress conditions suggesting their level of drought tolerance at the anthesis stage.



Figure 9. Principal component analysis (PCA) showing the relationship between two-grain yield-related traits after harvest for six winter wheat varieties (Rujana, Silvija, Fifi, Anđelka, Bubnjar, and Pepeljuga) under two drought regimes (T1 = VSMC-45% and T2 = VSMC-65%) and the control ©. PCA was performed on the correlation matrix of average values of grain yield-related traits (number of grains per ear, 1000 kernel weight).

At final, after harvest, no significant correlations were observed between traits (Table S3).

PCA considering grain yield-related traits (grains per ear and 1000 kernel weight) that were measured after harvest (Figure 9) revealed that PC1 accounted for 71.88% and PC2 for 28.12% of the total variability explaining together 100.00% of the total variability. The angle between the vectors of grains per ear and 1000 kernel weight on the PCA biplot is close to 90°, which means that the correlation between the two traits is close to zero. Furthermore, based on grain yield related-traits, the relative position of the control and T1 and T2 treatment differs among varieties. As can be seen, there is a small shift in Bubnjar under drought treatments compared to the control, indicating its good performance under drought stress. Pepeljuga and Anđelka also showed a relatively small reduction in grain yield-related traits under stress conditions. On the other hand, Rujana, Fifi, and particularly Silvija showed a more drastic reduction in grain yield-related traits under stress conditions. In all varieties, except Bubnjar, the reduction in grain yield-related traits follows stress severity.

3. Discussion

To adapt to drought stress, wheat plants have developed mechanisms that manifest themselves in morphological, physiological, developmental, and molecular changes. Under drought conditions, the plant produces ROS, while the antioxidant protective enzyme system, flavonoids, and secondary metabolites play a role in the protection of the plant by detoxifying ROS [35,36]. Aside from their role in irreversible DNA damage and cell death, ROS are important signaling molecules that regulate normal plant growth and responses to stress [37]. For example, ROS species are involved in the regulation of stomatal behavior [38], which is further controlled by ABA and SA [39,40].

In the present study, we investigated the mechanisms underlying the correlation of two endogenous levels of stress hormones, ABA and SA, and morpho-physiological traits at stem elongation and anthesis stages under two intensities of drought stress. In our previous research, morpho-physiological and biochemical responses of six winter wheat varieties (Silvija, Rujana, Bubnjar, Fifi, Anđelka, and Pepeljuga) to osmotic stress treatments caused by 10 and 20% PEG at germination and seedlings stage were examined [10]. Accordingly, all varieties significantly reduced germination energy at 20% PEG. The reduction of germination energy ranged from 6.6% for the variety Rujana to 17.0% for the variety Silvija. Seedling growth was also reduced for all varieties in a dose-dependent manner of applied PEG. The highest shoot length reduction was observed for the variety Silvija, followed by the variety Fifi while the smallest reduction was obtained for varieties Bubnjar, Pepeljuga, and Anđelka, compared to the control. Thus, results suggested Silvija as the most sensitive while Bubnjar is the most tolerant variety to osmotic stress at germination and early seedlings stage [10]. Herein, we proceeded with the research of stress response of the same varieties to two drought regimes at further developmental stages: stem elongation and anthesis. Finally, grain yield-related traits of six wheat varieties under drought conditions were measured after harvest.

3.1. Drought Response of Wheat Varieties at Stem Elongation Stage

The onset of stem elongation coincides with the transition from the vegetative to the reproductive stage when spikelet primordia are formed from leaf primordia when the apex meristem differentiates [41]. Therefore, this period is critical for spike development [42], where a significant reduction in the number of spikelets and, thus, the final number of grains per spike under stress can occur [43]. As a result, grain yield formation could be affected when 50% of grain yield potential based on the maximum number of floret primordia could be lost [44]. Further, at the beginning of stem elongation starts the highest water consumption by plants [8]. Drought stress during the stem elongation stage also reduced the elongation of the stem and cell expansion, which was related to changes in the metabolism of some hormones [45]. Previously it was reported that parallel with the increase in water deficit, there was a decrease in the RWC and water potential in the leaf [46]. RWC in leaves is, furthermore, a parameter reported to be significantly lower in plants under drought treatments compared to the control plants [47]. However, this is variety specific, and in the current study, RWC values were reduced under drought only in varieties Fifi and Andelka. Unfortunately, drought during the stem elongation phase has been much less studied, although it is an important phase in the study of drought stress.

In the current research, the number of tillers per plant was significantly reduced in varieties Silvija and Anđelka at a 65% reduction of VSMC, compared to the control, with a more pronounced reduction in variety Silvija. Rujana and Anđelka significantly reduced the number of tillers per plant already at a 45% reduction of VSMC, with a stronger reduction in tiller number of Anđelka under that treatment, compared to a 65% reduction of VSMC. In all varieties, leaf expansion (length and width) decreased significantly with increasing drought severity, compared to control, although differences were found among varieties. This is in agreement with the previous study, which reported genotypic variation in growth response to temperature for wheat leaf elongation rate [48]. In the current research, it is important to note that the reduction of leaf length was least pronounced for variety Bubnjar

at 65% reduction of VSMC, compared to other varieties. Our results are in agreement with those of Qaseem et al. [49], who reported a reduction in tillering under drought conditions at the stem elongation stage. According to Urbanavičiūtė et al. [50], the decrease in the number of leaves and tillers under drought was variety specific, which is consistent with our results. In addition, Urbanavičiūtė et al. [50] concluded that varieties were more tolerant to drought due to their successful development of tillers under stress conditions. Tiller formation can be affected mainly by drought and nutrient deficiency [51,52]. Moreover, the number of tillers and leaves were significantly positively correlated in the current research, which is in agreement with the results of Miralles and Richards [53], suggesting that tiller and leaf growth were closely coordinated in wheat plants.

ABA showed a tendency to increase in all varieties with increasing drought severity. A significant increase was recorded in all varieties, except in Andelka, under more drastic drought conditions. As the increase in ABA is in accordance with the stress that wheat varieties experienced under drought treatments, the concentration of this hormone can serve as a good stress marker. ABA triggers stress signaling and tolerance in plants [54] and acts as an inhibitor of plant growth under water deficit [55]. Its concentration rapidly increases to initiate stomatal closure in the plant [56] and stimulate root cell elongation [57] but at the expense of the number of tillers and leaves. During drought stress, very rapid ABAmediated closure of stomata will occur to limit water loss by evapotranspiration [25,58]. Furthermore, as the stomata close, the entry of CO_2 into the mesophyll also decreases, with negative consequences for the net photosynthetic rate [59]. It was previously concluded that photosynthesis is one of the most sensitive processes to water deficit [60]. It has been previously reported that wheat plants accumulate inorganic solutes such as potassium, calcium, silicon, and SA in their cytosol to maintain cell turgor by lowering their osmotic potential under drought stress [61]. Our results showed that SA has a tendency to increase with drought stress, although the changes were not always statistically significant. Besides its role as a plant hormone and signaling molecule, SA is a phenolic acid and, therefore, has antioxidant activity. The increase in SA under drought conditions may participate in protection against water deficit at this developmental stage. It was previously reported that SA alters key plant functions, including water relations [62] and stomatal functioning [63]. We may speculate that the increase in SA under drought conditions may be involved in the tolerance mechanisms. Since variety Bubnjar did not show significant reductions in leaf width, number of tillers and leaves per plant, and relative water content under both drought treatments, compared to the controls, we may suggest it as relatively tolerant to drought stress during the stem elongation phase. Indeed, PCA analysis confirmed that variety Bubnjar exhibited the least changes under the drought treatments in comparison to the control, while variety Silvija underwent the most drastic changes indicating its sensitivity to drought, at the stem elongation stage.

3.2. Drought Response of Wheat Varieties at Anthesis Stage and Resulting Grain Yield-Related Data

Plants were also subjected to two drought intensities during the anthesis stage, one of the terminal phases of wheat development. According to the study by Morgun et al. [46,64], differences in investigated traits between varieties were more contrasting when drought was applied at the anthesis, compared to the stem elongation stage. The flowering stage or anthesis begins after heading, and at this stage, the anthers release their pollen, after which grains are formed [65]. According to previous research, drought stress during grain filling is the most yield-damaging to wheat due to impaired grain development associated with imbalanced levels of growth hormones [66]. In the present study, a significant increase in the number of leaves per plant was observed in varieties Silvija and Anđelka under both drought treatments, compared to the controls, while in Anđelka, a significant increase in the number of fertile tillers per plant was also recorded. The number of fertile tillers depended on environmental conditions and the time of tiller formation [67].

The shape, size, senescence, and waxiness of leaves can also contribute to drought tolerance [68]. In the present study, leaf size (width and length) did not change or showed a

tendency to increase under stress conditions. RWC was significantly decreased in leaves of all varieties except Silvija and Fifi, suggesting higher water loss under drought conditions.

It has been previously reported that wheat height decreased due to drought stress [49]. In the current study, a significant decrease in plant height due to heavier drought stress (65% reduction of VSMC) was observed in two varieties (Rujana and Bubnjar). This can be explained by the fact that cell elongation was disrupted by drought, affecting wheat's growth and height. The reduction in plant height caused by drought was about 7% at the grain-filling stage [69]. This is in accordance with the current study, where there was a reduction in plant height caused by drought by 10%, on average, for all varieties. Furthermore, drought-tolerant plants tend to maintain lower plant height and plant area index to reduce the moisture demand and prevent moisture loss due to transpiration [70]. Therefore, the variety Bubnjar seems to respond to heavier drought stress by the significantly shortened stem and plant height.

At the anthesis stage, all varieties showed an increase in ABA under drought stress with significant changes at more severe drought treatments (except Pepeljuga). Pepeljuga was the only variety that did not significantly change ABA concentration between treatments, but it also was the only variety that increased SA at a 45% reduction of VSMC.

Stress hormones such as ABA and SA are among the main signaling molecules that orchestrate plant stress response. The correlation between ABA's endogenous level and stress tolerance is not unambiguous in the plant kingdom. The endogenous level of this hormone oscillates according to its metabolism, plant species, and organ/tissues, as well as the duration and severity of the drought stress. Application of exogenous ABA under water stress accelerated the accumulation of osmolytes and improved the water status of plants that, resulted in higher grain weight in susceptible wheat varieties [71]. Correlations between endogenous ABA increase and plant tolerance are somehow controversial in literature and obviously depend on plant species and developmental stage. There were examples of positive correlations between ABA level and tolerance (sunflower and switchgrass) which suggested that constitutively high ABA levels in tolerant varieties confer a better ability to cope with an adverse water deficit [72]. On the other hand, some native species from the arid regions showed that the highest ABA levels were found in drought-sensitive Poa *ligularis*, while the lowest ABA levels were identified in the highly tolerant xerophytic species *Papostypa speciosa* [72]. Besides the ABA level, ABA sensitivity is also an important trait for plant survival. Experiments on Arabidopsis and wheat suggested that plants with a high drought tolerance showed a significantly higher ABA sensitivity than the sensitive lines [73,74]. Previously, it was demonstrated that the overexpressor rice line in the OsSta2 gene (Oryza sativa Salt tolerance activation 2-Dominant) exhibited hypersensitivity to ABA and showed increased tolerance to drought and salt stress [75]. Our data demonstrated that ABA is a good stress marker in all wheat varieties under drought. SA is another well-known stress hormone, although the role of SA may be even more controversial than ABA under abiotic stress conditions since some investigators have reported an enhancement of drought tolerance by SA application, whereas others claimed a reduction in drought tolerance. Generally, the impact of SA in stress conditions was highly dependent on the concentration applied. Experiments with exogenous treatments showed that low concentrations of SA decreased oxidative stress and enhanced drought tolerance in maize, wheat, tomato, bean, etc. [30]. Furthermore, SA-accumulating mutants of A. thaliana (adr1, myb96-1d, siz1, acd6, and cpr5) exhibited stomatal closure and improved drought tolerance [30,34]. Moreover, it was reported that SA is able to improve the stability of photosynthetic apparatus [76]. Our results showed that all varieties at later developmental stages showed a slight increase or did not change the level of SA or under drought conditions significantly. Since SA is phenolic acid with reported antioxidant activity, its presence in the plant may also be positive as a ROS scavenger. It is necessary to keep in mind that tolerance is the result of a complex network of action and cross-talk of different plant hormones in which ABA and SA play an important role.

PCA analysis summarized all changes that six varieties exhibited under two stress regimes. Accordingly, it may be concluded that Bubnjar is the most tolerant variety while Silvija is the most sensitive under applied drought stress treatments.

It has been reported that the period of seven to ten days before anthesis and five days after anthesis is the most critical period for reproductive development [77]. The most damaging to the grain size of wheat was drought stress at and just after the anthesis stage [42], which coincides with the period of a drought treatment applied in the current study. Moreover, drought during the anthesis stage mainly caused a reduction in grain size [78]. All varieties showed a tendency to reduce the number of grains per ear as well as 1000 kernel weight under stress conditions, although the changes were not statistically significant in all cases. For example, varieties Bubnjar, Anđelka, and Pepeljuga did not significantly change the number of grains per ear compared to the controls. Previously it was reported that under drought stress, the average kernel weight was significantly reduced in all tested varieties compared to their controls [78]. This is partially in agreement with the current research where 1000 kernel weight was significantly reduced in four out of six tested wheat varieties, but a slight non-significant reduction was observed in all varieties at 65% reduction of VSMC, compared to the control. Only Andelka significantly reduced grain yield-related traits at a 45% reduction of VSMC. Khalili et al. [79] reported that increased drought intensity significantly decreased the grain yield and harvest index of maize, which is in agreement with our results. During reproductive development, drought stress reduced the grain number in the ear of wheat [80], where premature abortion of flowers occurred, which resulted in a reduced number of potential grains in the ear [81]. In previous studies, the number of grains per ear and the weight of grains was also affected by the environment, including drought [82]. Drought occurrence during anthesis affected the number of grains per spikelet and the total number of grains per spike [42], which was also demonstrated in the current research.

Correlation of grain yield-related traits and varieties under stress conditions presented by PCA clearly showed that Bubnjar is the most drought-tolerant variety, followed by Pepeljuga and Anđelka, while Rujana, Fifi, and particularly Silvija, are more sensitive to drought.

4. Materials and Methods

4.1. Plant Material

Six winter wheat varieties (Rujana, Silvija, Fifi, Bubnjar, Anđelka, and Pepeljuga) of the Agricultural Institute Osijek were examined under drought conditions. Rujana is a taller variety and is later in maturity than the other studied varieties. Silvija is a variety with a longer vegetation period and has a tolerance to low temperatures. Fifi is a medium-early variety with higher grain protein content. Bubnjar is a medium-early variety that was previously characterized by better germination under drought stress [10]. Anđelka, a medium-early variety, is characterized by good tolerance to lodging and lower temperatures, while Pepeljuga is a medium-early variety with medium height.

4.2. Chemicals

Salicylic acid (SA) and (+)-*cis, trans* abscisic acid (ABA) were purchased from Fluka and Duchefa-Biochemie, respectively. The internal isotope labeled standard SA-d₆ was purchased from Sigma-Aldrich, (+)-*cis, trans* ABA-d₆ from Trc. MiliQ[®] water (18.2 M Ω cm⁻¹; purified by MiliQ water purification system (Millipore, Bedford, MA, USA)) and HPLC gradient-grade methanol (J.T. Baker) were used with analytical-grade formic acid (FA) (Acros Organics) for mobile phase preparation. Acetic acid (AcOH) for extraction was purchased from Sigma-Aldrich.

4.3. Drought Stress during Two Growth Stages

After germination in distilled water, five-day-old wheat seedlings of each wheat variety were placed in a plant growth chamber to undergo a period of vernalization under conditions of 12 h day/12 h night $(4/3 \,^{\circ}C)$ for a period of six weeks. After that, two separate experiments were set up in a greenhouse (Gis Impro d.o.o., Vrbovec, Croatia) where each experiment included three treatments: (1) two intensities of drought during stem elongation stage (GS31) [66] and controlled treatment with regular irrigation, (2) two intensities of drought during anthesis stage (GS61) and controlled treatment with regular irrigation. Within each treatment, varieties were randomized according to the random block design in six replicates, each containing 4 plants/2.5 L pot filled with soil (pH-H₂O: 5.5–7.0, organic matter: 70.0–85.0%, N (1/2 vol.): 100–200 mg L^{-1} , P₂O₅ (1/2 vol.): 100–150 mg L^{-1} , K₂O (1/2 vol.): 200–400 mg L⁻¹). Nitrogen (N) fertilization was carried out at the two-leaf development stage (GS12) using calcium ammonium nitrate (CAN) (27%N) of grain/plant and two protections against diseases and pests. The first one was carried out with the fungicide Falcon Forte (spiroxamine 224 g L^{-1} , tebuconazole 148 g L^{-1} , prothioconazole 53 g L^{-1}) at the stem elongation stage (GS30), and the second one a week later with the insecticide Vantex $(gamma-cyhalothrin 60 \text{ g L}^{-1})$ after the emergence of aphids (GS31). During tillering, stage temperatures were maintained during the night at 8–12 $^{\circ}$ C (14 h) and daytime temperatures at 10–14 °C (10 h) with the maximum light intensity of 250 μ mol m⁻² s⁻¹. When the stem started to elongate, the length of day and night were adjusted to become equal, and daytime temperatures were maintained at 15–18 °C, while night temperatures were set up at 11–14 °C. Before anthesis, the length of the day was increased to 14 h, and the temperatures were maintained at 21–24°C and night temperatures at 17–20 °C with the maximum light intensity of 750 μ mol m⁻² s⁻¹. During stem elongation and anthesis stages, in two separate experiments, plants were subjected to different intensities of drought by reducing water content by 45% (T1) and 65% (T2) of the volumetric soil moisture content (VSMC) for two weeks. In both treatments, non-lethal, moderate drought stress was produced. In the controlled treatment, the VSMC was maintained at 30–35%, where along with other treatments, VSMC was measured daily by a soil moisture measuring device (TDR 150 Soil Moisture Meter, Spectrum Technologies, Aurora, CO, USA).

4.4. Morphological Traits and Relative Water Content (RWC)

During the stem elongation stage, the number of leaves and tillers was counted, while leaf length and width (mm) were measured by the ruler on the 14th day of the experiment, in six replicates.

During the anthesis stage on the 14th day of the experiment, leaf length and width (mm), stem height (mm), and plant height (mm) were measured by the ruler in six replicates, while the tiller and leaf number were counted. Stem and plant height were measured from the ground to the base and to the top of the ear, respectively. After maturity, six wheat ears were collected from each treatment for further analysis of seed morphology using a MARViN seed analyzer (MARViTECH GmbH, Wittenburg, Germany), where 1000 kernel weight and the number of seeds per ear were measured.

For relative water content (RWC) measurement, leaf samples were collected from control and drought treatments after 14 days in drought-treated plants during stem elongation and anthesis stage (10×10 mm diameter of the leaf/flag leaf) in six replicates. Leaf discs were weighed (FW) and immersed for 24 h in deionized water, after which the turgid weight (TW) was recorded. After 24 h of drying in a dryer at 105 °C, the dry biomass (DW) was recorded [83]. For RWC calculation, the following formula was used [84]:

$$RWC(\%) = \frac{FW - DW}{TW - DW} \times 100$$

4.5. Stress Hormone Analysis: ABA and SA

4.5.1. Sample Preparation

After plant tissue sampling, the samples were frozen in liquid nitrogen and lyophilized in three replicates from the stem elongation and anthesis stages. Further, lyophilized samples were shredded by mortar and pestle in liquid nitrogen. 30 mg of powdered sample were extracted in 1 mL extraction solution (10% MeOH and 1% acetic acid containing

38.5 ng mL⁻¹ of each internal isotope labeled standards SA-d₆ and ABA-d₆). After vortexing, the samples were placed in a Mixer Mill (Roche) (2 min, frequency 30,000 RPM), after which they were homogenized for 1 h at 4 °C. The samples were then centrifuged (10 min, 13,000 RPM), and 100 μ L of clear solution was used for liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis of stress hormones.

4.5.2. Preparation of Standard and Calibrant Solutions

Stock solutions of each analyte, including internal labeled standards, were prepared as 1 mg mL⁻¹ solutions in methanol. Stock solutions were diluted together in 10% MeOH + 0.1% AcOH to yield a working solution of 1 µg mL⁻¹ and 100 ng mL⁻¹ of each substance. 100 ng mL⁻¹ solutions of ABA and SA in 10% MeOH + 0.1% AcOH was used as a QC sample. In the QC sample, a mixture of isotope-labeled standards ABA-d₆ and SA-d₆ to a final concentration of 38.5 ng mL⁻¹ was also added. All standard solutions and QC samples were stored at -20 °C.

The calibration samples were prepared from stock solutions of each analyte in 10% MeOH + 0.1% AcOH with the addition of internal standard solution (40 μ L of spike mixture solution ABA-d₆ and SA-d₆ 1 μ g mL⁻¹, final concentration 38.5 ng mL⁻¹). Particular calibration points were as follows: calibrant 1 ABA and SA 9.6 ng mL⁻¹, calibrant 2 ABA and SA 24 ng/mL, calibrant 3 ABA and SA 48 ng mL⁻¹, calibrant 4 ABA and SA 96 ng mL⁻¹, calibrant 5 ABA and SA 192 ng mL⁻¹ and calibrant 6 ABA and SA 480 ng mL⁻¹, respectively. 5 μ L of each calibrant was injected into the LC column. The calibration curve was obtained by linear regression; the peak area ratio (analyte/internal standard) was plotted versus the analyte concentration. Least-squares linear regression gave Spearman correlation coefficients of r² = 0.9989 for ABA/ABA-d₆ (regression lines y = 0.0223 + 0.0783) and r² = 0.9969 for SA/SA-d₆ (regression lines y = 0.359 - 1.5305). Quantification was performed by adding the unknown area in the calibration curve plotted peak area ratio (analyte/internal standard) versus analyte concentration.

QC sample and instrumental blank were injected after every few runs. During analysis, all instrumental blank samples were negative, and the area of each analyte in the QC samples was repeatable.

4.5.3. LC-MS/MS Conditions

LC–MS/MS analysis was carried out using an Agilent Technologies 1200 series HPLC system equipped with a binary pump, a vacuum membrane degasser, an automated autosampler, and an injector interfaced with 6420 triple quadrupole mass spectrometer with electrospray ionization source (ESI) (Agilent Technologies Inc., Palo Alto, CA, USA).

The separation was performed on the Zorbax XDP C18 column (75×4.6 mm, 3.5μ m particle size) (Agilent Technologies Inc., Palo Alto, CA, USA). Solvents for the analysis were 0.1% formic acid (FA) in water (solvent A) and methanol (solvent B). The gradient was applied as follows: 0 min 50% A, 5–15 min 50% A–0% A, 15–17 min 0% A, 17.1–22 min 60% A. Flow rate was 0.3 mL min⁻¹.

The electrospray ionization source was operated in negative mode, and samples were detected in the multiple reaction monitoring (MRM) modes with a dwell time of 10 ms per MRM transition. The desolvation gas temperature was 350 °C with a flow rate of 6.0 L min⁻¹. The capillary voltage was 3.5 kV. The collision gas was nitrogen. The MRM transitions of precursor to product ion pairs were m/z 263–153 for ABA (quantifying ion), m/z 263–219 for ABA (qualifying ion), m/z 137–93 for SA, m/z 269–159 for ABA-d₆ and m/z 141–97 for SA-d₆ respectively. Fragmentor voltages were 100 V for ABA and ABA-d₆ and 70 V for SA and SA-d₆. The collision energy was set to 15 V for SA, 12 V for SA-d₆, 3 V for ABA quantifying and ABA-d₆, and 2 V for ABA qualifying transition.

All data acquisition and processing was performed using Agilent MassHunter software. ABA and SA concentrations were calculated and expressed as $ng mg^{-1}$ DW.

4.6. Statistical Analysis

A randomized complete block design was applied both in the plant growth chamber and greenhouse to minimize the effect on the environment. Samples were collected from each pot, whereas morpho-physiological measurements were done in six biological replicates. Collected data were statistically analyzed using the Statistica software (version 14). Fisher's LSD test at a 5% probability level was used to test differences among mean values. The results of analyzed morpho-physiological parameters were expressed as the mean value of six replicates \pm standard deviation (SD). ABA and SA concentration was measured in three replicates of leaves and flag leaves at stem elongation and anthesis stage, respectively, and expressed as the mean value of three replicates \pm standard deviation (SD). Correlation analyses were done by Spearman coefficient at p < 0.05 and p < 0.001. Principal component analysis was performed using Addinsoft XLSTAT (New York, NY, USA).

5. Conclusions

It was observed that certain morpho-physiological and hormonal changes were observed during drought stress, depending on varieties and stress intensity. ABA was increased in all six winter wheat varieties under drought, confirming the stress status of the plants. SA was increased at the stem elongation stage, while it did not change at the later developmental stage (anthesis stage). Correlation analysis and PCA showed that the variety Bubnjar, followed by Anđelka and Pepeljuga, experienced the least changes in morphophysiological traits under stress conditions resulting in good grain yield-related traits after harvest. On the other hand, Fifi, Rujana, and particularly Silvija were more sensitive to drought and underwent more drastic changes in morpho-physiological traits at the stem elongation and anthesis stages, resulting in a greater reduction in grain yield-related traits. These results are in agreement with our previous study investigating the response of the same varieties to drought at germination and young seedlings stages. Overall, our findings indicated that wheat varieties differ in their ability to produce ABA under drought during all growth stages, whereas tolerance to drought is variety specific but remains the same for all developmental stages. Understanding the responses of different wheat to drought stress can help breeders to develop genetically improved drought-tolerant varieties.

Supplementary Materials: The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/plants12030418/s1, Table S1: Correlation analysis of seven investigated traits in three treatments during the stem elongation stage; Table S2: Correlation analysis of ten investigated traits in three treatments during the anthesis stage; Table S3: Correlation analysis of two investigated traits in three treatments after harvest.

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Article Analysis of the Photosynthetic Parameters, Grain Yield, and Quality of Different Winter Wheat Varieties over a Two-Year Period

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Abstract: Due to increasingly frequent unfavorable climate changes, achieving a high grain yield of wheat is a challenge for breeders. The relationships between wheat productivity and photosynthesis traits are not very well understood during the growing season. This study investigated the effect of chlorophyll a fluorescence parameters (maximum quantum yield of primary photochemistry (TR_O/ABS) and performance index on absorption basis (PI_{ABS})) on grain yield and the yield-related and technological quality traits of six wheat varieties over two growing seasons. In the first growing season (2021/2022), grain yield was significantly positively correlated with 1000 kernel weight and TR_O/ABS at the second measurement point (growth stage 25 (GS 25)). Only the highest-yielding variety Bubnjar (104.0 dt ha^{-1}) showed values of TR_O/ABS at the same significance level between the second and third measurement points. Due to elevated virus and disease infections in the second growing season (2022/2023), the grain yield of the investigated varieties decreased between 37.9% (Bubnjar) and 67.6% (Andelka) relative to the first growing season. The three highest-yielding varieties (Bubnjar, Rujana, and Silvija) in 2022/2023 were the tallest, were later in maturity, escaped yellow rust pressure at the stem elongation stage more efficiently, and also showed the lowest increase in TR_O/ABS at this stage (fourth measurement point at GS 47, compared to the third at GS 32). In addition, the highest-yielding variety Bubnjar showed the highest increase in PI_{ABS} at the last measurement (seventh) at GS 71 compared to the sixth (GS 69), thus maintaining the vitality of flag leaves at the grain-filling stage, while the other varieties showed a very small increase or even a significant decrease. Therefore, plant photosynthetic activity over the entire growing season contributes to crop productivity.

Keywords: grain yield; photosynthesis; quality traits; wheat

1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most important staple foods consumed globally [1], contributing 20% of the caloric and protein intake of the human population [2]. Wheat productivity is hindered by the climate change that represents danger for food security due to increased extreme weather events leading to abiotic stresses such as floods, drought, and heat stress [3]. Climate change scenarios predict increasing occurrences of irregular rainfall, whereas the frequency of drought is certain to increase in the future as a result of global warming, which will result in a decline in overall food production. For example, grain yield reductions for wheat are predicted to range from 25 to 50%, depending on latitude and different soil properties and agronomical management features [4]. In the sowing structure of Croatia, wheat is the second most represented crop, sown on approximately 161,000 hectares in 2022 [5]. According to the Global Facility for Disaster



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Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). Reduction and Recovery, there is an up to 20% chance that droughts will occur in the next ten years in Croatia. Worldwide, drought stress is already considered a major limitation of crop productivity [6], and affects crop quality as well [7]. According to the study by Nouri-Ganbalani et al. [8], grain yield can decrease by up to 70% due to drought. Further, conditions of reduced water by 40% resulted in grain yield decreasing by 20.6% [9]. A risk assessment of the possible impacts of climate change on wheat grain quality in an irrigated area showed that grain protein content decreased between 7.3% and 27.2% [10].

Drought can occur at all stages of wheat growth, with various consequences depending on the growth stage or drought intensity [11,12], but drought occurrence is more critical during the flowering and grain-filling stages, resulting in significant grain yield losses [13]. Drought is expected as a result of prolonged water deficiency, but drought-tolerant plants maintain high internal water content [14]. Therefore, the morpho-physiological traits of plants that improve vegetative growth and root development are used to increase drought resistance [15], but these traits do not guarantee higher grain yields in terminal drought conditions [16,17].

On the other hand, increasing rainfall and carbon dioxide (CO_2) concentrations are beneficial for crop production to some extent, but high temperatures may diminish this effect [18]. It is well known that temperature and water play an important role in the spread of pathogens and insects. It seems that an increase in rainfall enhances pathogen life cycles and helps in pathogen colonization and growth during initial infection [19]. Temperature and rainfall alone are not the only factors influencing wheat grain yields. Higher levels of CO_2 in the atmosphere have a positive effect on photosynthesis and water retention, as the CO_2 assimilated by the photosynthetic apparatus is the basis of crop production, whereas the Calvin cycle reactions of photosynthetic CO₂ fixation take place in the chloroplast stroma [20]. Also, in the last few years, many areas of the world have been faced with serious ozone (O_3) pollution [21]. O_3 not only affects the health of the human population, but also affects the photosynthesis of crops, resulting in a decrease in crop yields [22]. The primary determinant of grain yield could be the cumulative rate of photosynthesis over the vegetative season. It has been reported that even small increases in the rate of net photosynthesis can translate into large increases in biomass, and hence grain yield, since carbon assimilation is integrated over the entire vegetative season of wheat plants [23]. Hence, a positive correlation between grain yield and net photosynthetic rate has been reported previously [24]. Improvements in photosynthesis to increase grain yields within climate change scenarios should become one of the main targets in wheat breeding [25]. For example, according to the recent research by Kubar et al. [26], fertilization with nitrogen resulted in increased leaf area and significantly prompted the photosynthetic rate, which resulted in an improvement in grain yield. In addition, plants exhibit genetic variations in photosynthetic response under abiotic and biotic stresses [27,28]. When plants are exposed to disease stress factors, especially during the flowering stage, disruption in the photosynthetic apparatus can occur, causing a decrease in plant productivity and overall grain yield [29]. Some wheat genotypes showed a reduction in grain yield as a consequence of higher Fusarium head blight pressure due to increased precipitation during the anthesis stage [30].

Modern agriculture is faced with different climatic changes, and it needs faster selection for the creation of varieties with high, stable, and high-quality grain yields in different growing conditions. However, recognized winter wheat varieties cannot always guarantee high-quality grain crops. The solution to this problem can be obtained by the deeper study of photosynthetic activity (using chlorophyll *a* fluorescence measurements throughout the whole growing season in field conditions) of wheat plants and the use of indicators in selection. Therefore, the objectives of this study were to examine the effects of different weather conditions on photosynthetic parameters, grain yield, yield-related, and technological quality traits of six bread wheat varieties in two-year field experiments, as well to check the effects of photosynthesis on grain yield and quality of winter wheat.

2. Materials and Methods

2.1. Plant Material and Field Trial

Six winter wheat varieties (Rujana, Silvija, Fifi, Bubnjar, Anđelka, and Pepeljuga) from the Agricultural Institute Osijek were used for the experiment. They were previously characterized in the study of Duvnjak et al. [31]. This study was conducted at the experimental site of the Agricultural Institute Osijek (45°27' N, 18°48' E) during two growing seasons (2021/2022 and 2022/2023). The soil of the experimental site is eutric cambisol (Table 1). The previous crop was maize in the 2021/2022 growing season and soybean in the 2022/2023 growing season. In the basic fertilization in the first growing season, nitrogen, phosphorus, and potassium (NPK 0-20-30) were added in the amount of 400 kg ha^{-1} , and urea (46% nitrogen) in the amount of 100 kg ha^{-1} , while in the second season, $200 \text{ kg ha}^{-1} \text{ NPK } 7-20-30 \text{ and } 100 \text{ kg ha}^{-1} \text{ urea were applied. The field experiment was laid$ out in eight row plots 1.08 m wide and 7 m long with a surface area of plots 7.56 m^2 in four replicates in a randomized complete block design. Through two fertilization events (growth stage 25 (GS 25) and GS 35), the total amount of 300 and 200 kg ha⁻¹ calcium ammonium nitrate (CAN) was applied in the first and second growing seasons, respectively. The agro-technical measures used during this study are listed in Table 2. Fungicidal treatment was omitted.

Table 1. Soil properties of eutric cambisol at location Osijek.

Component	Quantity		
pHKCl	6.25		
Humus	2.00-2.20%		
P_2O_5	$39.70 \text{ mg } 100 \text{ g}^{-1}$		
H ₂ O	$37.70 \text{mg} \ 100 \text{ g}^{-1}$		

Table 2. Agro-technical measures during the growing seasons 2021/2022 and 2022/2023.

Agro-Technical Measure	Agent and Concentrations of Active Compounds		
	2021/2022		
Pre-sowing seed treatment in 2021	Maxim Extra 050 FS (fludioxonil 25 g L^{-1} ; difenoconazole 25 g L^{-1})		
Weed control in November 2021	Sharpen 330 EC (pendimethalin 330 g $ m L^{-1}$)		
Pest control in April 2022	Vantex 60 CS (gamma-cyhalothrin 60 g L^{-1})		
Weed control in May 2022	Lodin (fluroxypyr-metil 295.5 g L^{-1}) + Tribe 75 WG (tribenuron 750 g kg ⁻¹)		
Pest control in May 2022	Cythrin Max (cypermethrin 500 g L^{-1})		
	2022/2023		
Pre-sowing seed treatment in 2022	Maxim Extra 050 FS (fludioxonil 25 g L^{-1} ; difenoconazole 25 g L^{-1})		
Weed control in February 2023	Alister New (diflufenican 120 g L^{-1} ; jodosulfuron 7.5 g L^{-1} ; mesosulfuron 9 g L^{-1})		
Weed control in May 2023	Sekator (amidosulfuron 100 g L ⁻¹ ; jodosulfuron metil 25 g L ⁻¹) + Tribe 75 WG (triborumon 750 c lo^{-1})		
Pest control in April 2023	Cyclone (lambda-cyhalothrin 50 g L^{-1})		
Pest control in May 2023	Cyclone (lambda-cyhalothrin 50 g L^{-1})		

The amount of rainfall (mm) and average temperatures (°C) during the two growing seasons (October 2021–June 2022 and October 2022–June 2023) are shown in Figure 1A, obtained from the Croatian Meteorological and Hydrological Service. The total accumulated rainfall during the first growing season was 440.8 mm with an average temperature of 9.3 °C. The total accumulated rainfall in the second growing season was 564.2 mm with an average temperature of 10.2 °C. The daily rainfall and temperatures at the time when chlorophyll *a* fluorescence was measured are shown in Figure 1B.



Figure 1. Total monthly rainfall (mm) and average temperatures (°C) during the growing season (from October to June calculated for the period 1990–2023 and singled out for the growing seasons 2021/2022 and 2022/2023 in which this study was conducted) (**A**); and daily rainfall (mm) and temperatures (°C) during the period of chlorophyll *a* fluorescence measurements at seven points in 2021/2022 and 2022/2023 (21 January, 2 February, 17 March, 28 April, 12 May, 26 May and 6 June) (**B**).

The measurements of chlorophyll *a* fluorescence were conducted at seven measurement points: 1st on 21 January (GS 23), 2nd on 2 February (GS 25), 3rd on 17 March (GS 32), 4th on 28 April (GS 47), 5th on 12 May (GS 59), 6th on 26 May (GS 69), and 7th on 6 June (GS 71) in both growing seasons. The growth stages were determined according to the growth stages of cereals [32]. After the seventh measurement point, parameters of chlorophyll *a* fluorescence were no longer measurable as the plants entered senescence. Wheat plants were harvested on 28 June in 2021/2022 and 30 June in 2022/2023 when grain moisture was below 14%.

2.2. Measurement of Agro-Morphological Traits

The barley yellow dwarf virus symptoms were scored at the tillering stage, while yellow rust incidence was scored at the beginning of the stem elongation stage. These traits were supplementary due to the high pressure of virus and yellow rust in the second growing season. Plant height was measured in cm from the ground to the top of spikes, excluding awns, whereas stem height was measured from the ground to the base of spikes. The differences between plant and stem height was used to calculate spike length (cm). The heading date was recorded when more than 50% of the wheat plants in the plot were in the anthesis stage. Harvesting was performed using a Wintersteiger grain harvester, with grains taken from the entire plot. Grain yield was recorded and corrected to 14% moisture and expressed in dt ha⁻¹. Test weight (kg hL⁻¹) was determined using a GAC 2100 (DICKEY-john, AUBURN, US), while the MARVIN grain analyzer (MARVITECH GmbH, Wittenburg, Germany) was used to calculate the 1000 kernel weight (g).

2.3. Measurement of Grain and Flour Quality Traits

Technological quality parameters were measured in the Wheat Quality Laboratory of the Department for Breeding & Genetics of Small Cereal Crops (Agricultural Institute Osijek). Protein content was measured with the Infratec 1241, Foss Tecator. Wet gluten content was determined using ICC method No. 155, while ICC method No. 116/1 and ICC method No. 107/1 were used to measure the Zeleny sedimentation volume and Hagberg falling number [33].

2.4. Chlorophyll a Fluorescence Measurement

The chlorophyll *a* fluorescence of leaves was measured seven times (1st measurement: main shoot and three tillers stage (GS 23); 2nd measurement: main shoot and five tillers stage (GS 25); 3rd measurement: second node detectable stage (GS 32); 4th measurement: flag leaf sheath opening stage (GS 47); 5th measurement: ear complete emergence above flag leaf ligule (GS 59); 6th measurement: flowering complete stage (GS 69); and 7th measurement: watery ripe stage (GS 71)) in both growing seasons in the time frame from January till June using the Plant Efficiency Analyser (Handy PEA, Hansatech, Pentney, UK). Before measurement, five representative leaves per plot were fully dark-isolated for 30 min by using a lightweight leaf clip shutter plate. The chlorophyll *a* fluorescence was induced with a saturated red light pulse (3200 µmol m⁻² s⁻¹, peak at 650 nm). Parameters were calculated with JIP test to calculate biophysical parameters that quantify the stepwise energy flow through Photosystem II (PSII). The parameters calculated and included in this study were: maximum quantum yield of primary photochemistry (TR_O/ABS) and performance index on absorption basis (PI_{ABS}) [34,35]. The mean values of the two parameters were calculated from five measurements for each plot.

2.5. Statistical Analysis

For the agronomic and qualitative traits (grain yield, test weight, 1000 kernel weight, plant height, spike length, heading date, protein content, sedimentation value, wet gluten content, and Hagberg falling number), a combined analysis of variance (ANOVA) was performed over two years, whereas for the chlorophyll *a* fluorescence parameters (TR_O/ABS and PI_{ABS}), ANOVA was combined over growing seasons and measurement points. Fisher's least significant difference (LSD) test was used to compare trait means and Spearman's rank correlation analysis was performed to determine the association among traits. The statistical analyses were performed using Statistica software (version 14.0). Principal component analysis was performed using Addinsoft XLSTAT (New York, NY, USA).

3. Results

The analysis of variance (ANOVA) for agro-morphological and quality traits showed a significant effect of variety (V) for all traits, a significant effect of year (Y) for all traits except protein content and wet gluten content, and a significant V \times Y interaction for all traits except 1000 kernel weight (Table 3). Due to significant differences between years, the results will be presented separately for each growing season.

Table 3. Analysis of variance for agro-morphological and quality traits of six wheat varieties evaluated across two years.

		Mean Square									
Source of Variability	DF	Grain Yield	Test Weight	1000 Kenel Weight	Heading Date	Plant Height	Spike Length	Protein Content	Sedimentation Value	Wet Gluten Content	Hagberg Falling Number
Variety (V) Year (Y) $V \times Y$ Error	5 1 5 36	712.8 *** 24,797.5 *** 186.2 *** 9.1	64.0 *** 314.7 *** 15.4 *** 0.6	206.0 *** 278.4 *** 6.58 2.76	40.6 *** 80.1 *** 3.0 *** 0.4	1204.1 *** 475.7 *** 63.5 *** 4.7	13.6 *** 1160.3 *** 3.43 ** 1.9	1.1 *** 0.1 0.8 *** 0.1	67.1 *** 125.5 *** 54.0 *** 2.5	36.3 *** 1.2 15.6 *** 1.4	829.0 ** 1210.0 ** 464.0 ** 183

***, **, = significant at *p* < 0.001, 0.01, respectively; DF—degrees of freedom.

Analysis of variance for photosynthetic parameters showed a significant effect of measurement point (M) and a significant $Y \times M$ interaction for TR_O/ABS , whereas for PI_{ABS} variety, year, and measurement point, as well as $V \times M$, $Y \times M$, and $V \times Y \times M$, interactions were significant (Table 4).

Table 4. Analysis of variance for photosynthetic parameters TR_O/ABS and PI_{ABS} of six wheat varieties evaluated across two years and seven measurement points.

Source of Variability	DE	Mean Square			
Source of variability	DF	TR _O /ABS	PI _{ABS}		
Variety (V)	5	0.01	1.72 **		
Year (Y)	1	0.03	32.93 ***		
Measurement point (M)	6	0.32 ***	206.87 ***		
$V imes \dot{Y}$	5	0.01	0.82		
V imes M	30	0.01	2.16 ***		
$\mathbf{Y} imes \mathbf{M}$	6	0.13 ***	65.73 ***		
V imes Y imes M	30	0.08	3.29 ***		
Error	1596	0.01	0.05		

***, **, = significant at *p* < 0.001, 0.01, respectively; DF—degrees of freedom.

3.1. Agro-Morphological and Technologically Quality Traits in 2021/2022 Vegetative Season

In the 2021/2022 growing season, the highest grain yield was recorded for the varieties Bubnjar (104.0 dt ha^{-1}) and Rujana (102.5 dt ha^{-1}), while the lowest grain yield was recorded for variety Anđelka (88.8 dt ha^{-1}) (Figure 2A). Varieties Silvija (98.3 dt ha^{-1}), Fifi (97.6 dt ha^{-1}), and Pepeljuga (97.1 dt ha^{-1}) had a significantly lower grain yield, compared to Bubnjar.

The highest test weight (86.5 kg hL^{-1}) was observed in Fifi, followed by Rujana (85.9 kg hL^{-1}), while Anđelka had a significantly lower test weight (81.8 kg hL^{-1}), compared to all other varieties (Figure 2B).

The 1000 kernel weight of the variety Bubnjar was 43.4 g, which was significantly higher than the 1000 kernel weight of Fifi (39.8 g), Silvija (39.0 g), Pepeljuga (34.3 g), and Anđelka (31.6 g) (Figure 2C). The heading date of Bubnjar (May 10) was two days after the earliest heading date of Fifi and Anđelka (8), and two days earlier from the latest heading date of Rujana (12) (Figure 2D). The highest plant height (100.3 cm) was observed in Rujana, followed by Bubnjar (93.5 cm), while Anđelka had the lowest plant height (69.3 cm), which was significantly different from the other varieties (Figure 2E). The highest spike length (20.5 cm) was recorded in Pepeljuga, followed by Anđelka (20.0 cm), while varieties Bubnjar, Rujana, Silvija, and Fifi had significantly different spike length from the previous two varieties (Figure 2F).

Protein content in grains was highest in Fifi (14.5%) and Rujana (14.4%), and lowest in Bubnjar (13.5%), whose protein content was not significantly different from the protein content of Pepeljuga and Anđelka (Figure 3A). The sedimentation value was the highest in Bubnjar (42.0 mL) and Silvija (40.5 mL) (Figure 3B). Rujana had the highest wet gluten content (33.2%), which was significantly different from that of all other varieties, of which Bubnjar had the lowest value (27.1%) (Figure 3C). The Hagberg falling number was highest in Bubnjar (350.8 s), followed by Fifi (333.8 s), and the lowest falling number was observed in Rujana (305.5 s) (Figure 3D).



Figure 2. Grain yield in dt ha⁻¹ (**A**), test weight in kg hL⁻¹ (**B**), 1000 kernel weight in g (**C**), heading date that represents the date of heading in May for each variety (**D**), plant height in cm (**E**), and spike length in cm (**F**) of six winter wheat varieties in the 2021/2022 growing season. Data are presented as mean value of four replicates \pm standard deviation. Trait means followed by the same letter are not significantly different at *p* < 0.05 according to LSD test.



Figure 3. Protein content in % (**A**), sedimentation value in ml (**B**), wet gluten content in % (**C**), and Hagberg falling number in s (**D**) of six winter wheat varieties in the 2021/2022 growing season. Data are presented as mean value of four replicates \pm standard deviation. Trait means followed by the same letter are not significantly different at *p* < 0.05 according to LSD test.

3.2. Agro-Morphological and Technological Quality Traits in 2022/2023 Vegetative Season

During tillering in 2022/2023, all varieties were affected by the barley yellow dwarf virus to a similar extent, with incidence ranging from 2.3% in Anđelka to 4.8% in Silvija (Table 5). On the other hand, a greater variation was observed among varieties in the incidence of yellow rust during the stem elongation stage, ranging from 0% (Bubnjar) to 90% (Anđelka). In the previous growing season, there were no visible symptoms of barley yellow dwarf virus or yellow rust, so the varieties were not screened for symptoms. Further, a reduction in grain yield was observed in all varieties in the 2022/2023 growing season compared to 2021/2022, with a mean reduction of 46.4% (Table S1).

Table 5. Incidence of barley yellow dwarf virus and yellow rust in the 2022/2023 growing season.

Variety	Barley Yellow Dwarf Virus (%)	Yellow Rust (%)
Bubnjar	3.8	0
Silvija	4.8	3
Rujana	2.8	5
Pepeljuga	4.0	10
Fifi	3.2	20
Anđelka	2.3	90

The score was taken as the mean of four replicates.

In the second growing season, the highest grain yield was recorded for the variety Bubnjar (64.6 dt ha⁻¹), followed by Silvija (61.1 dt ha⁻¹) (Figure 4A). Grain yield of Rujana and Pepeljuga was not significantly different from Silvija, but was significantly different from Bubnjar. The lowest grain yield was recorded for the variety Anđelka (28.7 dt ha⁻¹). The highest test weight was recorded in Pepeljuga (84.6 kg hL⁻¹), and the lowest in Anđelka (72.3 kg hL⁻¹) (Figure 4B). The highest 1000 kernel weight was observed in Bubnjar (39.1 g) and Rujana (38.5 g), while Pepeljuga (27.4 g) and Anđelka (25.2 g) had the lowest 1000 kernel weight (Figure 4C). The latest heading date was recorded for Bubnjar (9 May) and Rujana (9 May), followed by Silvija (8 May), Pepeljuga (8 May), Anđelka (3 May), and Fifi (3 May) (Figure 4D). The highest plant height (111.3 cm) was recorded in Rujana and Bubnjar (103.8 cm), followed by Silvija and Fifi, while Pepeljuga and Anđelka had the lowest plant heights (79.0 and 77.3 cm, respectively) (Figure 4E). The spike length of the variety Pepeljuga (13.0 cm) was significantly greater than the spike length of all other varieties (Figure 4F).

Protein content in grains was the highest (14.9%) in Pepeljuga, followed by Fifi and Anđelka, while the lowest protein content (13.2%) was observed in Bubnjar (Figure 5A). The sedimentation value was highest in Pepeljuga (49.4 mL), followed by Bubnjar (42.0 mL) and Silvija (42.0 mL), while the lowest sedimentation value was found in Fifi (38.5 mL) and Rujana (36.8 mL) (Figure 5B). Pepeljuga and Rujana had the highest wet gluten content (33.0 and 31.2%, respectively), although that of Rujana was not significantly different from the wet gluten content of Fifi and Anđelka, while Bubnjar had the lowest wet gluten content (24.7%) (Figure 5C). The Hagberg falling number was highest in Fifi (322.8 s) followed by Anđelka (318.5 s), while the lowest falling number was observed for Pepeljuga (302.3 s) (Figure 5D).

3.3. Photosynthetic Parameters of the Leaves during the 2021/2022 Growing Season

In the 2021/2022 growing season, all wheat varieties had a significant gradual increase in TR_O/ABS from the 1st to the 3rd measurement point, except the variety Bubnjar, where the values of TR_O/ABS remained at the same level at the 2nd and 3rd measurement points (Figure 6A–F). Further, Bubnjar, Rujana, Pepeljuga, and Anđelka kept TR_O/ABS at the same level at the last three measurements, while Silvija significantly reduced it at the last measurement point (7th), compared to the previous one (6th). It is also evident that Rujana, Fifi, and Anđelka significantly increased TR_O/ABS at the 7th measurement point, compared to the 2nd measurement point, Bubnjar and Pepeljuga significantly increased it

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compared to the 4th and 3rd measurement points, while Silvija significantly increased it at the 7th measurement point, compared to the 6th. All varieties significantly increased TR_O/ABS at the 7th measurement point, compared to the 1st measurement point.

Figure 4. Grain yield in dt ha⁻¹ (**A**), test weight in kg hL⁻¹ (**B**), 1000 kernel weight in g (**C**), heading date that represents the date of heading in May for each variety (**D**), plant height in cm (**E**), and spike length in cm (**F**) of six winter wheat varieties in the 2022/2023 growing season. Data are presented as mean value of four replicates \pm standard deviation. Trait means followed by the same letter are not significantly different at *p* < 0.05 according to LSD test.



Figure 5. Protein content in % (**A**), sedimentation value in ml (**B**), wet gluten content in % (**C**), and Hagberg falling number in s (**D**) of six winter wheat varieties in the 2022/2023 growing season. Data are presented as mean value of four replicates \pm standard deviation. Trait means followed by the same letter are not significantly different at *p* < 0.05 according to LSD test.





Figure 6. The maximum quantum yield of primary photochemistry (TR_O/ABS) of varieties Bubnjar (**A**), Rujana (**B**), Silvija (**C**), Fifi (**D**), Pepeljuga (**E**), and Anđelka (**F**) at seven measurement points (1st—GS 23, 2nd—GS 25 2, 3rd—GS 32, 4th—GS 47, 5th—GS 59, 6th—GS 69, and 7th—GS 71) in the 2021/2022 growing season. Data are presented as mean value of 20 replicates \pm standard deviation. Trait means followed by the same letter are not significantly different at *p* < 0.05 according to LSD test.

The variety Bubnjar had PI_{ABS} at the same level at the 2nd and 3rd measurement, while Rujana remained the values of PI_{ABS} at the same level at the 1st and 2nd measurement (Figure 7A,B). Silvija and Fifi significantly gradually increased PI_{ABS} from the 1st to the 3rd measurement point, Pepeljuga to the 4th, and Anđelka to the th measurement point (Figure 7C–F). It can also be seen that only variety Fifi kept the PI_{ABS} value at the same level from the 3rd to the 5th measurement point. All varieties significantly decreased PI_{ABS} at the last measurement point (7th) compared to the previous one (6th).

3.4. Photosynthetic Parameters of the Leaves during the 2022/2023 Growing Season

In the 2022/2023 growing season, a significant decrease in TR_O/ABS was observed at the 3rd measurement, compared to the 1st measurement in the varieties Bubnjar, Silvija, Rujana, and Anđelka (Figure 8A–C,F). All varieties significantly increased it at the 5th measurement point, compared to the 1st, except Pepeljuga (Figure 8A–F). All varieties significantly decreased TR_O/ABS at the last measurement point (7th) compared to the 5th, except Fifi.

a

ab

ab

RUJANA

b

ab


Figure 7. Performance index on absorption basic (PI_{ABS}) of varieties Bubnjar (**A**), Rujana (**B**), Silvija (**C**), Fifi (**D**), Pepeljuga (**E**), and Anđelka (**F**) at seven measurement points (1st—GS 23, 2nd—GS 25 2, 3rd—GS 32, 4th—GS 47, 5th—GS 59, 6th—GS 69, and 7th—GS 71) in the 2021/2022 growing season. Data are presented as mean value of 20 replicates \pm standard deviation. Trait means followed by the same letter are not significantly different at *p* < 0.05 according to LSD test.

The PI_{ABS} value significantly decreased at the 2nd measurement point compared to the 1st in varieties Bubnjar and Silvija, while other varieties remained PI_{ABS} at the same level during the first three measurements (Figure 9A–F). At the 5th measurement point, all varieties significantly increased PI_{ABS}, compared to the previous measurements. Further, Bubnjar, Silvija, Pepeljuga, and Anđelka significantly decreased it at the 6th measurement point, compared to the 5th, while Fifi and Rujana kept it at the same level. Only Fifi and Pepeljuga significantly decreased it at the 7th measurement point, compared to the 6th.



Figure 8. The maximum quantum yield of primary photochemistry (TR_O/ABS) of varieties Bubnjar (**A**), Silvija (**B**), Rujana (**C**), Pepeljuga (**D**), Fifi (**E**), and Anđelka (**F**) at seven measurement points (1st—GS 23, 2nd—GS 25 2, 3rd—GS 32, 4th—GS 47, 5th—GS 59, 6th—GS 69, and 7th—GS 71) in 2022/2023 vegetative season. Data are presented as mean value of 20 replicates \pm standard deviation. Trait means followed by the same letter are not significantly different at *p* < 0.05 according to LSD test.

3.5. Correlation between Traits

The principal component analysis (PCA) and correlation analysis for the 2021/2022 and 2022/2023 growing seasons are presented in Figures 10A and 10B, Table S2 and Table S3, respectively.

In the 2021/2022 growing season, the 1000 kernel weight, heading date, and TR_O/ABS (2nd, measured on 2 February) were found to be significantly positively correlated with grain yield, as was PI_{ABS} (3rd, measured on 17 March) with test weight (Figure 10A; Table S2). There was also a positive correlation of TR_O/ABS (2nd and 7th, measured on 2 February and 6 June) with 1000 kernel weight, TR_O/ABS (2nd, measured on 2 February) with plant height and heading date, and PI_{ABS} (1st and 5th, measured on 21 January and 26 May) with spike length. Protein content showed a positive correlation with wet gluten content and TR_O/ABS (6th, measured on 26 May), but a negative correlation with PI_{ABS} (2nd, measured on 2 February). In addition, sedimentation value was significantly negatively correlated with PI_{ABS} (4th, measured on 28 April), but positively correlated with TR_O/ABS and PI_{ABS} (3rd, measured on 17 March), but negatively correlated with PI_{ABS} (2nd, measured on 2 February). TR_O/ABS (1st, measured on 21 January) and PI_{ABS} (2nd, measured on 2 February). TR_O/ABS (1st, measured on 21 January) and PI_{ABS} (2nd, measured on 2 February). TR_O/ABS (1st, measured on 21 January) and PI_{ABS} (2nd, measured on 2 February). TR_O/ABS (1st, measured on 21 January) and PI_{ABS} (2nd, measured on 2 February). TR_O/ABS (1st, measured on 21 January) and PI_{ABS} (2nd, measured on 2 February). TR_O/ABS (1st, measured on 21 January) and PI_{ABS} (2nd, measured on 2 February).

measured on 26 May). A positive correlation was found between TR_O/ABS and PI_{ABS} at the 3rd measurement (measured on 17 March), and between PI_{ABS} in the 1st and 5th measurement (measured on 21 January and 12 May). In the 2022/2023 growing season, a significant negative correlation was observed between grain yield and TR_O/ABS (4th, measured on 28 April), while 1000 kernel weight was in positive correlation with plant height and TR_O/ABS (2nd, measured on 2 February) and negatively correlated with PI_{ABS} (4th, measured on 28 April) (Figure 10B; Table S3).



Figure 9. Performance index on absorption basic (PI_{ABS}) of varieties Bubnjar (**A**), Silvija (**B**), Rujana (**C**), Pepeljuga (**D**), Fifi (**E**), and Anđelka (**F**) at seven measurement points (1st—GS 23, 2nd—GS 25 2, 3rd—GS 32, 4th—GS 47, 5th—GS 59, 6th—GS 69, and 7th—GS 71) in the 2022/2023 growing season. Data are presented as mean value of 20 replicates \pm standard deviation. Trait means followed by the same letter are not significantly different at *p* < 0.05 according to LSD test.

Plant height was in positive correlation with TR_O/ABS (2nd, measured on February 2), but negatively correlated with PI_{ABS} (4th, measured on 28 April). Heading date correlated negatively with TR_O/ABS (4th, measured on 28 April), as did spike length with Hagberg falling number and PI_{ABS} (1st, measured on 21 January). Protein content correlated negatively with TR_O/ABS (3rd, measured on 17 March), and positively with PI_{ABS} (4th, measured on 28 April), while sedimentation value correlated negatively with PI_{ABS} (7th, measured on 6 June) and TR_O/ABS (7th, measured on 6 June). Wet gluten content correlated positively with PI_{ABS} (6th, measured on 26 May). TR_O/ABS (1st, measured on 21 January) was in positive correlation with TR_O/ABS (3rd, measured on 17 March), while TR_O/ABS (2nd, measured on 2 February) showed a positive correlation with TR_O/ABS



Biplot (axes F1 and F2: 64.94 %)

Figure 10. Principal component analysis (PCA) showing the relationship between grain yield, test weight, 1000 kernel weight, plant height, heading date, spike length, protein content, sedimentation value, wet gluten content, Hagberg falling number, and photosynthetic parameters (TRO/ABS 1st, $TR_O/ABS\ 2nd,\ TR_O/ABS\ 3rd,\ TR_O/ABS\ 4th,\ TR_O/ABS\ 5th,\ TR_O/ABS\ 6th,\ TR_O/ABS\ 7th,\ PI_{ABS}\ 1st,\ NR_O/ABS\ 7th,\ PI_{ABS}\ 7t$ PIABS 2nd, PIABS 3rd, PIABS 4th, PIABS 5th, PIABS 6th, and PIABS 7th) of six winter wheat varieties (Rujana, Silvija, Fifi, Anđelka, Bubnjar, and Pepeljuga) at (A) 2021/2022 and (B) 2022/2023 growing season.

4. Discussion

Wheat productivity is highly influenced by climate changes. Both temperature and rainfall variations have a significant impact on plants' phenology [36]. One of the most effective adaptation strategies to climate change is the development of new genetic wheat varieties with improved tolerance to biotic and abiotic stresses, where plants could take advantage of periods of optimal temperatures and rainfall [37]. It is believed that the efficiency of photosynthetic energy conversion could be increased under field conditions [38], thereby contributing to a higher grain yield [39]. To analyze the relationship between photosynthesis and other investigated traits, we systematically measured photosynthesis-related parameters in a time-course manner in six winter wheat varieties.

4.1. Vegetative Season 2021/2022

In the 2021/2022 growing season, after sowing, an increase in rainfall was recorded, compared to the multi-year average, but this trend did not continue during the growing season. From January to April in 2022, a rainfall deficit was recorded, so this period was declared dry. This period includes root growth, leaf emergence on the main shoot, tillering, and the beginning of the stem elongation stage. However, despite this four-month long drought period, wheat plants were likely able to absorb water from the soil reserves accumulated during the previous period (October to December 2021), which allowed them to achieve normal development of tillers and stem elongation. A recent study has shown that adequate levels of soil water storage in the early stage can ensure the promotion of tillering in winter wheat, and finally increase the effective number of tillers to increase the grain yield [40]. This could especially be the case for the varieties Bubnjar and Rujana, previously declared drought tolerant [31], which in the 2021/2022 growing season had higher grain yields (above 100 dt ha $^{-1}$) than the other four varieties with yields between 88 and 98 dt ha $^{-1}$. Therefore, the observed differences in grain yield among the six varieties could be, to some extent, the result of their different tolerance to drought. According to the previous research of Eitzinger et al. [41], drought has significant negative effects on the grain yield of winter wheat, especially during the flowering and grain-filling stages. Furthermore, drought stress during the flowering stage can negatively impact net photosynthetic rate, reduce the period of photosynthesis, and considerably increase flag leaf senescence [42]. In the current study, drought was not pronounced during the flowering period through April–June in 2022. Obembe et al. [1] reported that a one cm reduction in precipitation from the average decreases grain yield by 1.35% in the fall, 1.11% in the winter, and 0.3% in the spring. Due to climate changes in Southern Europe, the increase in temperatures accompanied by drought intervals have resulted in a reduction in wheat yield of about 5% [43].

The correlation matrix showed that grain yield was significantly positively correlated with 1000 kernel weight and plant height, which was consistent with previous studies [44–46]. Further, grain yield was significantly correlated with TRo/ABS at the 2nd measurement that coincides with tillering stage. The previous study indicated that enhanced photosynthesis, even at the level of a single leaf, may increase plant yields [47]. Tillering is the stage when plants start to produce side shoots (tillers), which are very important for wheat productivity [48]. This growth stage is controlled by the environment from the three-leaf stage to the jointing stage (GS 13–GS 30). The tillering stage depends on the genetic background, and it has previously been observed that fertile tillers per plant are associated with more total shoots initiated, faster tillering rate, delayed tillering onset, and higher survival [49]. It is believed that the growth of tillers is regulated by the concentration and ratio of phytohormones such as indole-3-acetic acid (IAA), abscisic acid (ABA), and zeatin (ZT) in tiller nodes [50]. ABA application reduced the effect of drought stress, increased photosynthetic parameters, and decreased the decline in the functions of photosystem II [51]. It is important that those tillers first initiated at the plant will always have an advantage in growth and development, compared to those initiated later [48]. In the current research, from January to April in 2022, there was a lack of precipitation and it can be assumed that plants were in mild drought stress not in strong one as a result of sufficient water accumulation in the soil in previous months. High yield is still possible even if there is less precipitation during the growing season [52]. In a previous study of Duvnjak et al. [31], ABA was increased under drought stress, suggesting its role as a

hormone involved in the regulation of stress response, such as the increase in the number of leaves and tillers in drought stress conditions, and further maintaining turgor pressure and osmotic adjustment in leaves. In the same study, the ABA increase was particularly pronounced in the variety Bubnjar. In the current study, the same variety was the most yielding and also the only variety where TR_O/ABS and PI_{ABS} remained at the same level during the 2nd and 3rd measurements. We can assume that Bubnjar maintained lower photosynthetic activity during that phase, compared to other varieties, thus enabling more productive tillering. It was previously concluded that more drought-tolerant wheat varieties could conserve water content in photosynthetic tissue, that in our case are represented by the leaves, where evapotranspiration water losses could be less pronounced [53]. This means that all wheat varieties, except Bubnjar, accelerated photosynthesis during tillering and probably went through this stage faster than in Bubnjar. Also, Bubnjar had the highest 1000 kernel weight. This could also be explained by the fact that tillers with a larger leaf area will produce more kernels, heavier kernels, and are less likely to be lost due to tiller mortality [48]. The 1000 kernel weight strongly correlated with the values of TR_O/ABS at the 2nd measurement, but also at the last (7th) measurement, which coincided with the grain-filling stage. Zhang et al. [54] suggested that increasing flag leaf photosynthesis improves the 1000 kernel weight, contributing to high grain yield.

Plant height and grain yield were also significantly positively correlated, and it was observed that the two highest yielding varieties (Bubnjar and Rujana) were also the tallest. This was in accordance with the research of Mahdy et al. [55], while Spanic et al. [56] reported a negative correlation between plant height and grain yield, but in the case of very tall varieties used in the past.

Further, the current study showed that the highest yielding varieties were the latest in maturity, as the heading date was in significant positive correlation with plant height, which in turn was positively correlated with grain yield. Heading date is critical as this is the stage immediately followed by flowering that could be delayed and reproductive development accelerated, resulting in reduced grain-filling [57]. It is interesting to note that the heading date was closely related to TR_O/ABS at the 2nd measurement, but also with the grain yield, 1000 kernel weight, and plant height. It was observed that spike length was significantly positively correlated with PI_{ABS} at the 1st and 5th measurement. Zhou et al. [58] did not found a significant difference between the spike length and spikelet number of two wheat varieties, although they differed in the number of grains per spike and therefore greater total grain volume per spike.

Protein content and wet gluten content were significantly positively correlated, which was in line with the previous research of Kaushik et al. [59], as the gluten–protein complex is derived from the storage proteins of wheat grain. However, the correlation matrix showed that protein content was strongly positively correlated with TR_O/ABS at the 6th measurement, which coincided with the beginning of the grain-filling stage. This was expected since proteins are formed at this stage [60]. In addition, the higher sedimentation values indicate a high protein quantity and/or stronger gluten [61]. It was observed that the sedimentation value was in significant positive correlation with the Hagberg falling number, which is an indicator of α -amylase activity. In the study of Laidig et al. [62], there were strong relationships between protein content, sedimentation value, and loaf volume.

4.2. Vegetative Season 2022/2023

In contrast to the first growing season, in the second growing season (2022/2023), significantly less rainfall was recorded at the time of sowing. According to the previous research of Iizumi and Ramankutty [63], wheat sowing under severe drought has the shortest grain-filling duration. However, in the present study, a higher amount of rainfall was recorded from November 2022 to May 2023, compared to the multi-year average.

Although increased amounts of rainfall were recorded, grain yield drastically decreased in all varieties studied, largely owing to a sharp increase in leaf and spike diseases associated with a high incidence of aphids in the autumn, which are vectors of barley yellow dwarf virus and weakened the plants already in the autumn. Aphids have been reported to be responsible for transmitting 50% of the virus [64] and virus infection reduced grain yield in wheat by up to 84% [65]. In the current study, disease development was favored by large amounts of rainfall during April and May 2023. In addition, high pressure of yellow or stripe rust (Puccinia striiformis) was recorded starting from April. It was concluded that yellow rust can cause more than a 25% reduction in grain yield [66]. Other studies have reported 10-16% grain yield losses due to diseases and pests [67,68]. In addition, yellow rust, Septoria spp. were present throughout the growing season, and furthermore, Fusarium head blight (FHB) occurred during flowering, but data for FHB and Septoria attacks are not shown due to the severe attack of yellow rust. The frequency of either very wet days (>10 mm rainfall) or consecutive wet days (three days with at least 1 mm of rain) during the early growth of the wheat crop has been found to be one of the most important factors in the distribution of Septoria tritici [69]. Grain yield losses can reach 25 to 50% under severe epidemics with Septoria spp. [70]. FHB infection was also expected in the current study as a result of the increased temperatures and rainfall in April and May 2023, as the most susceptible period for *Fusarium* infection of wheat is the flowering stage, with optimal temperatures for infection between 20 and 25 °C and moisture content of 95% [71]. Further, FHB-infected grains become shriveled thus reducing grain yield and quality of wheat [72].

TRo/ABS at the 4th measurement was in significant negative correlation with grain yield, indicating that lower TR_O/ABS at this stage resulted in higher grain yields. The stage at the 4th measurement corresponds with the stem elongation when severe infection with yellow rust occurred. This is also the stage when the transition from the vegetative to the reproductive stage occurs and when spikelet primordiums are formed, showing its importance for spike development [31]. It is likely that the varieties that retained TR_O/ABS at lower rates during this period maintained the energy for the generative development stage. It has been previously reported that the number of spikelets and grains will be reduced when stress occurs in the stem elongation stage [73]. Three varieties (Bubnjar, Silvija, and Rujana) showed the lowest increase in TR_O/ABS from the 3rd to the 4th measurement, compared to other varieties with a lower grain yield. The presence of any type of stress can inactivate or damage PSII, leading to a decrease in TR_O/ABS [29]. However, a drastic decline in the activities of PSII and PSI was not the case for any of the varieties that could inhibit photosynthesis in the last four measurements.

Further, varieties Bubnjar, Silvija, and Rujana had higher 1000 kernel weight, plant height, and were later in maturity. Heading dates showed a negative correlation with TR_{O}/ABS at the 4th measurement where those varieties that were later in maturity could have escaped virus/disease pressure more efficiently than earlier varieties. Further, the varieties with the highest grain yield (Bubnjar, Silvija, and Rujana) showed an increase in PI_{ABS}, or a slight decrease in the case of Silvija, from the 6th to the 7th measurement, compared to other varieties showing a pronounced decrease between these two measurements. In the previous research, it was reported that longer integrity of functional PSII units and extended preservation of the optimal level of energetic connectivity among PSII units during grain filling resulted in better agronomic performance [28]. Higher grain yield resulted from increased 1000 kernel weight, which could be related to the source/sink ratio established during the early grain-filling stage [74]. Usually, high or low photosynthetic activity may be used as a very important quality indicator in wheat varieties. Furthermore, photosynthetic efficiency is especially important during flowering and the early grainfilling stage as its reduction at these stages can lead to spikelet sterility and lower grain yield [29]. Also, it was reported that 70% of grain yield is produced by photosynthesis in leaf and spike tissues after the heading stage [75]. In the current study, biotic stress as a result of viral and disease attack occurred much earlier than the heading stage began (around stem elongation stage due to yellow rust pressure). After this stage, varieties with better photosynthetic efficiency were able to save up energy better.

The lowest protein content was observed in Bubnjar, Rujana, and Silvija, which was to be expected, since these varieties were the most yielding. It is very well known that higher grain yields are associated with lower protein concentration [76]. It is difficult to achieve high grain yield and high protein content at the same time, because of the negative correlation between these two traits [77–79]. It is hypothesized that the negative correlation is the result of competition between carbon and nitrogen for energy [80] and the dilution effect of nitrogen by carbon-based compounds [81]. However, based on the observed negative correlation between TR_O/ABS at the 3rd measurement during tillering and PI_{ABS} at the last measurement (7th) in the dough stage, it can be assumed that photosynthesis had an influence on protein formation, especially at the last measurement. This could also be observed for the sedimentation value, which negatively correlated with the parameters of photosynthesis at the last measurement (7th). It has previously been found that stress during the grain-filling stage in wheat significantly modified kernel traits, grain protein content, and composition [82]. In the current study, temperatures during June were above the 33-year average, which could result in a decline in technological quality. It has been observed that heat stress (>27 °C) is common during the grain-filling stage that deteriorates cellular organelles and reduces photosynthesis [83].

5. Conclusions

In both years, Bubnjar, Rujana, and Silvija may have delayed leaf senescence during the reproductive period, resulting in a higher 1000 kernel weight and grain yield. In this experiment, we determined that grain yield was in a significant positive correlation with 1000 kernel weight and TR_O/ABS at the 2nd (GS 25) and the 7th measurement points (GS 71) in the first growing season. In addition, our results showed that the variety Bubnjar was the highest yielding (104.0 dt ha⁻¹) and the only variety where TR_O/ABS and PI_{ABS} remained at the same level at the 2nd and 3rd measurement (GS 32), which coincided with the tillering stage when a mild drought was recorded. In the second growing season (2022/2023), grain yield drastically decreased (46.4% for all varieties together), compared to the first one, due to infestations of leaf and ear diseases on plants already weakened by viruses in this season. Furthermore, our results indicated that the varieties with the highest grain yield showed the smallest change in PI_{ABS} from the 6th (GS 69) to the 7th measurement, compared to the other varieties. Overall, grain yield, 1000 kernel weight, plant height, and heading date were positively associated. Increased knowledge about the extent of genetic variation in the chlorophyll fluorescence will increase our understanding of wheat adaptations in relation to different weather conditions. Understanding the physiology of wheat in future investigations will also aid in the identification of better and more stable varieties for adaptation to stressed environments.

Supplementary Materials: The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/agronomy14030478/s1, Table S1: Reduction in grain yield in the 2022/2023 relative to the 2021/2022 growing season; Table S2: Spearman's rank correlation coefficients between twenty-four traits in 2021/2022 growing season; Table S3: Spearman's rank correlation coefficients between twenty-four traits in 2022/2023 growing season.

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Article Effects of Drought at Anthesis on Flag Leaf Physiology and Gene Expression in Diverse Wheat (*Triticum aestivum* L.) Genotypes

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Abstract: The current study aimed to quantify the effects of two drought intensities achieved by deprivation of watering for 45 and 65% of the volumetric soil moisture content (VSMC) for 14 days after wheat anthesis to identify physio-biochemical and molecular changes associated with drought tolerance in six genotypes with different drought tolerance. Drought at 65% of VSMC induced a significant decrease in the chlorophyll a content in the drought-sensitive genotype, which indicated a strong loss of photosynthetic reaction centres. Further, in the drought-tolerant genotype, the content of carotenoids, which could play a vital role in resisting water shortage stress, tended to increase. The increased production of malondialdehyde showed that the antioxidant system in the drought-sensitive genotypes was not properly activated. A significant decrease in catalase (CAT; EC 1.11.1.6) was observed at a 45% reduction in VSMC, compared to the control, in the drought-sensitive genotype, and at a reduction in VSMC of 65%, in all medium sensitive genotypes. Further, the drought-tolerant and -medium tolerant genotypes responded to drought with a decline in total glutathione concentrations with the intention to reinforce their defence system. Thereby, dehydroascorbate reductase (DHAR; EC 1.8.5.1), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), and glutathione reductase (GR; EC 1.6.4.2) were critical enzymes involved in the ascorbate-glutathione cycle together with CAT, showing their main role in the detoxification of ROS produced with the reduction in VSMC by 65%. The results of gene expression analysis showed that severe drought increased the levels of the DHN5 and WZY2 genes (that were significantly positively correlated) in the drought-tolerant genotype, whose grain weight, area, and length did not change in maturity. Also, it was seen that DHN5 expression showed a significant positive correlation with grain length and proline content at a 45% reduction in VSMC. The identification of different mechanisms under drought can contribute to the selection of drought-tolerant genotypes.

Keywords: drought; molecular response; physiology; wheat

1. Introduction

Wheat (*Triticum aestivum* L.) is the basic food for an estimated 35% of the population, with a global production of 700 million tons [1]. Also, wheat is one of the four major crops grown worldwide, and drastic environmental and climatic changes dramatically influence grain yields [2]. Among stresses, drought is a major abiotic stress limiting wheat productivity worldwide and resulting in grain yield losses of up to 86% [3]. The fact is that climate change has altered the average amount of precipitation on land, which has increased the frequency of droughts [4]. Drought is also characterized by a reduction in water in the atmosphere and soil, which causes wastage of water transpiration and evaporation [5]. Hence, global food security is threatened by drought events that are declared a major stress on crop production, due to the low amount of precipitation and



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Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). high temperatures associated with them [6]. Therefore, to satisfy the needs of the world's fast-increasing population and to ensure food security, wheat production must double by 2050 [7]. In the current context of both climate changes and increasing population over the globe, the main challenge for breeders is to enlarge the wheat yields.

During the growing season, wheat plants are under the influence of many biotic or abiotic stresses, each affecting the development of plants. As a consequence, severe morphological, biochemical, and physiological changes might occur in wheat plants. Drought affects all growing stages of wheat causing a delay in the germination of seeds, tillering, booting, heading, anthesis, grain filling, and maturity [8]. Hence, it has a negative impact on physio-morphological traits such as shoot and root length, relative water content, photosynthesis activity, and leaf area in wheat plants [9]. It was previously reported that the most sensitive periods to drought are the anthesis and grain filling stages when the highest grain yield losses are expected [10]. It was described that the initiation of flowering and inflorescence are badly affected by drought [11]. Thus, the reduction in grain yield may vary from 1% to 30% during mild drought in the post-anthesis stage or even reach 92% in the case of prolonged mild drought in the anthesis stage and during grain formation. Finally, a water deficit can negatively affect plant growth and development by modifying different agro-physiological and biochemical processes and pathways [12]. However, plants can cope with drought by different mechanisms: (i) by finishing the life cycle before the occurrence of severe drought; (ii) through water-conserving mechanisms such as the closure of stomata and a reduction in leaf area; (iii) through osmotic adjustment and increased cell wall elasticity; (iv) through increased antioxidant metabolism [13]. Drought influences photosynthesis due to limitations in the CO₂ influx, resulting in damage to chloroplast and chlorophyll structure, thylakoid membrane, and photosystem II and in the disruption of electron transport. Also, the earliest affected process under drought is photosynthesis [14]. The first modification that occurs in wheat tissue during drought events is the closure of the stomata as a result of other processes, such as a reduced water content in guard cells [15]. The activity of Rubisco is also disturbed, resulting in reduced photosynthesis rate [16]. Further, the rate of photorespiration increases dramatically, causing the production of reactive oxygen species (ROS) and lipid peroxidation [17]. Mitochondria are the most important sources of ROS, such as superoxide and hydrogen peroxide (H_2O_2), and ROS-scavenging systems try to eliminate them [18]. After ROS production, an increase in the expression of genes encoding antioxidants will start, leading to the intensification of the antioxidative system activity. ROS can be eliminated by both enzymatic and non-enzymatic antioxidative defence [19]. Enzymatic defence involves superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferases (GST), ascorbate peroxidase (APX), monodehydroascorbate reductase (MD-HAR), and dehydroascorbate reductase (DHAR) [20]. Usually, MDHAR, DHAR, and GR are important in maintaining redox homeostasis under drought [21]. One of the most common non-enzymatic antioxidants is glutathione (GSH), which influences the stability of the redox state in all parts of the plant cell [22]. Further, a non-enzymatic antioxidant defence system includes proline, tocopherol, phenolic compounds, and carotenoids. In the case of genotypic sensitivity to drought, the plant response will fail, resulting in cell damage. It was previously reported that traits associated with drought tolerance include high soluble sugar and chlorophyll content, low gas exchange, increased proline and carbohydrate content, and decreased superoxide dismutase concentration [12]. However, the physiological responses of plants to drought can vary in different stages of plant development, and the responses in the final stages of wheat growth are not very much explored.

As mentioned, the physiological as well as the biochemical responses to drought are controlled by an extensive modification of gene expression [23]. One of the most characterised transcription factor families involved in plant abiotic stress is the apetala2/ethylene-responsive element-binding protein (AP2/EREBP) family including dehydration-responsive element-binding proteins (DREBs), which regulates developmental, physiological, and metabolic processes [5,24]. The DREB subfamily can influence the expression of multiple

dehydration-regulated (DRE)/C-repeat element (CRT) genes through their dehydration- or cold-regulated (RD/COR) gene promoters, which respond to drought and low temperatures [25,26]. It was shown that in wheat, many genes encoding different DREB transcription factors are regulated under drought, thereby improving tolerance to drought [27–29]. According to research by Abedini et al. [30], the accumulation of dehydrin transcripts or proteins was positively correlated with drought tolerance. Several studies reported that overexpression of the dehydrin gene DHN5 resulted in tolerance to osmotic and salt stress in Arabidopsis plants as a consequence of the regulation of proline content and improved antioxidative response [31,32], while the dehydrin gene WZY2 may have a main role in the signalling pathway of ABA through interaction with 2C protein phosphatases, influencing the expression of stress-responsive genes in wheat [28]. It was previously observed that proline accumulation is the cause of increased osmotic stress tolerance in wheat [33]. Therefore, the expression of the *P5CS* gene encoding the enzyme pyrroline-5-carboxylate synthase (P5CS) in wheat was enhanced under osmotic stress in positive correlation with the proline content [33]. Also, it was observed that in drought, the overexpression of the *P5CS* gene in wheat transgenic plants resulted in increased stress tolerance [34].

The traditional drought tolerance assessments are largely based on determining survival rates, yield, and other growth indicators, with less attention given to physiological and cellular-level oxidative stress responses, such as chlorophyll content, malondialdehyde (MDA) levels, proline content, and changes in the antioxidant enzyme system. These enzymes, including SOD, CAT, and APX, play crucial roles in regulating plant adaptation to drought. The existing standards may not fully consider these biochemical parameters, potentially overlooking the link between plant antioxidant capacity and drought tolerance.

Furthermore, the choice of flag leaves as the study subject and the initiation of measurements 14 days post-anthesis is due to the significant role of flag leaves in photosynthesis and biomass accumulation in wheat, directly affecting grain weight and yield. The two weeks post-anthesis represent a critical period in wheat development, during which drought can directly impact the grain yield. Given this, the current study aims to develop a new set of evaluation standards that consider both enzymatic and non-enzymatic physiological indicators to more comprehensively assess the drought tolerance of wheat genotypes. By systematically analysing the activity of antioxidant enzymes and key physiological indicators in flag leaves, we hope to reveal the intrinsic connections between these parameters and wheat drought tolerance, providing more precise selection criteria for breeding work.

2. Materials and Methods

2.1. Plant Material and Experimental Layout

In the current study, six winter wheat genotypes (Bubnjar, Pepeljuga, Anđelka, Rujana, Fifi, and Silvija) from the Agriculture Institute Osijek were investigated at different levels of drought in the anthesis stage. These genotypes were previously characterised in the study of Duvnjak et al. [8] and sorted according to their drought tolerance (Table 1). After the germination stage, seedlings of each wheat genotype were moved in a plant growth chamber for a period of vernalisation of six weeks under conditions of 12 hours of day/ 12 hours of night $(4/3 \,^{\circ}\text{C})$. After vernalisation, the plants were moved into a greenhouse (Gis Impro d.o.o., Vrbovec, Croatia). Each genotype was subjected to three treatments: a control treatment (C) with regular irrigation and two drought treatments at different intensities (T1 and T2). In the control treatment (C), the volumetric soil moisture content (VSMC) was maintained at 30–35%, while in the drought treatments, the water content was reduced by 45% (T1) and 65% (T2). The measurement of VSMC was carried out daily by a soil moisture measuring device (TDR 150 Soil Moisture Meter, Spectrum Technologies, Aurora, IL, USA). After simulating drought during two weeks, the flag leaves of the plants were sampled, frozen in liquid nitrogen, and stored at -80 °C. Prior to extraction for further analyses, the flag leaves were ground in 10 mL stainless steel jars together with a grinding ball for 1 min at 30 Hz in a TissueLyser II bead mill (Qiagen, Hilden, Germany).

Wheat Cultivar	Origin	Registration Year	Drought Tolerance
Bubnjar	AIO, HR	2016	tolerant
Pepeljuga	AIO, HR	2017	medium tolerant
Anđelka	AIO, HR	2008	medium tolerant
Rujana	AIO, HR	2017	medium sensitive
Fifi	AIO, HR	2016	medium sensitive
Silvija	AIO, HR	2010	sensitive

Table 1. Origin and tolerance of wheat genotypes to drought.

Abbreviations: AIO, Agricultural Institute Osijek; HR, Croatia.

2.2. Determination of the Photosynthetic Pigments

The photosynthetic pigments were determined by a method described by Lichtenthaler [35]. The mass of sample for pigment analysis was 0.01 g. The lyophilized wheat flag leave tissue powder obtained after grinding was homogenised in absolute acetone. Further, extraction was carried out for 15 min at 4 °C and centrifugation for 15 min at 16,000× g and 4 °C. This procedure was repeated until the plant material was uncoloured. The absorption of the extracted material was measured spectrophotometrically at 470, 645, and 662 nm, and the photosynthetic pigment concentrations are reported as mg g⁻¹ DW.

2.3. Determination of Malondialdehyde (MDA)

The malondialdehyde (MDA) content was measured using thiobarbituric acid (TBA) [36]. The procedure was based on the production of a red colouration from lipid peroxidation breakdown products with TBA. Frozen flag leaf tissue powder (0.2 g) was homogenised in a 0.1% trichloroacetic acid (TCA) solution (1/5, w/v) and centrifugated at $10,000 \times g$ for 10 min at 4 °C. The incubation of the reaction mixture that consisted of 0.5 mL of tissue extract and 1 mL of reagent (0.5% TBA in 20% TCA) was carried out for 30 min at 95 °C on a TS-100 Thermo-Shaker (Biosan, Riga, Latvia). The reaction mixture was cooled in an ice bath, and the red colouration was measured at 532 and 600 nm on a LAMBDA 25 UV-Vis equipped with UV WinLab v6.0.4 software package (PerkinElmer, Waltham, MA, USA). The results were calculated as nmol g^{-1} FW.

2.4. Determination of the Proline Content

The proline content was evaluated according to the method described by Carillo et al. [37]. To extract proline, 0.03 g of lyophilized tissue powder incubated in 40% ethanol overnight at 4 °C was used. The homogenate was centrifuged at 14,000 × *g* for 5 min at 4 °C after cold extraction. Incubation of an aliquot of extract (50 µL) with 0.1 mL of ninhydrin reagent (1% (w/v) in 60% (v/v) acetic acid and 20% ethanol (v/v)) was performed at 95 °C for 20 min on a TS-100 Thermo-Shaker (Biosan, Riga, Latvia). Then, 100 µL of the reaction mixture was transferred to a microplate after cooling for 5 min and a brief centrifugation at 2500 × *g* for 1 min at 4 °C. The absorbance was measured at 520 nm and 25 °C using a Spark multimode microplate reader with SparkControl software (Tecan, Männedorf, Switzerland). The proline content was measured using a proline standard curve and is expressed in nmol mg⁻¹ DW.

2.5. Determination of the Glutathione Content

The total glutathione (tGSH) content was calculated by a modified microplate assay using a kinetic method based on the continuous reduction of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) by reduced glutathione (GSH), where NADPH reduces GSSG in the presence of GR [38]. Greiner UV Star 96-well plates on a Spark multimode microplate reader were used for the measurements. For tGSH content determination, frozen flag leaf tissue powder was homogenised in a 5% 5-sulfosalicylic acid solution (1/10, w/v) and centrifuged for 10 min at 16,000× g and 4 °C. The subsequent reaction mixture consisted of 10 µL of the resulting supernatant, 0.03 mg mL⁻¹ of DTNB, 0.12 U mL⁻¹ of GR, 1 mM EDTA, and 100 mM phosphate buffer (pH 7.0). Incubation was performed for 5 min at room temperature, and after that, the addition of NADPH initiated the reaction. For 5 min every 15 s, the formation of TNB was measured at 412 nm and 25 °C. The final amount of tGSH was calculated using a standard curve of GSH and is expressed as nmol g^{-1} FW.

2.6. Antioxidant Enzyme Activity Determination

Homogenisation of flag leaf tissue powder obtained by grounding the leaves in cold 100 mM phosphate buffer (pH 7.0) containing 1 mM EDTA (1/5, w/v) was performed. The homogenates were moved onto ice for 15 min and centrifuged at 19,000× g for 15 min at 4 °C. The supernatants were stored at -80 °C until further analysis. Then, 96-well plates were used for the measurement of enzyme activities with a Spark Multimode microplate reader with SparkControl software version 2.1 (Tecan, Männedorf, Switzerland). Bovine serum albumin was used as a protein standard for the determination of protein concentration in the enzyme extracts by the Bradford method [39] modified for microplate assay analysis. Incubation was performed with the Bradford reagent (Sigma-Aldrich, Steinheim, Germany) for 5 min at room temperature. After the incubation was finished, a Spark multimode microplate reader was used for the measurement of the intensity of the resulting blue colour at 595 nm.

For the measurement of ascorbate peroxidase (APX, EC 1.11.1.11) activity [40], the reaction mixture consisted of the enzyme extracts (10 μ L), 0.7 mM ascorbic acid, 5 mM H₂O₂, and 0.1 mM EDTA in 50 mM potassium phosphate buffer (pH 7.0). Incubation was performed for 3 min at room temperature, after which, the decrease in absorbance was monitored at 290 nm for 3 min every 15 s. APX activity was measured using a molar extinction coefficient (ϵ = 1.71 mM cm⁻¹) and is expressed in U mg⁻¹ protein.

For catalase (CAT, EC 1.11.1.6) activity [41], the reaction mixture consisted of 0.036% H₂O₂ in 50 mM phosphate buffer pH (7.0), while the reaction started with the addition of 10 μ L of diluted protein extract. The decrease in absorbance was measured at 240 nm for 3 min every 15 s. CAT activity was measured using the molar extinction coefficient ($\epsilon = 0.04$ mM cm⁻¹) and is expressed as U mg⁻¹ protein.

Glutathione S-transferase (GST, EC 2.5.1.18) activity was measured based on the formation of glutathione-2,4-dinitrobenzene due to the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH [42]. The reaction mixture consisted of 1 mM GSH, 2 mM CDNB, 1 mM EDTA, and 10 μ L of protein extract in 100 mM phosphate buffer (pH 6.5). The increase in absorbance was recorded at 340 nm for 3 min every 15 s. GST activity was measured using the molar extinction coefficient (ε = 5.71 mM cm⁻¹) and is expressed as U g⁻¹ protein.

To determine dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity, the method described by Ma and Cheng [43] was used, after modification for microplate assay [44]. The reaction mixture was composed of 0.1 mM EDTA, 2.5 mM GSH, 0.2 mM dehydroascorbate (DHA), and 10 μ L of protein extract in 50 mm HEPES buffer (pH 7.0). The increase in absorbance was measured at 265 nm for 3 min every 15 s. The calculation of DHAR activity was performed using the molar extinction coefficient (ϵ = 8.33 mM cm⁻¹), and the activity is expressed in U g⁻¹ protein.

The measurement of monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity was performed according to [45] with modifications for the microplate assay. The reaction mixture was composed of 2.5 mM ascorbate, 0.5 mM NADH, and 10 μ L of protein extract in 50 mM Tris-HCl buffer (pH 7.6). The incubation was performed for 3 min at room temperature, while the reaction was started by the addition of ascorbate oxidase at a final concentration of 0.14 U. The decrease in absorbance was measured at 340 nm for 3 min every 15 s. The calculation of MDHAR activity was performed using the molar extinction coefficient (ϵ = 3.7 mM cm⁻¹), and the activity is expressed in U g⁻¹ protein.

For the determination of glutathione reductase (GR, EC 1.6.4.2) activity [44,46], the reaction mixture consisted of 50 mM HEPES buffer (pH 8.0), 0.5 mM EDTA, 0.25 mM NADPH, and 10 μ L of protein extract. After 10 min of equilibration at room temperature, the reaction was initiated by adding oxidised glutathione (GSSG). The decrease in

absorbance was recorded at 340 nm for 5 min every 15 s. GR activity was finally measured by the molar extinction coefficient for NADPH ($\epsilon = 3.7 \text{ mM cm}^{-1}$) and is expressed in U g⁻¹ protein.

2.7. Molecular Analysis

2.7.1. RNA Extraction and cDNA Synthesis

RNA was isolated using the NucleoZOL reagent (Macherey-Nagel, Dueren, Germany), according to the manufacturer's instructions, using 50 mg of frozen wheat flag leaf tissue powder. Further, in the obtained RNA solution, residual DNA was eliminated using rDNase (Macherey-Nagel). For DNA digestion, the rDNase buffer premix (1/10, v/v) was added to the RNA solution, and the mixture was incubated at 37 °C for 10 min. Further, the repurification of RNA was conducted by ethanol precipitation (0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol were added to one sample volume). The incubation of the samples lasted for 2 h at -20 °C, after which, the samples were centrifuged at maximum speed for 10 min. At this point, 70% ethanol was used to wash the RNA pellet, which was then dried and resuspended in RNase-free water. The NanoPhotometer NP-80 (Implen, München, Germany) was used for the estimation of the purity and concentration of RNA.

The synthesis of first-strand cDNA was carried out according to the manufacturer's instructions from 3 µg of total RNA using the GoTaq[®] 2-Step RT-qPCR System (Promega, Madison, WI, USA). The RNA template and the Oligo(dT)15 primer premix were denaturized at 70 °C for 5 min, and cDNA was synthesised in a final volume of 20 µL by combining the denatured premix with the reaction mixture (1× GoScript buffer, 2.5 mM MgCl₂, 0.5 mM nucleotide mix, 20 U of ribonuclease inhibitor, and 1U of reverse transcriptase). The MiniAmp Plus Thermal PCR Cycler (Applied Biosystems, Waltham, MA, USA) was used for cDNA synthesis (primer annealing for 5 min at 25 °C, extension for 1 h at 42 °C, and enzyme inactivation for 5 min at 70 °C). Prior to being used in the quantitative PCR (qPCR) step, all cDNAs were diluted 5-fold with nuclease-free water.

2.7.2. QPCR Analysis

The StepOnePlusTM Real-Time PCR System with StepOnePlusTM Software v2.3 (Applied Biosystems, Waltham, MA, USA) and the GoTaq[®] 2-Step RT-qPCR System (Promega) were used for qPCR analysis to analyse the transcript levels of six genes (*P5CS*, *DHN5*, *WZY2*, *DREB1*, *DREB2*, and *actin*). Specific oligonucleotide primers were designed based on sequences in the GeneBank database using Primer3 software (Table 2). All target sequences were amplified in a 25 µL reaction mixture containing 5 µL of five-fold diluted cDNA template, 200 nmol of each primer, 12.5 µL of GoTaq qPCR Master Mix (2×), and 0.25 µL of the CXR reference dye. Amplification was performed with the following cycling program: GoTaq Hot Start Polymerase activation for 2 min at 95 °C, followed by 40 cycles consisting in denaturation for 15 s at 95 °C, primer annealing, and extension for 1 min at 60 °C. For the quantification, three replicates were used, and the expression of each gene was recorded using three biological replicates. A relative standard curve based on five points was used for relative gene expression and normalised using the geometric average of the reference gene *actin* [47].

Table 2. Oligonucleotide primer sequences.

Target Gene	GenBank Accession No.	Product Length (bp)	Forward Primer	Tm and %CG	Reverse Primer	Tm and %CG
P5CS	KT868850	85	ccggtgaatggcagagtaat	60 °C, 50%	ccccacggagaactttaaca	60 °C, 50%
DHN5	AY619566	99	agaagaagggcatcatggac	59.1 °C, 50%	ggcacctccactctcagaag	60 °C, 60%
WZY2	KF112871	142	tcgttcgtcgtggtagtctg	59.9 °C, 55%	atgaccttgctgtccgtagg	60 °C, 55%
DREB1	DQ195070	80	gttggtacccaacccaagtg	60.1 °C, 55%	aacagaacgaagcagggcta	60 °C, 50%
DREB2	AY781345.1	121	ccacagctcgttcaaagtga	60 °C, 50%	atgccattcaaaaaccaagc	60 °C, 40%
actin	AK457930	215	tgaccgtatgagcaaggag	58 °C, 53%	ccagacaactcgcaacttag	60 °C, 50%

2.8. Grain Morphology

After ripening, the grains of the investigated genotypes from sampled ears in each treatment were analysed. The analyses of grain morphology (weight (g), area (\emptyset), length (mm), width (mm), and circularity (\emptyset)) were performed with the MARViN seed analyser (MARViTECH GmbH, Wittenburg, Germany). The grain area and circularity represent 2D projection of a grain to an area and a circle.

2.9. Data Analysis

A randomised complete block design was applied in the greenhouse to minimise the effect of the environment. Six and three replications of pooled tissue of the flag leaf samples, each derived from at least six or three different pots with four plants per pot, were used for the biochemical and molecular analyses, respectively, i.e., the data are based on 18 biological replications. For grain morphology analysis, six biological replications provided the mean values for each treatment (C, T1, and T2). Fisher's Least Significant Difference (LSD) test ($\alpha = 0.05$) was used to calculate whether the observed difference in performance between treatments for each genotype separately (control plants vs. plants under two drought stresses) was significant (Statsoft Inc., Tulsa, OK, USA). The error bars represent standard deviations. Correlation analyses were performed at p < 0.05 [48].

3. Results

3.1. Content of Chlorophylls and Carotenoids

At a reduction in VSMC of 45%, compared to the control, the genotypes Rujana, Bubnjar, and Anđelka showed significant increases in the content of chlorophyll a (Chl a), chlorophyll b (Chl b), and chlorophyll a + b (Chl a+b), while at the same time, the content of total carotenoids increased by 43% in the flag leaves of Bubnjar (Figure 1A–D). Bubnjar also showed the highest significant increases in Chl a, Chl b, and Chl a+b, corresponding to 33, 34, and 32%, respectively, compared to the other genotypes. The drought-stressed-plants of Silvija at a 65% reduction in VSMC showed a strong decrease in Chl a (25%), compared to the control, while more drought-tolerant and -medium tolerant genotypes reported the same Chl a level as the control.



Figure 1. Content of (**A**) chlorophyll a (Chl a), (**B**) chlorophyll b (Chl b), (**C**) chlorophyll a + b (Chl a+b), and (**D**) carotenoids (Car) in the wheat flag leaves of six winter wheat genotypes under control and two drought treatments (T1 = VSMC – 45%, T2 = VSMC – 65%). Data are average values of six biological replicates \pm SD. Significant differences among treatments for each genotype, separately, were assessed by the Fisher LSD test. Trait means with the same letter do not significantly differ at p < 0.05.

3.2. Content of Malondialdehyde (MDA) and Proline

At a reduction in VSMC of 45%, the MDA content in most genotypes was the same as in the control conditions, while it was significantly reduced in Anđelka and Pepeljuga (Figure 2A). The drought-sensitive and -medium sensitive genotypes showed significantly elevated MDA levels at a reduction in VSMC by 65%, in contrast to the drought-tolerant and -medium tolerant genotypes that showed the same MDA level as the control or a significantly reduced level, as found for Anđelka. The highest significant increase in MDA content was observed in Rujana (40%), followed by Silvija (12%) and Fifi (11%).



Figure 2. Content of (**A**) malondialdehyde (MDA) and (**B**) proline in wheat flag leaves of six winter wheat genotypes under control and two drought treatments (T1 = VSMC - 45%, T2 = VSMC - 65%). Data are average values of six biological replicates \pm SD. Significant differences among treatments for each genotype, separately, were assessed by the Fisher LSD test. Trait means with the same letter do not significantly differ at *p* < 0.05.

The proline content was at the same significant level in the control and at a 45% reduction in VSMC in all genotypes, except for Rujana and Anđelka, in which it significantly increased by 267 and 30%, respectively (Figure 2B). At 65% reduction in VSMC, Bubnjar, Rujana, Silvija, Anđelka, Pepeljuga, and Fifi showed a significantly increased proline content by 378, 306, 227, 168, 93, and 72%, respectively.

3.3. Content of Total Glutathione (tGSH)

The content of tGSH significantly increased in the drought-sensitive and -medium sensitive genotypes by 226% (Rujana), 78% (Fifi), and 67% (Silvija) at a 45% reduction in VSMC (Figure 3). On the contrary, in the drought-tolerant and -medium tolerant genotypes, the tGSH level significantly decreased, such as by 42% in Anđelka, or remained at the same level, such as in the flag leaves of Bubnjar and Pepeljuga. Further, at a 65% reduction in VSMC, Rujana, Fifi, and Silvija showed significant increases in the tGSH content by 230, 162, and 42%. Pepeljuga and Anđelka reported significantly reductions of 74 and 62%, while in Bubnjar, it remained at the same level.



Figure 3. Content of total glutathione (tGSH) in wheat flag leaves of six winter wheat genotypes under control and two drought treatments (T1 = VSMC – 45%, T2 = VSMC – 65%). Data are average values of six biological replicates \pm SD. Significant differences among treatments for each genotype, separately, were assessed by the Fisher LSD test. Trait means with the same letter do not significantly differ at *p* < 0.05.

3.4. Enzymatic Activity

3.4.1. Activity of Ascorbate Peroxidase (APX)

At a 45% reduction in VSMC, APX activity in the flag leaves was significantly reduced in the drought-sensitive genotype Silvija by 26%, while in other genotypes, APX did not significantly change (Figure 4A). APX showed higher activity by 15% in the flag leaves of Silvija at a 65% reduction in VSMC, while in the other genotypes, APX activity significantly decreased, compared to the control, except in Pepeljuga, in which it remained at the same level.



Figure 4. The activity of (**A**) ascorbate peroxidase (APX), (**B**) glutathione reductase (GR), (**C**) dehydroascorbate reductase (DHAR), (**D**) monodehydroascorbate (MDHAR), (**E**) catalase (CAT), and (**F**) glutathione S-transferase (GST) in wheat flag leaves of six winter wheat genotypes under control and two drought treatments (T1 = VSMC – 45%, T2 = VSMC – 65%). Data are average values of six biological replicates \pm SD. Significant differences among treatments for each genotype, separately, were assessed by the Fisher LSD test. Trait means with the same letter do not significantly differ at p < 0.05.

3.4.2. Activity of Glutathione Reductase (GR)

In Silvija, GR activity was significantly reduced by 39% at a reduction in VSMC by 45%, while in the other genotypes, it remained at the same level as in the control (Figure 4B). At a 65% reduction in VSMC, the drought-sensitive and -medium sensitive genotypes, namely, Rujana, Silvija, and Fifi, showed significant reductions in GR activity by 24, 23, and 12%, respectively.

3.4.3. Activity of Dehydroascorbate Reductase (DHAR) and Monodehydroascorbate Reductase (MDHAR)

DHAR activity was significantly reduced in Silvija and Rujana by 21 and 16% at a reduction in VSMC by 45%. No significant difference was obtained in MDHAR activity between control conditions and the drought treatment with a reduction in VSMC of 45% for Anđelka, Bubnjar, Pepeljuga, and Fifi (Figure 4C). Rujana and Fifi exhibited significantly reduced DHAR activity by 34 and 32% at a reduction in VSMC of 65%.

MDHAR activity was significantly reduced in Silvija and Rujana by 30 and 10% at a reduction in VSMC of 45% (Figure 4D). Rujana, Fifi, and Silvija showed significantly reduced MDHAR activity by 26, 18, and 10%, respectively, at a reduction in VSMC of 65%.

3.4.4. Activity of Catalase (CAT)

At a reduction in VSMC of 45%, CAT activity significantly decreased in Silvija by 25% and an increased in Fifi by 21% (Figure 4E). At a reduction in VSMC of 65%, the drought-sensitive and -medium sensitive genotypes displayed significantly reduced CAT activity: Rujana by 27%, Fifi by 26%, and Silvija by 10%. On the other hand, the drought-tolerant and -medium tolerant genotypes showed the same CAT activity level in control and drought conditions.

3.4.5. Activity of Glutathione S-Transferase (GST)

GST activity was significantly reduced in Silvija by 22% at a reduction in VSMC of 45% (Figure 4F). Rujana presented significantly reduced GST activity by 28% at a reduction in VSMC of 65%.

3.5. Relative Expression of Genes

Only Rujana showed significantly increased *DHN5* expression in milder drought conditions, i.e., at a reduction in VSMC by 45%, while the other genotypes showed the same expression level (Figure 5A). A significant increase in severe drought, at a reduction in VSMC of 65%, was recorded in the genotypes Silvija, Fifi, and Bubnjar. Although *WZY2* gene expression under both drought treatments was upregulated in all tested genotypes, significant increases in milder drought were recorded only in the genotypes Rujana and Silvija, while in severe drought, a significant increase was recorded in all genotypes except Rujana and Pepeljuga (Figure 5B). The highest expression of both genes in severe drought was recorded in the genotype Bubnjar (577-fold higher for *DHN5* and 58-fold higher for *WZY2*).

In milder drought, at a reduction in VSMC of 45%, a significantly increased expression of *P5CS* was recorded only in the genotype Rujana (Figure 5C). *P5CS* gene expression was significantly increased due to severe drought at a reduction in VSMC of 65% in the genotypes Silvija, Fifi, and Anđelka. In both drought treatments, no significant changes in gene expression were recorded only in the genotypes Bubnjar and Pepeljuga.

Significant changes in *DREB1* expression were recorded only in Rujana, which showed significantly increased expression at a reduction in VSMC of 45% (Figure 5D). Considering the expression of *DREB2*, only Rujana reported a significantly increased level at a reduction in VSMC of 45%, while Bubnjar exhibited significantly decreased expression (Figure 5E). The other genotypes did not show significant differences.



Figure 5. Relatively expression levels of *DHN5* (**A**), *WZY2* (**B**), *P5CS* (**C**), *DREB1* (**D**), and *DREB2* (**E**) in wheat flag leaves of six winter wheat genotypes under control and two drought treatments (T1 = VSMC - 45%, T2 = VSMC - 65%). Data are average values of three biological replicates \pm SD. Significant differences among treatments, for each genotype, separately, were assessed by the Fisher LSD test. Trait means with the same letter do not significantly different at *p* < 0.05.

3.6. Grain Morphology

Rujana, Anđelka, and Fifi showed a significant decrease in grain weight at a reduction in VSMC of 45%. There were no significant differences in grain weight in Bubnjar at a reduction in VSMC of 65%, while in all other genotypes, a significant decrease was observed (Figure 6A). The highest significant decrease in grain weight was observed in Fifi (47%) and Silvija (41%).

No significant differences were obtained for grain area between control and 45% reduction in VSMC, for all genotypes (Figure 6B). At a 65% reduction in VSMC, the grain area of all genotypes showed no significant changes, except for those of Bubnjar, which significantly increased by 9%, and Fifi, which significantly decreased by 13%.

At a reduction in VSMC of 45%, the grain width of all genotypes was not significantly different, compared to that in control conditions (Figure 6C). At a reduction in VSMC of 65%, Silvija, Fifi, and Pepeljuga showed a significant reduction in grain width by 10, 5, and 5%, respectively.

At a reduction in VSMC of 45%, the grain length of all genotypes showed no significant differences, except for that of Fifi, which was significantly reduced by 4% (Figure 6D). At a reduction in VSMC of 65%, the drought-sensitive genotype Silvija showed a significantly decreased grain length by 3%, while the drought-tolerant genotype Bubnjar reported a significantly increased grain length by 5%.



Figure 6. Grain weight (**A**), grain area (**B**), grain width (**C**), grain length (**D**), and grain circularity (**E**) after harvest of six winter wheat genotypes under control and two drought treatments (T1 = VSMC – 45%, T2 = VSMC – 65%). Data are average values of six biological replicates \pm SD. Significant differences among treatments, for each genotype, separately, were assessed by the Fisher LSD test. Trait means with the same letter do not significantly differ at *p* < 0.05.

The grain circularity of all genotypes showed no significant differences between control and 45% and 65% reductions in VSMC, except for that of Silvija, which showed a significant reduction of 2% at a reduction in VSMC of 65% (Figure 6E).

3.7. Correlation Analysis

Under control conditions, APX showed a significant positive correlation (p < 0.05) with DHN5 (Supplementary Figure S1A). CAT, DHAR, and MDHAR displayed a significant positive correlation, while DHAR and MDHAR reported a significant negative correlation with tGSH and a significant positive correlation with *DHN5*. In contrast, *DHN5* was negatively correlated with tGSH. GR was positively correlated with MDA, and MDA with carotenoids. Chlorophylls were significantly positively correlated, while Chl b showed a significant positive correlation with *P5CS*. Grain area and width were significantly negatively correlated with Chl a and Chl a+b, while grain area and length showed a significant positive correlation with proline content. Grain length and circularity were significantly positively correlated. Grain weight presented a significant positive correlation with grain length and circularity, and length with grain area.

Under drought with 45% reduction in VSMC, APX had a significant negative correlation with tGSH and a significant positive correlation with MDA, DHAR, and MDHAR (Supplementary Figure S1B). CAT, DHAR, and MDHAR displayed a significant positive correlation, and CAT was positively correlated with GST. DHAR and MDHAR were significantly negatively correlated with tGSH, while DHAR exhibited significant positive correlation with MDA. Chlorophylls were significantly positively correlated, while Chl b was significantly positively correlated with Car. Proline was significantly positively correlated with *DHN5*, *P5CS*, *DREB1*, and *DREB2*. *P5CS*, *DREB1*, and *DREB2* displayed a significant positive correlation. Grain area was significantly negatively correlated with chlorophylls, while grain length showed a negative correlation with Chl b and Chl a+b and a positive correlation with *DHN5* and grain area.

At a 65% reduction in VSMC, MDHAR reported a significant positive correlation with APX (Supplementary Figure S1C). GR was significantly negatively correlated with *DREB1*, and *DREB1* was significantly positively correlated with *P5CS*. tGSH showed a significant negative correlation with Chl a+b and a significant positive correlation with *DHN5* and *WZY2*, while Chl a was significantly positively correlated with Chl a+b. MDA exhibited a significant positive correlation with *DREB2*, while *DHN5* showed a significant negative correlation with CAT and a significant positive correlation with *WZY2*. Grain area showed a negative correlation with MDHAR and Chl a+b and a significant positive correlation with grain length and width, while grain width revealed a negative correlation with GR. Grain length was negatively correlated with Chl a, Chl a+b, and carotenoids and positively correlated with proline, while proline exhibited a significant negative correlation with Chl a+b and significant positively correlated with Chl a+b, and carotenoids and positively correlated with proline, while proline exhibited a significant negative correlation with Chl a+b and significant positive correlation with Chl a+b and significant positively correlated with Chl a, Chl a+b, and carotenoids and positively correlated with proline, while proline exhibited a significant negative correlation with Chl a+b and significant positive correlation with Chl a+b and positively correlated with Chl a+b, and carotenoids and positively correlated with proline, while proline exhibited a significant negative correlation with Chl a+b and significant positive correlation with Chl a+b and positive correlation with Chl a+b

4. Discussion

Drought in wheat refers to external influences that adversely affect plant growth, development, or grain productivity. However, plants have defence mechanisms consisting of enzymatic and non-enzymatic defence systems such as carotenoids and proline. Pompelli et al. [49] reported that the elevation in the activity of enzymatic and non-enzymatic systems, defending plant tissues against oxidative injury, is the result of decreased amounts of MDA, H₂O₂, and proline. The ascorbate–glutathione (AsA-GSH) cycle is a major antioxidative system that detoxifies ROS and is composed of MDHAR, DHAR, APX, and GR.

4.1. Pigments Involved in Photosynthesis during Drought

Photosynthates and assimilates are transported to the developing grain after photosynthesis in the flag leaf and from pre-anthesis reserves in tissues such as the stem and the ear. The chloroplast is an organelle that contains the photosynthetic pigment chlorophyll and is the site of the earliest abiotic injury visible in plants [50] due to the photo-oxidation of pigments and the degradation of chlorophyll. In the current research, in most of the wheat genotypes, Chl a, Chl b, and Chl a+b tended to increase in the flag leaves at a 45% reduction in VSMC, while some genotypes showed no significant changes. García-Valenzuela et al. [51] showed that an increase in chlorophyll accumulation may be the result of osmotic stress. In the present study, at a reduction in VSMC of 65%, all wheat genotypes exhibited the same amount of chlorophylls as in the control conditions, except the most sensitive genotype, Silvija, which showed a significantly reduced level of Chl a. It was demonstrated that drought in wheat reduced the chlorophyll content and photosynthesis in the leaf [3]. In previous research, a reduced content of Chl a, Chl b, total Chl, and carotenoids was reported under drought [52]. Thus, the chloroplast structure could be changed, or inhibition of the biosynthesis of Chl or its precursors might occur. Thereby, decreased concentrations of pigments involved in photosynthesis can directly limit the photosynthetic efficiency.

One of the main causes of a reduced photosynthetic activity is the formation of ROS such as superoxides and hydroxyl radicals, which impair the photosynthetic machinery

where the ROS-scavenging system is not induced properly [53]. Thus, in the current research, mild stress, with a reduction in VSMC of 45%, resulted in an increase in chlorophyll content. If the concentration of the chlorophyll pigment increases, the photosynthetic systems should be efficient in ROS scavenging [54]. Further, other pigments, such as carotenoids, protect photosystems as a result of a reaction with lipid peroxidation products and scavenging singlet oxygen [55]. In the present study, only the drought-tolerant genotype Bubnjar showed a significantly increased level of carotenoids in mild drought, with a production of MDA that did not significantly change in both types of drought stress. It was reported that carotenoids, with ascorbate, GSH, and α -tocopherol, might be good indicators of drought tolerance [56].

In the current research, different metabolic processes in severe drought, at a reduction in of by 65%, were affected, leading to a significant reduction in chlorophyll content in the drought-sensitive genotype (Silvija). The loss of chlorophyll content can be the first sign of the inactivation of photosynthesis. Those genotypes that exhibited higher chlorophyll contents under drought could lead us to the conclusion that they turned on the ROSscavenging system to some extent. It was also shown that chlorophylls were significantly positively correlated in mild stress, while Chl b showed a significant correlation with carotenoids, while in severe stress, only Chl a+b presented a positive correlation with Chl a. Similar findings were obtained by Ahmed et al. [9], who showed that chlorophylls were positively related among themselves under drought conditions. This shows the significance of these attributes for drought in future wheat breeding programs.

4.2. Content of Malondialdehyde (MDA) and Proline

The structure and function of cellular membranes could be damaged during drought via lipid peroxidation, in particular, the thylakoid membranes within the chloroplast [57]. Hence, the stability of the cell membrane is determined in the screening of drought-tolerant genotypes [58]. Lipids, the main components of the cellular membrane, are the primary target of ROS, undergoing lipid peroxidation. The cell membranes are damaged the earliest during oxidative stress by ROS, which will result in lipid peroxidation and, consequently, membrane injuries, enzyme inactivation, and protein degradation. The product of lipid peroxidation is MDA, which is usually used as a marker for oxidative hurt and antioxidant status. From the data of the current research, it is obvious that the drought-sensitive and -medium sensitive genotypes displayed elevated MDA levels at a reduction in VSMC of 65% in contrast to the drought-tolerant and -medium tolerant genotypes. Similar results were obtained by Sultan et al. [59], who reported that drought-tolerant genotypes showed significantly increased proline and relative water content, while the MDA content decreased under drought conditions. Vuković et al. [22] also highlighted that lipid peroxidation was induced by drought in wheat genotypes, with a higher increase in MDA in seedlings of drought-sensitive genotypes. In the current research, with both drought intensities, the MDA level was reduced or significantly unchanged in the genotypes Bubnjar, Pepeljuga, and Fifi, thus indicating a stronger antioxidative response in them. The enhancement of enzymatic and non-enzymatic systems that have a role in plant tissues protection against oxidative injury was evidenced by the lower amounts of MDA in the flag leaves in mild than in severe drought, except for the drought-tolerant and -medium tolerant genotypes. However, the drought-sensitive and -medium sensitive genotypes under mild drought did not differ significantly in MDA content. The differences between drought-tolerant or -medium tolerant and drought-sensitive or -medium sensitive genotypes became evident as the degree of the stress increased, where the drought-sensitive genotypes experienced more stress injury.

Proline is an amino acid that accumulates under different stresses. It is an osmolyte and a reservoir of carbon and nitrogen, but it also protects plants against free-radicalinduced damage, and its accumulation is related to high temperatures and drought [60]. The increase in osmolytes, like proline or glycine betaine, and in late-embryogenesisabundant proteins, which have a role in the protection of lipid membranes, stabilises the membrane [61]. Peršić et al. [62] reported that there are contrasting results on the relation between proline content and drought tolerance. According to some research, it is believed that an increased proline content is an indicator of drought-induced stress, while other authors believe that proline is associated with sensitivity to drought [63–65]. In the current research, all genotypes showed a significantly higher proline content in severe drought, and four genotypes in mild stress. Similarly, Anjum et al. [55] observed that the accumulation of proline and other osmolytes in maize plants increased with the severity of drought. Also, the increased proline content in plants under severe drought conditions could have important role in recovery after stress [66]. In the current research, it was observed that proline displayed a significant positive correlation with *P5CS*, *DREB1*, and *DREB2* in mild drought but not in severe drought. However, it was observed that the proline content was significantly positively correlated with grain area and length. It was previously reported that there was a strong correlation between elevated enzymatic activity of *P5CS* and proline content [33].

4.3. ROS-Scavenging System

An increased content of ROS at the cell level influences protein degradation, the inhibition of enzymes, oxidative damage to DNA and RNA, and lipid peroxidation in membranes, causing the death of cells [67]. Among the antioxidant enzymes involved in the degradation of ROS during drought, the best described are CAT, SOD, GPX, APX, and GR [22,68,69]. For instance, the activity of the enzymes of the AsA-GSH scavenging pathway (APX and GR) was elevated under drought [66]. Drought led to the upregulation of APX in the endosperm, while GR, CAT, and POD activity increased in the shoots of seedlings in drought-tolerant genotypes of wheat [70]. The same authors concluded that a genotype is likely to be drought-tolerant if any of the specified enzymes' activities is upregulated in specified tissues under drought. According to Foyer and Noctor [71], the main ROS detoxification process is under the influence of the enzymes, including APX, GR, DHAR, and MDHAR, involved in AsA-GSH metabolism. CAT predominantly scavenges H_2O_2 in the peroxisomes. Therefore, CAT can neutralise H_2O_2 by decomposing it into molecular oxygen and water. Under stress conditions, a strong elevation in CAT activity in the leaves may protect the chloroplasts, the principal generators and targets of ROS, thus supporting persistent electron fluxes [72]. Thus, the stability of CAT activity in the leaves is likely responsible for the elimination of photorespiratory H_2O_2 . In the current research, significant reductions in CAT in response to drought appeared in the drought-sensitive and -medium sensitive genotypes, especially at a 65% reduction in VSMC. Hence, we may assume that CAT activity in drought-tolerant genotypes is a critical and accessory component of photosynthesis that prevents ROS accumulation. Similar results were obtained by Chakraborty and Pradhan [52], who reported that the CAT and SOD activity decreased in all periods of drought in more drought-sensitive wheat genotypes. Anjum et al. [55] also observed that the activities of some enzymes, such as POD and CAT, decreased with drought severity. Further, Vuković et al. [22] reported a negative correlation between reduced CAT activity and lipid peroxidation levels, suggesting that the absence of CAT induction resulted in increased lipid peroxidation. Similar results were obtained in the current research, where the drought-sensitive and -medium sensitive genotypes showed significantly elevated MDA levels in severe drought, with a decrease in CAT activity. It could be seen that CAT exhibited a significant negative correlation with DHN5 in severe drought.

APX is an integral component of the AsA-GSH cycle that has the ability to reduce H_2O_2 to H_2O and DHA, using ascorbic acid in the cytosol and chloroplasts [68]. DHA is further reduced to ascorbate by the action of DHAR, with an expenditure of GSH or NADPH. The second line of antioxidant defence is more activated with an increase in stress when APX activity is elevated [73]. Silvija could increase APX activity in flag leaves, trying to detoxify ROS and minimise the photooxidative damage during severe drought. Similar results were observed by D'Arcy-Lameta et al. [74] when the transcript levels of

the cytosolic and peroxisomal APX genes appeared elevated in a genotype sensitive to drought. According to that, we can assume that APX was expressed much earlier in the more drought-tolerant genotypes that managed to decrease the MDA content.

Glutathione is involved in multiple metabolic functions, such as the protection of membranes, by maintaining the reduced form of both α -tocopherol and zeaxanthin, preventing the oxidative denaturation of proteins under stress [75]. GSH is also the substrate of GPX reactions and GST, which also participates in the removal of ROS. It was reported that an elevation in tGSH in the flag leaves of wheat implies its role in drought tolerance [76]. In the current research, the tGSH content significantly increased in the drought-sensitive and -medium sensitive genotypes in both types of drought stress, thus showing that the decline in tGSH concentration in the drought-tolerant and -medium tolerant genotypes indicated sufficient defence capacities. Loggini et al. [77] reported that more drought-tolerant genotypes did not increase the enzyme activity because its present activity was enough to tolerate stress, whereas the more drought-sensitive genotype reinforced its defence systems. However, GSH is also a protector of chlorophyll biosynthesis enzymes, which might be related to a higher chlorophyll content [78]. In the current research, the drought-sensitive and -medium sensitive genotypes showed a significantly elevated tGSH level, which might protect the chlorophyll structure. For example, adding exogenous GSH enhanced non-enzymatic and enzymatic components in some plants [79]. Further, genotypes with different drought tolerance showed a decrease in the tGSH level and a higher GSH/GSSG ratio after one month of drought [77]. However, in the current research, it was observed that an increase in tGSH activity was only observed in the drought-sensitive and -medium sensitive genotypes. It might be assumed that different genotypes depend on tGSH to decrease oxidative stress, whereas enhanced antioxidative functioning in drought-tolerant and -medium tolerant genotypes might occur early to withstand drought, compared to drought-sensitive and -medium sensitive genotypes, where tGSH content significantly increased later. After two weeks of drought, the drought-sensitive and -medium sensitive genotypes were still not able to defend themselves, thus elevating tGHS content

GR catalyses GSSG reduction to GSH using NADPH, and the reduced GSH is further utilised for the regeneration of ascorbic acid [19]. This helps in regulating the ratio of GSH/GSSG and suppling GSH to GPX and DHAR. Due to the maintenance of a favourable GSH/GSSG ratio, GR provides stress tolerance in plants [80]. The drought-sensitive and -medium sensitive genotypes showed significantly reduced GR activity at a 65% reduction in VSMC, which thus did not contribute to the detoxification of ROS. Due to the decreased GR activity in these three genotypes, GSH could not be recycled sufficiently, as shown by the significant increase in tGSH in the drought-sensitive and -medium sensitive genotypes in both types of drought stress. In contrast, the drought-tolerant genotype showed no significant differences in tGSH in control and drought conditions, thus minimising the formation of ROS. It was observed previously that tGSH increased contribution to the redox potential could compensate for the modest increase in GR activity in roots under drought [19]. Similar results were observed in the current research, where the droughttolerant and -medium tolerant genotypes had a tendency to decrease the tGSH level, maintaining GR activity significantly unchanged in flag leaves. Also, it could be seen that GR showed a significant negative correlation with DREB1 as well as with grain area and width in severe drought. According to the research of Chakraborty and Pradhan [52], increased POD and GR activity was the most influential factor conferring drought tolerance.

GST is an enzyme that catalyses the reaction of electrophilic substrates in the removal of ROS and is also related to tolerance to abiotic stresses [54]. It can repair phospholipid damages in the membrane [81]. However, previous research about the importance of GST in drought is not consistent [22]. In the current research, no clear picture was obtained about the role of GST in drought tolerance in wheat genotypes. However, some studies found that an increase in GST might reduce the accumulation of H_2O_2 and MDA and help to maintain the GSH/GSSG ratio under salt stress [20].

Ascorbate is an efficient primary scavenger of ROS, being oxidised and further recycled back by the activities of MDHAR and DHAR [71]. It was previously concluded that higher ascorbate levels in transgenic plants of rice were due to increased MDHAR and DHAR activities [82]. In the plant antioxidant system, MDHAR is important in maintaining the ascorbate pool by catalysing the reduction of monodehydroascorbate (MDHA) to ascorbate and, therefore, maintains a pool of reduced ascorbate [20]. In the research of Shokat et al. [21], higher MDHAR activity within the leaves of wheat was a predictive biomarker for a higher grain number under drought. In the current research, the drought-tolerant and -medium tolerant genotypes did not show significant changes in MDHAR activity at both drought intensities, while the drought-sensitive and -medium sensitive genotypes showed a tendency to a significant reduction.

DHAR participates in the catalysis of the reduction of DHAR using reduced GSH, yielding ascorbic acid and GSSG and thus keeping an ascorbate redox state [20]. The main role of the AsA-GSH cycle, particularly of DHAR, is in minimising the drought-induced grain yield loss in rice [83]. Therefore, in the current research, the drought-tolerant and -medium tolerant genotypes showed no significant changes in DHAR in the two drought treatments and in control conditions. This indicated that in those genotypes, DHAR activity was sufficient to overcome drought. Also, DHAR activity maintains high levels of chlorophyll and photosynthetic functioning, resulting in delayed leaf ageing [84]. Further, the decrease in DHAR was associated with reduced photosynthesis and increased oxidative injury.

4.4. The Expression of Genes (P5CS, DHN5, WZY2, DREB1, and DREB2) under Drought

Knowledge about the relation of antioxidant defence at both protein and gene expression levels to genetic variation in drought tolerance is important for the identification of predominant or major protection pathways to improve drought tolerance. Thus, in the current research, the expression patterns of the drought-sensitive gene *P5CS*, of genes encoding dehydrins (*DHN5* and *WZY2*), and of genes encoding transcription factors (*DREB1* and *DREB2*) were analysed.

The elevation in the relative expression of the analysed stress-responsive genes *DHN5* and *WZY2* was mostly evident under drought conditions. Only Rujana showed a significant increase in the expression of both genes in mild stress, and Silvija showed an increase in *WZY2* expression. It is evident from the current research that the most drought-tolerant genotype, Bubnjar, highly overexpressed *DHN5* and *WZY2* in severe drought, compared to all other genotypes. Previous studies reported that the *DHN* genes are important in abiotic stress tolerance [85–87]. Moreover, the *TaDHN* genes respond strongly to stresses such as drought, cold, and high salinity [88]. Saibi et al. [32] reported that a higher expression of *TtDHN5* elevated tolerance to osmotic and salt stress in transgenic Arabidopsis plants. Also, dehydrin *WZY2*, whose relative expression was increased in most plants during cold, drought, heat, or other abiotic stresses, was identified as a drought stress-responsive gene [89,90].

Ma et al. [91] showed that under osmotic stress, *TaP5CS* was overexpressed in transgenic Arabidopsis, which further showed increased proline content and decreased lipid peroxidation. In addition to osmotic stress, the transcription of *P5CR* was also increased under cold and biotic stress [92,93]. According to the current research, in severe drought (65% reduction in VSMC) *P5CS* gene expression was significantly increased in Silvija, Fifi, and Anđelka, but only Anđelka displayed a significant reduction in MDA. Rujana showed a significant increase in *P5CS* already in mild drought, together with an increase in proline content. Furthermore, many studies previously reported that increased P5CS enzyme activity strongly correlated with proline accumulation [33,94], which ultimately led to an increase in stress tolerance [34]. According to Bohnert et al. [95], the *DREB* genes are grouped into classes based on similarities in their functioning. Two main subgroups of the *DREB* subfamily include the genes *DREB1* and *DREB2* [96]. Currently, both genes *DREB1* and *DREB2* are used in molecular breeding studies to improve the tolerance of wheat to abiotic stresses. It was concluded that the *DREB* transcription factor plays crucial roles in the abiotic stress response [97]. For example, increased proline content was related to *CpDreb2* ectopic overexpression in tobacco [98]. This was similarly observed in the current research, where Rujana was the only genotype with a significant increase in *DREB1* and *DREB2* in mild drought and in *DREB2* in severe drought. This genotype also showed a significant and strong increase in proline in both drought treatments. However, as mentioned before, there is not a clear understanding of the role of proline in drought tolerance.

It was previously reported that the expression of *DREB2B* under stress was similar to or lower than that in control plants [99], suggesting that this gene does not have a role in tolerance. A decreased expression may simply reflect impairment of the normal metabolic functioning of a plant. In the current research, only Bubnjar (drought-tolerant genotype) showed a significantly decreased *DREB2* expression in mild drought. According to the expression of *DREB2*, we might conclude that the expression levels might serve as indicators of the degree of stress in relation to plant metabolism. Further, the transcriptional activity of several APX genes and the activity of the APX protein were increased in *DREB2C* overexpressors [100]. It is evident that the most drought-sensitive genotype Silvija showed significantly increased APX activity in severe drought.

According to previous research, more drought-tolerant genotypes of wheat accumulated more *DREB1* gene transcripts under drought than sensitive genotypes [27,101]. In contrast, Yousfi et al. [98] reported that drought-sensitive genotypes increased *DREB1A* expression, compared with tolerant ones, suggesting that the expression of this gene is not related to drought tolerance. It shows that for sensitives genotypes, it is not possible to deal with stress. Due to the significant expression of *DREB1* only in one genotype, it is not possible to make a proper conclusion about the role of this gene, although *DREB1* showed a negative correlation with GR activity and grain area and width in severe stress. Other genes not investigated in the current research might influence the antioxidant defence.

4.5. Postharvest Traits of Grain Morphology Influenced by Drought

One of the ways to increase the grain yield is the selection for enhanced grain size, as it is directly related to 1000 kernel weight, a component of grain yield. Grain size is related to grain filling and includes grain length, grain width, grain thickness, and grain surface area. Brinton et al. [102] reported that the 5A locus was responsible for an increase in grain weight influencing grain length. Along with that goes the fact that grain length is genetically controlled and stable across environments, has a pleiotropic effect on grain width in the final stage of grain development, and is more variable across environments. In this study, five traits of grain morphology (grain weight, grain area, grain width, grain length, and grain circularity) were analysed after harvest. For milling, the best morphology is that of large and spherical grains [103], while small and shrivelled grains will decrease the yield of milling. Furthermore, grain yield is positively affected by grain size, as the latter increases the grain weight [104,105]. During the grain filling stage, drought affects the accumulation of starch and protein in the grains, which results in a decrease in grain size, thus affecting grain yield and quality [106]. Simmonds et al. [107] reported that grain width genes on chromosome 6A are under the influence of increased grain weight, suggesting that increased grain size could contribute to a higher grain yield. Further, during anthesis, drought will provoke partial or complete sterility of the florets [108]. Grain formation lasts 12–14 days after anthesis and fertilisation of the florets. This is also the time when most of the endosperm is formed, which corresponds to the cell division phase. It is evident that high post-anthesis temperatures or drought reduce the mature grain weight in wheat [109]. On the other hand, terminal drought will reduce the grain number, rather than the grain size, which causes a significant decrease in grain yield in wheat [110].

According to the current research, only the drought-tolerant genotype did not significantly change the grain weight and even significantly increased grain area as well as grain length under both intensities of drought. According to the above, we can conclude that the genotype Bubnjar proved to be more tolerant to drought than the other tested genotypes, as was already concluded in previous research [22]. Further, it was seen that *DHN5* showed a significant positive correlation with grain length and proline content in mild drought, while in severe drought, proline content showed a significant positive correlation with grain area and length. On the other hand, the drought-sensitive genotype Silvija showed a significant decrease in almost all traits of grain morphology in severe drought. In the early stage of grain filling, the number of endosperm cells and starch granules per cell will be reduced under drought, resulting in a decrease in grain size [111]. It could be seen that when drought occurred at the grain filling stage, grain weight was reduced in almost all genotypes, thus meaning that the rate of grain filling was not optimal. Photosynthetic activity and remobilisation of sugars were probably decreased in other plant parts up to the grain. Drought also triggers early senescence, thus shortening the accumulation period of dry weight in the grains.

5. Conclusions

Compiling all data, the extinguishing of chlorophyll may be a stress indicator as a first-line defence system in the flag leaves of wheat, with increased MDA accumulation. On the other hand, the accumulation of carotenoids may help plants cope with drought. APX and GST expression under drought was genotype-specific and dependent on drought intensity. The results suggest that drought induced oxidative stress and that besides CAT, the enzymes of the AsA-GSH cycle (GR, MDHAR, and DHAR) appeared to function as important components of the antioxidative defence system under severe drought. The relative expression of the *DHN5* and *WZY2* genes in severe drought was higher in the drought-tolerant genotype (Bubnjar) compared to the other genotypes. These two genes showed a significant positive correlation in severe drought, while in control conditions and mild drought, no correlation was recorded between them. Our results suggest that drought-tolerant and -medium tolerant wheat genotypes can better acclimatize to drought and induce antioxidant systems earlier than drought-sensitive genotypes. Wheat breeders should use these results in the selection of drought-tolerant genotypes and in the development of high-yielding wheat genotypes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14071522/s1, Figure S1: Correlation analysis of the investigated traits in (A) control conditions, (B) mild drought (VSMC – 45%), and (C) strong drought (VSMC – 65%).

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