NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF EARTH SCIENCES

EXPLORING BREAD WHEAT DIVERSITY IN WATERLOGGING RESPONSES: ROOT SYSTEM, PLANT DEVELOPMENT, ECOPHYSIOLOGICAL TRAITS AND YIELD IMPLICATIONS

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`To Elisa, my lovely daughter

To my family

ACKNOWLEDGEMENTS

To Full Professor Fernando Lidon, I am grateful for the opportunity, the shared knowledge, the support, encouragement, friendship, tremendous confidence, and to be one of my advisers.

To my advisers Doctors Paula Scotti-Campos and Benvindo Maçãs, for the privilege of conducting research at INIAV, as well as for their support and guidance.

To the CAT members, Full Professor Fernando Lidon, Professor Maria Glória Esquível, Doctor Benvindo Maçãs, Doctor Paula Scotti-Campos, and Doctor Ana Sofia Bagulho, for their availability and support in outlining the activities.

To NOVA School of Science and Technology, NOVA University Lisbon.

To the National Institute of Agricultural and Veterinarian Research, IP.

To all the colleagues with whom I shared the activities that led to the writing of this document.

I am grateful to Rita Moreira for her unwavering support, cheerful spirit, companionship, professionalism, and friendship.

I am grateful to José Semedo for his indispensable involvement in the gas exchanges determinations.

Thank you Zé for save me with the respiration analysis, conducted in ISA. I also thank you the encouragement and the constant challenge of scientific problems that have always accompanied us. Despite all the ups and downs, I am thankful for the friendship that has lasted many years.

To all my colleagues at INIAV for the encouragement and harmonious coexistence.

To my colleagues at FCT Nova, thank you for the companionship, mutual support, and pleasant and enjoyable moments. A special thanks to Rita and Claudia for their steady availability and affection.

To Paula Sampayo for being a good listener, for her friendship, and for the kilometres we walked together while clearing our minds.

To my dear Denis for all the messages of confidence and for the affection and support.

To Elisa, my daughter. I thank you for all the support, encouragement, trust, pride... always wrapped in a tight hug.

Thank you sister Fátima, for your wise words of encouragement. Thank you for providing moments of relaxation during times when stress was mounting. You have consistently guided me along a path I never imagined possible.

To all my family for being my support.

"Too much water drowns the miller"

ABSTRACT

Waterlogging is an abiotic stress that has increasingly affected wheat production, compromising food security stability. With the predicted climate changes, extreme events of heavy rainfall will increase in intensity and frequency, further worsening the declines already felt in production. To maintain the energy contribution of this cereal in human nutrition with the projected population increase, it is necessary to develop varieties that can cope with the adverse environmental conditions resulting from climate change. To address this topic, it is essential to understand plants responses at various levels. The root system plays a crucial role in plant development. However, in waterlogging conditions, it suffers the first impacts and sometimes the strongest ones, with significant repercussions on the plants above-ground part. This study was conducted under environmentally controlled conditions, focusing on differences in the seminal root architecture at the seedling stage and the ability to emit adventitious roots in response to 14-day waterlogging starting at the tillering stage. Additionally, the impacts in the plant above-ground part were studied, covering aspects related to growth and development, senescence, changes in ecophysiological traits, and impacts on yield. In the final chapter, a thorough analysis that encompassed all the studied traits highlighted some contributes for the screening of bread wheat waterlogging tolerance. The study comprised genotypes from five groups with distinct genetic backgrounds. Results showed a substantial variability between and within groups concerning the majority of the studied traits. Several genotypes depicted a good performance under water stress, exhibiting stable or even higher yields; this was evident in the Australian group but was found across all groups, with the exception of the Italian. On the other hand, strong yield decreases were found, reaching 86.6%. Several traits showed significant correlations with yield. Among them, and considering those evaluated during stress and the recovery period, SPAD values and F_v/F_m and F_v'/F_m' showed the strongest correlation.

Keywords: *Triticum aestivum* L.**,** water stress, physiological traits, senescent, breeding.

This document has been written in U.K. English

RESUMO

O alagamento é um stress abiótico que tem afetado cada vez mais a produção de trigo, comprometendo a estabilidade da segurança alimentar. Com as alterações climáticas previstas, eventos extremos de chuvas intensas tendem a aumentar em intensidade e frequência, agravando as quebras já observadas na produção. Para garantir a contribuição energética do trigo na nutrição humana, é essencial desenvolver variedades tolerantes às condições ambientais adversas resultantes destas alterações. Compreender as respostas das plantas em vários níveis torna-se assim fundamental. O sistema radicular desempenha um papel crucial no desenvolvimento das plantas, sendo o primeiro a sofrer os impactos do alagamento, muitas vezes com repercussões significativas na parte aérea. Este estudo foi realizado em condições ambientais controladas, analisando diferenças na arquitetura radicular seminal na fase de plântula, assim como na capacidade de emitir raízes adventícias em resposta a 14 dias de alagamento, iniciado em pleno afilhamento. Os impactos na parte aérea da planta foram analisados, abrangendo aspetos relacionados com o crescimento e desenvolvimento, senescência, mudanças em características ecofisiológicas e os seus impactos na produção. No capítulo final, uma análise minuciosa de todos os parâmetros estudados permitiu identificar os que mais contribuíram para o *screening* entre variedades tolerantes e suscetíveis. O estudo incluiu genótipos de cinco grupos com diferentes *backgrounds* genéticos. Os resultados revelaram variabilidade entre e dentro dos grupos relativamente à maioria das características analisadas. Vários genótipos apresentaram bom desempenho sob alagamento, com produções por planta estáveis ou até superiores; isso foi evidente no grupo australiano, mas abrangeu todos os grupos, com exceção do italiano. Por outro lado, quebras de produção atingiram 86,6% nalguns casos. Vários parâmetros mostraram correlações significativas com o rendimento. Entre eles, e considerando aqueles avaliados durante o stress e o período de recuperação, os valores de SPAD e F_v/F_m e F_v'/F_m' mostraram a correlação mais forte

Palavras chave: *Triticum aestivum* L., stress hídrico, parâmetros fisiológicos, senescência, melhoramento

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ABBREVIATIONS, ACRONYMS, SYMBOLS

°C	Celsius degrees
6th SR	6th Seminal root
AdvL	Advanced lines obtained through the PCBP or CIMMYT
AR	Adventitious root
Austrl	Australian germplasm
ca.	*circa*
C_i	Intracellular CO_2 concentration
CIMMYT	International Maize and Wheat Improvement Center
DAS	Days after sowing
DBI	Lipid unsaturation, double bond index
DW	Dry weight
EC	Electrical conductivity
Eh	Redox potential
FA	Fatty acid
e.g.	*exempli gratia*
FAO	Food and Agriculture Organization.
FC	Field capacity
F_v/F_m	Maximal photochemical efficiency of PSII
F_v'/F_m'	Actual PSII photochemical efficiency of energy conversion under light-adapted conditions
FW	Fresh weight
HPLC	High-Performance Liquid Chromatography

GR	Post-Green Revolution varieties with introduced CIMMYT germplasm, developed by the PCBP and released between 1980 and 1989
g_s	Stomatal conductance
I%	Membrane injury index
i.e.	*id est*
INIAV	National Institute of Agricultural and Veterinarian Research, IP
PL	Portuguese landraces from Vasconcelos ancient collection
IT	Varieties with introduced Italian germplasm, developed by the PCBP and released between 1950 and 1970
LatSL-1	Lateral seminal root length measured 1 day after sowing
LatSL-2	Lateral seminal root length measured 2 days after sowing
LatSL-3	Lateral seminal root length measured 3 days after sowing
LatSL-6	Lateral seminal root length measured 4 days after sowing
LT	Total lipids
MC	Main culm
MDA	Malondialdehyde
PCBP	Portuguese Cereal Breeding Programme
P_n	Photosynthetic rate
PSII	Photosystem II
RadL-1	Radicle length measured 1 day after sowing
RadL-2	Radicle length measured 2 day after sowing
RadL-3	Radicle length measured 3 day after sowing
RadL-6	Radicle length measured 6 day after sowing
R_d	Respiration rate
RGA	Root growth angle
rpm	Revolutions per minute

ROS	Reactive oxygen species
RWC	Relative water content
SPAD	Soil Plant Analysis Development (value related to chlorophyll measurement)
T	Tiller
T0	Beginning of waterlogging
T14	14th day of waterlogging
T14R	14th day of the recovery period
T2	2nd day of waterlogging
T4	4th day of waterlogging
T7	7th day of waterlogging
T7R	7th day of the recovery period
TFA	Total fatty acids
Tr	Transpiration rate

1 THE OVERALL INTRODUCTION*

Wheat is the third most widely cultivated cereal worldwide, being only surpassed by rice and corn. With a world production of 808 million tonnes per year (FAOSTAT, 2022), of which 95% correspond to bread wheat (*Triticum aestivum* L.), this crop occupied an area of 219 million ha (FAOSTAT, 2022), providing *ca.* 20% of the energy and protein needs of the human diet (Hawkesford *et al*., 2013; Shewry, 2015).

Waterlogging is an important abiotic stress with a global impact on crop productivity (Zampieri *et al*., 2017; Tian *et al*., 2021). The occurrence of flooding events has risen in recent decades due to climate change, which results from intensified and unpredictable rainfall (Pais *et al*., 2020; IPCC 2023). If the projected global warming scenarios occur, the frequency of severe weather events will increase, leading to an elevated vulnerability to flooding in areas that were previously unaffected (Fatica *et al*., 2022). Flooding impacted both urban and agricultural areas, already affecting annually 15-20% of the global wheat-cultivated land (Kaur *et al*., 2020). Globally, floods were responsible for two-thirds of all damage and loss to crops between 2006 and 2016 (FAO, 2017).

Water plays a vital role in plant growth, being crucial in plants interaction with the environment (Yu *et al*., 2014; Gavrilescu, 2021). Excessive water in the soil may alter its properties by reducing oxygen availability and disrupting its diffusion to plant tissues (Pan *et al*., 2021). This can result in hypoxic or anoxic conditions (Sasidharan *et al*., 2017). The effects of waterlogging are dependent on multiple factors, such as the depth and duration of the stress (Herzog *et al*., 2016), the crop growth stage (de San Celedonio *et al*., 2014; Pampana *et al*., 2016), and the weather conditions (Herzog *et al*., 2016).

In a waterlogged environment, oxygen deficiency can restrict root growth and ultimately lead to their death. Such conditions hinder energy-dependent processes, such as the absorption and transport of water and nutrients to the shoot, thereby compromising plant growth and development (Herzog *et al*., 2016; Kaur *et al*., 2020; Pan *et al*., 2021). Moreover, this impairment has a direct impact on the final yield (Zheng *et al*., 2009; Arguello *et al*., 2016; Herzog *et al*., 2016; Kaur *et al*., 2020; Ding *et al*., 2020). Decreases in leaf nitrogen content, leaf water potential, stomatal conductance, CO_2 assimilation rate, and photosynthesis, as well as accelerated occurrence of leaf chlorosis and senescence, may also be observed (Pan *et al*., 2021).

Waterlogging-sensitive plants experience a decrease in the activity of their photosynthetic machinery, leading to an excessive production of reactive oxygen species (ROS). This excessive ROS production causes severe oxidative damage and degradation of cellular structures, interfering with normal metabolism (Herzog *et al*., 2016; Lal *et al*., 2019).

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*Chapter based in the review paper:
Pais IP, Moreira R, Semedo JN, Ramalho JC, Lidon FC, Coutinho J, Maçãs B, Scotti-Campos P (2023). Wheat Crop under Waterlogging: Potential Soil and Plant Effects. *Plants*, 12, 149. https://doi.org/10.3390/plants12010149

ROS lead to lipoperoxidation, resulting in cell membrane injury, protein degradation, enzyme inactivation, and damage to nucleic acids. Ultimately, this can result in cell death (Gill and Tuteja, 2010; Sharma *et al*., 2012; Hasanuzzaman *et al*., 2017; Bali *et al*., 2019). Considering predictable population growth and climate change scenarios, it is necessary to increase wheat productivity (Hawkesford *et al*., 2013) and ensure production stability, both crucial elements of food security.

1.1 Document rationale

The current study focuses on phenotypical, ecophysiological, and biochemical aspects of bread wheat plants in response to waterlogging. These features are crucial for understanding how plants interact with the environment, evaluating their ability to adapt, and identifying key traits among germplasm with different genetic backgrounds. The study is divided into seven chapters.

The present chapter provides an introduction, outlining the relevant elements within the context of the findings that will be elaborated upon in subsequent chapters.

Chapter 2 describes the research methodology. The respective sections detail the distinct methodologies employed in the *in vitro* and *in vivo* studies, which form the two main components of the work. The methods used in the *in vivo* studies, which examine the effects of 14 days of waterlogging imposed during the tillering stage, will be categorized as follows: soil changes, premature senescence, ecophysiological traits of the waterlogging response, and yield and yield traits.

Chapter 3 details the *in vitro* examination of the seminal root systems of the 23 selected bread wheat genotypes.

Chapter 4 presents the outcomes of the *in vivo* investigation regarding the impacts on soil, plant growth, and senescence.

Chapter 5 focuses on the effects of waterlogging on ecophysiological traits.

Chapter 6 discusses the effects of waterlogging on yield and yield traits. Additionally, it attempts to synthesise the diverse findings by establishing connections between *in vivo* and *in vitro* research and examining the correlations between yield impacts and the studied traits.

Chapter 7 provides the final remarks and future perspectives.

1.2 Bread wheat

Wheat is an annual plant belonging to the genus *Triticum* of the *Poaceae* family, kingdom *Plantae*. Bread wheat belongs to the species *Triticum aestivum* L., and its genome includes three diploid subgenomes (AA, BB, and DD) that originated from different ancestors.

Around 7 million years ago, an unidentified wheat-like diploid ancestor gave rise to two diploid lineages, *Triticum urartu* (2n = 14, AA) and *Aegilops speltoides* (2n = 14, BB). After *ca*. 1 million years, the two linages hybridised and produced a new diploid lineage, *Aegilops tauschii* (2n = 14, DD). The two lineages, AA and BB, hybridised *ca*. 500,000 years ago, resulting in the formation of the tetraploid *Triticum turgidum* (2n = 28, AABB). After *ca*. 400,000 years, this tetraploid hybridised with *Aegilops*

tauschii (2n = 14, DD), resulting in the hexaploid *Triticum aestivum* (AABBDD, 2n = 42) (Marcussen *et al*., 2014; Brewster *et al*., 2019; Gruet *et al*., 2022). These events are summarised in Figure 1.1).

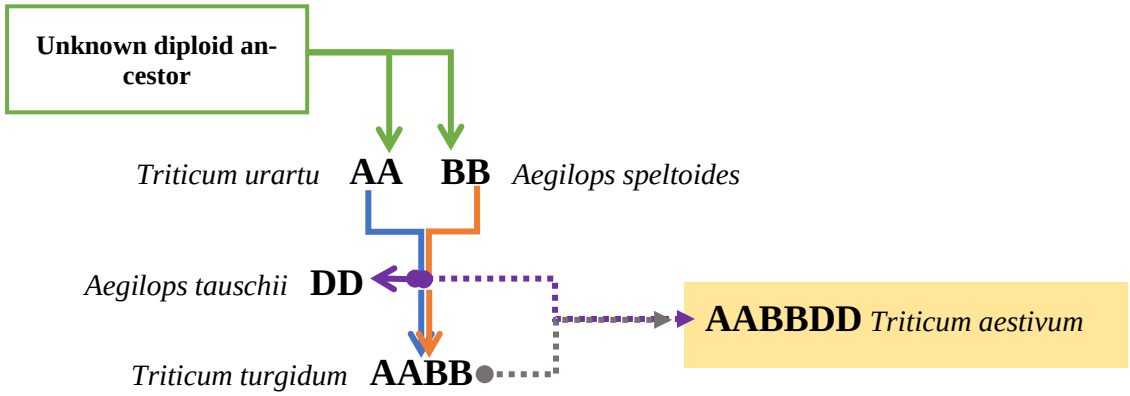


Figure 1.1 – Putative scheme of hybridization events that led to the creation of *Triticum aestivum* L.

The recent sequencing of the hexaploid (AABBDD) and tetraploid (AABB) wheat genomes (IWGSC, 2014), along with their diploid ancestors (AA, BB, DD), provides a valuable resource for improving this species (Jia *et al*., 2013; Ling *et al*., 2013; Walkowiak *et al*., 2020), making it an additional tool in wheat breeding programmes. Moreover, the genetic diversity present in wild relatives provides significant tolerance traits for both biotic and abiotic stresses (Brewster *et al*., 2019; Kapazoglou *et al*., 2023).

The wheat plant (*Triticum* sp.) is structured into roots, culm, leaves, and inflorescences. The root system consists of seminal, permanent (crown), and adventitious roots.

The development of leaves begins with the emergence of the coleoptile (Figure 1.2 A), a false leaf that functions as a protective covering for the initial leaf (Figures 1.2 A and B).

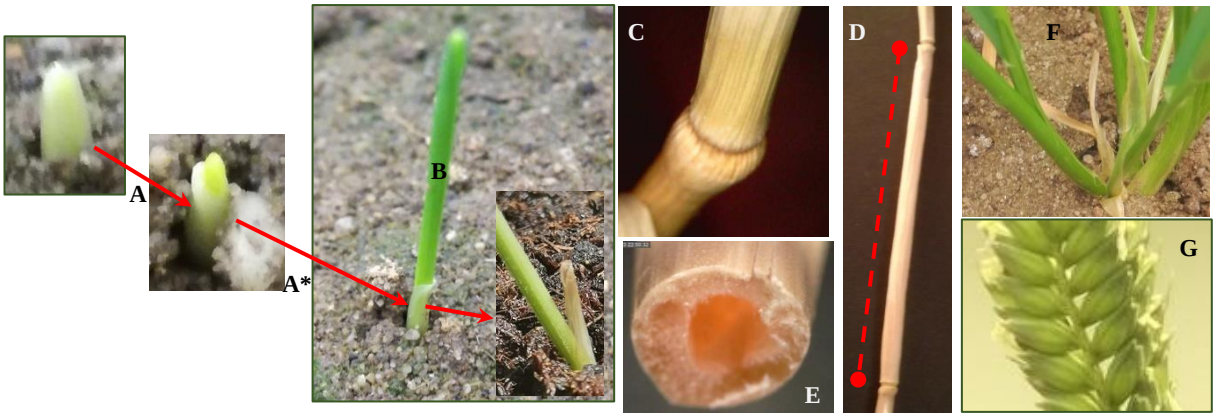


Figure 1.2 - Some anatomical aspects of wheat in different development stages. Emergency of the coleoptile (A); coleoptile opening and the first leaf emergency (A*); Complete coleoptile opening and developing first leaf (B); Detail of a culm node (C), and culm internode distance (D); Wheat plant at tillering stage (F); Detail of wheat spike, with the spikelets attached alternately and oppositely on the rachis (G). (Photos by the author).

By the end of the growth cycle, wheat plants typically have 5 to 6 leaves per culm (Scheeren *et al*., 2015), which corresponds to the number of nodes (Figure 1.2 C). Depending on the growth conditions, the culm height can be different between genotypes and even within the same genotype, influenced

by the number and length of the internodes (distance between two consecutive nodes, Figure 1.2 D). The culm is a tubular structure, hollow inside (Figure 1.2 E) with 4 to 7 internodes (Scheeren *et al*., 2015). About 15 days after germination, new culms, known as tillers (Figure 1.2 F), are produced. The appearance of the first tiller depends on the genotype, plant density, sowing depth, air temperature, radiation, and seed size. The environment greatly influences this stage of development, with tillers being produced only when appropriate conditions occur (Shang *et al*., 2021). The wheat inflorescence is a compound and distichous spike, formed by spikelets attached alternately and oppositely on the rachis (Figure 1.2 G). There is considerable variation in the density, shape, length, and width of the spike.

The seminal roots originate directly from the seed while simultaneously developing the coleoptile (Figure 1.3 A). In most plants, around 20 days after emergence, approximately 1-2 cm below the soil surface, the crown forms from which the permanent roots emerge (Scheeren *et al*., 2015) (Figure 1.3 B). In certain conditions, adventitious roots can emerge above the soil surface from the first or second nodes of the plant (Figure 1.3 C).

Figure 1.3 - Details of wheat root system. Seminal root system emerging from the seed simultaneously with the coleoptile (A); Permanent roots (B); Adventitious roots emerging from the culm 1st node above the soil surface (C). (Photos by the author).

To accurately assess the developmental stage of wheat, the Zadoks scale (Zadoks *et al*., 1974) is the most detailed and universally accepted. A two-digit code represents this scale, with the first digit representing one of the ten main developmental stages and the second digit representing one of the ten steps that subdivided each main stage (Scheeren *et al*., 2015). This detailed categorisation allows for accurate monitoring and assessment of wheat growth. The Zadoks scale describes the developmental stages of wheat as follow:

Stage 0 – Germination: From 00 (dry seed) to 09 (leaf at the apex of the coleoptile).

Stage 1 - Seedling growth: From 10 (first leaf through the coleoptile) to 19 (nine or more leaves unfolded).

Stage 2 – Tillering: From 20 (only the main culm) to 29 (main culm and nine or more tillers).

Stage 3 - Stem elongation: From 30 (erect pseudoculm) to 39 (flag leaf ligule just visible).

Stage 4 – Booting: From 40 (flag leaf sheath extending) to 49 (first awns visible).

Stage 5 - Inflorescence emergence: From 50 (first spikelet of the inflorescence just visible) to 59 (complete emergence of the inflorescence).

Stage 6 – Anthesis: From 60 (beginning of anthesis) to 69 (complete anthesis).

Stage 7 - Milk development: From 70 (watery caryopsis) to 79 (end of the milk stage).

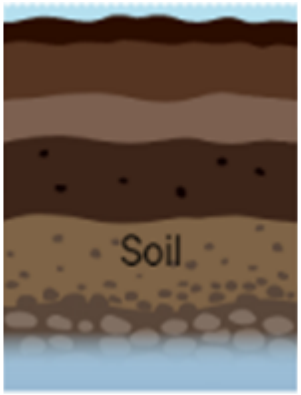
Stage 8 - Dough development: From 80 (beginning of the dough stage) to 89 (hard dough stage).

Stage 9 – Ripening: From 90 (hard caryopsis) to 99 (secondary dormancy lost).

1.3 Waterlogging events and their impacts in the soil

Waterlogging can occur whenever soil moisture levels exceed the field capacity. In these situations, the soil profile surrounding the plant root system becomes oversaturated with an excessive amount of water, saturating the soil pores (Walne and Reddy, 2021). This abiotic stress has a negative effect on the majority of terrestrial plants, limiting crop yield. Changes in the physical, chemical, electrochemical, and biological properties of the soil (Table 1) can lower root biomass, hampering vegetative development (Herzog *et al*., 2016; de San Celedonio *et al*., 2016) and inducing plant organ senescence (Jimenez *et al*., 2012; Pan *et al*., 2021).

Table 1.1 - Effects of waterlogging on the physical, electrochemical, chemical and biological properties of the soil. Elements of the figure were created using biorender.com and published in Pais *et al*., 2023.

Impacts in soil properties due to waterlogging		
	Physical	Changes in ideal solid:pore and soil:air volume ratios; Decreased [O_2]; Increased [CO_2]; Lowered diffusion coefficient for gases.
	Electrochemical	Decreased redox potential (Eh); Changes in soil pH and EC.
	Chemical	Changes in solubility, mobility and bioavailability of nutrients and potentially toxic elements.
	Biological	Changes in microbial activity and in the nitrogen cycle (mineralization and immobilization of organic N).

In waterlogged soil, all pores are filled with water, changing the ideal solid:pore material (50:50) and soil:air volume (75:25) ratios, which have implications for plant physiological performance (Morales-Olmedo *et al*., 2015). The atmosphere contains 21% oxygen, whereas the soil should maintain a minimum concentration of 10% (Goud *et al*., 2022). The dissolved concentration of oxygen in well-cultivated soil is approximately 0.23 mol m^{-3}, while in waterlogged areas it is less than 0.05 mmol m^{-3} (Pan *et al*., 2021). The soil atmosphere is usually rich in CO_2 and deficient in O_2 (Morales-Olmedo *et al*., 2015) as a result of the aerobic respiration carried out by roots and microorganisms. Nevertheless, effective aeration enables swift O_2 intake and CO_2 release, ensuring an adequate supply of oxygen to meet the nutritional plants requirements and support their development (Morales-Olmedo *et al*., 2015). Waterlogging significantly reduces gas exchanges between soil and atmosphere, as the diffusion of gases (such as oxygen and carbon dioxide) in water is significantly slower than in air (Sasidharan *et al*., 2017; Upreti *et al*., 2020). Additionally, plant root respiration and microbial activity consume the trapped oxygen in the soil, promoting a hypoxia/anoxic situation in the rhizosphere (Sasidharan *et al*., 2017), resulting in both insufficient O_2 levels and toxic CO_2 concentrations in the soil.

Soil redox potential (Eh) can undergo significant changes due to waterlogging, and it is the most important physicochemical parameter for assessing a flooded soil oxidation or reduction level (Matilla, 2024). Eh measures the difference between oxidation (the release of electrons from a chemical compound) and reduction (the uptake of electrons by a chemical compound), thereby providing the overall electron availability in the soil (Zhang and Furman, 2021; Matilla, 2024). Therefore, a high Eh favours the process of oxidising reduced compounds, while a low Eh level promotes the reduction of oxidised compounds (Wang *et al*., 2018). In flooded soils, oxygen deficiency leads to biological reduction processes and a decrease in Eh values. At [O_2] of *ca.* 10%, Eh will be approximately 250 mV, and 0 mV for [O_2] of 1-2%. Under optimal aeration conditions (>1 mg O_2 L^{-1}) (Matilla, 2024), cultivated soils usually have Eh values ranging from +300 to +500 mV (Macías *et al*., 2010), with values between +400 and +450 mV appearing to be the most favourable (Husson *et al*., 2013). Although Eh can reach values between -300 mV and +100 mV (Husson *et al*., 2013) in waterlogged soils. When [O_2] drops below 1% with Eh values ≤ 100 mV, a transition from aerobic to anaerobic metabolism in plants roots takes place (Søndergaard *et al*., 2009), explaining why growth decreases in plants susceptible to this water stress.

The ideal pH range for most cultivated plants is usually between 6.5 and 7, which ensures optimal nutrient availability. Nonetheless, growth conditions are still favourable within the range of values between 5.5 and 8.0 (Husson *et al*., 2013; Warner *et al*., 2023). In waterlogged soils, pH tends toward neutrality, with increases in acidic soils and decreases in alkaline ones (Parent *et al*., 2008; Husson *et al*., 2013). Soil pH strongly influences the ability of nutrients and potentially toxic elements to dissolve, move, and become available for plants to absorb. Soil acidity is linked to deficiencies in molybdenum, phosphorus, magnesium, and calcium and more availability of elements like aluminium, manganese, and iron. However, excessive levels of aluminium, iron, and manganese may be toxic to plants (Liu *et al*., 2020). Conversely, alkaline soils generally lack sufficient amounts of Cobalt and Zinc and show reduced availability of phosphorus due to its binding with calcium. Under these conditions, plants tend to be under developed, depicting poor growth and lower yield (Setter *et al*., 2009; Mantri *et al*., 2012; Xiao *et al*., 2014; Herzog *et al*., 2016; Rizvi *et al*., 2020).

Nitrogen, an essential element for plant growth and a key nutrient that significantly limits crop yield, is typically extracted from the soil in the form of inorganic compounds such as ammonium (NH_4^+) or nitrate (NO_3^-). The presence of nitrogen in the soil can impact on various plant processes, including nutrient uptake, enzyme activity, photosynthesis, respiration rate, water balance, and signalling pathways. This impact is influenced by both the quantity and the form of nitrogen available in the soil (Guo *et al*., 2007; Guo *et al*., 2019). Waterlogging significantly reduces gas diffusion, leading to an increase in NH_4^+ in the soil. While this ion functions as an intermediary in numerous metabolic reactions, its sole presence as the nitrogen source can induce a strong inhibition of potassium uptake. Potassium is an essential nutrient that plays a crucial role in several important plant physiological processes (Guo *et al*., 2019). Wheat grows preferentially on NO_3^- nutrition. In waterlogged soils, substantial decreases (15-20%) in wheat growth and yield were reported (Herzog *et al*., 2016), possibly due to high NH_4^+ levels.

Electrical conductivity (EC) serves as a reliable indicator of soil quality (Bünemann *et al*., 2016; Carmo *et al*., 2016) and is closely related with levels of NO_3^-, sulphate (SO_4^{2-}), NH_4^+, potassium, sulphur,

and chloride, as well as the soil nutrient availability and crop yield. Waterlogged soil can cause substantial changes in EC since it inhibits oxygen-based reactions. The anaerobic reactions that follow convert NO_3^- into NO_2^- and then into N_2O or N_2. Additionally, insoluble MnO_2 and $Fe(OH)_3$ turn into Mn^{2}+ and Fe^{2}+, respectively. When all ferric iron has been reduced to ferrous, accomplished when the Eh < +120 mV at a pH of 7, SO_4^{2-} turns into H_2S, followed by CO_2 converted into CH_4. Thus, anaerobic reactions result in nitrate loss from the soil as gaseous N_2O or N_2 (denitrification), an increase in phytotoxic substances (*e.g.*, H_2S), and very high concentrations of soluble Fe^{2+} and Mn^{2+} ions (Fitter and Hay, 2002). During the initial stages of stress, the electrical conductivity (EC) of the soil tends to rise, reach its peak, and subsequently decline to a stable level. This rise is due to the mobilisation of Fe^{2+} and Mn^{2+}, the accumulation of NH_4^+, HCO_3, and RCOO-, as well as the replacement of cations adsorbed on colloids by available Fe^{2+}, Mn^{2+}, and NH_4^+. Due to nutrient leaching, the soil may be less fertile after a flood (Santos *et al*., 2002), displaying low EC values. However, EC values below 0.10 dS m^{-1} suggest soil deterioration (Sharma *et al*., 2018).

1.4 Waterlogging events – impacts at plant level

Plants responses to waterlogging are highly dependent on multiple factors, including the plant´s developmental stage, the depth of water level, and the duration of exposure (de San Celedonio *et al*., 2014; Wu *et al*., 2015; Fukao *et al*., 2019; Langan *et al*., 2022).

Various studies have reported different findings regarding the wheat reproductive phases as the most susceptible to waterlogging stress. Researchers have identified the period from stem elongation to anthesis and post-anthesis as the most vulnerable (Setter *et al*., 2003; Araki *et al*., 2012a; de San Celedonio *et al*., 2014). Similarly, the seedling stage (Wu *et al*., 2015), as well as the jointing and tillering stages (Ding *et al*., 2020), have also been referred to as susceptible. Conversely, other studies found the period after anthesis to be the most tolerant to waterlogging, followed by the jointing stage (Araki *et al*., 2012a). Additionally, decreases in leaf area at the anthesis and at the milk-ripe stage were also found. At the tillering stage, reported yield decreases were due to reduced spike number and, as a result, a decline in the kernel number per plant (Wu *et al*., 2015). Furthermore, waterlogging during the booting stage mainly led to reduced yield due to decreased single kernel weight. Other authors found that waterlogging throughout the three- and four-leaf stages did not induce yield changes (Pampana *et al*., 2016).

In the field, waterlogging depths can change, influencing the severity of plant damage. Waterlogging can be classified as either total or partial, depending on how deeply water penetrates the soil. Total waterlogging occurs when there is water throughout the soil, from the bottom to the surface, affecting all plant organs below ground. On the other hand, partial waterlogging refers to a scenario where water does not reach the soil surface and only affects a portion of the root systems (Sasidharan *et al*., 2017). In bread wheat, waterlogging stress impacts the tiller emission differently, with pronounced declines as the water level approaches the soil surface (Malik *et al*., 2001). Furthermore, plants subjected to total

waterlogging experienced more severe reductions in the length of adventitious root main axes per plant than those that were partially waterlogged.

The duration of waterlogging events has a significant effect on the damage inflicted on crops. Overall, plants experience more pronounced negative effects as the duration of exposure increases (Tian *et al*., 2021). The reported impacts of waterlogging include changes in plant photosynthesis, respiration, transpiration, and the antioxidative system. It also promotes senescence and reduced accumulation and remobilization of photosynthetic products. These effects ultimately result in decreased yield components, such as the number of spikes, kernels per spike, and kernel weight (Olgun *et al*., 2008; Hossain *et al*., 2011; Li *et al*., 2012; Araki *et al*., 2012a; Araki *et al*., 2012b; Herzog *et al*., 2016). The primary cause for the observed negative effects is the reduced availability of O_2 in the soil (Colmer *et al*., 2009; Voesenek *et al*., 2015; Herzog *et al*., 2016). However, plants may undergo anatomical, morphological, or physiological changes to mitigate the effects of this deficiency (Ploschuk *et al*., 2018).

1.4.1 Root system

Roots act as vital organs for water and nutrient uptake, storage of photoassimilates, anchoring, mechanical support, and interaction with the rhizosphere interface (Hendel *et al*., 2021; Takahashi and Pradal, 2021). To accomplish this, roots rely on energy obtained through cellular respiration (Fagerstedt *et al*., 2013). Waterlogging primarily impacts wheat plants at the root level, where the initial responses are triggered (Chen *et al*., 2020). Subsequent reactions reflect the severe impairments in shoot growth (Robertson *et al*., 2009; Herzog *et al*., 2016; Pan *et al*., 2021) (Figure 1.4).

In waterlogged conditions, the low concentration of oxygen in the rhizosphere increases anaerobic respiration. As a result, there is a decrease in ATP production, which leads to arrested root growth and root death (Herzog *et al*., 2016; Pan *et al*., 2021). Additionally, the lack of energy at the root level hinders the phosphorylation of aquaporins, which regulate the flow of water in cells, resulting in a drastic reduction in root hydraulic conductivity (Malik *et al*., 2002; Tan *et al*., 2018) and in the ability of roots to absorb water and nutrients. Furthermore, the soil Eh drop in may increase the availability of Mn^{2+} and Fe^{2+} to toxic levels and their accumulation in the roots. In anoxic soil conditions, organic acids and other potentially toxic metabolites may increase through the decomposition of organic matter. Anaerobic respiration can lead to the accumulation of lactic acid, ethanol, aldehydes, and other substances. It also results in ROS production, namely hydrogen peroxide, which can cause cellular damage (Colmar *et al*., 2019; Pan *et al*., 2021). Organic acid toxicity may inhibit root respiration, reduce nutrient uptake, promote suberisation and/or lignification in the outer cell walls, and can cause root occlusions (Pang *et al*., 2007). In barley, adverse effects of organic acids on K^+ fluxes in roots were reported (Blokhina *et al*., 2003). Endogenously produced CO_2 and ethylene can also adversely affect roots. Excess CO_2 can cause root cells to become more acidic, while high levels of ethylene can inhibit root extension (Herzog *et al*., 2016; Nguyen *et al*., 2018).

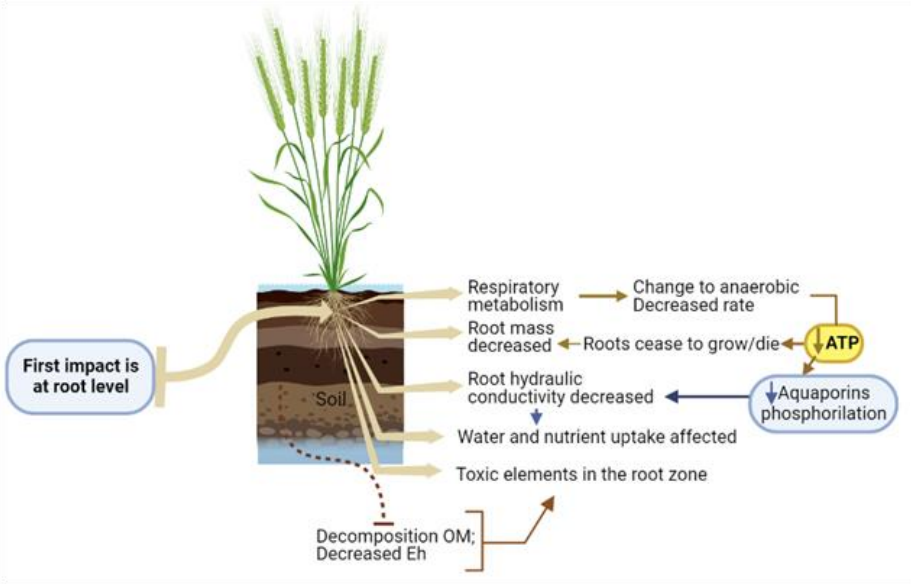


Figure 1.4 - Major effects of waterlogging occurring at root level in wheat plants. Figure elements created using biorender.com and published in Pais *et al.,* 2023.

Some plants tolerant to hypoxia (low O_2 levels) or anoxia (complete absence of O_2) are able to develop morphological adaptations to compensate for the lack of O_2 in the root zone (Ayi *et al*., 2016; Nguyen *et al*., 2018). Adventitious roots commonly originate from either the culm or the branches and are a common response (Ayi *et al*., 2016). These roots promote gas transport (*e.g*., O_2) as well as nutrient and water uptake, thereby making them available to the submerged roots (Steffens and Ranmussen, 2016). This process greatly improves the survival and productivity of plants in waterlogging conditions (Steffens *et al*., 2013) by mitigating the O_2 deficiency. Furthermore, it was reported that in wheat plants subjected to waterlogging, adventitious roots exhibit a higher efficiency in absorbing phosphorus and potassium compared to seminal roots (Wiengweera and Greenway, 2004).

When plants are subjected to waterlogging, ethylene accumulation can trigger programmed cell death of root cortical cells, leading to the formation of aerenchyma in adventitious roots. In waterlogged conditions, aerenchyma development in wheat can boost plants tolerance and survival. The interconnected, large, gas-filled intercellular spaces of this specialised parenchymal tissue reduce the stress caused by oxygen deprivation. These cavities create a low-resistance pathway, enhancing gas diffusion between the roots and shoot (Steffens and Rasmussen, 2016). As the O_2 levels improve, the amount of energy available increases (Yamauchi *et al*., 2014; Steffens and Rasmussen, 2016). Furthermore, aerenchyma can discharge CO_2 and toxic volatile substances from submerged tissues (Pan *et al*., 2021). However, the internal O_2 movement to the apex, which enables root expansion, is limited, and adventitious root growth not fully compensates seminal root loss. Waterlogged wheat plants can experience a decrease in the root:shoot ratio from 0.4 to 0.2. This reduction is due to a stronger decrease in root dry mass, up to 62%, compared to the shoot, which can decline up to 33% (Herzog *et al*., 2016).

In addition to the emission of adventitious roots and aerenchyma formation, root architecture can also play an important role as it determines root distribution in the soil (Pan *et al*., 2021). Shallower root

systems can be advantageous in flooded areas for oxygen uptake, as the upper soil layers usually contain a higher O_2 concentration than deeper ones (Omori *et al*., 2007; Pais *et al*., 2022a).

1.4.2 Shoot development and productivity

The absorption of water through the roots and the release of water vapour through the leaves (transpiration) enables plant growth. However, when exposed to waterlogging, susceptible genotypes may present severe impairments of some key metabolic processes, such as photosynthesis, respiration, and transpiration. The detrimental effect on these processes leads to severe energy deficiency, poor growth, and enhanced leaf and organ senescence. This may result in decreased accumulation and remobilization of photoassimilates, ultimately leading to a reduction in grain yield (Gibbs *et al*., 2003; Shao *et al*., 2013; Herzog *et al*., 2016; de San Celedonio *et al*., 2018) (Figure 1.5).

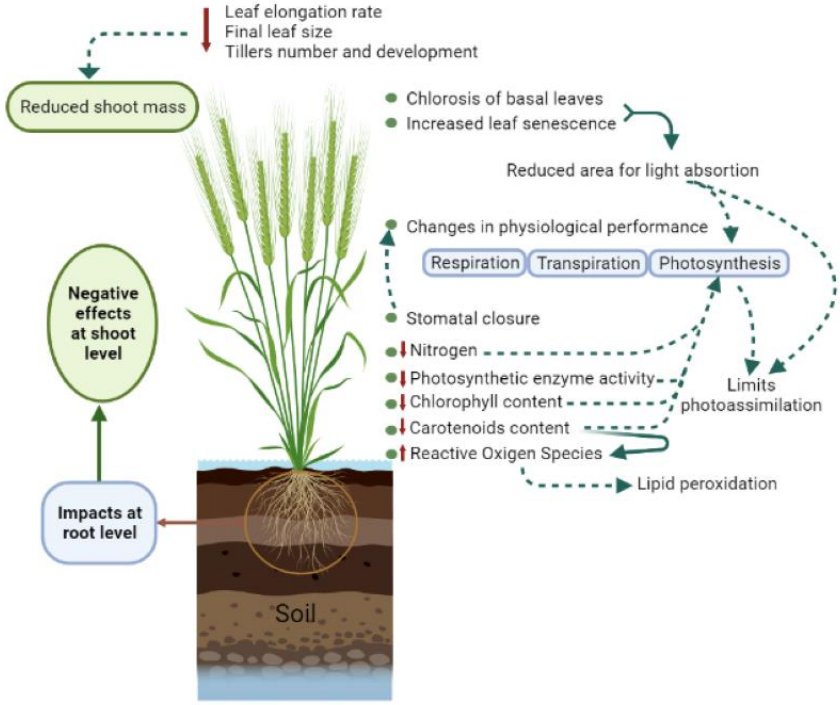


Figure 1.5 – Effects of waterlogging at shoot level in wheat plants. Figure elements created using biorender.com and published in Pais *et al.,* 2023.

Waterlogging significantly retards the growth of above-ground plant parts. This is mostly due to a reduced rate of leaf elongation, resulting in smaller leaves. Additionally, waterlogging decreases the number of tillers and impairs their development (Collaku and Harrison, 2002; Dickin *et al*., 2008; Robertson *et al*., 2009; Amri *et al*., 2014; Herzog *et al*., 2016; Pampana *et al*., 2016). Malik *et al*. (2002) found that subjecting three-week-old plants to waterlogging for 3-21 days, followed by a recovery period of 21 or 7 days, resulted in a decrease in shoot mass ranging from 43% to 72% compared to plants that were well-drained during the same time frame. According to Herzog *et al*. (2016), the average dry mass of wheat shoots grown in waterlogged soil was decreased by 67%. This decline significantly

reduces the surface area available for light absorption. This, along with premature leaf senescence and overall plant wilting, limits photoassimilation.

In wheat, the process of natural senescence, which marks the final stage of leaf development, typically starts during grain filling and occurs after a period of intense photosynthesis and biomass accumulation (Agüera and Haba, 2018). During senescence, leaves suffer chlorophyll loss and the photosynthetic apparatus breaks down. This leads to a decrease in the capacity and efficiency of photosynthetic energy conversion (Miloud and Ali, 2020). The proportion of dry mass of senescent leaves in bread wheat may be increased during waterlogging as well as after the stress terminus (Pais *et al*., 2022b).

1.4.3 Impacts in physiological performance

1.4.3.1 Gas exchanges and sugars metabolism

Stressful conditions lead to changes in respiration and photosynthesis (P_n), which are often used as indicators of productivity in stressful conditions (Gill and Tuteja, 2010; Scotti-Campos *et al*., 2014). Waterlogging-susceptible plants significantly diminished their physiological activities, which can lead to cell death (Manghwar *et al*., 2024). In contrast, tolerant plants showed less severe effects or may even show an enhancement in the response of some traits (Malik *et al*., 2001).

Waterlogging may cause a reduction in shoot physiological performance in wheat plants due to impaired root function (Bhagat *et al*., 2014; Herzog *et al*., 2016; Manghwar *et al*., 2024). Stomatal closure, reduced transpiration, and photosynthesis inhibition are common responses to this environmental stress (Bhagat *et al*., 2014; Herzog *et al*., 2016; Manghwar *et al*., 2024). Stomatal conductance (g_s) is a key factor in photosynthesis, with a significant effect on photosynthetic rates during waterlogged conditions (Herzog *et al*., 2016; Manghwar *et al*., 2024) Stomatal closure and a decrease in stomatal conductance (g_s) enable the down-regulation of leaf transpiration (Herzog *et al*. 2016). However, this also lowers the internal CO_2 concentration (C_i), which in turn restricts the process of carbon fixation, resulting in a reduction in photosynthesis and an increase in respiration. This has a negative impact on plant production (Malik *et al*., 2001; Wu *et al*., 2014). Nevertheless, photosynthetic rates can also decline as a result of non-stomatal factors, such as chlorophyll degradation and reduced chlorophyll synthesis. This can lead to leaf senescence and a yellow appearance (Amri *et al*., 2014; Herzog *et al*., 2016; Ploschuk *et al*., 2018).

The decline in photosynthetic activity and the detrimental effects of waterlogging can be exacerbated by the damage caused to photosystem II by reactive oxygen species (ROS), decreased photosynthetic enzyme activities, and low nitrogen content (Amri *et al*., 2014; Herzog *et al*., 2016; Ploschuk *et al*., 2018; Langan *et al*., 2022). Through P_n, plants convert carbon dioxide and water into sugars, which serve as their primary energy source for various cellular activities (Anjum *et al*., 2011). Waterlogging can lead to changes in P_n and respiration, with consequences for sugar metabolism and energetic balance (Yadav *et al*., 2015). Lower P_n rates reported at the onset of waterlogging may be related to the accumulation of sugars in the leaves of wheat plants (Malik *et al*., 2002; Herzog *et al*., 2016) rather than to

stomatal closure (Malik *et al*., 2002; Herzog *et al*., 2016). The accumulation of sugars in the leaves may be caused by root hypoxia resulting from waterlogging. Under these circumstances, the growth of both the roots and the aboveground part is frequently hindered or suspended, resulting in the production of sugars in the leaves exceeding their consumption (Malik *et al*., 2001; Malik *et al*., 2002; Herzog *et al*., 2016). Simultaneously, constraints in the root system diminish the capacity of the roots to transport phloem (Herzog *et al*., 2016; Manghwar *et al*., 2024), which also contributes to the accumulation of photoassimilates in the leaves. Thus, the sugar overproduction combined with a reduced ability for phloem transport in hypoxic roots leads to further decrease in P_n as a negative feedback of carbohydrate accumulation (Herzog *et al*., 2016). Nevertheless, an accumulation of sugars during the initial phase of stress may also play a vital role in wheat plants ability to withstand and survive long-term waterlogging. In this situation, plants experience a lack of energy and carbohydrates due to decreased photosynthesis and aerobic respiration. Stored glucose reserves may be used to enable the maintenance of metabolic activity under anaerobic conditions (Yadav *et al*., 2015; Alizadeh-Vaskasi *et al*., 2018). In addition, sugars are also involved in plant stress responses and adaptation by contributing to the stabilisation of membrane structures and maintaining cell turgor through osmotic adjustment and osmoprotection (Ozcubukcu *et al*., 2013).

1.4.3.2 Chlorophylls and carotenoids

Photosynthetic pigments are fundamental molecules in the photosynthetic process, serving primarily to absorb light and produce reducing compounds (Cessna *et al*., 2010; Pan *et al*., 2021). Changes in pigment content and composition directly impact photosynthetic rate.

Chlorophylls (Chl) are essential for the conversion of light radiation into chemical energy. They are strictly linked to the photosynthetic efficiency and, therefore, to plants growth and their environmental adaptability (Cessna *et al*., 2010; Pan *et al*., 2021). Chlorophyll a is located at the reaction centres of both photosystems (PSI and PSII), whereas chlorophyll b serves as the most important accessory pigment in light-harvesting complexes. Waterlogging may decrease Chl levels in wheat, as previously reported in studies conducted at the emergence (Lazár *et al*., 1999) or at tillering stages (Amri *et al*., 2014).

Carotenoids play multiple roles in plant metabolism. In photosynthesis, they act as accessory antenna molecules, harvesting and transferring light energy to chlorophylls. Carotenoids also play an important role in oxidative stress tolerance. They protect the photosynthetic apparatus by scavenging ROS and repressing lipid peroxidation (Simkin *et al*., 2022). Waterlogging has been found to impact the concentration of carotenoid pigments, with reductions in wheat susceptible plants (Collaku *et al*., 2002; Lazár *et al*., 2006; Ploschuck *et al*., 2018), while the amount remains high in tolerant genotypes (Singh *et al*., 2018). Overall, the decline in carotenoid content is more severe in plants subjected to longer waterlogging periods, and it strongly depends on the crop development phase (Lazár *et al*., 2006).

1.4.3.3 Chlorophyll fluorescence

The light-harvesting antenna captures light photons through chlorophyll and partially (*ca*. 2%) reemits them as fluorescence (Fukao *et al*., 2019). Chlorophyll fluorescence is a reliable and sensitive tool to assess light-harvesting efficiency in plants (Sharma *et al*., 2018; Sharma *et al*., 2019), which complements the information obtained from gas exchanges. Under stressful conditions, this parameter can decrease, allowing a quantitative assessment of stress responses and indirectly providing information on leaf photosynthetic performance.

The maximal photochemical efficiency of PSII (F_v/F_m) evaluates the proportion of functional PSII reaction centres. Reductions in this ratio can indicate damage to the photosynthetic apparatus, which may result in a decrease in P_n (Shao *et al*., 2013). In wheat, declines in the F_v/F_m ratio have been reported due to the imposition of waterlogging, indicating impairment of PSII (Amri *et al*., 2014; Wu *et al*., 2015; Herzog *et al*., 2016) and, consequently, a decreased use-efficiency of captured photon energy (Shao *et al*., 2013).

1.4.3.4 Membrane integrity and the role of lipid composition

Cellular compartmentalisation and metabolic processes functionality rely on the ability to maintain membrane integrity even in stressful conditions. This is crucial for protoplasmic tolerance (Scotti-Campos *et al*., 2014). Waterlogging, along with other biotic and/or abiotic stresses, can cause changes at the membrane level, resulting in structural effects that impact membrane permeability. This can be assessed by an increase in electrolyte leakage from cells (Dias *et al*., 2010; Shabala *et al*., 2011; Scotti-Campos *et al*., 2014; Scotti-Campos *et al*., 2015), which may indicate significant membrane damage and reduced ability to survive (Blokhina *et al*., 2010). Therefore, membrane stability is frequently used as an indicator of tolerance or susceptibility to environmental stresses (Scotti-Campos *et al*., 2014; Demidchik *et al*., 2014).

Lipids are fundamental plant macromolecules, playing key roles in membrane structure, energy storage, and metabolic signalling (Wang *et al*., 2004; Xiao *et al*., 2011; Xu *et al*., 2019; Xie *et al*., 2021). In response to abiotic stresses, qualitative and quantitative changes may occur in the lipid matrix composition, with remodelling plasticity being crucial for the maintenance of membrane integrity and significantly contributing to its functionality (Colmer *et al*., 2019; Xu *et al*., 2019). Hypoxia/anoxia can induce changes in membrane lipids, and it was shown that tolerant plants could increase the degree of unsaturation of membrane lipids and also enhance lipid biosynthesis under such conditions (Blokhina *et al*., 2010; Xie *et al*., 2021).

Lipid remodelling influences the fluidity, integrity, and permeability of plant cell membranes by changing the composition of lipid classes, the lengths of their carbon skeletons, or the saturation of their fatty acids (Xu *et al*., 2019; Xie *et al*., 2021). Hypoxia treatment significantly altered wheat lipid composition, with tolerant genotypes exhibiting more efficient lipid remodelling, thereby preserving membrane bilayer structure during hypoxia stress (Xu *et al*., 2019). The phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) decreased by hypoxia. However, the PC to PE ratio increased in the tolerant genotype, limiting the synthesis of non-bilayer membrane phases and conserving fluidity.

Non-susceptible plants exhibited considerable increases in phosphatidylglycerol (PG) and phosphatidic acid (PA) as a result of hypoxia. Even though tolerant and susceptible genotypes had different amounts of PE, changes caused by hypoxia followed the same pattern in both. This suggests that PE did not play a role in hypoxia tolerance. Regarding the glycolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) changes in response to flooding have been reported (Xie *et al*., 2021). After 4 days of exposure to hypoxia, a 31.6% and 20.0% decrease in MGDG concentrations in sensitive and tolerant genotypes, respectively, were reported (Xu *et al*., 2019). DGDG content was unchanged by hypoxia in the sensitive genotype, while the tolerant genotype experienced an increase of 25.3% after 2 days of treatment, followed by a drop of 31.1% on the fourth day of hypoxia. Changes in glycolipids can have a significant effect on plants tolerance to waterlogging, since MGDG is essential for photosynthetic reactions and DGDG is essential for maintaining the maximum efficiency of photosynthetic electron flow by altering PSI and PSII activity (Xu *et al*., 2019).

1.4.3.5 Oxidative stress

Although ROS are a normal product of plant cell metabolism, both biotic and abiotic stresses often lead to oxidative stress, which is characterised by an increase in intracellular ROS levels. ROS can be categorised into two groups: free radicals, which include the superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), and perhydroxyl radical ($HO_2^{\bullet}$); and non-radicals, which comprise singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Pan *et al*., 2021). When accumulated in mesophyll cells, their strong oxidising activity can cause lipoperoxidation and degradation of membrane lipids, leading to oxidative damage to proteins and DNA, which in turn causes severe cell injuries (Sharma *et al*., 2012; Baxter *et al*., 2014; Pan *et al*., 2021). ROS preferentially target carbon-carbon double bonds in lipids, making cell membranes rich in polyunsaturated fatty acids (PUFA) highly susceptible to lipoperoxidation. These PUFA are particularly abundant in chloroplasts (Ayala *et al*., 2014; Duhan *et al*., 2019). In waterlogging-susceptible plants, the downregulation of the photosynthetic machinery leads to overproduction of ROS in the leaf. Rapid chlorosis of basal leaves precedes premature leaf senescence due to the remobilization of nitrogen to younger leaves (Herzog *et al*., 2016; Agüera and Haba, 2018). Decreased chlorophyll content in the remaining leaves is an indicator of oxidative stress (Anjum *et al*., 2011). Excessive water can strongly increase ROS content in plant cells, causing severe oxidative damage (Lal *et al*., 2019). This rise suggests the occurrence of lipoperoxidation events (Nikolaeva *et al*., 2010; Sachdev *et al*., 2021) and is commonly linked to higher levels of malondialdehyde (MDA), one of several by-products of lipid oxidation (Ayala *et al*., 2014; Duhan *et al*., 2019). Genotypes showing reduced levels of MDA under stress may be more resistant to oxidative stress (Duhan *et al*., 2019). ROS accumulation also causes a significant disruption to plant ionic homeostasis, directly influencing the functioning of various cations (Shabala *et al*., 2014) and anion channels (Pottosin *et al*., 2018). Wheat plants can overcome oxidative stress through activation of antioxidative defence systems involving both enzymatic and non-enzymatic mechanisms. These mechanisms interact in order to neutralise excessive ROS and minimise the extent of oxidative damage (Gill and Tuteja, 2010; Li *et al*., 2012; Lal *et al*., 2019; Bali *et al*., 2019). Antioxidant enzymes, such as superoxide dismutase, catalase, glutathione reductase, and ascorbate

peroxidase, prevent uncontrolled oxidation by removing, neutralising, or scavenging ROS and their intermediates. These enzymes catalyse the conversion of ROS into harmless compounds (Alizadeh-Vaskasi *et al*., 2018). Non-enzymatic antioxidants, such as reduced glutathione, ascorbic acid, carotenoids, and tocopherols, play a crucial role in membrane stabilisation and cellular component protection. Several reports of increased antioxidant enzyme activity in waterlogging-tolerant wheat plants have also been reported (Lázar, 2006; Pan *et al*., 2021).

1.4.3.6 Waterlogging effects in yield components

Waterlogging is known to cause decreases in grain yield per plant, as widely reported (Collaku and Harrison, 2002; Olgun *et al*., 2008; Robertson *et al*., 2009; Pampana *et al*., 2016; Ploschuck *et al*., 2018). Throughout the sowing, seedling, tillering, flowering, and grain-filling stages, continuous waterlogging can strongly reduce grain yield (Lázar, 2006; Olgun *et al*., 2008; Ding *et al*., 2020). Reduction can be attributed to poor seed formation, a lower number of spikes per unit area (Olgun *et al*., 2008; Ding *et al*., 2020) reduced spikes per plant (Marashi and Chinchanikar, 2010), a declined number of kernels per spike, and a lower thousand kernel weight (Arguello, 2016).

Determining if the number of fertile tillers is the main factor that influences yield changes in waterlogged wheat genotypes is crucial. The final yield is directly affected by the number of spikes per unit area, which in turn is impacted by the number of emitted tillers, which developed, and survived. Several studies reported yield decreases associated with low tiller survival due to waterlogging during the tillering stage (Malik *et al*., 2001; Malik *et al*., 2002). However, different wheat genotypes did not consistently reduce the number of fertile tillers, suggesting that plants had a successful strategy for maintaining production despite an energy deficit (Malik *et al*., 2001; Collaku and Harrison, 2002; Robertson *et al*., 2009). When the number of fertile tillers remains unaltered, the decline in productivity results from a small contribution of fertile tillers to the formation of the final yield (Valério *et al*., 2008). In such cases, smaller fertile tillers, smaller grains (Condon and Giunta, 2003; Yaduvanshi *et al*., 2012), or a decreased seed number per spike (Lázar, 2006; Olgun *et al*., 2008; Ding *et al*., 2020) may be involved.

1.5 Breeding for waterlogging-tolerant genotypes

It is crucial to develop genotypes that can tolerate waterlogging in order to sustain agricultural productivity in regions prone to excessive water. Research studies may contribute to a comprehensive understanding of the mechanisms that confer this tolerance. Waterlogging tolerance is a complex feature that is related to morphological adaptations, physiological responses, biochemical adjustments, and genetic control (Collaku and Harrison, 2005; Unay *et al*., 2020). The first step in breeding for waterlogging tolerance is to screen existing germplasm to identify tolerant varieties. This process also ensures the presence of sufficient genetic variability for waterlogging tolerance traits (Zhou *et al*., 2007; Amin *et al*., 2014). Screening refers to the evaluation of plant development and physiological performance under stress conditions (phenotypic screening), either in controlled environments or field trials. It can also

involve genotypes characterization using molecular markers associated with tolerance traits (genetic screening). Portuguese breeders typically employ the conventional selection approach for wheat breeding, choosing the best-performing progenies over several generations to develop new adapted varieties. Plant breeding still relies on phenotyping, not only due to its longstanding tradition but also because it is an essential tool for wheat breeders (Fasoula *et al*., 2020). Addressing the phenotypic wheat characteristics in breeding programmes allows for a well-informed, accurate, and efficient selection of genotypes based on observable and measurable traits.

Breeding for waterlogging tolerance requires a comprehensive approach that combines both traditional and modern techniques. Phenotyping and physiological characterisation provide important insights on the role of plant shoot and root systems in developing resilient wheat varieties that can cope with waterlogging. This is an effective way to ensure food security and sustainability in affected regions. Nevertheless, the process of identifying a new variety requires a minimum of ten successive generations of deliberate selection. Furthermore, after developing waterlogging-tolerant genotypes, it is necessary to test them in various environments to verify their performance and traits stability.

1.6 Work objectives

The present work aimed to characterise, under environmentally controlled conditions, bread wheat (*T. aestivum* L.) germplasm with different genetic backgrounds. The main goal was to study the seminal root system *in vitro* and the root and above-ground ecophysiological and morphological traits in plants subjected to waterlogging *in vivo* in order to predict the impact of such stress on yield traits and to aid in the early selection of genotypes. This will also enable the identification of genotypes suitable as progenitors in the Portuguese wheat breeding programme. We primarily examined the physiological performance and phenotypic variations during a 14-day total waterlogging period imposed at the tillering stage, as well as the following 7 and 14 days of recovery. The impacts at harvesting were also discussed. We aimed to understand the traits and/or eco-physiological mechanisms that contribute to the tolerance/susceptibility to this stress, as well as the most effective indicators for identifying germplasm with the highest tolerance, to be used as parental genotypes for crosses in Portuguese cereal breeding programme. Early seminal root traits were also evaluated *in vitro* to find eventual correlations with behaviour of adult stressed plants.

The study focused on 23 bread wheat genotypes, classified into five distinct groups based on their genetic backgrounds. Germplasm comprised genotypes from four distinct phases of the Portuguese wheat breeding programme and one group of Australian germplasm that was the result of an exchange between the Portuguese and Australian breeding programmes.

1.7 Research questions formulation

Within the context of projected climate change, with an increase in both the frequency and intensity of heavy rainfall events that may lead to flooding of agricultural areas, the following concerns emerge:

Is there genetic variability within the bread wheat germplasm under study that may be used to select tolerant genotypes?

What are the mechanisms or traits that enable tolerance to these conditions?

Which eco-physiological parameters are most effective for evaluating waterlogging tolerance or sensitivity?

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2 MATERIALS AND METHODS

2.1 Bread wheat (*Triticum aestivum* L.) germplasm

The studied *T. aestivum* L. germplasm was supplied by the Portuguese Cereal Breeding Programme (PCBP) taking place at the National Institute of Agricultural and Veterinary Research (INIAV) and selected from groups with different genetic backgrounds (Table 2.1).

Table 2.1 -Bread wheat (*T. aestivum* L.) germplasm. Genotypes supplied by the PCBP and belonging to five groups according to genetic background.

Germplasm Group		Genotype	
Portuguese landraces from Vasconcelos ancient collection	PL	Alentejano	PL-1
		Ardito	PL-2
		Mocho Cabeçudo	PL-3
		Mocho de Espiga Quadrada	PL-4
		Mocho de Espiga Branca	PL-5
Varieties with introduced Italian germplasm, developed by the PCBP and released between 1950 and 1970	IT	Restauração	IT-1
		Chaimite	IT-2
		Mara	IT-3
		Pirana	IT-4
Post-Green Revolution varieties with introduced CIMMYT germplasm, developed by the PCBP and released between 1980 and 1989	GR	Caia	GR-1
		Nabão	GR-2
		Roxo	GR-3
		Mondego	GR-4
Advanced lines obtained through the PCBP or CIMMYT	AdvL	Ducula/Gondo//Sokol [1]	AdvL-1
		Katunga × (Centauro/Vega) [2]	AdvL-2
		Kennedy × Roxo [3]	AdvL-3
		KLDR/Pewit1//Milan/Ducula [1]	AdvL-4
		GUS/3/Prl/Sara/Tsi/Vee#5/… [1]	AdvL-5
Australian germplasm	Austrl	BT-Schomburgk	Austrl-1
		Escalibur	Austrl-2
		Sunvale	Austrl-3
		Sunlin	Austrl-4
		Trident	Austrl-5

[1] CIMMYT material; [2] Australian × Italian; [3] Australian × Portuguese.

The research focused on 23 bread wheat genotypes, which include Australian varieties and genotypes from different stages of wheat cultivation in Portugal.

The Portuguese landraces (PL) varieties are part of the Vasconcelos collection (Vasconcelos, 1933), compiled in 1933, which then accurately reflected the genetic diversity of regional Portuguese wheat. Genotypes released between the 1950s and 1970s comprise a group of varieties containing Italian germplasm (IT). During this time, Portuguese breeders crossed PL with Italian agronomic varieties, creating the first Portuguese cultivar, Pirana. Following that, bread wheat varieties such as Restauração and Chaimite were cultivated along with highly improved Italian cultivars (Mara). The next phase was characterised by the introduction of CIMMYT germplasm and took place after the Green Revolution.

Those cultivars (GR) incorporated dwarfism genes and showed promising attributes for agriculture and in the bakery and industry (*e.g.*, Caia and Mondego), as well as remarkable adaptability to Mediterranean climates and tolerance to rust strains. Through exchanges with the Mexican programme of CIMMYT, resources, including advanced lines (AdvL), have been regularly incorporated into the national breeding programme along with AdvL obtained by Portuguese breeders. In addition to developing new or improved genotypes that are tolerant to major abiotic and biotic stresses, selecting new germplasm has also been a top priority. Regarding the Australian group (Austrl), the underlying interest in this choice is due to the Mediterranean climate prevalent in certain regions of Australia with similarities with that of Portugal. Additionally, Australia has made significant strides in developing waterlogging-tolerant varieties.

As the supply of certified seeds was limited, seed multiplication was previously conducted, ensuring their availability, vigour, and germination capacity.

2.1.1 Seeds multiplication

Seed multiplication was conducted in growth chambers (Fitoclima 10000 EHHF, ARALAB, Portugal) under controlled conditions of temperature (22/15°C, day/night), irradiance (*ca*. 500–600 μmol m^{-2} s^{-1}), relative humidity (70/75%, day/night), photoperiod (14 hours), and CO_2 levels (400 μL L^{-1}). Seeds were sown in 5 L pots containing loamy clay soil obtained from the field. The newly obtained seeds were used for the study.

2.2 *In vitro* evaluation of phenotypic diversity of the seminal root traits

Seeds were previously treated with ethanol (96%) for 10 sec, rinsed with sterile water, soaked in 0.5% sodium hypochlorite for 1 min, and then washed twice with sterile water. For each genotype, 60 seeds were sown in polystyrene petri dishes (12 x 12 cm) filled with sterilised bacto agar (2%). Six seeds per plate (10 plates) were sown with the germ-end facing down (Figure 2.1), and plates were positioned vertically at 21°C in the dark (modified from Gonçalves and Lynch, 2014).

Radicle length, the number of seminal roots, and length of the first pair of lateral seminal roots were assessed at 1, 2, 3, and 6 days after sowing (DAS). The root growth angle (RGA) was evaluated by measuring the angle between the first pair of seminal roots, each with a minimum length of 3 cm.

Figure 2.1 - Disinfection and sowing of bread wheat seeds and the visual aspect of a germinated seed for phenotypic evaluation of the seminal root system. (Photos by the author).

2.3 Effects of 14-day waterlogging: experimental design

Approximately 120 seeds from each genotype were soaked in water, placed on moist filter paper in Petri dishes (Figure 2.2 A), and kept at room temperature until the radicle and the first two lateral seminal roots emerged (Zadoks growth scale 05 to 06, ZGS 05 to ZGS 06, Zadoks *et al*., 1974) (Figure 2.2 B). The newly germinated seeds were then placed at a depth of 2 cm (Figure 2.2 C) with the germ-end facing down in 5L pots (7 seeds per pot) filled with sieved loamy clay soil.

Figure 2.2 - Germination and sowing of *T. aestivum* L. seeds. **A**: Petri dish with moist filter paper and seeds placed with the germ visually accessible; **B**: emergence of the radicle and the first pair of lateral seminal roots; **C**: sowing with the germ end facing down. (Photos by the author).

For each genotype, 12 pots were prepared with 7 seeds per pot. Six pots were assigned for control plants (WW) and the remaining six for waterlogged plants (WL). Thus, each genotype had a total of 6 pots (biological unit) and 42 plants (biological subunit) per treatment (Figure 2.3). Plants were grown in walk-in growth chambers (EHHF 10000, ARALAB, Portugal), under the same conditions described in Section 2.1.1, and maintained with a field capacity of *ca*. 85% (adjusted every 2 days), except for stressed plants during the waterlogging period. The plants were fertilised weekly with 250 mL of a 12% nitrogen, 4% phosphorus, and 6% potassium solution (NPK, Complesal, Bayer), except during water stress, two weeks immediately before and after stress, and the final stages of maturation.

Treatments were established at the tillering stage (ZGS 22 to 25). WW plants were maintained at *ca*. 85% field capacity, and WL plants were subjected to 14 days of waterlogging (WL). For that, pots were placed in plastic containers and filled with water until a water layer of *ca*. 0.5 cm was formed above the soil surface. When necessary, the water level was manually refilled with extreme caution to ensure minimal air intake. The water stress was suspended by removing the pots from the boxes. Subsequently, WW and WL plants were kept in the same conditions until harvest (Figure 2.3).

All plants were grown in the same walk-in growth chamber, although due to space limitations, the study was conducted in three consecutive series. Series 1 was conducted with 10 genotypes (PL-2, PL-4, PL-5, IT-2; AdvL-1, AdvL-2; AdvL-3, AdvL-5, Austrl-4, and Austrl-5), Series 2 with 5 genotypes (IT-1, GR-2, Autrl-1, Austrl-2, and Austrl-3), and Series 3 with 8 genotypes (PL-1, PL-3, IT-3, IT-4, GR-1, GR-3, GR-4, and AdvL-4).

Soil and plant-level analysis were conducted for all genotypes, as summarised in Figure 2.3 and described in detail in the following sections.

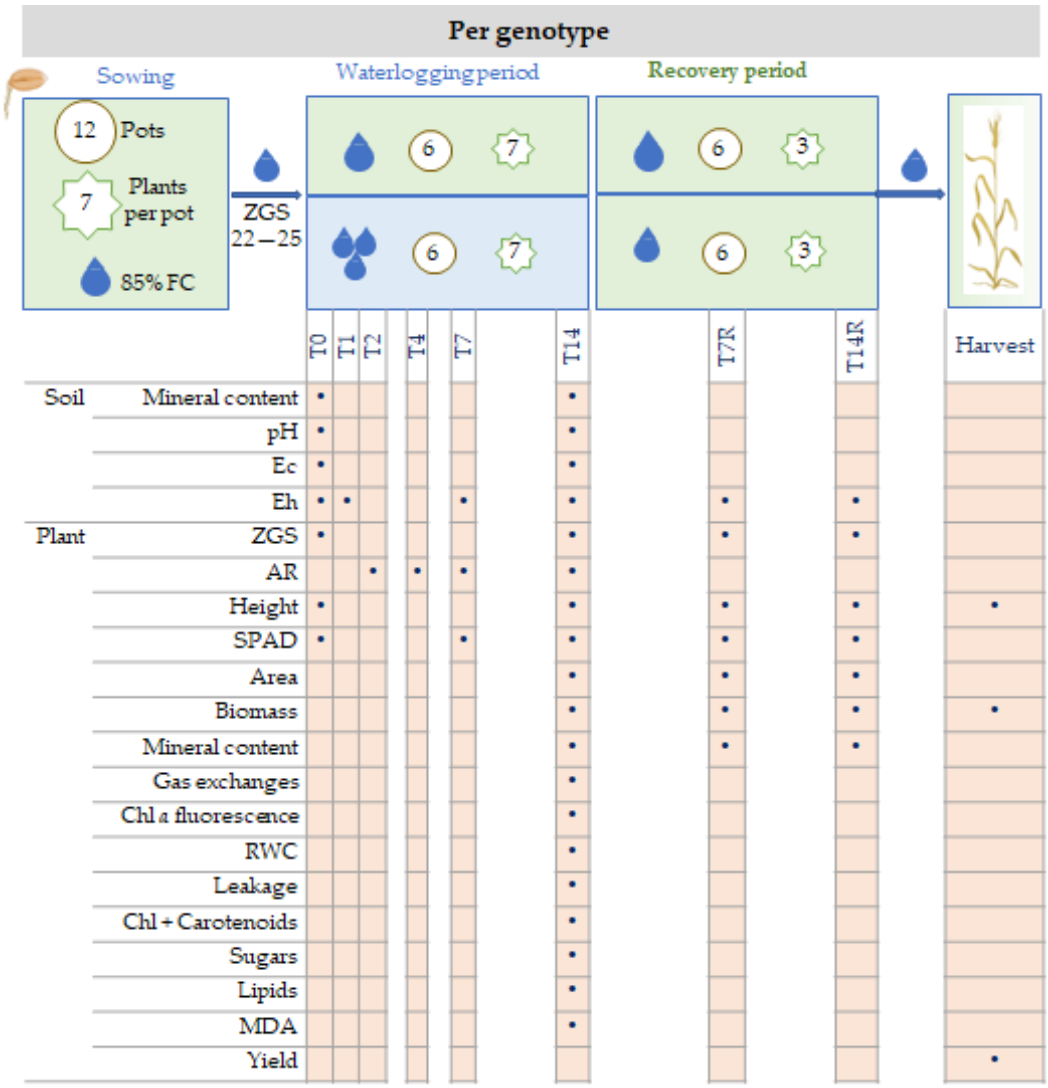

Figure 2.3 - Diagram illustrating the main measurements performed for each genotype performed at either the soil or plant level.

2.4 Soil properties and changes due to water stress

The soil used in this study was collected from one of the regions that represent the national cereal culture system, where poor and shallow soils prevail. After air drying, the soil was sieved and the percentage of humidity determined. The soil used was classified as a loamy clay soil with the following properties (Table 2.2).

Table 2.2 - Properties of the soil used in this study

Soil properties		
Texture	Sand (%)	40
	Clay (%)	35
	Silt (%)	25
Organic matter (%)		1.15
Phosphorus (%)		0.05
Potassium (%)		0.95
Nitrogen (%)		0.03
EC (µS cm^{-1})		334.93
pH_{Ca}		6.68

2.4.1 Field capacity

The gravimetric method was employed to quantify the field capacity (FC). The pots were initially weighed without any contents (tare weight) and then filled with 7.5 kg (dry weight) of sieved soil. Afterwards, the pots were placed in pot-dishes, and water was added until the soil was fully soaked. The pot-dish (used to collect any draining water), was left for 12 hours to ensure complete saturation through capillarity, and then it was removed. Pots were left to drain for 48 hours, individually weighed, and the tare weight deducted. The result was considered to be 100% of the field capacity (Dumroese *et al*., 2015). Individual calculations were performed for each pot to maintain plants at 85% FC.

2.4.2 Soil samples

Soil samples were extracted from three pots per genotype and treatment, from two opposite zones of each pot, using a hole punch with a 1.5 cm diameter and a 10 cm length. The samples were collected at the beginning and end of the waterlogging period (T0 and T14, respectively) and dried at 105 °C for 24 hours. After cooled in a desiccator, samples were homogenised in a porcelain mortar, sieved (nylon sieve, 2 mm pore size), and submitted to an extra dehydration in the same conditions as explained above. Soil samples were stored in a desiccator until analysis.

2.4.3 Nitrogen content

For determination of nitrogen content, the Kjeldahl method was used. The soil sample (1 g) was mixed with 12 mL of H_2SO_4 and *ca*. 4 g of a catalyst mixture ($CuSO_4$ and K_2SO_4, 87:13 w / w) and digested at 420 °C for 60 minutes. After cooling to 50-60 °C, 50 mL of distilled water was added prior to distillation on a Kjeldahl distillation apparatus (Velp Scientifica, UDK129 Kjeldahl distillation unit, Italy). The solution was distilled with 50 mL of NaOH (40%) for 5 minutes into an acidic solution of 30 mL of H_3BO_3 (4%) with two drops of Tashiro indicator. The amount of nitrogen present in the sample was determined by titration with a HCl solution (0.2 N).

2.4.4 pH

The determination of soil pH was conducted at the start (T0) and end (T14) of the waterlogging period, being the later assessed in both waterlogged (WL) and non-waterlogged (WW) pots. The determinations followed the method described by Minasny *et al*. (2011), with some adjustments.

In glass bottles with a screw cap, soil and $CaCl_2$ (0.01 M) were combined in a ratio of 1:5 (w/v). The mixture was vortexed for 5 minutes and stirred (100 rpm) in an orbital agitator for 60 minutes at 25°C. The bottles were placed in a temperature-controlled bath set at 25 °C and shaken gently for 30 minutes. After 2 hours of decantation, the pH was measured using a potentiometer (InoLab pH Level 1, WTW, Germany) and recorded as pH_{Ca}.

2.4.5 Electric conductivity (EC)

The determination of soil EC was conducted in the same pots as pH measurements following

Norm AFNOR NFX 31–103 and Pessoa *et al*. (2016). The EC was assessed using a conductivity meter (Crison GLP 31, Crison Instruments, Spain), and expressed as $\mu S\ cm^{-1}$.

2.4.6 Soil redox potential (Eh)

Soil reduction-oxidation potential was measured in 1 pot of each genotype in each treatment at the beginning of waterlogging (T0), after 24 hours (T1), at 7 days of waterlogging (T7), at the end of the stress period (T14), and after 7 and 14 days of recovery (T7R and T14R, respectively). A portable Eh meter (XS-Instruments, ORP-5, Italy) was used, and measurements were performed at 6 cm deep, and the value (mV) registered after stabilisation (*ca*. 15 min).

2.5 Effects of 14-day waterlogging during the tillering stage: Impacts on plant development and senescence

2.5.1 Adventitious roots

For each genotype and treatment, the number of adventitious roots was determined by examining every plant in every pot at 2, 4, 7, and 14 days following the onset of waterlogging (T2, T4, T7, and T14, respectively).

2.5.2 Number of tillers

For each genotype and treatment, the number of living tillers was counted at T7, T14, T7R, and T14R in 6 pots (18 plants) per treatment. At the end of the growth cycle, the number of productive tillers (Prod) and the maximum number of emitted tillers were also obtained in 3 pots (9 plants) per treatment.

2.5.3 Phenotypic development and height of the main culm

For each genotype, the phenotypic development of three plants per pot (WW and WL plants) was recorded using the Zadoks growth scale (Zadoks *et al*., 1974). Evaluations were performed at the beginning (T0) and end (T14) of the stress period, as well as after 7 (T7R) and 14 days (T14R) of recovery.

The height of the main culm (in centimetres) was measured in three pots (one plant per pot) for each treatment and genotype. Measurements were taken at the end of the waterlogging period (T14), as well as at 7 and 14 days of recovery (T7R and T14R, respectively), and at harvest (FC).

2.5.4 Leaf area and leaf biomass

On the last day of the waterlogging (T14), and following a recovery period of 7 days (T7R) and 14 days (T14R), one plant from three pots per treatment and genotype was collected. The leaves were individually detached from the stem for each culm (main culm and tillers).

The surface area of the green leaves was assessed for all genotypes in both treatments using a

foliar area meter (LI-COR, LI-3000A, USA).

The green leaves and chlorotic leaves were divided within each culm, subjected to drying in a ventilated oven at 80 °C until a stable weight was achieved, subsequently cooled in a desiccator, and finally weighed.

2.5.5 Leaf mineral elements by X-ray fluorescence spectrometry

X-ray fluorescence spectrometry was used to evaluate the phosphorus (%), potassium (%), iron (%), aluminium (%), and manganese (ppm) content in the leaves used for area and biomass evaluations. Prior to the measurements with an Olympus Vanta C Series XRF Analyzer (Olympus, Model VCA, USA), the dried leaves were grounded and sieved (1 mm pore size). Analyses were conducted at the end (T14) of the waterlogging period, as well as at two recovery periods (T7R and T14R), for all genotypes in both water treatments.

2.5.6 SPAD measurements

The relative chlorophyll content of all leaves from the main culm was analysed using a SPAD-502 Plus device (Konica Minolta, Tokyo, Japan). Measurements were collected for each genotype in all pots at five time points: the beginning (T0), the 7th day (T7), the end of the water stress period (T14), and after 7 and 14 days of recovery (T7R and T14R, respectively).

2.6 Effects of 14-day waterlogging during tillering stage: Impacts on some ecophysiological leaf traits

2.6.1 Relative water content

The plant water status, namely the relative water content (RWC), was determined at the end of the waterlogging period (T14) as described in Scotti-Campos *et al*. (2013). Briefly, 12 leaf discs of 0.35 cm^2 were taken from the upper second leaf of the main culm of 4 plants per pot. Three replicates of each treatment and genotype were used. The fresh weight of the discs was assessed immediately after their cutting, the turgid weight was determined following their overnight water saturation in a humid chamber at 25 °C, and the dry weight was obtained after 48 hours at 80 °C.

2.6.2 Gas exchanges

Leaf gas exchanges, including net photosynthetic rate (P_n), stomatal conductance (g_s), transpiration (Tr), and intercellular CO_2 concentration (C_i), were measured using a portable CO_2/H_2O infrared gas analyser exchange system LI-6400 (LI-Cor, Inc., Lincoln, NE, USA) at the end of the waterlogging period (T14). An external CO_2 supply of *ca.* 400 µL L^{-1}, artificial irradiation by an LED lamp of 500 µmol m^{-2} s^{-1}, and a measuring chamber at 25 °C in an open system were used. The CO_2 and H_2O values of the surrounding environment were used as references. Measurements were

conducted at the end of the waterlogging period (T14), in the morning, after 2 hours of exposure to light, on the second top leaf of the main culm, in three plants per pot, for each treatment and genotype.

At the end of the waterlogging period (T14), measurements of O_2 evolution, expressing respiration rate (Rd), were performed in plants after one night at darkness, using leaf discs (1.86 cm^2) collected from the second top leaf of the main culm in one plant of five pots, under dark conditions, at 25 °C, using a Clark-type leaf-disc O_2 electrode (LD2/2, Hansatech, UK).

2.6.3 Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence parameters were acquired from the same leaves that were used for gas exchange measurements with a FluorPen FP110/D (PSI, Drásov, Czech Republic). The F_v/F_m and F_v'/F_m' represented the maximal photochemical efficiency of photosystem II (PSII) in converting light energy into chemical energy and the actual PSII photochemical efficiency of energy conversion under light-adapted conditions, respectively. F_v/F_m and F_v'/F_m' were obtained before dawn (dark-adapted) or photosynthetic steady-state conditions, respectively.

2.6.4 Chlorophylls and carotenoids

Chlorophylls and carotenoids were determined at the end of the stress period (T14) and extracted from pooled samples of the top two leaves of the main culm of four plants per pot. In triplicates, 50 mg of fresh weight (FW) were placed in vials containing 10 mL of pure methanol and stored at 4 °C in the dark for 72 h, as described in Scotti-Campos *et al*. (2015). Thereafter, the extract concentration was measured spectrophotometrically (Shimadzu UV160A, Kyoto, Japan) at wavelengths of 665.2, 652.4, and 470 nm. The concentration was calculated using the Lichtenthaler equations (Lichtenthaler, 1987). Three replicates of each treatment and genotype were used.

2.6.5 Soluble sugars

The quantification of soluble sugars was conducted following Damesin and Lelarge (2003) with some modifications, at the end of waterlogging (T14) and extracted from pooled samples as explained in Section 2.6.4. Approximately 500 mg of FW (in triplicates) were frozen in liquid nitrogen and stored at -80 °C until extraction. With 5 mL of ultra-pure cold H_2O (Type I, Milli-Q, Merck Millipore, France) and 100 mg of polyvinylpolypyrrolidone, leaves were macerated and left to extract on ice for 20 minutes with agitation of 100 rpm to enhance extraction. After being centrifuged (Biofuge 28 RS centrifuge, Heraeus Sepatech, Germany) at 12000 *g* and 4 °C for 5 minutes, the supernatant was sequentially exposed to a 100 °C water bath for 3 minutes and then cooled on ice for 6 minutes. After centrifugation for 15 minutes at the same conditions described above, the resulting supernatant was filtered through a nylon filter with a pore size of 0.45 μm.

Aliquots of 50 μL of the filtered solution were analysed using an HPLC system (Waters, USA) equipped with a refractometric detector set to 30 °C (Waters 2414, USA). The system included a pre-column (SugarPak II inserts, Waters 015209) and a reverse-phase SugarPak l column (Watters

300x6.5mm) maintained at 90 °C. The sugar separation occurred during an isocratic run with a constant flow rate of 0.5 mL^{-1}, using water with EDTA-Ca (50 mg L^{-1}) as the mobile phase.

The main sugars were quantified using sucrose (Sigma), glucose (Merck), raffinose (Merck), and fructose (Merck) as reference standards.

2.6.6 Membrane permeability and injury index

For each variety, 8 sections (*ca*. 1 cm^2 each) from pooled samples prepared as explained in Section 2.6.4, were washed with deionised water and floated for 22 h at 25 °C in 15 mL of deionised water. Conductivity values resulting from electrolytes released by cells were read using a conductivity meter (Crison GLP 31, Crison Instruments, Barcelona, Spain), at *ca*. 25 °C. Total conductivity was measured after sample exposure to 90 °C in an oven for 2 h, followed by cooling at 25 °C. Membrane injury index (I%) was expressed as a percentage of the total conductivity, according to Scotti-Campos *et al*., 2011.

2.6.7 Lipid composition of the leaf membranes

The extraction of total lipids (LT) from cell membranes was carried out as described in Scotti-Campos *et al*. (2014) from pooled samples (see Section 2.6.4). Leaf samples of *ca*. 1 g FW, previously frozen in liquid nitrogen and kept at -80 °C, were boiled in 10 mL of ultra-pure water (Milli-Q, Type I) for 2 min to stop lipolytic activity. The extraction of the lipid fraction was carried out by maceration in a mortar with 30 mL of a mixture of chloroform/methanol/water (1/1/1; v/v/v), followed by centrifugation at 15000 *g* for 15 min at 4 °C. The lower phase (containing the lipids) was collected in a glass vial and dried in a thermostatised bath at 40 °C under N_2 flow. The dry residue was resuspended in 1 mL of ethanol/toluene (1/4, v/v) and stored at -30 °C.

To analyse the total fatty acids (TFA), 100 µL of the LT were saponified with 4 mL of 0.5 M sodium hydroxide in methanol (NaOH 0.5 M-Methanol) at 65 °C for 15 min. The reaction was then stopped by cooling the mixture in running water. An internal standard of 100 µg of heptadecanoic acid (C17:0) was added, and samples were methylated with 2 mL of boron trifluoride (BF_3-Methanol) under identical saponification conditions. The upper phase, which contained the methyl esters of fatty acids (TFA), was collected after adding 10 mL of pentane and 2 mL of water, stirring in a vortex (30 s), and decanting for 60 min. The residue was dried (thermostatised bath at 40 °C under N_2 flow), dissolved with 200 µL of ethanol:toluene (1/4, v/v), and stored at -30 °C. Prior to injection, the ethanol:toluene mixture was dried in a N_2 flow at ambient temperature and the residue mixed with 30 µL of n-Hexane (GC-grade).

Methylated samples were injected (1 µL) into a GC-FID (CP-3380, Varian, CA, USA). The fatty acids were separated with a DB-Wax capillary column (J & W Scientific, US) with a 0.25 mm internal diameter, 30 m length, and 0.25 µm film thickness. The temperature was increased from 80 to 200 °C at a rate of 12 °C min^{-1}, with an initial cut-off of 2 min. Injector and detector temperatures were 200 and 250 °C, respectively. The carrier gas was hydrogen, with a flow rate of 1 mL min^{-1} and a split of 1:100 of the sample. FAs were identified by comparison with known standards (Sigma, USA). TFA values correspond to the sum of the following individual FAs: linolenic acid (C18:3), linoleic acid (C18:2),

oleic acid (C18:1), stearic acid (C18:0), palmitoleic acid (C16:1), and palmitic acid (C16:0).

The degree of unsaturation of the TFA was calculated by the Double Bond Index (DBI), according to the formula: DBI = [(% monoens + 2 × % dienes + 3 × % trienes)/% saturated FA] (Mazliak, 1983).

2.6.8 Membrane lipoperoxidation

The extent of lipid peroxidation in cell membranes was estimated by quantifying MDA (malondialdehyde) according to Hodges *et al*. (1999) in pooled samples (see Section 2.6.4). Leaf samples of *ca*. 200 mg FW, previously frozen in liquid nitrogen and stored at -80 °C, were macerated with 5 mL of ethanol/H_2O (80/20, v/v) and centrifuged for 10 min at 3000 *g* and 4 °C. For the quantification, two reactions were made:

A) supernatant + ethanol/H_2O (80/20, v/v, 750 µL) + trichloroacetic acid (TCA, at 20%, w/v) with hydroxytoluene butylated (BHT, 0.01%, w/v) or

B) supernatant + ethanol/H_2O (80/20, v/v) + TCA at 20% (p/v) with BHT (0.01%, w/v) and thiobarbituric acid (TBA, 0.65%, v/v).

After vortex agitation, the tubes were placed in a thermostatised bath at 95 °C for 25 min, cooled in ice, and centrifuged for 10 min at 3000 *g* and 4 °C. The absorptions were spectrophotometrically read (Shimadzu UV160A, Japan) at 440, 532, and 600 nm. For a blank, ethanol/H_2O (80/20, v/v) and the corresponding reaction mixture were processed in the same way as the samples.

2.7 Effects of 14-day waterlogging during tillering stage: yield and yield traits

Three pots with 3 plants each, per treatment and per genotype were allowed to grow until they reached full maturity. These pots were the basis for the results presented in this chapter. Plants were detached from their root system, oven-dried for 72 hours at 35 °C to eliminate excess humidity.

2.7.1 Number of spikes

The number of spikes was counted for each genotype and treatment, and the result was recorded individually for the main culm and tillers. The number of spikes per plant resulted from the sum of the obtained values.

2.7.2 Number of kernels and kernel weight

Following manual threshing, the number of kernels per spike, their total weight, and the single kernel weight were recorded.

2.7.3 Yield

The yield per plant, as well as the individual contributions of the main culm and each tiller, were assessed using the data obtained in Section 2.7.2.

2.8 Statistical analysis

All data were analysed using the software PAST (Paleontological Statistics software, version 3, University of Oslo, Norway).

For the *in vitro* study, a one-way ANOVA followed by a Tukey's pairwise test was used at a 95% confidence level and performed independently for each genotype. Biological replicates were used in this study, with n = 10 plates (with 60 biological subunits, seeds).

For the *in vivo* evaluations, a two-way or one-way ANOVA was applied to evaluate the differences between water treatments (WW or WL) and/or tissue (main culm or tillers) and their interaction, followed by a Tukey's test for mean comparisons. A 95% confidence level was adopted for all tests, which were performed independently for each genotype. Biological replicates were used in this study, with n = 6 pots for the assessment of phenotypic development and n = 3 pots for the remaining evaluations.

PCA analyses were performed in PAST software, and data from the two main principal components was plotted.

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3 *IN VITRO* EVALUATION OF PHENOTYPIC DIVERSITY OF THE SEMINAL ROOT TRAITS*

Abstract

It is challenging to breed genotypes that can cope with the unpredictable and unevenly distributed effects of climate change. When breeding, it is crucial to take root system traits into account, since root architecture affects the plants capacity to spatially explore the soil and, therefore, its ability to uptake water and nutrients. Genetic diversity in the wheat (*Triticum aestivum* L.) root system may be assessed at the early stages of growth. This study assesses the genetic variability of root growth angle (RGA), seminal root number (SRN), and radicle length (RadL) in 23 bread wheat genotypes with distinct genetic backgrounds. The evaluations were performed *in vitro* at the seedling stage. The SRN and RadL were analysed at 1, 2, 3, and 6 days after sowing (DAS), and RGA was measured through the angle formed by the first pair of lateral seminal roots. A large variability was found in RGA values, with narrower angles tending to occur among landraces, while the higher RGA values were observed in advanced lines and Australian varieties. Differences were also observed in the SRN and RadL, with variability occurring between and within groups.

This study aims to assess the existence of genetic variability for breeding programmes, as well as determine whether the root traits evaluated at the seedling stage may enable faster selection of genotypes that are better adapted to environmental and soil constraints. If so, it will also contribute to the establishment of wheat ideotypes with improved performance under Mediterranean climate conditions, which is essential for the Portuguese Cereal Breeding Programme.

Key-words: Seedling root system, root growth angle, root number, root length.

*Chapter based in the research paper:
Pais IP, Moreira R, Semedo JN, Reboredo FH, Lidon FC, Coutinho J, Maçãs B, Scotti-Campos P (2022). Phenotypic diversity of seminal root traits in bread wheat germplasm from different origins. *Plants*, 11, 2842. https://doi.org/10.3390/plants11212842

3.1 Introduction

Bread wheat (*Triticum aestivum* L.) is one of the world staple crops, with a high economic and social impact for human food and livestock feed. Current climate change projections pose a significant challenge in breeding genotypes that can cope with the upcoming environmental conditions, such as water deficit, waterlogging, increasing temperatures, and rising CO_2 concentration (Martins *et al*., 2016; Semedo *et al*., 2021; Pais *et al*., 2023, 2024). Apart from the unpredictable and uneven occurrences of extreme climatic events across the globe (Pais *et al*., 2020), rises in urbanisation, soil degradation (Lynch, 2007), and shortages of global fertiliser stocks (St.Clair *et al*., 2010) will lead to a decreased arable land per capita. However, population growth will still increase food demand. Since the 1990s, there have been slight increases in crop yields, worsening the concerns about ensuring food security. In the previous century, the green revolution was primarily driven by dwarf plant varieties selected for their response to soil fertility, fertiliser application, and water availability, allowing population growth to keep pace. The next "agricultural revolution" must select plants with plasticity to maintain or increase yields under less-than-optimal conditions.

For the Mediterranean basin, losses of 60% in bread wheat yield have been estimated due to the expected effects of global warming (Sanchez-Garcia *et al*., 2012; Uga *et al*., 2015). On the Iberian Peninsula, a significant climate change hotspot, precipitation and temperature patterns have already impacted wheat production (Bento *et al*., 2021).

Water and nutrients availability have a profound effect on plant growth. These resources are heterogeneously distributed in the soil, with the greatest variations occurring with depth (Haque, Oyanagi, and Kawaguchi, 2012). In the rhizosphere, the top layer is typically nutrient-richer and holds fewer mobile elements, such as phosphorus and potassium (Lynch 2007). Usually it also has a lower water content (Uga *et al*. 2015) and higher oxygen concentrations (Omori and Mano 2007). With roots playing a crucial role in water and nutrient uptake by plants, the initial negative impact on a plant due to rhizosphere changes, as well as the first response, occurs at the root level (Chen *et al*., 2020). Among abiotic stresses, drought, waterlogging, nutrient deficiency, high salinity, and/or mineral toxicity are the most significant.

Root system architecture (RSA) comprises the shape and spatial arrangement of a plant root system within the soil and sets the plant ability to spatially explore it (Takahashi and Pradal, 2021). Determined by the interaction of plant genetics and soil characteristics (Takahashi and Pradal, 2021), RSA also differs considerably between wheat genotypes (Oyanagi *et al*., 2004) and, consequently, in their pattern of water and nutrient uptake.

Some authors reported that in flooded soils, genotypes with a shallow root system produced higher yields than genotypes with a deep root system (Oyanagi *et al*., 2004), reflecting some tolerance to this stress. Advantages in obtaining O_2 (Omori and Mano, 2007) as well as phosphorus, potassium, and ammonium, which are relatively immobile in the soil (Takahashi and Pradal, 2021) may account for this enhanced performance. On the other hand, genotypes with a deeper root system present a better ability to cope with water deficiency as they are more able to absorb water and soluble nutrients from

the soil, such as nitrogen, calcium, and magnesium, which tend to move to deeper soil layers (Gonçalves and Lynch, 2014). Additionally, deeper RS may improve soil structure and its carbon steady-state, as well as water and nutrient retention, thereby contributing to a more sustainable crop production (Kell, 2011).

The root system begins as a single root generated during embryogenesis and develops as the plant matures (Rich and Watt 2013). The wheat root system consists of seminal (*i.e*., embryonic) and nodal roots that remain functionally active throughout the plant life cycle. Seminal roots originate from the seed embryo, and the later from stem nodes (Rich and Watt, 2013). Due to their ability to develop earlier and deeper into the soil, seminal roots may be as significant as or even more important than nodal roots for yield maintenance. Additionally, under insufficient soil moisture, plants achieve maturity primarily via their seminal roots, since nodal roots do not form or their development is restricted (Sanguineti *et al*., 2007; Maccaferri *et al*., 2016).

Being an important agronomic trait in acclimation to several environmental constraints (Nagel *et al*., 2020), it is advantageous that RSA play an important role in wheat breeding programmes. This enables the development of new varieties with a suitable root ideotype for each specific environment (Takahashi and Pradal, 2021). Given the difficulties of accessing mature root systems in soil, it is possible to select genotypes based on traits that are expressed in the early stages of development (Manschadi *et al*., 2006). At the seedling stage, seminal root growth, specifically root growth angle (RGA), is closely linked to the architecture of mature plant root systems (Manschadi *et al*., 2008; Hohn and Bektas, 2020). Apart from RGA, these genetically determined features include the number of seminal roots (NSR) and the primary root length (RadL) (Rich and Watt, 2013; Uga *et al*., 2015; Golan *et al*., 2018; Chen *et al*., 2020). Therefore, the uptake efficiency of the root apparatus as well as the identification of root features at the seedling stage may lead to an earlier selection of genotypes that cope better with a variety of adverse environments.

3.1.1 The configuration of the wheat seminal root system

The wheat (*Triticum aestivum* L.) seminal root system is composed of the primary seminal root (or radicle, Figure 3.1 A), the first pair of lateral seminal roots (Figure 3.1 B), and the second pair of lateral seminal roots (Figure 3.1 C). Occasionally a sixth seminal root may appear (Figure 3.1 D).

Figure 3.1 - Configuration of the seminal root system of wheat (*T. aestivum* L.). A – Radicle emission; B – Radicle and emission of the first pair of lateral seminal roots: C – Radicle and two pairs of lateral seminal roots; D – Bread wheat seed with six seminal roots. (Photos by the author).

The primary seminal root (or radicle) develops first during germination and emerges within 1-2 days after imbibition (DAI). At the scutellar node of the embryonic hypocotyls, two pairs of lateral seminal roots emerge to form the seminal root system in wheat. The first pair emerges within 1 to 4 DAI, followed by a second pair (5-9 DAI). A sixth seminal root may appear in 5-10 DAI (Golan *et al*., 2018; Hohn and Bektas, 2020; Pigolev *et al*., 2021).

Regarding seminal root number (SRN), domesticated wheats have a higher number than their wild relatives (Golan *et al*., 2018; Pigolev *et al*., 2021), and it has been suggested that this variation plays a role in wheat adaptation to water stress. Genotypes with a higher number of seminal roots presented a larger root surface and a denser and deeper root system, which promotes soil resource exploitation (Richard *et al*., 2015). However, a reduction in the number of seminal roots increases hydraulic resistance and slows early water usage, and soil water will be used in more critical periods such as flowering and grain filling (Golan *et al*., 2018). According to several authors (Robertson, *et al*., 1979; Bektaş and Waines, 2020), the SRN are closely determined by the endosperm reserves; however, other studies refer that this trait is regulated by embryo-expressed rather than non-endosperm-expressed factors (Golan *et al*., 2018).

The total length of roots in the soil impacts the absorption of water and nutrients and the overall performance of the crop (Sanguineti *et al*., 2007). When plants are grown in soils with insufficient water or nutrient content, extensive root systems are essential.

3.1.2 Root growth angle

The Root Growth Angle (RGA) has been used as an early screening tool in cereal breeding programmes since it can serve as a proxy for gravitropic root system tendency (Wasson *et al*., 2012). It determines root distribution and elongation direction, *i.e*., whether a plant has a shallow or deep root system (Uga *et al*., 2015). Narrow seminal root angles have been associated with deeper root systems that reach lower soil levels, which can be advantageous in drought conditions. Conversely, wide angles were related to superficial root systems that promote lateral root growth, resulting in some benefits under wetter conditions and artificial irrigation (Lynch, 2013; Uga *et al*., 2015). Some authors observed a significant genetic variation in wheat RGA, which was related to the genetic background and geographical adaptation of varieties, being a valuable breeding resource for boosting crop yield (Manschadi *et al*., 2006, 2008; Hohn and Bektas, 2020).

3.2 Objective of this study

The main goals of this study were to evaluate *in vitro*, at seedling stage, the extent of potential genetic diversity for the root growth angle, seminal root number and radicle length in a set of 23 genotypes of *Triticum aestivum* L. from five germplasm groups with distinct genetic backgrounds.

3.3 RESULTS

3.3.1 Root Growth Angle

Phenotypic diversity was observed in Root growth angle (RGA), with values ranging from 63.1° to 122.2° (Figure 3.2).

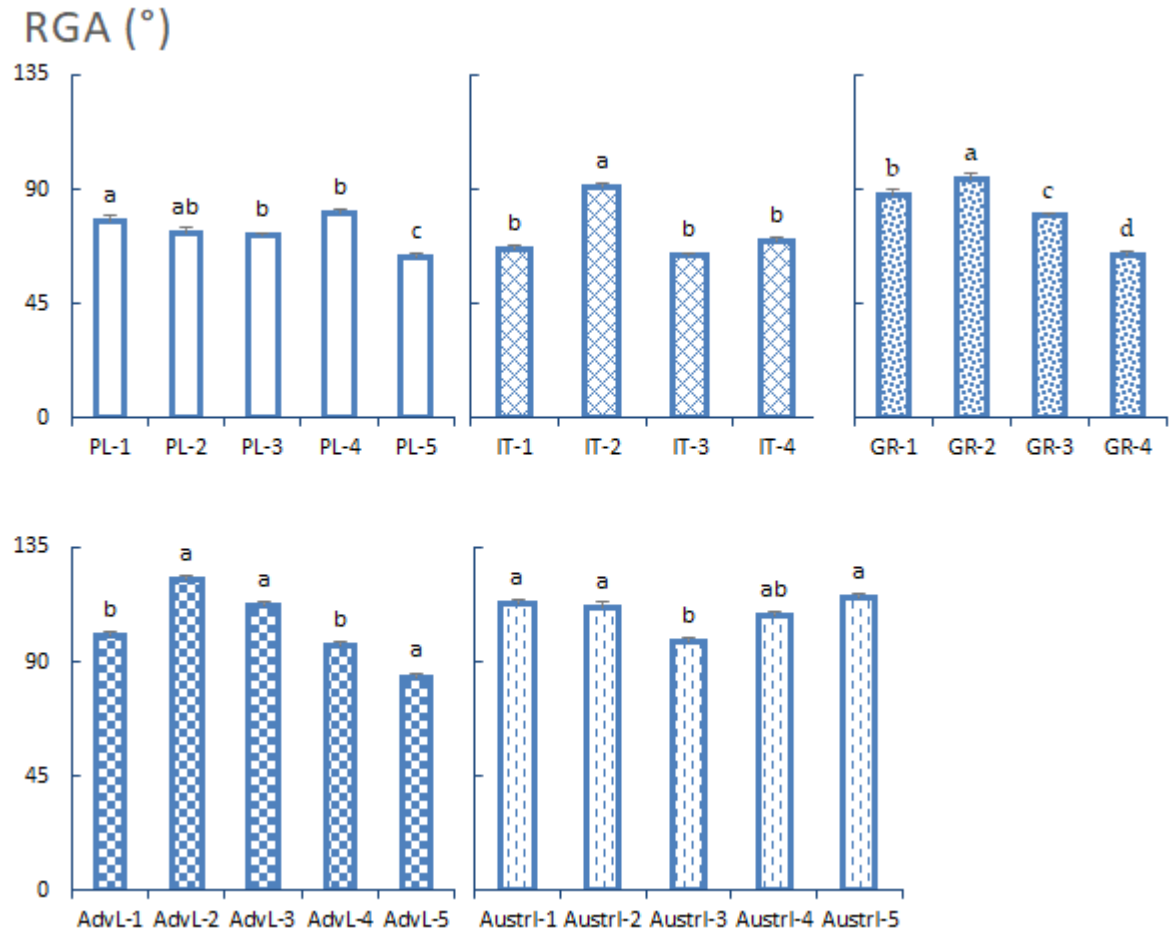


Figure 3.2 - Root growth angle (RGA, °) between the first pair of lateral seminal roots. Evaluations performed in bread wheat genotypes belonging to five groups with different genetic background. (PL - Portuguese landraces from Vasconcelos ancient collection; It - Varieties with introduced Italian germplasm; GR - Post-Green Revolution varieties with introduced CIMMYT germplasm; AdvL - Advanced lines; Austrl - Australian germplasm). For each genotype, the mean values ± SE (n =10), followed by different letters (a, b, c, and d) express significant differences between genotypes within each group, for a 95% confidence level. The highest value corresponds to letter a.

Although variability was evident within each germplasm group, it was particularly pronounced in the GR and AdvL. The latter group exhibited the greatest intragroup variation (38.1°) and was followed by GR (30.1°), IT (26.8°), Austr (17.5°), and PL (17.4°). A tendency to smaller angles (63.1° to 80.5°) was observed in PL genotypes, and despite the smaller amplitude, significant variation in RGA values was found, with PL-5 and PL-4 presenting the lowest and the greatest values, respectively (Figure 3.2). The Austr and AdvL genotypes exhibited the wider angles, measuring 98.0° to 115.5° and 84.1°

to 122.2°, respectively. Among the IT germplasm, IT-2 stood out with the highest value (90.5°). In GR, each variety had distinct RGA values, which ranged from 63.9° (GR-4) to 94.0° (GR-2). Within the Austrl germplasm, Austrl-3 and Austrl-4 showed similar RGA values (98.0° and 108.0°) but were significantly different from Austrl-2 (111.4°), Austrl-1 (112.5°), and Austrl-5 (115.5°). Diversity was also present within AdvL germplasm, with AdvL-5 showing the lowest RGA (84.1°), significantly differing from AdvL-4 and AdvL-1 (96.4° and 99.9°, respectively) and from AdvL-3 (112.3°) and AdvL-2 (122.2°) (Figure 3.2).

Results revealed that several genotypes exhibited comparable RGA values, regardless of their distinct genetic background (Figure 3.2). Based on the RGA similarity, the genotypes were grouped into three clusters (Figure 3.3). Cluster 1 consisted of genotypes with RGA values ranging from 63.1° to 80.5°; Cluster 2 joined genotypes with RGA values between 84.1° and 99.9°, Cluster 3 comprised genotypes with larger angles, depicting RGA values varying from 108° to 122.5°.

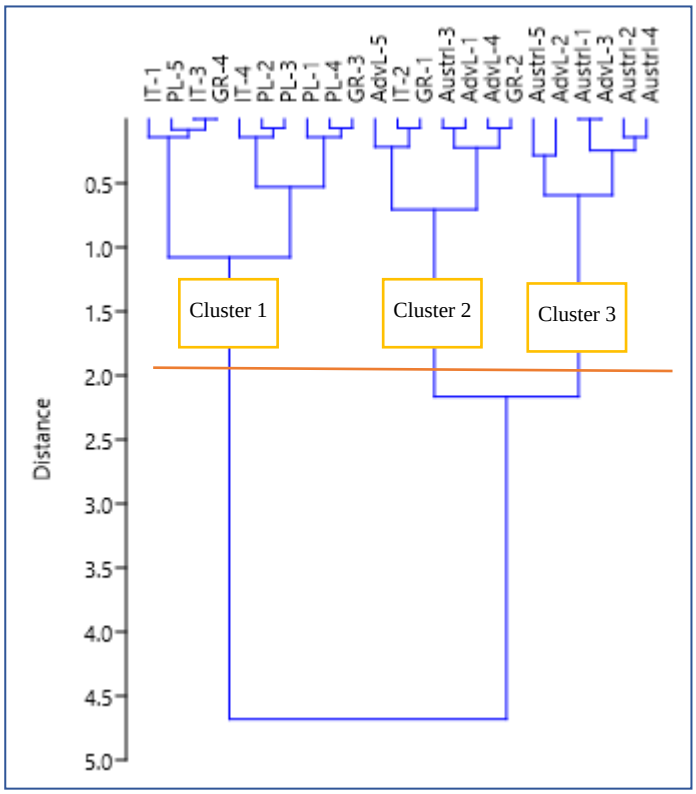


Figure 3.3 - Dendrogram of similarity clusters of the root growth angle (RGA), generated using Ward´s method. The horizontal line represents the cut-off used to form the clusters within genotypes of *T. aestivum* L. belonging to five groups with distinct genetic background. PL - Portuguese landraces; IT - Varieties with introduced Italian germplasm; GR - Post-Green Revolution varieties with introduced CIMMYT germplasm; AdvL - Advanced lines; Austrl - Australian germplasm.

3.3.2 Length of the radicle and of the 1st pair of lateral seminal roots

All genotypes exhibited a consistently high germination rate throughout the study, ranging from 40 to 100% one day after sowing (DAS), 87 to 100% at 2 DAS, and 97 to 100% at 3 and 6 DAS (Table 3.1).

Table 3.1 - Radicle length (RadL, cm) and length of the 1st pair of lateral seminal roots (LatSL, cm). Evaluations were performed at 1, 2, 3 and 6 days after sowing (RadL1, RadL2, RadL3, and RadL6, or LatSL1, LatSL2, LatSl3, and LatSL6, respectively) in bread wheat genotypes belonging to five groups with distinct genetic background. Germination (%, in parenthesis) was evaluated at same days after sowing. One-way Anova followed by a Tukey test was performed within each germplasm group in each day of observation. For each genotype, the mean values ± SE (n=10) followed by different letters express significant differences (a, b, c) for a 95% confidence level. Highest value corresponds to the letter *a*. PL - Portuguese landraces from Vasconcelos ancient collection; IT - Varieties with introduced Italian germplasm; GR - Post-Green Revolution varieties, with introduced CIMMYT germplasm; AdvL - Advanced lines; Austrl - Australian germplasm.

Genotype	Radicle length (cm)				Length (cm) - 1st pair of lateral seminal roots			
	RadL1	RadL2	RadL3	RadL6	LatSL1	LatSL2	LatSL3	LatSL6
PL-1	1.29±0.12 *a* (97)	4.97±0.06 *a* (100)	6.15±0.14 *a* (100)	8.12±0.41 *a* (100)	0.28±0.02 *ab*	3.72±0.09 *a*	5.17±0.11 *a*	7.06±0.36 *ab*
PL-2	0.54±0.04 c (90)	3.98±0.03 *bc* (100)	5.42±0.09 *a* (100)	6.59±0.11 *ab* (100)	0.17±0.04 *b*	2.75±0.20 *b*	4.21±0.21 *b*	6.33±0.38 *ab*
PL-3	1.29±0.04 *a* (80)	4.68±0.09 *ab* (87)	5.25±0.09 *a* (97)	5.24±0.17 *b* (97)	0.44±0.04 *a*	3.34±0.22 *ab*	4.35±0.23 *b*	5.95±0.37 *b*
PL-4	0.90±0.03 *b* (80)	3.78±0.12 c (100)	4.55±1.15 *b* (100)	7.92±0.19 *ab* (100)	0.30±0.05 *ab*	2.93±0.17 *b*	4.66±0.21 *ab*	7.39±0.24 *a*
PL-5	1.49±0.02 *a* (80)	4.28±0.10 *a* (97)	5.42±0.38 *a* (97)	6.02±0.49 *ab* (100)	0.42±0.02 *a*	3.15±0.15 *ab*	4.41±0.16 *b*	6.04±0.20 *ab*
IT-1	0.53±0.03 *ab* (90)	4.45±0.07 *a* (100)	5.45±0.16 *ab* (100)	6.86±0.38 *a* (100)	0.13±0.03 *a*	3.23±0.18 *a*	4.05±0.17 *b*	6.31±0.31 *a*
IT-2	0.31±0.02 *b* (47)	3.46±0.10 *b* (100)	4.36±0.12 *b* (100)	6.84±0.43 *a* (100)		2.30±0.14 *b*	3.86±0.11 *b*	5.91±0.20 *a*
IT-3	0.67±0.02 *a* (97)	5.01±0.08 *a* (100)	6.15±0.16 *a* (100)	8.85±0.26 *ab* (100)	0.12±0.01 *a*	3.40±0.08 *a*	4.38±0.19 *ab*	6.12±0.56 *a*
IT-4	0.71±0.04 *a* (100)	3.48±0.07 *b* (100)	5.20±0.10 *ab* (100)	7.97±0.45 *ab* (100)	0.18±0.03 *a*	3.44±0.17 *a*	4.73±0.16 *a*	7.16±0.30 *a*
GR-1	0.51±0.03 *b* (90)	3.59±0.06 *c* (97)	4.17±0.14 *b* (97)	6.25±0.24 *b* (97)	0.17±0.01 *bc*	2.70±0.06 *b*	3.48±0.05 *c*	4.31±0.34 *b*
GR-2	0.49±0.03 *b* (100)	4.20±0.05 *b* (100)	5.26±0.17 *ab* (100)	9.8±0.41 *a* (100)	0.11±0.00 *c*	2.91±0.10 *b*	4.23±0.10 *b*	5.81±0.35 *a*
GR-3	0.90±0.03 *a* (97)	4.42±0.06 *ab* (97)	5.74±0.19 *a* (97)	8.49±0.34 *b* (97)	0.27±0.05 *b*	3.37±0.06 *a*	5.20±0.14 *a*	6.83±0.38 *a*
GR-4	0.98±0.04 *a* (100)	4.78±0.06 *a* (100)	5.84±0.15 *a* (100)	7.93±0.23 *b* (100)	0.47±0.03 *a*	3.51±0.04 *a*	4.97±0.14 *a*	6.30±0.25 *a*
AdvL-1	1.07±0.01 *a* (100)	4.18±0.06 *a* (100)	5.34±0.20 *a* (100)	8.08±0.30 *ab* (100)	0.42±0.02 *a*	2.98±0.09 *a*	4.52±0.12 *a*	5.01±0.21 *b*
AdvL-2	0.19±0.01 *b* (80)	2.33±0.07 *b* (97)	4.68±0.08 *ab* (98)	7.83±0.35 *ab* (98)	0.10±0.00 *b*	1.57±0.14 *b*	3.58±0.15 *ab*	5.07±0.20 *b*
AdvL-3	0.17±0.01 *b* (40)	2.35±0.09 *b* (100)	4.67±0.07 *ab* (100)	9.02±0.42 *ab* (100)	0.10±0.00 *b*	1.60±0.24 *b*	3.92±0.14 *ab*	6.98±0.15 *a*
AdvL-4	0.13±0.01 *b* (46)	2.28±0.11 *b* (73)	4.68±0.08 *b* (100)	7.05±0.26 *b* (100)	0.10±0.00 *b*	1.82±0.16 *b*	3.24±0.17 *b*	6.04±0.38 *b*
AdvL-5	0.13±0.01 *b* (80)	2.14±0.11 *b* (98)	4.43±0.06 *ab* (100)	10.8±0.43 *a* (100)	0.10±0.00 *b*	1.58±0.21 *b*	3.83±0.40 *ab*	7.78±0.28 *a*
Austrl-1	0.96±0.02 *a* (100)	4.16±0.06 *a* (100)	5.26±0.19 *a* (100)	8.26±0.30 *a* (100)	0.39±0.04 *a*	2.94±0.14 *a*	5.34±0.11 *a*	6.98±0.25 *a*
Austrl-2	0.93±0.04 *a* (100)	3.85±0.08 *a* (100)	4.84±0.14 *a* (100)	7.42±0.23 *a* (100)	0.30±0.03 *ab*	2.89±0.15 *a*	4.57±0.07 *b*	6.06±0.51 *a*
Austrl-3	0.99±0.02 *a* (98)	4.08±0.06 *a* (100)	5.02±0.18 *a* (100)	7.47±0.28 *a* (100)	0.39±0.04 *a*	3.08±0.04 *a*	4.85±0.11 *ab*	6.18±0.47 *a*
Austrl-4	0.51±0.03 *b* (77)	2.80±0.06 *b* (98)	4.84±0.10 *a* (98)	5.06±0.13 *b* (98)	0.14±0.02 *b*	2.03±0.15 *b*	4.29±0.05 *bc*	5.36±0.19 *a*
Austrl-5	0.82±0.04 *a* (97)	3.68±0.08 *a* (100)	4.90±0.13 *a* (100)	7.08±0.28 *ab* (100)	0.25±0.05 *ab*	2.26±0.12 *b*	4.03±0.25 *c*	6.27±0.68 *a*

Radicle length (RadL) showed some intra-group variation, particularly during the initial stage of germination (RadL1), as shown in Table 3.1. This was particularly evident among PL genotypes, with values varying from 0.54 cm (PL-2) to 1.49 cm (PL-5). After 6 days (RadL6), the differences within and between groups were attenuated and tended to be similar in all genotypes. The minimal RadL6 values ranged from 5.06 to 7.05 cm, while maximal values were between 8.26 and 9.66 cm. The radicle growth rate reached its peak between 1 and 2 DAS, with genotypes RadL depicting a 2.9 to 16.9-fold increase. This was followed by a 1.1 to 2.1-fold rise between 2 and 3 DAS (Table 3.1).

The lateral seminal roots exhibited the same pattern of increasing length, starting from values <0.5 cm at 1DAS and reaching 1.57−3.72 cm at 2DAS. Subsequently, there was a further increase of 1.3-2.1 times between 2 and 3 DAS, as shown in Table 3.1.

3.3.3 Number of seminal roots

At the initial stage of germination (NSR1), all genotypes had between one and three seminal roots (Table 3.2). At this stage, the most homogeneous groups were GR and Austrl, with values ranging from 2.63-3.0 and 2.90-3.0, respectively. However, this pattern was not evident 6 days after sowing (NSR6), when all groups exhibit comparable values (5.35-5.72 in PL; 4.73-5.35 in It; 4.93-5.12 in GR, 5.10-5.80 in AdvL, and 5.48-5.83 in Austrl).

Table 3.2 - Number of seminal roots (NSR) and percentage of the 6th seminal root formation (in parenthesis). Evaluations performed at 1, 2, 3 and 6 days after sowing (NSR1, NSR2, NSR3, and NSR6, respectively), in bread wheat genotypes belonging to 5 groups with distinct genetic background. For each genotype, the mean values ± SE (n=10) followed by different letters express significant differences (a, b, c, d, e) for a 95% confidence level. Highest value corresponds to the letter a. PL - Portuguese landraces; IT - Varieties with introduced Italian germplasm; GR - Post-Green Revolution varieties, with introduced CIMMYT germplasm; AdvL - Advanced lines; Austrl - Australian germplasm

Genotype	Number of seminal roots			
	NSR1	NSR2	NSR3	NSR6
PL-1	2.65±0.07 *ab*	3.92±0.09 *a*	4.52±0.10 *a* (4)	5.35±0.09 *ab* (49)
PL-2	1.83±0.13 *b*	3.87±0.10 *a*	4.65±0.09 *a* (6)	5.72±0.06 *a* (72)
PL-3	2.92±0.04 *a*	4.65±0.09 *a*	4.83±0.12 *a* (26)	5.70±0.06 *a* (72)
PL-4	2.08±0.12 *b*	3.00±0.07 *b*	3.30±0.08 *b* (26)	5.70±0.06 *b* (72)
PL-5	2.92±0.04 *a*	4.22±0.14 *a*	4.52±0.09 *a*	5.67±0.09 *a* (77)
IT-1	1.33±0.09 *ab*	3.37±0.09 *a*	4.45±0.09 *a* (4)	5.12±0.11 *a* (43)
IT-2	1.00±0.00 *b*	3.00±0.00 *a*	3.17±0.05 *b*	4.85±0.12 *a* (29)
IT-3	2.10±0.11 *a*	3.00±0.00 *a*	3.47±0.10 *b*	5.35±0.09 *a* (47)
IT-4	1.60±0.10 *ab*	3.00±0.00 *a*	3.37±0.08 *b* (3)	4.73±0.10 *a* (10)
GR-1	2.63±0.09 *b*	3.07±0.05 *a*	4.17±0.10 *ab*	5.07±0.14 *a* (55)
GR-2	3.00±0.00 *a*	3.00±0.05 *a*	3.45±0.08 *bc*	4.98±0.05 *a* (7)
GR-3	2.97±0.02 *a*	3.00±0.00 *a*	3.22±0.08 *c*	4.93±0.08 *a* (14)
GR-4	2.97±0.02 *a*	3.00±0.00 *a*	4.55±0.12 *a* (7)	5.12±0.08 *a* (24)
AdvL-1	3.00±0.00 *a*	3.27±0.09 *a*	4.63±0.08 *a*	5.60±0.06 *ab* (60)
AdvL-2	1.50±0.11 *c*	2.67±0.08 *b*	3.30±0.10 *b*	5.10±0.11 *b* (40)
AdvL-3	2.83±0.07 *a*	2.83±0.07 *ab*	3.75±0.09 *ab*	5.80±0.06 *a* (81)
AdvL-4	2.07±0.07 *b*	2.65±0.09 *b*	4.00±0.12 *ab*	5.70±0.07 *ab* (73)
AdvL-5	2.77±0.08 *a*	2.90±0.05 *ab*	4.40±0.11 *a*	5.30±0.07 *ab* (33)
Austrl-1	2.90±0.05 *a*	3.53±0.10 *a*	4.35±0.10 *a*	5.67±0.07 *a* (68)
Austrl-2	3.00±0.00 *a*	3.13±0.06 *a*	4.85±0.05 *a*	5.63±0.06 *a* (63)
Austrl-3	3.00±0.00 *a*	3.00±0.00 *a*	4.87±0.04 *a*	5.48±0.07 *a* (48)
Austrl-4	2.93±0.05 *a*	3.07±0.05 *a*	4.55±0.09 *a*	5.60±0.09 *a* (70)
Austrl-5	2.97±0.02 *a*	3.27±0.12 *a*	4.97±0.08 *a* (13)	5.83±0.05 *a* (83)

The number of seminal roots increased to average maxima of 4.22, 4.97, and 5.83 after 2 (NSR2), 3 (NSR3), and 6 (NSR6) days after sowing, respectively (Table 3.2).

Regarding the emission of the sixth seminal root, all genotypes exhibited this trait, although the incidence differed, ranging from 7 to 83% at the 6th day after sowing. Among the PL and Austrl germplasm, a frequency closer or higher than 50% was observed in all genotypes, with the remaining germplasm groups being heterogeneous regarding this trait (Table 3.2).

3.3.4 Seminal root traits – understanding their correlations

The correlation between the studied seminal root traits is essential for understanding the early root development and its potential impact on plant establishment. Evaluating traits at multiple time points (1, 2, 3, and 6 days after sowing) allows us to capture the dynamics of root elongation and how it relates to the overall growth pattern.

The root growth angle plays a critical role in determining how roots explore the soil, directly influencing nutrient and water uptake. Meanwhile, the number and length of seminal roots provide insights into the early establishment of the root system, which is vital for anchoring the plant and accessing deeper soil layers. By correlating these parameters, we aim to identify whether early root traits, are linked to specific growth angles that could enhance or hinder resource acquisition, ultimately affecting plant performance.

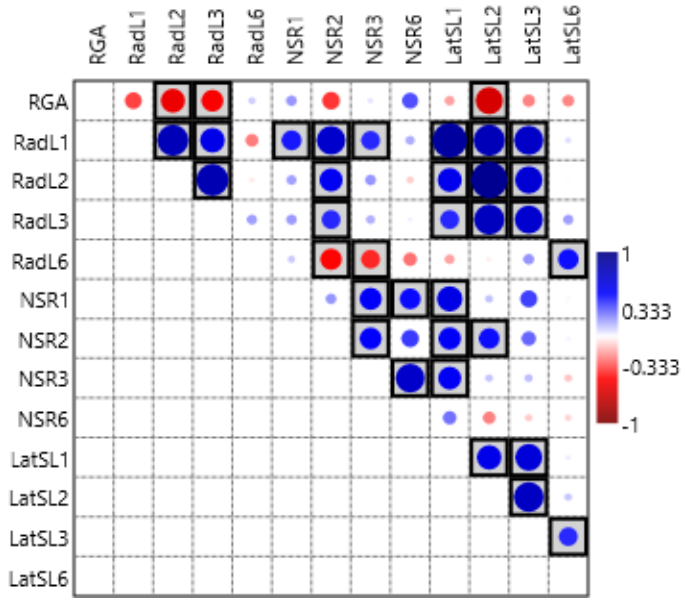


Figure 3.4 – Pearson correlation plot between root growth angle (RGA), radicle length (RadL, 1, 2, 3, and 6 DAS), number of seminal roots (NSR, 1, 2, 3, and 6 DAS), and length of the 1st pair of lateral seminal roots (LatSL, 1, 2, 3, and 6 DAS). Correlations $p<0.05$ are boxed.

Overall, RadL, NSR, and LatSL, analysed on different days after sowing (DAS), showed positive correlations (r values from 0.6 to 0.9) within each parameter across the measurements (*e.g.*, RadL1 correlates with RadL2 and RadL3, and RadL2 with RadL3) with the strongest values observed for 2 or 3 DAS. Genotypes with longer radicles also exhibited more extended lateral seminal roots. On the other hand, RGA showed negative correlations with RadL and LatSL (r values ranging from -0.6 to -0.8), indicating that genotypes with smaller angles have longer seminal roots, whereas wider angles correspond to shorter roots.

3.3.5 Seminal root traits - Clustering analysis

The seminal root traits study revealed similar genotypes among groups, regarding not only RGA values but also the remaining studied traits. A hierarchical cluster analysis was performed (Figure 3.4),

with standardised data regarding the precocity of the seminal root system (RadL1, NSR1, and LatSL1), the length of the radicle (RadL6), the length of the 1st pair of lateral seminal roots (LatSL6), the RGA values, and the percentage of the 6th seminal root emission.

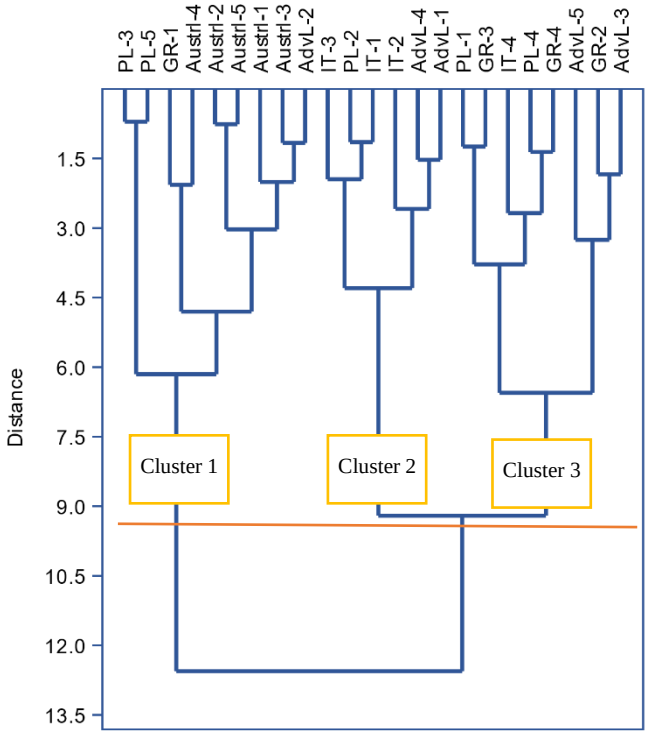


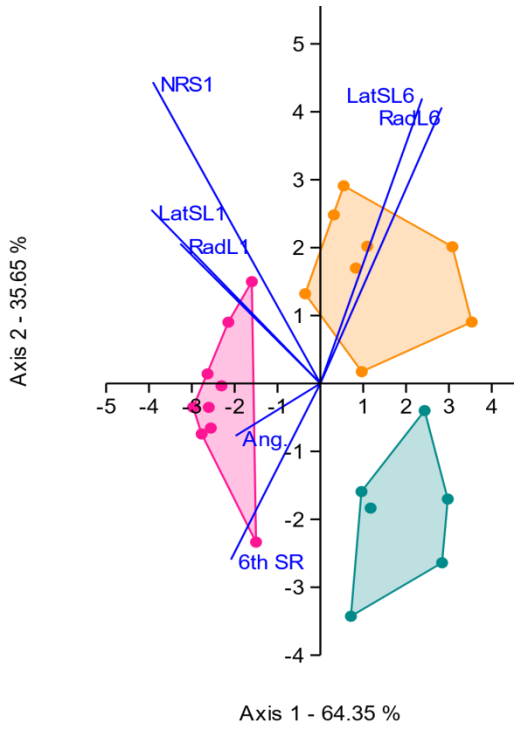
Figure 3.5 - Dendrogram of similarity clusters performed with: precocity of the seminal root system; length of the radicle and of the 1st pair of lateral seminal roots; RGA values; percentage of the 6th seminal root emission.
Dendrogram generated using Ward´s method. Prior to analysis, the following values were standardised: root growth angle (RGA); length of the radicle after one day (RadL1) and six days (RadL6) of imbibition; length of the first pair of lateral seminal roots (LatSL1, LatSL6) measured at the same time of the radicle; number of seminal roots (NSR1) counted one day after sowing; percentage of the 6th seminal root emission (6th SR). The horizontal line represents the cut-off used to form the clusters within genotypes of *T. aestivum* L. Germplasm belongs to five groups with distinct genetic background. PL - Portuguese landraces from Vasconcelos ancient collection; It - Varieties with introduced Italian germplasm; GR - Post-Green Revolution varieties with introduced CIMMYT germplasm; AdvL - Advanced lines; Austrl - Australian germplasm.

Similar to the cluster analysis carried out in section 3.3.1 (Figure 3.3) three clusters were formed. However, analysing not only the RGA but also the traits mentioned above, the similarity between genotypes is not the same, forming different sets of genotypes (Figure 3.4). Cluster 1 covers all Austrl genotypes together with two PL, one GR and one AdvL in a total of 9 genotypes. Cluster 2, with five genotypes, joins three IT genotypes, one PL and 2 AdvL while Cluster 3 shows the similarity between two PL genotypes, three GR, one IT, and two AdvL.

Linear discriminant analysis (LDA) was used to identify the most effective linear feature combinations for clusters differentiation (Figure 3.4). The variation was explained by 64.35% and 35.65% along discriminant axis 1 and 2, respectively.

The eigenvalues of 4.122 (Axis 1) and 2.284 (Axis 2) reflect the discriminant power of axis 1 which effectively distinguished clusters (Figure 3.5). The coefficients of linear discriminants (loadings) provide insight into what features contribute the most to the separation of classes. Since data was standardized, coefficients can be directly compared (absolute value) showing that all traits except RGA,

RadL1 and 6th SR, had a stronger contribution (|value|>0.3) to the discriminant function. The confusion matrix indicates 100% correctly classified data into the different clusters (Figure 3.5).



Coefficients of linear discriminants (Loadings)		
	Axis 1	**Axis 2**
RGA	-0.164	-0.064
RadL1	-0.271	0.170
RadL6	0.235	0.337
NRS1	-0.3241	0.368
LatSL1	-0.327	0.212
LatSL6	0.197	0.348
6th SR	-0.173	-0.215

	Axis 1	**Axis 2**
Eigenvalue	4.122	2.284

Confusion matrix			
	Cluster 1	**Cluster 2**	**Cluster 3**
Cluster 1	9	0	0
Cluster 2	0	6	0
Cluster 3	0	0	8
% correctly classified			100

Figure 3.6 - Linear discriminant analysis with the clusters obtained in hierarchical clustering as the independent variable and the seminal root traits as the dependent variable. Prior to analysis, the following values were standardized: the root growth angle (RGA); the length of the radicle after one day (RadL1) and six days (RadL6) of inhibition; the length of the first pair of lateral seminal roots (LatSL1, LatSL6) measured at the same time of the radicle; the number of seminal roots (NSR1) counted one day after sowing; the percentage of emission of the 6th seminal root (6th SR). Coefficients of linear discriminants, eigenvalues and the confusion matrix are presented.

3.4 Discussion

Root system architecture is determined by the angle at which roots emerge from the seed and penetrate the soil (Wasson *et al*., 2012). Studying the root system in the field is laborious, time-consuming, and expensive. However, field findings and laboratory study results regarding RGA were consistent (Alahmad *et al*., 2019; Hendel *et al*., 2021), and RGA patterns at the seedling stage also correlated with adult root morphology (Rufo *et al*., 2020). Therefore, the variability observed in our study has the potential to be replicated in a field environment, with regards to the studied root traits.

The present results indicate that RGA variability exists between genotypes across groups as well as within groups. The observed genetic diversity in wheat RGA appears to be partially related to the genetic background and local adaptation of varieties, which is in accordance with several authors

(Manschadi *et al*., 2006, 2008; Hohn and Bektas, 2020). RGA cluster analysis confirmed that most Austrl varieties and AdvL genotypes depicted wider angles, in contrast with PL varieties all joint due to narrower RGA. PL varieties are part of a collection that represents the genetic diversity of regional wheat varieties from Portugal and are well adapted to the Mediterranean climate, characterised by a very long, hot, and dry summer and concentrated precipitation in autumn and winter (Almeida *et al*., 2016). In such environmental conditions, narrow angles that typically indicate a deeper root system would be beneficial to the yield stability under these extreme conditions, enabling plants to reach water and nutrients from lower soil layers and a better water extraction capacity from the subsoil (Wasson *et al*., 2012; Leigh *et al*., 2022). Additionally, it is estimated that a 30 cm increase in root depth could capture an extra 10 mm of rainfall water during the critical grain filling stage (Kirkegaard and Lilley, 2007) and that each additional mm of water extracted could generate an additional 55 kg ha^{-1} of grain yield (Manschadi *et al*., 2006). Three IT and two GR varieties showed similar lower RGA values. This could be due to the cross-breeding of Italian germplasm and germplasm from the Green Revolution with PL varieties, resulting in the expression of similar root characteristics as the latter. Conversely wider angles of seminal roots are associated with superficial root systems, where the bulk of the root is concentrated in the upper soil layers. This can play a crucial role in tolerance to wetter soil conditions, such as artificial irrigation or waterlogging, since this RSA facilitates water and nutrient uptake from a wider sub-surface area. Also, root proliferation in superficial soil layers enhanced phosphorus capture and access to oxygen, which are more available in this region of the rhizosphere (Lynch, 2013; Uga *et al*., 2015; 2019; Rufo *et al*., 2020). Although some authors suggest that the Green Revolution strongly contributed to the reduction of wheat genetic diversity, our results showed variability in RGA values within the GR group. These results may be linked to the cross-breeding of local germplasm with CIMMYT materials, which characterized that germplasm group, in agreement with Trethowan *et al*. (2007). These authors refer to the open exchange of large numbers of diverse materials across the wheat breeding programmes worldwide, resulting in genetic diversity that is at least as significant as that shown by CIMMYT-bred germplasm.

Regarding radicle length, results revealed some variability within groups. This was especially clear from the 1DAS observations. The fast development of seminal roots may be beneficial in areas such as the Mediterranean basin with limited upper-layer soil moisture and where uneven rain distribution can cause young seedlings to become dehydrated (Hendel *et al*., 2021). In such environmental conditions, earlier root system deepening can increase water and nutrient uptake efficiency (Sanguineti *et al*., 2007) and thus contribute to the overall plant development. Since a deeper root is known to be a major component in improved drought avoidance (Manschadi *et al*., 2006), this characteristic, along with narrower RGA, could be considered suitable markers for drought resistance.

Between the genotypes studied, no differences were found in the maximum number of seminal roots (NSR). This aligns with the low variation in NSR in domesticated wheat, which typically reaches a maximum of 5-6, compared to 3 in wild wheat (Golan *et al*., 2018). The higher number of seminal roots in domesticated wheat leads to a larger root surface area, a longer root system, and greater root biomass, enhancing soil resource utilisation. In later wheat growth stages, when water demand rises due

to increased leaf area and higher transpiration, root area becomes a limiting factor, making an enlarged root system advantageous. However, fewer seminal roots can increase hydraulic resistance, slower water usage and extending availability (Manschadi *et al*., 2006). Under water-limited conditions, adventitious root development is inhibited (Steinemann *et al*., 2015), and seminal roots may sustain plant growth until maturity.

Genetic variation in populations is needed to ensure appropriate breeding work. Root trait identification and characterisation have advantages for progress to be made in root trait-based selection. This germplasm may be used for future breeding work, allowing the selection of targeted root types. Knowledge concerning root angles and seminal roots in the different evolutive groups may also be used to design ideotypes more adapted to contrasting environments or watering conditions, namely waterlogging.

3.5 Conclusions

This study highlighted the variability in seminal root traits between and within different germplasm groups. At the seedling stage, narrower angles occurred mainly in PL varieties, while larger angles were found in Austrl and AdvL genotypes. However, the seminal root system exhibited greater complexity than just the RGA, and both the length of the radicle and the 1st pair of seminal roots displayed diversity. Additionally, the early development of the seminal root system and the occurrence of the 6th seminal root emission also played a role in the observed variability. The RGA has been identified as a fundamental trait that significantly influences root system architecture. However, our results suggested that multiple traits need to be considered for effectively grouping genotypes based on seminal root system similarity. Germplasm diversity identified in root traits may be used in breeding for yield stability/resilience under contrasting water situations occurring in Mediterranean regions.

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4 WATERLOGGING DURING TILLERING: EFFECTS ON SOIL PROPERTIES, PLANT GROWTH, AND SENESCENCE*

Abstract

Wheat yield losses, currently estimated between 20 and 50%, will be exacerbated by anticipated increases in the intensity, frequency, and unpredictability of waterlogging events. Evaluating germplasm from diverse genetic backgrounds may uncover variability in waterlogging tolerance, allowing a more accurate selection of genotypes with desirable traits for wheat breeding programmes. This chapter addressed the effects of waterlogging on soil properties, plant growth, senescence, and foliar content of nitrogen, phosphorus, and potassium, as well as iron, manganese, and aluminium after 14-day waterlogging at the tillering stage in a climatised growth chamber. All traits exhibited intra- and inter-groups variability. Waterlogged plants exhibited 0-7.6 adventitious roots plant^{-1}, with four Portuguese landraces depicting the highest number, making their root features valuable for genotype selection and cross-breeding. Water stress led to lower tiller emission and/or increased death, more senescent foliar mass, and decreased green foliar area. At the end of the stress period, the number of living tillers varied widely, with changes ranging from 100% reductions to 35% increases. Kernel-producing tillers at harvest also showed significant variation, with reductions up to 100% and increases up to 138%. Foliar mass was impacted, with senescent biomass increasing (24.1-100%), and SPAD values decreasing (11.8-87.7%) in green leaves. During recovery, senescent mass negatively correlated with nitrogen content, which positively correlated with the ratio between the number of kernel-producing tillers and the highest number of emitted tillers (K-PT/Max). Both main culm and tillers were affected, with the latter experiencing the most significant effects. Responses to waterlogging varied in intensity and precocity, with genotypes depicting more evident effects in the first days and others later on. Furthermore, some genotypes were able to recover from damage, whereas others showed accelerated senescence. The recovery period revealed the strongest impacts, suggesting that the capacity to survive the re-entry of oxygen into the tissues is just as crucial as the ability to cope with waterlogging. Genotypes IT-1, IT-4, GR-4, AdvL-4, and AdvL-5 showed severe tiller death, increased senescent mass, and reduced SPAD values, while PL-1, GR-3, AdvL-3, and Austrl-5 demonstrated potential for waterlogging tolerance through effective stress recovery. Identifying variability in key traits for tolerance will aid in developing adapted wheat plants for stable or improved yield in a changing environment.

*This chapter is based in the research papers:

Pais IP, Moreira R, Semedo JN, Reboredo FH, Coutinho J, Lidon FC, Maçãs B, Scotti-Campos P (2023). Waterlogging effects in adventitious roots, tillering and yield of bread wheat germplasm. *Agric, Res, Technol, Open Access J*, 27:556383. htts://doi.org/10.19080/ARTOAJ.2023.27.556383.

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Key-words: Adventitious roots; Biomass; Mineral elements; Senescence; *Triticum aestivum* L.

4.1 Introduction

Overall, soil components are 50% solids, 25% water, and 25% air. Soil air contains nitrogen (78%), oxygen (18-20%), carbon dioxide (1-10%), vaporised water, and trace gases (Nawaz *et al*., 2014). Instead of the gas phase, waterlogging fills the soil pores with water, reducing the oxygen concentration immediately. Root respiration and microbial activity in the rhizosphere rapidly consume the remaining oxygen trapped in the soil. Moreover, the drastic reduction of gas exchange between the soil and the atmosphere exacerbates this lack of oxygen (Greenway *et al*., 2006; Pais *et al*., 2023a), resulting in hypoxia or even environmental anoxia. Under these conditions, soil chemical properties significantly change (Pierret *et al*., 2007; de San Celedonio *et al*., 2016; Pais *et al*., 2023a). Waterlogged soil typically has a lower redox potential (Eh) since the oxygen concentration in the soil is inversely proportional to the Eh (Husson, 2013). When oxygen concentration is approximately 1%, Eh is close to 0 mV (Søndergaard, 2009), and plant roots will switch from aerobic to anaerobic metabolism (Søndergaard, 2009; Husson, 2013). Waterlogging first affects roots, resulting in impaired root functioning and negative effects on shoots (Liu *et al*., 2020). Waterlogging-prone wheat genotypes frequently exhibit severe metabolic constraints with strong impacts on the plants shoot. Changes in tillers emission, survival and development, faster leaf and organ senescence, decrease accumulation and remobilization of photoassimilates, and reduced yield are common symptoms of waterlogging-susceptible genotypes (de San Celedonio *et al*., 2018; Gibbs and Greenway, 2003; Malik *et al*., 2002).

The formation of adventitious roots in response to waterlogging may be a strategy to reduce oxygen deficiency, as these roots facilitate gas transport between submerged tissues and the aerial portion of the plant. In addition, these morphological adaptations play a decisive role in nutrient and water uptake during waterlogging, significantly contributing to survival (Steffens *et al*., 2023) and maintaining productivity.

Tillers are directly proportional to the number of spikes per area unit (Valério *et al*., 2008), which has a direct effect on the final yield (Malik *et al*., 2001). Lower yields in waterlogged plants often correlate with low tiller emission and/or tiller survival (Condon and Giunta, 2003; Yaduvanshi *et al*., 2012; Amri *et al*., 2014; Herzog *et al*., 2016) and fewer fertile tillers (Malik *et al*., 2001, 2002). However, the reduction in total emissions and survival does not always correspond to a decrease in fertile ones, indicating some capacity to maintain production in an energy deficit situation (Malik *et al*., 2001; Collaku and Harrison, 2002; Robertson *et al*., 2009). On the other hand, preserving the number of fertile tillers does not guarantee yield maintenance, as their contribution to the final yield may be affected (Valério *et al*., 2008). Lower tillering, tiller impairment, reduced leaf elongation, poor growth, delayed development, and enhanced senescence, among other factors, can lead to a decrease in shoot mass in waterlogged plants (Malik *et al*., 2001; Dickin *et al*., 2008; Robertson *et al*., 2009; Amri *et al*., 2014; Herzog *et al*., 2016; Pais *et al*., 2022), thus reducing the green area. Furthermore, waterlogging can also induce chlorosis and decrease photosynthetic pigments in green tissues, impairing light absorption and consequently photoassimilation.

Water availability, soil pH, and Eh influence the solubility and availability of nutrients and

potentially harmful elements in the rhizosphere. Moreover, soil total concentrations of mineral elements are not reliable indicators of plant availability, as they are frequently inaccessible for plants. The most essential elements are present as ions in the soil solution, which flow into the plant by water absorption (Strawn *et al*., 2020). Waterlogging and root system impairment can impact the absorption of nutrients. Nitrogen, phosphorus, and potassium are the most common added fertilisers applied to soil in order to promote crop growth. However, leaching and denitrification can lead to the loss of nitrogen in waterlogged soils. A deficiency of this element, which is a key component of chlorophyll, amino acids, and proteins, leads to stunted growth, chlorosis, and reduced tillering. Phosphate strong adsorption with soil minerals often retains Phosphorous, making it unavailable for plant uptake and the most limiting for plant growth. Phosphorus is essential for energy transfer, root development, and flowering; therefore, deficiency can result in poor root growth and delayed flowering, among other symptoms. Soil also strongly retains potassium, particularly in clay minerals, resulting in typically low levels of this element in soil solutions. Potassium is crucial for numerous physiological processes within plants. Among them, it has a role in seed germination and emergence, enzyme activation, osmoregulation, phloem transport, protein synthesis, photosynthesis and stomatal regulation, nutritional balance, and stress tolerance (Marschner, 2012; Hasanuzzaman *et al*., 2018). A crucial mechanism during waterlogging is the avoidance of Potassium loss, providing tolerance during the low concentrations of oxygen. Deficiency leads to weak stalks, poor resilience to drought, and leaf scorching (Hasanuzzaman *et al*., 2018).

In waterlogged soils, the reduction of Fe^{3+} (solid) to Fe^{2+} (dissolved form in soil solution) increased this element bioavailability. Additionally, plants typically find manganese in the soil as Mn^{4+}, a form that is not readily available to them. However, in anaerobic conditions like waterlogged soils, Mn^{4+} undergoes reduction to Mn^{2+}, making it more soluble and available for plant uptake (Strawn *et al*., 2020). Apart from their role as essential microelements, when iron and manganese ions are available to plants at concentrations that do harm, they are considered potentially toxic (Strawn *et al*., 2020). In wheat, iron toxicity is mainly described for flooded soils, where it can co-occur with manganese toxicity (Faria *et al*., 2021), with the most commonly reported symptoms being the yellowing of older leaves (Herzog *et al*., 2016). Aluminium can also be toxic to plants when it is released from its soil-bound form into the bioavailable Al^{3+}, influenced by soil moisture, pH, temperature, and Eh (Faria *et al*., 2021). The increased bioavailability of aluminium, iron, and manganese leads to a decline in soil fertility by promoting deficiencies in plant essential nutrients (*e.g*., potassium) and decreased phosphorus solubility (Faria *et al*., 2021).

The selection of potential progenitors in breeding programmes largely depends on the evaluation and understanding of the existing genetic variability (Mustroph, 2018; Pfeiffer *et al*., 2004). Screening genotypes with distinct genetic backgrounds may provide the genetic diversity required for the development of varieties with yield stability and resilience in adverse environments (Heinemann *et al*., 2013; Leight *et al*., 2022). Studies conducted under controlled conditions provide the most reliable and reproducible results, which are essential to understand the relationship between the effects of stress and its impacts on plant development (Nóia Junior *et al*., 2023).

4.2 Objective of this study

This study aimed to assess the impact of 14 days of waterlogging on bread wheat (*T. aestivum* L.) germplasm with different genetic backgrounds under environmentally controlled conditions. Soil changes were evaluated during the water-stress period, with a specific focus on pH, Eh, and electric conductivity. In order to contribute to the understanding of the waterlogging impacts on plant development and senescence, the occurrence of adventitious roots, the survival of tillers throughout the periods of stress and recovery, and the number of fertile tillers at harvest were assessed. In addition, changes in the foliar biomass, the green leaf area, the development stage, and the main culm height were also investigated. The detection of senescence involved the quantification of the senescent biomass in the main culm and tillers, as well as SPAD measurements on the main culm leaves. Moreover, changes in nitrogen, phosphorus, potassium, iron, manganese, and aluminium were evaluated to identify any deficiencies or toxicities resulting from water stress. Linear correlations between the analysed parameters were studied, along with the interactions between the ability to emit adventitious roots. The existence of variability among germplasm and the identification of changes in key traits underlying tolerance to waterlogging will contribute to develop more adapted wheat plants and to improve wheat yield under a changing climate.

4.3 Results

4.3.1 Soil redox potential, pH and electrical conductivity

Regarding redox potential (Eh) values, no differences were found between control (WW) and waterlogged (WL) pots immediately prior to the imposition of waterlogging (T0) (Figure 4.1).

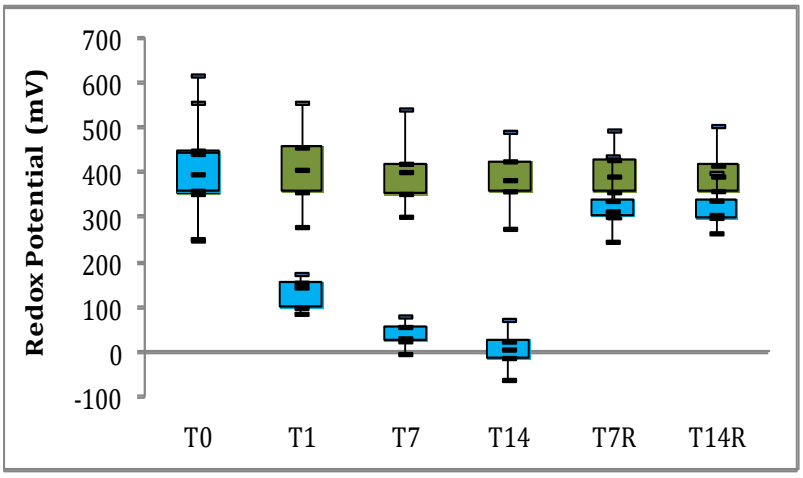


Figure 4.1 – Soil redox potential. Waterlogged (■) and well-watered pots (■) at the onset of waterlogging (T0), after 1 (T1), 7 (T7) and 14 (T14) days of stress and 7 and 14 days of recovery (T7R and T14R, respectively). (n=23).

Waterlogging induced an abrupt decrease of Eh (due to the depletion of oxygen) earlier at T1 (1-day waterlogging), which was accentuated (although in a softer way) in the following days, reaching minimum values close to 0 mV (even negative in some pots) at the end of the stress period (14-day

waterlogging). In the pots that remained at *ca*. 85% field capacity (WW), no changes were observed between the observed periods (T0 to T14R) with average values of *ca*. 400 mV, which is considered an ideal value for plant development. In the WL pots, Eh values rose with waterlogging suspension and the re-entrance of oxygen into the soil, approaching the values of WW pots after 7 days of recovery (Figure 4.1).

Similar to Eh, the electrical conductivity (EC) and pH measured at T0 showed no differences between WW and WL values (Table 4.1).

Table 4.1 – Soil pH and soil electrical conductivity (EC) of well-watered (WW) and waterlogged (WL) pots. Evaluations performed immediately prior of the beginning of the water stress (T0) and at the end of the 14t^h-day waterlogging (T14). Statistical analysis (Two-way Anova, n = 69 pots, $p<0.05$) was performed to compared T0 and T14 in each water treatment (letters *a* and *b*), as well as differences between WW and WL at T0 or T14 (letters *r* and *s*). Letters *a* and *r* represented the highest values.

	pH		**EC**	
	T0	T14	T0	T14
WW	6,69 ± 0,02 *a r*	6,71 ± 0,03 *a s*	386.0 ± 9.74 *a r*	176.9 ± 6.30 *b r*
WL	6,71 ± 0,03 *b r*	6,81 ± 0,04 *a r*	397.4 ± 8.73 *a r*	139.3 ± 2.59 *b s*

After 14 days, WW pots presented unaltered soil pH values, while WL pots showed a slight but significant increase, leading to a decrease in $[H^+]$ (Table 4.1). As plant growth progressed (T14), the EC value decreased in both WW and WL pots, with the latter being more pronounced. This resulted in a 21.3% reduction of EC due to water stress (Table 4.1).

4.3.2 Adventitious roots emission

Adventitious root (AR) emission was not observed in plants grown under control conditions for any genotype. Thus, the following results (Figure 4.2) will refer to waterlogged plants.

In plants exposed to waterlogging (WL) conditions, considerable heterogeneity was detected, with some genotypes exhibiting an early (T2) formation of AR (Figure 4.2). Overall, the IT and GR varieties showed less capacity for AR formation during the 14-day waterlogging. Among all germplasm studied, only two genotypes did not present any AR (IT-3 and GR-4). Additionally, IT-4, GR-1, GR-3, and AdvL-3 exhibited a very small number throughout stress treatment (Figure 4.2). The genotype PL-2 stood out by displaying the highest number of AR plant^{-1} across all analysed WL periods, reaching a value of 7.6 at the end of the stress period (T14). Among the 23 genotypes tested, seven (PL-1, PL-3, PL-5, GR-2, AdvL-4, Austrl-2, and Austrl-3) achieved an average of 2-4 AR plant^{-1}. On the other hand, PL-4, IT-1, IT-2, AdvL-1, AdvL-2, and Austrl-1 displayed values ranging from 1-2, while the remaining genotypes had less than one AR plant^{-1} (Figure 4.2).

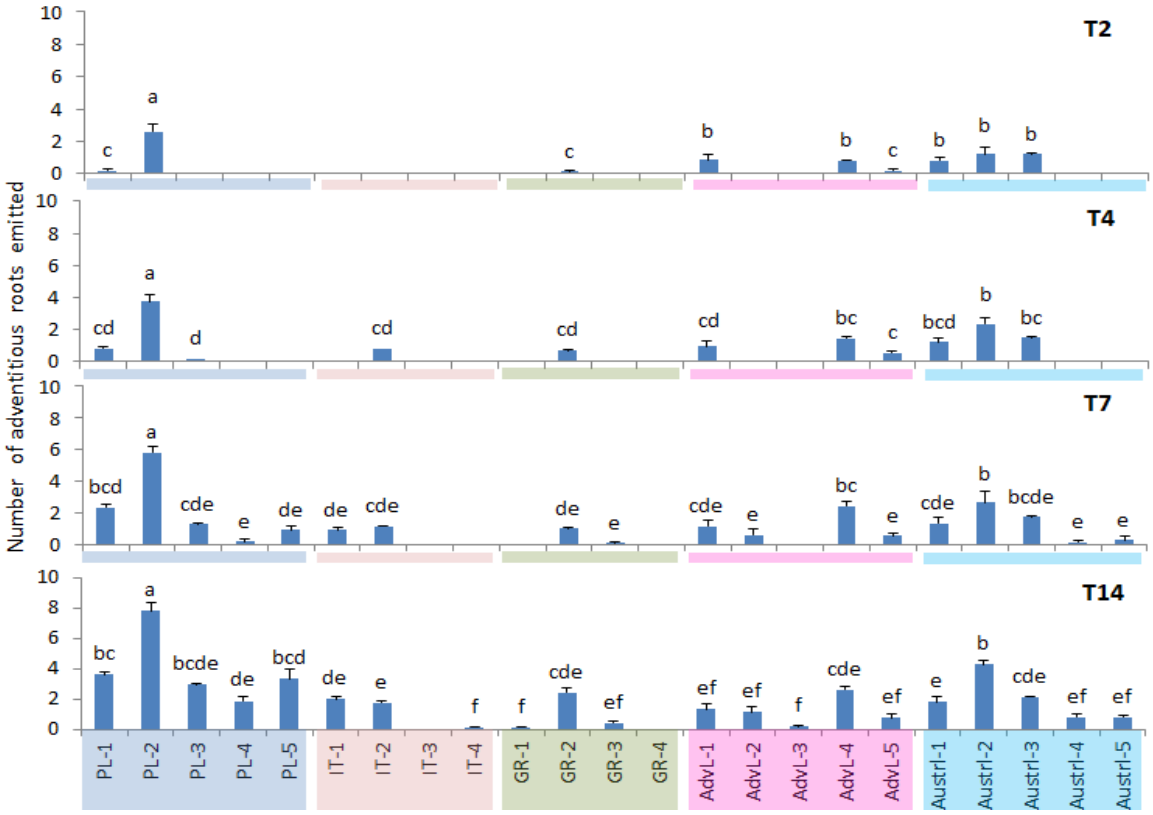


Figure 4.2 - Number of adventitious roots per plant induced by waterlogging. Observations performed in genotypes of *T. aestivum* L. with different genetic backgrounds. (PL) Portuguese Landraces; (IT) Varieties with introduced Italian germplasm; (GR) Post-Green revolution varieties with introduced CIMMYT germplasm; (AdvL) Advanced lines from Portuguese Cereal Breeding Programme (INIAV); (Austrl) Australian varieties; Observations were performed at 2, 4, 7 and 14 days of waterlogging (T2, T4, T7 and T14, respectively) and the statistical analysis for differences among germplasm, with One-way Anova (n=6 pots per genotype, $p<0.05$).

4.3.3 Tillers number and fertility

At the beginning of water treatment (T0), all genotypes were in a similar phenological stage (Figure 4.3), presenting 2-5 tillers (ZGS 22-ZGS 25) in the WW and WL plants.

A great variability was observed in the average tiller number throughout the growth cycle (Figure 4.3) of WW plants. Despite all plants of all genotypes initially having an equal number of tillers (T0), there was variability in their survival rate, in the plants capacity to emit additional ones, and in the fertility of tillers (kernel-producing tiller at harvest, K-PT). In these plants (WW) at T7, the number of living tillers ranged from 1.4 to 6.5, indicating either the loss of some tillers (as in Austrl-4, AdvL-1, and AdvL-5) or, in other cases, the progression of tillering (as in IT-3). The reduction or increase in tiller number compared to T0 was observed in all germplasm groups throughout the growth cycle (T7, T14, T7R, T14R, and K-PT) (Figure 4.3). At harvest, there was variability in the number of fertile tillers across genotypes from different or the same germplasm group. The average K-PT values in WW plants ranged from 0.5 to 6.8, with twelve genotypes displaying less than 2.0 and ten genotypes between 2.0 and 4.0. AdvL-4 and Austrl-3 the highest K-PT per plant, with values of 4.9 and 6.8, respectively, in contrast to AdvL-2, which had the lowest number (0.5) (Figure 4.3).

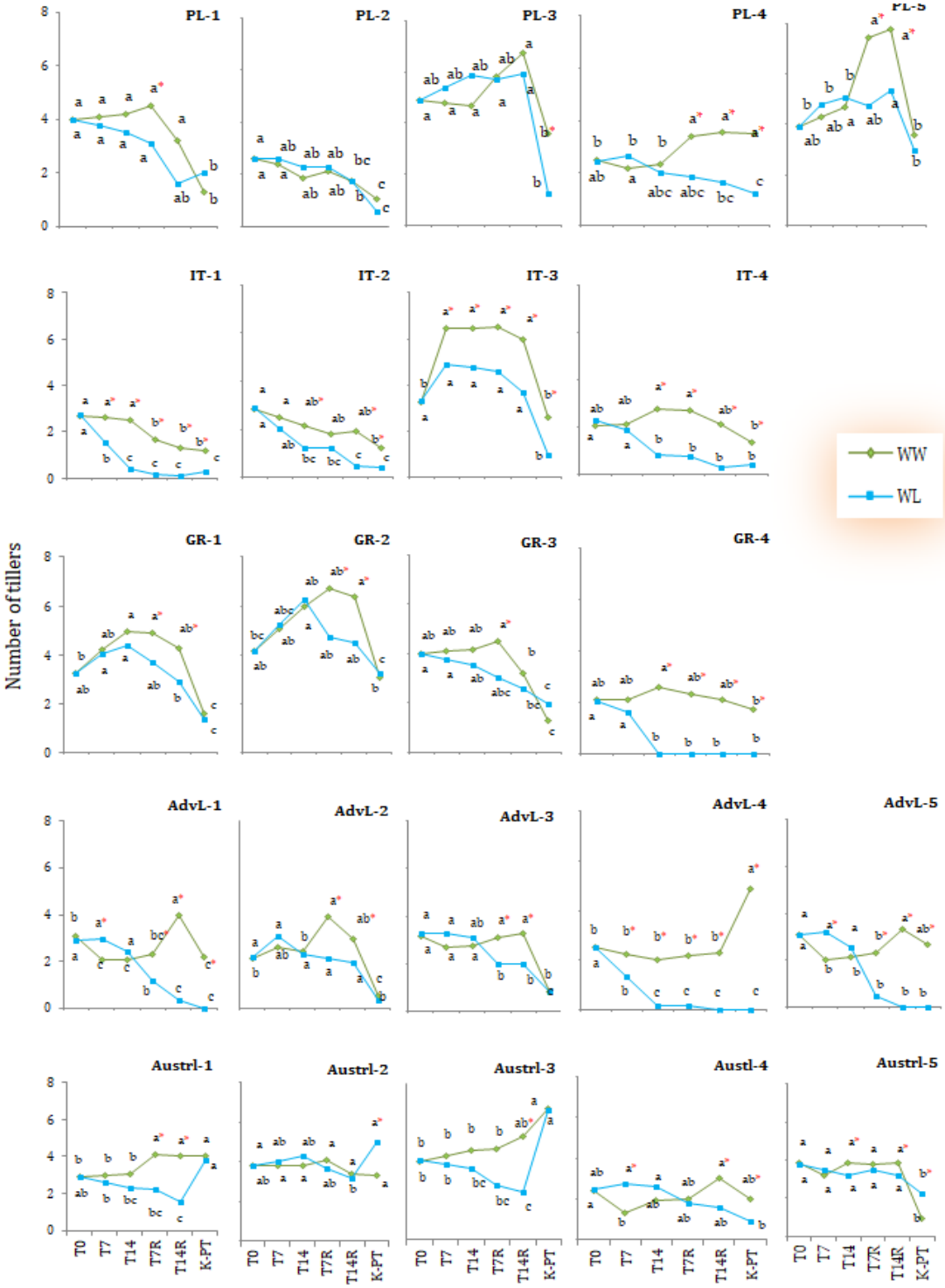


Figure 4.3 - Differences in fertility and the number of living tillers due to waterlogging were evaluated at the beginning (T0), 7 (T7) and 14 days (T14) of waterlogging, 7 and 14 days of recovery (T7R and T14R, respectively), and as kernel-producing tillers at harvest (K-PT), in well-watered (WW) and waterlogged (WL) plants of bread wheat germplasm: PL: Portuguese Landraces; IT - Varieties with introduced Italian germplasm; GR - Post-Green revolution varieties with introduced CIMMYT germplasm; AdvL - Advanced Lines from the Portuguese Cereal Breeding Programme; Austrl - Australian varieties. For each genotype, mean values (n=3-6 pots; $p<0.05$) with different letters (a, b, c) express significant differences between T0, T7, T14, T7R, T14R, K-PT under the same water regime (WW or WL) or, when followed by *, differences between WW and WL in the same period. Letter ***a*** or * corresponds to the highest values.

At the 7th day of the stress period (T7), the majority of genotypes (16) did not exhibit any changes in the number of living tillers due to waterlogging (Figure 4.3). However, IT-1 and AdvL-4 suffered decreases (38% and 43%, respectively), resulting in lower values in WL plants (Figure 4.3). Since stress did not promote either tiller death or arrested emission of new tillers, the lower value in WL plants in IT-3 was attributed to a less pronounced emission of new tillers (Figure 4.3). In AdvL-1, AdvL-5, and Austrl-4, a contrasting response was observed, with WL plants showing higher values (35, 43, and 61%, respectively). This difference was due to tiller mortality in WW plants, while the number of living tillers in WL plants remained unchanged compared to the beginning of the stress treatment (T0).

At the end of waterlogging (T14), no stress-induced differences appeared in PL genotypes, despite the non-significant trend for higher values in PL-2, PL-3, and PL-5. In contrast, compared to WW plants, the number of living tillers in WL plants decreased in all genotypes of the IT group (27% to 84%) and in Austrl-5 (18% reduction). Waterlogging strongly impacted GR-4 and AdvL-4, with no living tillers in the first genotype and a 90% reduction in the latter compared to WW plants (Figure 4.3).

The largest differences between WW and WL plants occurred after waterlogging suspension. At T7R, 17 out of the 23 genotypes significantly reduced the number of tillers (24-100%). At this point, PL-4, IT-1, IT-4, GR-4 AdvL-1, AdvL-4, and AdvL-5 had already reduced their values by over 50% compared to WW plants. On the other hand, Australian varieties (except Austrl-1) were unaffected, together with PL-2 and PL-3. At the end of the recovery period (14R), 18 genotypes had lower tiller number (18-100% reductions) in relation to WW plants, with PL-4, IT-1, IT-4, GR-4 AdvL-1, AdvL-4, and AdvL-5 still showing the strongest impacts, with repercussions in the number of K-PT (Figure 4.3).

Waterlogging affected the K-PT differently across groups, with some genotypes suffering severe effects, others remaining unaffected, and others, like Austrl-5 and Austrl-2, showing increases of 144% and 52%, respectively (Figure 4.3).

IT genotypes were severely impacted by water stress, with decreases of 68-88% in K-PT. With similar behaviour, Austrl-4, PL-3, and PL-4 showed 55%, 64%, and 66% reductions, respectively. GR-4, AdvL-1, AdvL-4, and AdvL-5 were unable to recover from the tillers mortality showed at the ending of stress, displaying a 100% decrease in K-PT (Figure 4.3).

Several genotypes presented similar K-PT in both WW and WL plants. However, those similarities covered distinct responses among genotypes. PL-2 remained unaffected during the whole studied periods of the growing cycle. On the other hand, PL-1, Austrl-1, and Austrl-3 manage to attain equal values of WW plants by recovering from the previous negative impacts. In addition, the reduced tillering survival observed during periods of water stress and/or recovery in GR-1, GR-2, GR-3, AdvL-2, and AdvL-3 did not result in lower final K-TP (Figure 4.3).

Since more tillers may result in more fertile spikes at harvest and differences in tillering capacity were observed among genotypes, we investigated whether waterlogging contributed to changes in the ratio of the number of kernel-producing tillers at harvest with the maximal number of emitted tillers (K-PT/Max), and results are presented in Table 4.2.

In addition to the observed variability in the number of tillers, the K-PT/Max ratio exhibited differences among genotypes and within each germplasm group for both WW and WL plants (Table 4.2).

In WW plants, PL-4, AdvL-4, Austrl-1, and Austrl-3 showed values of 1.0 or very close to it, while in 14 of the remaining genotypes, this ratio was ≤0.5 (Table 4.2).

Waterlogging led to a significantly decreased K-PT/Max ratio in 12 genotypes, with GR-4, AdvL-1, AdvL4, and AdvL-5 reaching a ratio of 0. Conversely, this ratio increased in 5 genotypes (PL-1, PL-5, GR-3, Austrl-2, and Austrl-5), whereas the remaining were unaffected. Austrl-1 and Austrl-3 stood out for their ability to maintain a good ratio of *ca.* 1.0 (Table 4.2).

Table 4.2 - Ratio of kernel-producing tillers and the maximal emitted (K-PT/Max) on well-watered (WW) and waterlogged (WL) plants of bread wheat germplasm with distinct genetic backgrounds: Portuguese Landraces (PL); Varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme; Australian varieties (Austrl); ***** highlight significant differences (n=3-6 pots; p<0.05) between WW and WL per genotype as well as the highest value.

	K-PT/Max	
Genotype	WW	WL
PL-1	0.28	0.49*
PL-2	0.40*	0.21
PL-3	0.53*	0.21
PL-4	0.99*	0.46
PL-5	0.46	0.56*
IT-1	0.45*	0.10
IT-2	0.43*	0.15
IT-3	0.40*	0.19
IT-4	0.50*	0.20
GR-1	0.33	0.32
GR-2	0.46	0.51
GR-3	0.28	0.49*
GR-4	0.68*	0.00
AdvL-1	0.56*	0.00
AdvL-2	0.13	0.11
AdvL-3	0.26	0.25
AdvL-4	1.00*	0.00
AdvL-5	0.80*	0.00
Austrl-1	0.97	1.00
Austrl-2	0.82	1.00*
Austrl-3	1.00	1.00
Austrl-4	0.66*	0.33
Austrl-5	0.24	0.59*

4.3.4 Changes in plant growth stage

Throughout the 14-day waterlogging period, all genotypes displayed growth progress in both well-watered (WW) and waterlogged (WL) plants. At the end of the water treatment, the genotypes exposed to stress and those maintained in control conditions environment showed no developmental delays. On the contrary, AdvL-4, Austrl-2, and Austrl-4 showed acceleration in their development in WL plants (Figure 4.4). In Austral-2 and Austrl-4 varieties, the main culms of WW plants were at the booting stage (Zadoks growth scale, ZGS 40-49), whereas in WL they had already reached the stage of spike emergence (ZGS 50-59). In the AdvL-4, the ZGS of WW plants corresponded with the spike emergence stage, progressing to the anthesis stage (ZGS 60-69) in WL plants (Figure 4.4).

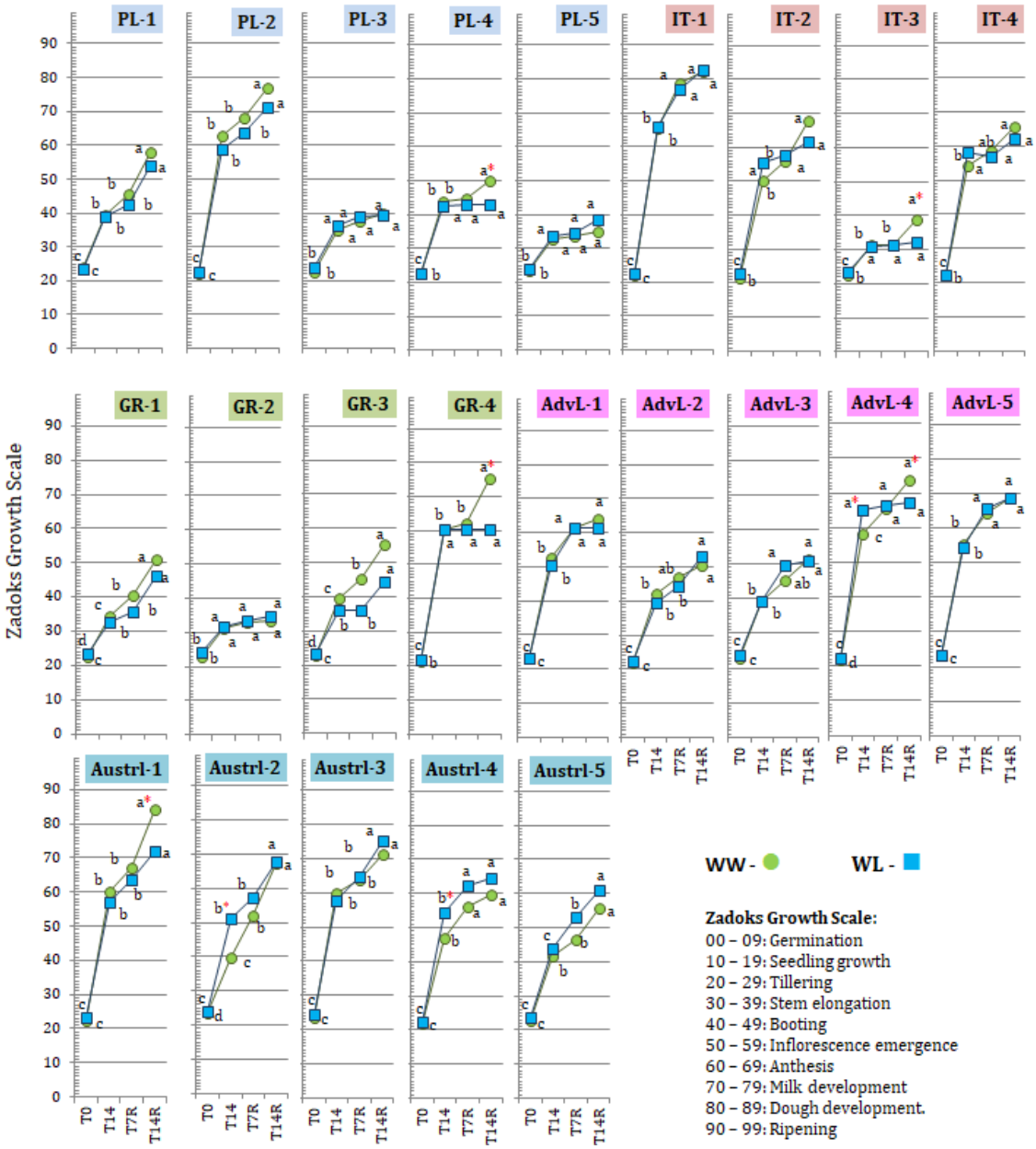


Figure 4.4 – Waterlogging-induced differences in plant growth development (Zadoks Growth Scale). Observations performed in the main culm of *T. aestivum* L. genotypes from five germplasm groups with distinct genetic backgrounds: Portuguese Landraces (PL); varieties with the introduction of Italian germplasm (IT); Post-Green Revolution varieties with the introduction of CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme and Australian varieties (Austrl). Comparisons were performed for each genotype regarding the main culm under control conditions (WW) or waterlogged (WL). Evaluations were made at the beginning (T0), after 14 days of stress (T14), and at the 7th and 14th days of recovery. For each genotype, different letters (a, b, c, d) indicate significant differences between various observation periods (T0, T14, T7R, T14R) for both water regimes (WW or WL). Differences between WW and WL, for each T, are highlighted with * (ANOVA, n = 3-6, $p<0.05$). The highest values are represented by the letter **a** or by *.

Despite all genotypes facing the same 14-day period of waterlogging, starting at the same developmental stage (tillering, ZGS 22-25), their growth rates were not uniform. As a result, nine genotypes suffered waterlogging during tillering and stem elongation (PL-1, PL-3, PL-5, IT-3, GR-1, GR-2, GR-3, AdvL-2, and AdvL-3), while others experienced stress also during the booting stage (PL-4 and Austrl-5). Furthermore, genotypes with faster development rates faced waterlogging events into later growth

stages, thereby suffering stress during the spike emergence stage (PL-2, IT-2, IT-4, AdvL-1, AdvL-5, Austrl-1; Austrl-2, Austrl-3, and Austrl-4) or even during anthesis (IT-1, GR-4, and AdvL-4) (Figure 4.4). During recovery, most genotypes showed no differences between the developmental stages of WW and WL plants. However, water stress hindered or stopped development in PL-4, IT-3, GR-4, AdvL-4, and Austrl-1. In the latter, slower growth progress was observed in WL plants, while the remaining genotypes showed similar values from the end of WL to the 14th day of recovery.

4.3.5 Changes in main culm height

In the beginning of water treatment (T0), plants off all genotypes from both water treatments (WW and WL) showed no differences in average plant height (Figure 4.5).

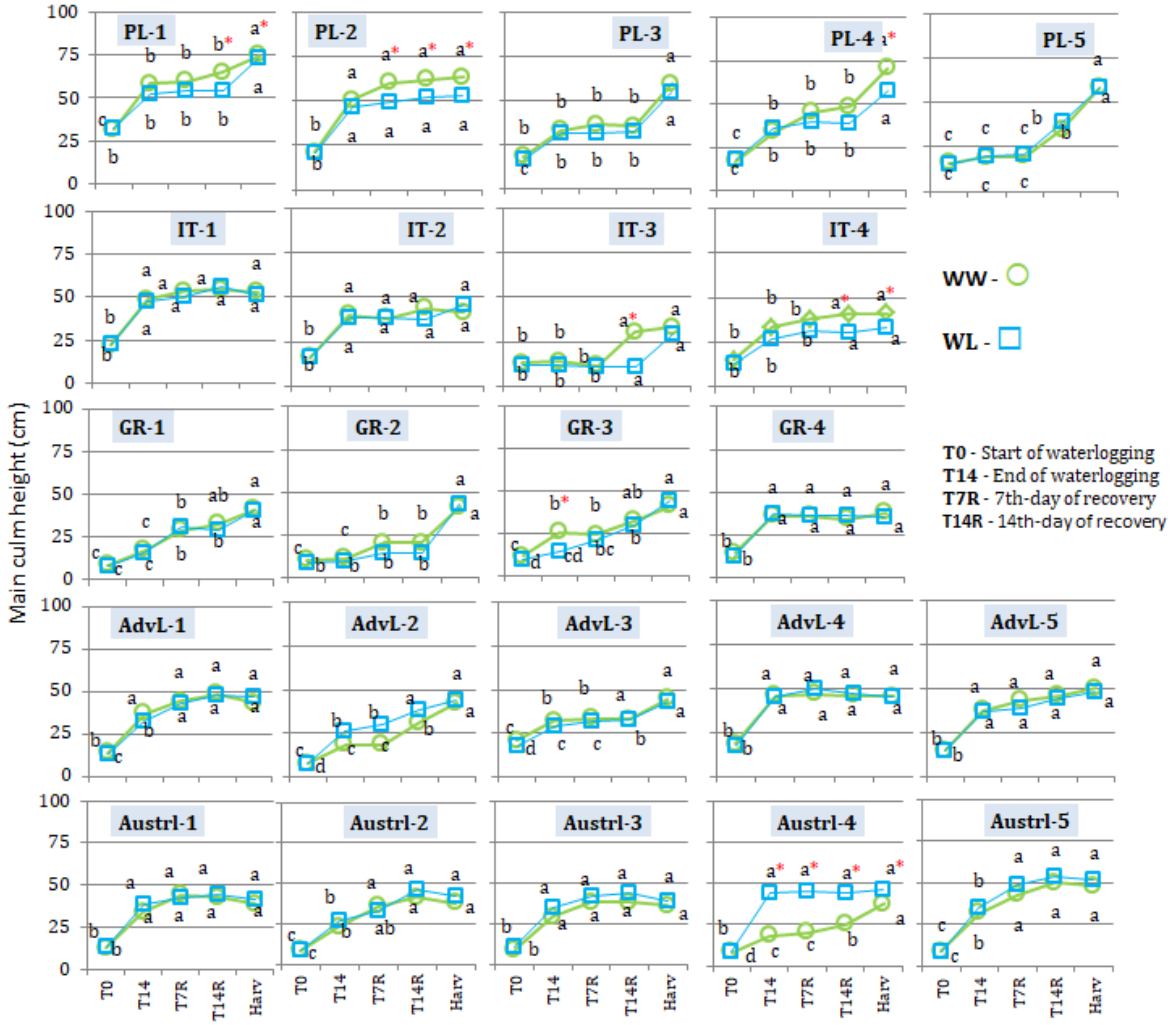


Figure 4.5 – Waterlogging-induced changes in the main culm height (cm) in *T. aestivum* L. genotypes from five germplasm groups with distinct genetic backgrounds: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with the introduction of CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme, and Australian varieties (Austrl). For each genotype, comparisons were made between well-watered (WW) and waterlogged (WL) plants at the beginning (T0) and the 14th day of stress (T14), at the 7th (T7R) and 14th days of recovery (T14R), and full maturity (Harv). For each genotype, different letters (a, b, c, d) indicate significant differences between T0, T14, T7R, T14R, and Harv, for both water regimes (WW or WL). Differences between WW and WL, for each observation time, are highlight with * (ANOVA, n = 3-6, $p<0.05$). The letter a or an * represents the highest values.

At maturity (Harv), the Portuguese landraces exhibited the highest values (Figure 4.5).

On the 14th day of waterlogging (T14), a reduction (44.5%) in the main culm height of GR-3 and a strong rise (143.5%) in Austrl-4 were observed. However, GR-3 showed similar results in both WW and WL plants from T7R until the last stage of the growing cycle, while Austrl-4 consistently exhibited higher values (123.8%, 75.1%, and 24.5%, at T7R, T14R, and Harv, respectively) in the WL plants (Figure 4.5).

Water stress also induced decreases in PL-2, IT-3, and IT-4 during the recovery period, with both the IT varieties being affected at T14R and PL-2 also earlier, at T7R. The IT-4 and PL-2 reductions also had an impact at Harv, resulting in decreases of 20.2% and 16.2%, respectively. Additionally, in PL-4, the reduced values trend (but not statistically significant) seen throughout the recovery phase ended in a drop of 18.2% at Harv (Figure 4.5).

4.3.6 Foliar biomass

The foliar biomass of the plant consists of both the green and chlorotic leaves from the main culm (MC) and all tillers (T). It is expressed as dry weight and shown separately for each genotype (Figure 4.6).

The foliar plant biomass was at its lowest at T14 in both the WW and WL treatments. However, only PL-1, PL-3, GR-2, and Austrl-3 exhibited this feature simultaneously (Figure 4.6). Stable values from T14 to T14R were observed in WW and WL plants of 14 genotypes. Nevertheless, at the end of the stress period, PL-5 and AdvL-5 plants showed more foliar biomass in comparison to WW plants, with a respective increase of 106% and 68%. Conversely, WL plants displayed lower values in GR-1, GR-2, and GR-4 (decreases of 31, 58, and 33%, respectively). On the 7th day of recovery (T7R), WL plants presented a lower foliar biomass in PL-1 (24% lower) and PL-2 (39% lower). This also occurred on the 14th day of recovery (T14R) in the PL-5 and IT-4 genotypes, with reductions of 53% and 54%, respectively, compared to WW plants (Figure 4.6).

Changes in plant foliar biomass are the result of variations in the values obtained for the main culm and/or tillers. The germplasm under study showed variability in response to waterlogging. In some cases, simultaneous but not significant variations in both the main culm and tillers led to differences in the foliar biomass of WW and WL plants. This was found at the end of the stress period (T14) for GR-1, GR-2, and GR-4, as well as in PL-1 at T7R. Austrl-2 displayed a 39% decrease in the main culm of WL plants, which was compensated by a rise in tillers, leading to similar values for WW and WL plants at T14 (Figure 4.6). Reductions in WL foliar biomass of the main culm of GR-3 (31%) and IT-4 (23%) were found at T14R. In the latter genotype, similar decreases were already observed at earlier stages (T7R). In PL-5, the plant increment (106% at T14) and the decrease (53% at T14R) referred above were mainly linked to a rise (108%) in tillers and a reduction (43%) in the main culm foliar biomass, respectively (Figure 4.6).

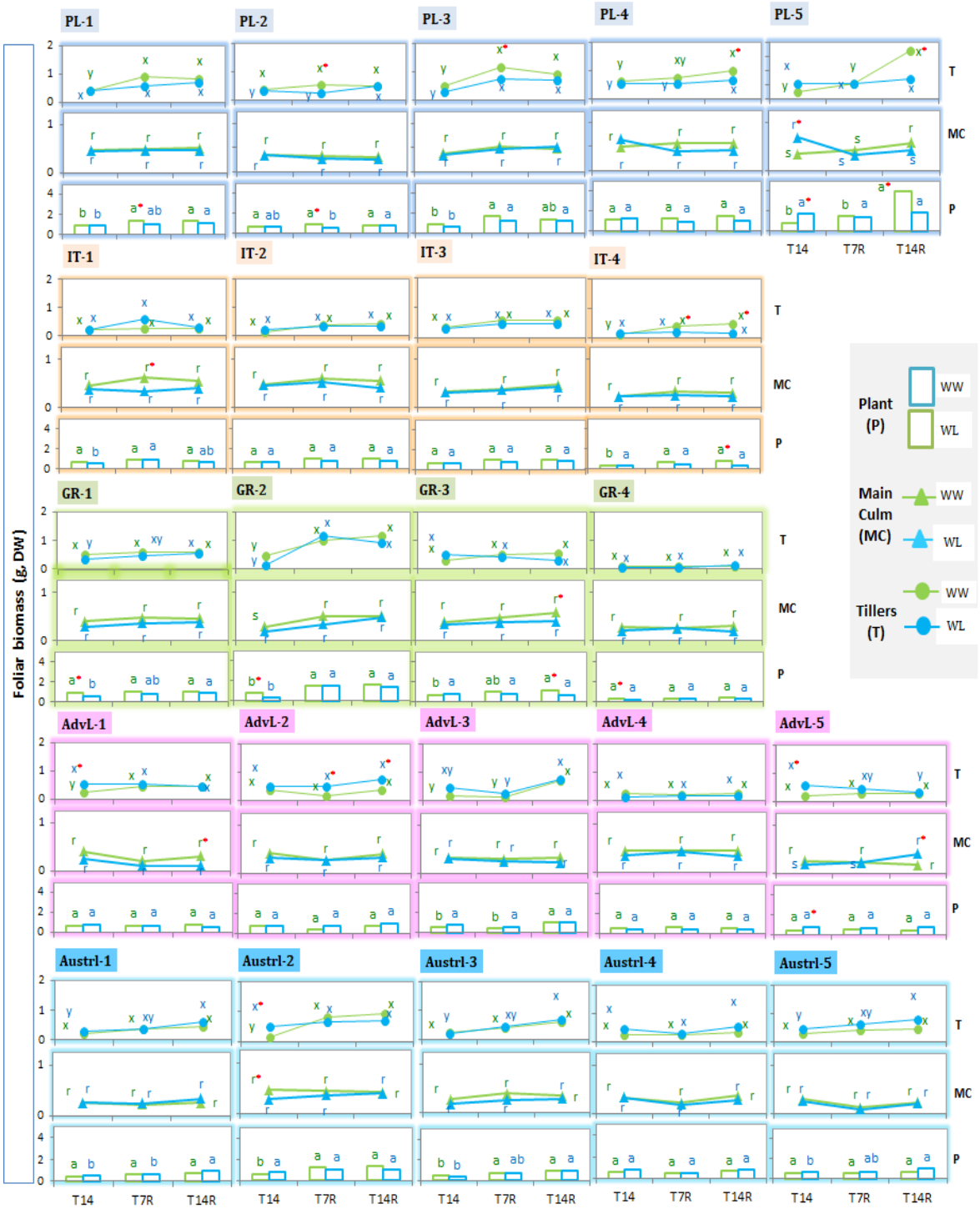


Figure 4.6 – Foliar biomass (g, DW) of the whole plant (P), the main culm (MC), or the total number of tillers (T). Evaluations performed in *T. aestivum* L. genotypes from five germplasm groups with distinct genetic background: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme; Australian varieties (Austrl); Comparisons were performed per genotype, at the end of 14-day waterlogging (T14) after 7 (T7R) and 14 (T14R) days of recovery. For each observation time, differences between well-watered (WW) and waterlogged (WL) plants are highlighted with *. Changes between the observation periods (T14, T7R, T14R) for both water regimes (WW or WL) in P, MC or T are indicated by (a, b, c), ANOVA, n = 3-6, $p<0.05$ with the highest values represented by the letter **a** or by an *.

4.3.7 Foliar senescence induced by waterlogging

Given that the foliar biomass presented in Section 4.3.6 included chlorotic and non-chlorotic leaves, this section addresses the impacts of waterlogging on premature leaf senescence. To better understand the impact on the plant as a whole, the proportion of senescent leaves within the plant foliar biomass, as well as their distribution between the main culm and tillers, were analysed by genotype. Comparisons were performed between WW and WL values at T14, T7R, and T14R (Figure 4.7).

Overall, waterlogging clearly resulted in an increase in senescent biomass in almost all genotypes throughout the observation periods (T14, T7R, and T14R). As expected, the percentage of senescent leaves in the control plants increased as the growth cycle advanced, reaching 3-30% at T14, 12-42% at T7R, and 11-56% at T14R (Figure 4.7).

In WL plants, values significantly rose, ranging from 24-72% for T14, 34-100% for T7R, and 29-100% for T14R. Apart from the variability observed within and between groups, waterlogging induced premature leaf senescence, with an average increase of 33% at the end of water stress (T14), 40% at T7R, and 38% at T14R, compared to WW plants.

Despite a slight tendency towards higher values, some genotypes did not exhibit any significant differences between WW and WL plants. This was particularly evident in the Australian germplasm, as Austrl-1 showed comparable values at T14, Austrl-4 at T7R, and Austrl-5 at both T14 and T14 R, while Austrl-2 had no variations. Among the remaining groups, only PL-2 exhibited similar values between WW and WL plants at T14 and PL-1 and IT-3 at T14R (Figure 4.7).

The rise in the plant senescence leaf mass was driven by changes in the main culm and/or tillers. However, the effects on tillers were more pronounced, impacting 19-22 genotypes, with average increases in senescence of 26.8% (T14), 37.9% (T7R), and 44.5% (T14R). The main culm exhibited similar values (23.6-30.8%) throughout all observation periods, with fewer genotypes being affected compared to tillers. The strongest impacts were observed during the recovery period. At T7R, three genotypes (IT-1, IT-4, and GR-4) presented over 90% of senescent leaves, which increased to seven genotypes (PL-2, IT-1; IT-4, GR-4, AdvL-4, AdvL-5, Austrl-3) by T14R (Figure 4.7).

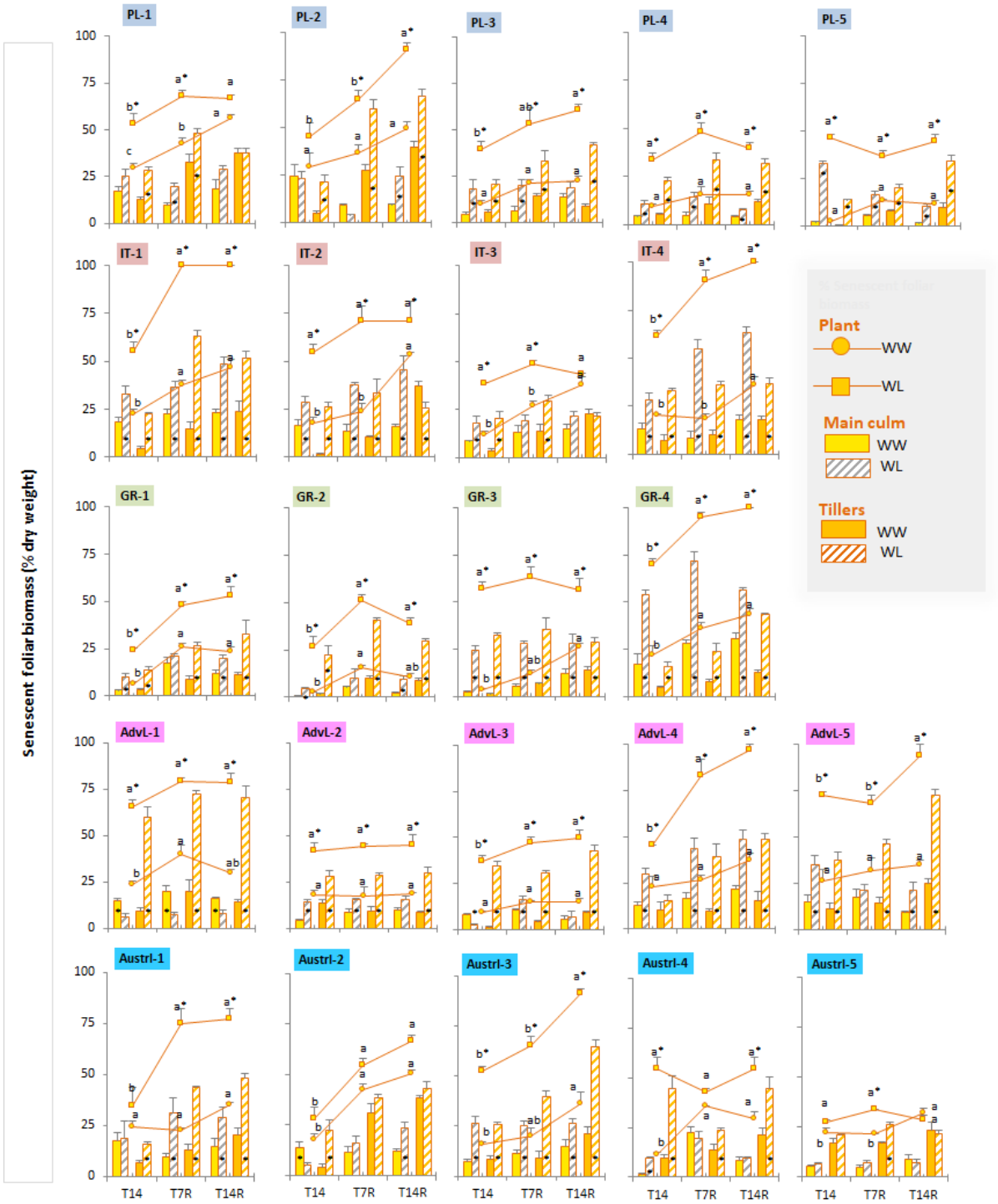


Figure 4.7 – Impact of 14-day waterlogging on the % of senescent foliar biomass (DW) evaluated in 23 *T. aestivum* L. genotypes, from five distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme; Australian varieties (Austrl), and conducted at the end of water stress (T14) and after 7 (T7R) and 14 (T14R) days of recovery. The senescent mass on the entire plant is indicated by —●— for well-watered (WW) and —■— for waterlogged plants. The column chart shows the distribution of those % among the main culm (MC) and tillers (T). The MC is represented by ■ and ■ (WW and WL, respectively), while tillers by ▨ and ▨ (WW and WL, respectively). Comparisons conducted per genotype and performed individually for Plant, MC, or T at T14, T7R, or T14R highlighting the differences between WW and WL plants with an * (highest value; ANOVA, n = 3-6, $p<0.05$). Additionally, the senescence progression of the whole plant was evaluated, with the differences in each water regime given by letters a, b, and c, with a for the highest value (ANOVA, n = 3-6, $p<0.05$).

4.3.8 Green leaf area

The analysed germplasm showed considerable heterogeneity in the green leaf area. Well-watered plants (WW) displayed green leaf area values ranging from 64 to 307 cm^2 (in T14), 48 to 402 cm^2 (in T7R), and from 45 to 1044 cm^2 (in T14R). Waterlogging resulted in green leaf area declines (28 to 100%), leading to extremely low values in some genotypes (*e.g.*, GR-4 and AdvL-4 at T14; GR-4 at T7R), or even values of zero (*e.g.*, IT-1 at T7R; IT-1, IT-4, GR-4, and AdvL-4 at T14R) (Figure 4.8).

Figure 4.8 - Impact of 14-day waterlogging on the green leaf area (cm^2). Determinations performed in *T. aestivum* L. genotypes, from five distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme; Australian varieties (Austrl). Analysis was performed at the end of water stress (T14) and at the 7th (T7R) and 14th (T14R) days of recovery. The green leaf area on the main culm and tillers are represented by column chart on the primary axis (left), and on the whole plant, by line chart on the secondary axis (right). The entire plant is indicated by —●— for well-watered regime (WW) and —■— for waterlogging. The main culm by ▭ in WW and by ▨ for WL; tillers by ■ and ▤ (WW and WL, respectively). Comparisons were conducted per genotype, highlighting the differences between WW and WL plants with an ***** (highest value; ANOVA, n = 3-6, $p<0.05$), and performed individually for Plant, Main culm, or Tillers at T14, T7R, or T14R. Additionally, the green leaf area of the whole plant was evaluated, with the differences in each water regime given by letters **a**, **b**, and **c**, with **a** for the highest value (ANOVA, n = 3-6, $p<0.05$).

At the end of the water stress (T14), nine genotypes showed unaffected green leaf area. However, only AdvL-2, AdvL-3, and Austrl-2 were able to maintain these levels until the 14th day of recovery

(T14R). Except for Austrl-5, which stood out by showing a trend of increased values (17-48%) across all observation points (with a significant rise only at T14R), the remaining five genotypes experienced strong decreases at T7R (56-67% for PL-1, PL-2, IT-2, and Austrl-1) and at T14R (54 and 85% for Austrl-1 and PL-2, respectively) or only at T14R (64% for PL-5).

Despite the reductions in waterlogging plants being high at T14 (ranging from 46% to 80%), the major decreases occurred during the recovery period. At T14R, seven genotypes exhibited reductions above 80%, with plants of three genotypes showing very low (PL-2 and AdvL-4) or even complete absence of green leaves (IT-1, IT-4, and GR-4) (Figure 4.8). The observed declines were due to decreases in the main culm and/or tillers. However, the diversity in germplasm led to several responses, as shown by eight genotypes (PL-3, IT-1, IT-4, GR-2, GR-3, AdvL-1, AdvL-4, and Austrl-3) exhibiting decreases in both the main culm and tillers throughout all observation periods (T14, T7R, and T14R), whereas AdvL-2, AdvL-3, AdvL-5, and Austrl-2 did not exhibit any reductions. Moreover, in the main culm of PL-2, PL-5, and IT-2, as well as in the tillers of PL-1, PL-2, PL-5, IT-2, and Austrl-1, the adverse effects were exclusively found during the recovery period (T7R and T14R). Overall, the impacts were more pronounced in the tillers, with average decreases ranging from 70-75% throughout T14, T7R, and T14R, whereas in the main culm, declines reached 47-58%. Results suggest that negative impacts were more evident on the 7th day of recovery, affecting a larger number of genotypes (17) (Figure 4.8).

4.3.9 Relative chlorophyll content – SPAD

The relative chlorophyll content was measured at the beginning (T0), after 7 and 14 days of waterlogging (T7 and T14, respectively), and at the 7th (T7R) and the 14th (T14R) day of recovery, in all leaves from the main culm. The average values are presented in Figure 4.9.

At T0, WW and WL plants presented similar values across all genotypes. Waterlogging induced reductions in SPAD values in all genotypes except Austrl-5, which remained unaffected. An overall deterioration occurred throughout the observations, with average decreases of 24.1%, 41.5%, 52.2%, and 55.9% at T7, T14, T7R, and T14R, respectively. Additionally, at T7, eleven genotypes were impacted, while at T14 and T7R, twenty genotypes showed reduced values, and at T14R, one additional genotype exhibited declines, resulting in a negative impact on twenty-one out of the twenty-three genotypes (Figure 4.9).

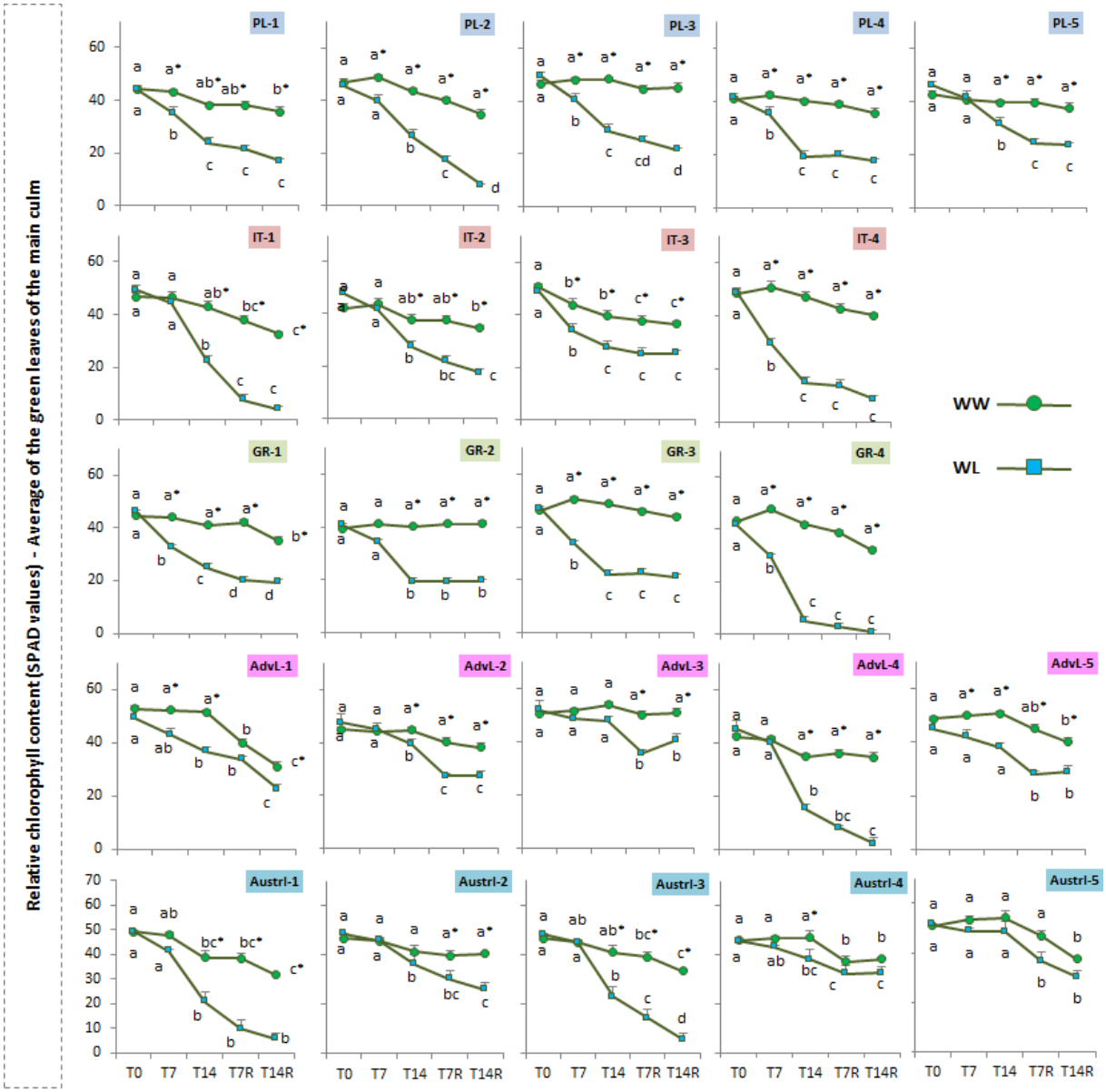
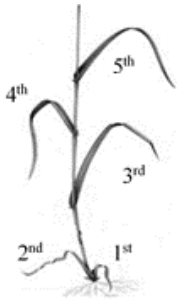


Figure 4.9 – Waterlogging induced changes in the average relative chlorophyll content (SPAD), determined in all leaves of the main culm. Determinations performed in well-watered (WW, —●—) and waterlogged plants (WL, —■—) of *T. aestivum* L. genotypes, representing five distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme; Australian varieties (Austrl). Analysis was performed at the beginning (T0), after 7 days (T7) and at the end of water stress (T14), as well as at the 7th (T7R) and 14th (T14R) days of recovery. Comparisons were conducted per genotype, highlighting the differences between WW and WL plants with an ***** (highest value; ANOVA, n = 3-6, $p<0.05$). Differences between T0, T7, T14, T7R, and T14R, were performed for each water regime and are given by letters a, b, c, and d, with a for the highest value (ANOVA, n = 3-6, $p<0.05$).

At T7, a strong impact on the SPAD values of IT-4 and GR-4 resulted in decreases of 42.3 and 38.1%, respectively. As water stress progressed (T14), values declined to 68.7 and 87.7% (respectively), along with SPAD reductions above 40% in PL-2, PL-3, PL-4, IT-1, GR-1, GR2, GR-3, AdvL-4, Austrl-1, and Austrl-3. On the 14th day of recovery, differences between WW and WL plants were even clearer. With reductions ranging from 78.0 to 97.9%, the genotypes PL-2, IT-1, IT-4, GR-4, AdvL-4, Austrl-1, and Austrl-3 presented the major impact in SPAD values. They were followed by all PL and GR genotypes (except PL-2 and GR-4), IT-2, IT-3, and Austrl-2, which lowered SPAD values by 31.0-52.6%

compared to WW plants. Except for AdvL-4, all genotypes in the AdvL group depicted SPAD reductions between 20.0% and 28.8% at the end of the recovery period (Figure 4.9). With a different behaviour, WL plants of Austrl-4 and Austrl-5 reached T14R with similar SPAD values as those found for well-watered plants (Figure 4.9).

To evaluate changes in the progression of senescence, next results focused on SPAD values measured in five leaves from the main culm: the basal leaves (1st and the 2nd), which are the first to appear during the plants initial growth stage and often the first to exhibit signs of senescence, and the subsequent three fully formed leaves (Figure 4.10). Measurements were performed only in leaves with green tissues indicating some level of photosynthetic activity. Fully senescent leaves are represented with the letter "s"in Table 4.3.



ution in the main culm, regarding the five leaves used for SPAD measures were conducted in 23 *T. aestivum* L. genotypes under well-watered and onditions.

At the beginning of stress (T0), SPAD values did not show any differences between well-watered (WW) and waterlogged (WL) plants on the five leaves examined, except for AdvL-5, which showed a 15.1% reduction in the 2nd leaf (Table 4.3).

In WW plants, the basal leaves undergo natural senescence, leading to a gradual decline in SPAD values across the observation periods (T0, T7, T14, T7R, and T14R). In T0, values ranged from 39.4 to 55.6. However, at T7 and T14, the minimum value reduced to 21.0 and 4.6, respectively, while the highest value remained stable, declining to *ca.* 35 at T7R and T14R. In WW plants, the complete senescence (SPAD value <10) of the 1st leaf was observed at T14 in five genotypes (PL-1, PL-2, IT-2, AdvL-4, and Austrl-2), which increased to nine and sixteen genotypes at T7R and T14R, respectively (Table 4.3). Regarding the 2nd leaf, SPAD values were lower than 10 (PL-1, PL-2, IT-1, IT-2, and AdvL-1) only at T14R.

Table 4.3 - Relative chlorophyll content of 23 genotypes of *T. aestivum* was measured with a SPAD meter in five leaves counted from the basis of the main culm, during waterlogging and the recovery period. Only leaves with green tissues, suggesting photosynthetic activity, were included. Senescent leaves are represented with the letter "s". Evaluations at the beginning (T0), after 7 days (T7) and at the end of water stress (T14), as well as at the 7th (T7R) and 14th (T14R) days of recovery were conducted per genotype and statistical analysis performed in each period of analysis, highlighting the differences between well-watered (WW) and waterlogged plants (WL) with an * (highest value; ANOVA, n = 6, $p<0.05$). Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme (INIAV, I.P.); Australian varieties (Austrl).

		1st Leaf					2nd Leaf					3rd Leaf					4th Leaf					5th Leaf				
		T0	T7	T14	T7R	T14R	T0	T7	T14	T7R	T14R	T0	T7	T14	T7R	T14R	T0	T7	T14	T7R	T14R	T0	T7	T14	T7R	T14R
PL-1	WW	44.1	31.7*	4.6	s	s	45.2	43.8*	26.8*	17.3*	10.7	44.2	47.0	38.8*	35.1*	25.2*	45.1	47.7	48.6*	50.8*	41.7*	42.2	47.7*	50.1*	55.4*	45.7*
	WL	43.2	13.9	s	s	s	44.8	33.5	7.6	1.6	s	43.1	40.5	17.2	12.6	3.8	47.0	48.3	32.3	26.0	12.5	44.3	40.9	39.6	37.2	24.1
PL-2	WW	44.1	22.8	6.1	s	s	52.4	51.8*	38.2*	16.4*	2.1	52.9	60.0*	51.1*	49.7*	32.5*	46.2	57.0*	54.1*	57.2*	53.5*	39.1	52.6*	57.7*	57.3*	59.5*
	WL	41.7	25.4	3.0	s	s	48.8	40.5	13.2	1.5	s	48.8	46.4	24.0	11.5	s	45.2	44.4	38.3	23.9	8.0	43.9	43.7	43.4	31.8	17.7
PL-3	WW	45.4	43.9*	39.1*	32.0*	24.3*	48.4	49.0*	48.9*	40.5*	37.7*	46.3	51.4*	52.2*	46.8*	49.3*	45.3	51.0*	49.8*	48.7*	43.1*	-	45.5*	50.9*	47.8*	51.6*
	WL	47.7	35.1	9.9	1.0	0.2	51.5	40.6	25.4	20.6	17.4	49.4	45.1	32.8	28.5	22.4	47.8	45.4	33.5	33.6	28.9	-	35.1	40.1	33.4	31.1
PL-4	WW	41.4	34.0	30.7*	20.3*	14.0*	44.1	47.0*	41.3*	32.1*	24.7*	40.9	48.0*	43.6*	44.0*	36.5*	37.8*	44.6*	44.1*	45.9*	45.6*	37.2	39.3*	40.0*	42.7*	46.4*
	WL	43.3	29.5	4.1	0.6	1.3	45.6	37.7	10.0	8.5	3.3	41.1	40.8	22.1	17.5	11.9	33.7	36.3	27.2	21.7	18.6	34.9	32.0	27.2	29.6	29.8
PL-5	WW	46.0	39.3	34.8*	22.7*	19.7*	43.9	42.1	39.1*	39.6*	37.8*	39.1	41.4	40.7	48.5*	42.6*	34.0	40.1	41.2	43.8*	44.2*	-	38.7	41.7*	43.0*	40.6*
	WL	47.6	38.6	20.6	7.1	6.3	49.5	45.2	28.8	18.4	15.7	43.5	43.0	35.6	30.5	26.5	36.7	40.9	35.7	31.8	26.6	-	34.7	32.7	29.1	31.9
IT-1	WW	48.6	20.7	11.6*	4.2	s	52.7	41.8	33.2*	16.4*	3.9	53.9	58.0	57.7*	53.5*	40.3*	48.5	55.2	56.0*	55.9*	56.6*	34.7	55.6	56.0*	58.1*	55.9*
	WL	46.3	21.1	1.1	s	s	54.7	48.7	6.4	s	s	57.7	54.5	25.1	s	s	47.5	52.4	36.8	s	s	31.1	49.5	42.1	s	s
IT-2	WW	44.1	36.0*	8.5*	2.3	s	44.3	41.6	21.4*	14.7*	7.0	43.6	45.5	38.4*	35.1*	18.8*	41.4	48.3	47.7*	48.8*	41.8*	37.3	46.9	51.6	52.9*	53.2*
	WL	45.7	26.5	s	s	s	49.3	37.1	3.1	s	s	50.0	47.6	16.7	7.2	s	47.3	49.3	32.2	17.1	2.7	44.9	48.1	45.6	28.4	12.6
IT-3	WW	55.5	38.1*	25.6*	20.7*	8.5*	53.8	45.1*	39.7*	33.6*	27.9*	42.1	49.4*	44.2*	43.2*	41.4*	-	44.6*	43.7*	46.1*	46.3*	-	41.3	44.5*	44.6*	52.3*
	WL	54.1	25.9	14.4	10.9	1.1	53.7	36.0	27.3	22.9	19.2	38.4	40.1	33.5	30.3	30.0	-	34.5	33.1	31.2	32.5	-	-	31.0	28.4	32.2
IT-4	WW	49.0	45.4*	27.1*	12.5*	2.9	50.6	53.4*	45.0*	33.7*	23.4*	49.0	56.1*	54.4*	52.8*	53.3*	39.4	54.0*	56.2*	53.5*	52.2*	-	47.4	51.1*	52.7*	54.8*
	WL	47.8	2.2	s	s	s	50.9	25.8	s	s	s	48.2	45.3	15.5	9.5	s	44.3	43.0	29.0	20.7	s	-	52.3	29.6	29.9	s
GR-1	WW	44.9	36.3*	25.9*	20.6*	4.1	46.0	47.3*	39.3*	37.5*	18.0*	42.8	46.0*	43.6*	45.9*	35.9*	-	45.4*	47.5*	50.5*	46.6*	-	44.8*	45.8*	49.8*	53.7*
	WL	46.3	24.6	9.0	s	s	46.6	31.4	17.4	2.1	1.4	45.6	36.7	27.7	21.4	11.0	44.5	35.8	35.4	34.9	28.4	-	37.2	32.3	33.4	33.4

GR-2	WW	43.8	38.8	35.8*	34.1*	33.6*	39.7	38.0	42.9*	41.1*	39.0*	36.6	42.7	41.7*	42.2*	42.4*	38.3	45.9*	41.1*	44.9*	45.1*	-	-	-	45.2*	45.2*
	WL	44.1	32.7	6.2	3.3	1.7	41.9	34.4	20.7	18.4	13.4	36.8	36.7	25.1	25.7	20.8	38.6	35.4	24.8	25.7	26.6	-	-	26.4	25.8	30.5
GR-3	WW	49.4	48.8*	42.3*	27.4*	15.5*	46.9	52.0*	47.0*	37.3*	27.9*	41.7	52.2*	53.9*	50.6*	43.5*	53.6	51.7*	51.7*	52.3*	52.2*	-	48.5	50.6*	53.5*	54.7*
	WL	48.2	24.6	2.5	2.4	1.5	47.6	34.4	8.2	8.1	6.0	45.2	38.7	16.6	19.1	16.2	-	36.2	29.3	25.8	23.2	-	-	36.0	32.9	32.5
GR-4	WW	41.6	38.4*	20.8*	15.9*	4.8*	47.4	48.9*	40.8*	32.4*	20.0*	44.5	51.2*	49.9*	47.8*	46.6*	40.3	51.7	51.2*	51.5*	48.9*	39.8	47.5	48.4*	50.1*	48.8*
	WL	39.9	5.5	s	s	s	42.1	20.5	s	s	s	46.5	43.0	s	s	s	38.0	46.9	6.6	6.2	s	40.8	45.1	7.6	6.7	s
AdvL-1	WW	47.5	53.0*	27.8*	s	s	53.7*	53.2*	49.5*	12.3*	s	55.6	57.4*	58.9*	33.8*	21.0*	54.0	55.9	57.1*	52.3	35.4*	54.2	53.5	57.5	58.8	48.8
	WL	45.3	18.9	8.3	s	s	45.6	41.8	20.8	6.8	s	49.4	48.4	32.0	22.6	8.5	52.5	53.3	49.3	49.9	26.3	51.3	48.4	50.4	60.0	48.2
AdvL-2	WW	48.5	39.9	35.8	13.3*	9.1*	47.6	46.1	46.3*	37.9*	24.5*	46.4	47.5	46.8	44.6*	40.9*	41.8	44.4	47.2	48.2	48.4	38.8	43.3	47.6	49.9	50.0
	WL	48.1	46.8	30.8	s	s	52.6	46.8	40.3	10.1	3.8	45.4	47.0	42.6	30.1	31.4	42.1	40.6	44.9	43.3	42.5	-	41.5	39.4	43.3	44.5
AdvL-3	WW	52.9	52.9	50.3*	37.4*	32.1*	51.0	55.3	58.8*	48.7*	47.2*	48.4	50.9	57.4*	54.0*	56.0*	51.4	50.1	55.9	54.6*	56.3*	-	46.1	53.7	55.2*	55.8
	WL	52.5	49.0	38.4	14.3	15.1	55.1	51.3	47.9	27.6	30.9	50.3	48.8	49.8	39.2	45.6	46.8	48.1	52.1	45.4	49.8	-	46.5	49.2	44.4	52.4
AdvL-4	WW	39.4	26.3*	5.7*	5.3*	3.0	40.4	40.2	22.0*	23.3*	22.8*	43.6	44.4	44.0*	45.5*	43.2*	45.1	47.1	52.0*	54.6*	53.7*	45.8	49.7	52.5*	53.5*	51.7*
	WL	39.8	18.7	s	s	s	45.8	39.4	2.3	s	s	47.5	48.5	13.6	6.8	s	46.4	50.6	36.2	23.3	s	47.2	49.1	29.5	8.3	s
AdvL-5	WW	41.7	41.5*	32.0*	19.4*	7.0	48.7	51.3*	51.6*	29.1*	29.2*	50.9	54.4*	55.4*	47.4*	30.9	50.0	54.4	57.0	55.0*	53.8*	55.2	50.9	55.0	54.5*	54.2*
	WL	39.3	22.7	12.3	1.9	s	41.7	39.6	26.7	12.1	11.8	45.7	44.8	37.8	26.1	29.0	45.2	49.2	48.9	34.3	36.7	48.6	47.5	48.8	47.6	44.4
Austrl-1	WW	50.7	42.6*	15.3*	9.5*	0.9	51.0	48.9	35.7*	34.5*	18.6*	49.0	52.5*	51.9*	57.7*	47.1*	44.3	46.9	58.2*	60.0*	56.5*			44.9	65.9*	51.7*
	WL	48.6	35.6	3.5	2.2	2.4	50.3	42.0	9.2	3.5	2.5	49.0	45.3	35.2	8.9	2.8	47.9	44.4	47.4	19.2	10.7			44.5	43.6	32.6
Austrl-2	WW	45.7	38.5	10.6	8.3	4.5	48.1	46.1	32.2	24.9	17.6*	47.8	49.5	52.5*	50.7	37.5*	47.0	51.1	58.0	56.2	57.3*	43.6	43.0	53.3	58.8	60.6*
	WL	47.9	43.9	7.5	2.3	0.6	51.2	47.1	34.8	20.7	7.4	47.7	46.5	44.5	38.0	29.0	47.2	45.9	48.3	48.7	45.1		46.1	51.6	51.6	46.9
Austrl-3	WW	48.8	36.3	19.9	12.5	4.7	49.7	45.8	38.9*	31.3*	29.4*	47.0	48.9	50.1*	51.5*	45.5*	41.7	47.7	53.8*	57.7*	50.4*		46.9	50.0	57.7	55.5
	WL	48.5	45.8*	38.9*	31.3*	29.4*	49.6	45.4	13.8	1.1	s	47.6	48.7	38.7	18.3	1.3	47.2	49.8	43.4	45.8	21.8					
Austrl-4	WW	47.1	44.6*	35.5*	6.2	6.0	48.2	48.8	48.4*	24.4*	31.6*	45.3	48.7	51.3	43.0	46.5*	42.1	45.6	50.8	46.4	47.2		44.7	48.1	48.6	46.3
	WL	45.1	31.3	10.3	3.3	s	47.7	44.5	36.3	11.4	10.0	45.5	47.9	47.4	39.2	38.1	42.8	47.5	47.8	43.7	47.6		44.5	48.2	47.0	45.5
Austrl-5	WW	55.6	55.3*	45.8*	16.0*	9.0*	52.9	54.0	56.8	44.3*	35.7*	48.3	54.1	58.3	54.6*	53.3*	40.3	51.9	57.1	56.2	52.7		50.3	54.9	55.6	54.5
	WL	54.7	45.9	32.9	2.7	s	53.8	54.3	52.9	28.7	12.1	51.2	51.5	51.7	42.2	29.7	43.3	47.5	52.7	47.8	45.1		41.4	53.6	52.8	48.4

Waterlogged plants showed a stronger decrease in SPAD values of the basal leaves, suggesting a premature senescence. At T7, sixteen genotypes had SPAD values <10 for the 1st leaf, which increased to twenty-one genotypes at T14R. In addition, the genotypes that exhibited some green tissues showed a gradual decrease in maximum values from 54.7 at T0 to 29.4 at T14R, resulting in a 49% decline. The 2nd leaf showed a similar trend, where waterlogging accelerated senescence, leading to values <10 in nine (T14), fourteen (T7R), and sixteen (T14R) genotypes. Additionally, the highest values decreased from 55 to *ca*. 30 at T14R, resulting in an identical reduction (45%) observed in the 1st leaf (Table 4.3).

Considering the 3rd to 5th leaves of WW plants, the highest SPAD values consistently ranged from 54-66 throughout T0 to T14R. However, PL-1, IT-2, and AdvL-1 denote the initial phases of senescence, with the lower value, particularly in the 3rd leaf, reduced from *ca*. 40 at T0 to 19-25 by T14R (Table 4.3). The 4th and 5th leaves, positioned above, exhibited minor fluctuations in the SPAD values across observations, ranging from *ca*. 35 to 45 for both.

With waterlogging, the 3rd leaf exhibited SPAD reductions in several genotypes. Earlier at T7, eleven genotypes decreased values by 13.7-25.9%. By the end of the water-stress period, the declines ranged from 24.2-71.5%, affecting twenty genotypes. In addition, the GR-4 3rd leaf had already undergone senescence. During the recovery period, SPAD declines intensified (20-90%), impacting 22 genotypes. Similar to GR-4, the genotypes PL-2, IT-2, IT-4, AdvL-4, Austrl-1, and Austrl-3 have already reached senescence on their 3rd leaf at T14R, differing from WW plants, which have SPAD values of 19-47 (Table 4.3).

The negative impacts were also evident in the 4th and 5th leaves. During waterlogging and the recovery period, SPAD values of the 4th leaf decreased in eight (T7), sixteen (T14), eighteen (T7R), and twenty (T14R) genotypes, while the 5th leaf was impacted in five (T7), twelve (T14), and seventeen (T7R and T14R). While at T14, PL-5 did not show reductions in SPAD values of the 4th leaf and IT-2, GR-2, AdvL-1, Austrl-1, and Austrl-3 on the 5th leaf, AdvL-2, AdvL-3, AdvL-5, Austrl-2, Austrl-4, and Austrl-5 were unaffected in both the 4th and 5th leaves. At T14, in GR-4, the 4th and 5th leaves showed the strongest decreases (68% and 84%, respectively), developing full senescence during the recovery period. Additionally, PL-2, IT-2, and Austrl-1 also suffered strong declines (81-94%) on the 4th leaf, and IT-1, GR-4, and AdvL-4 reductions of 84.5-100% on both the 4th and 5th leaves (Table 4.3).

4.3.10 Changes in Nitrogen, Phosphorus and Potassium leaf content

4.3.10.1 Nitrogen

In well-watered plants (WW), the nitrogen level on the leaves of most genotypes remained constant throughout the observations (T14, T7R, and T14R). However, for PL-1, PL-2, and GR-4, it decreased by 10.3% to 32.1%, and for GR-1, it rose by 16.0% (Figure 4.11).

Waterlogging caused significant reductions in the nitrogen content of the green leaves. Fifteen genotypes showed reductions at the end of the stress period (T14), ranging from 16.4% to 66.9%. This decrease was observed in all germplasm groups, with the exception of the Australian, where all genotypes remained unaffected.

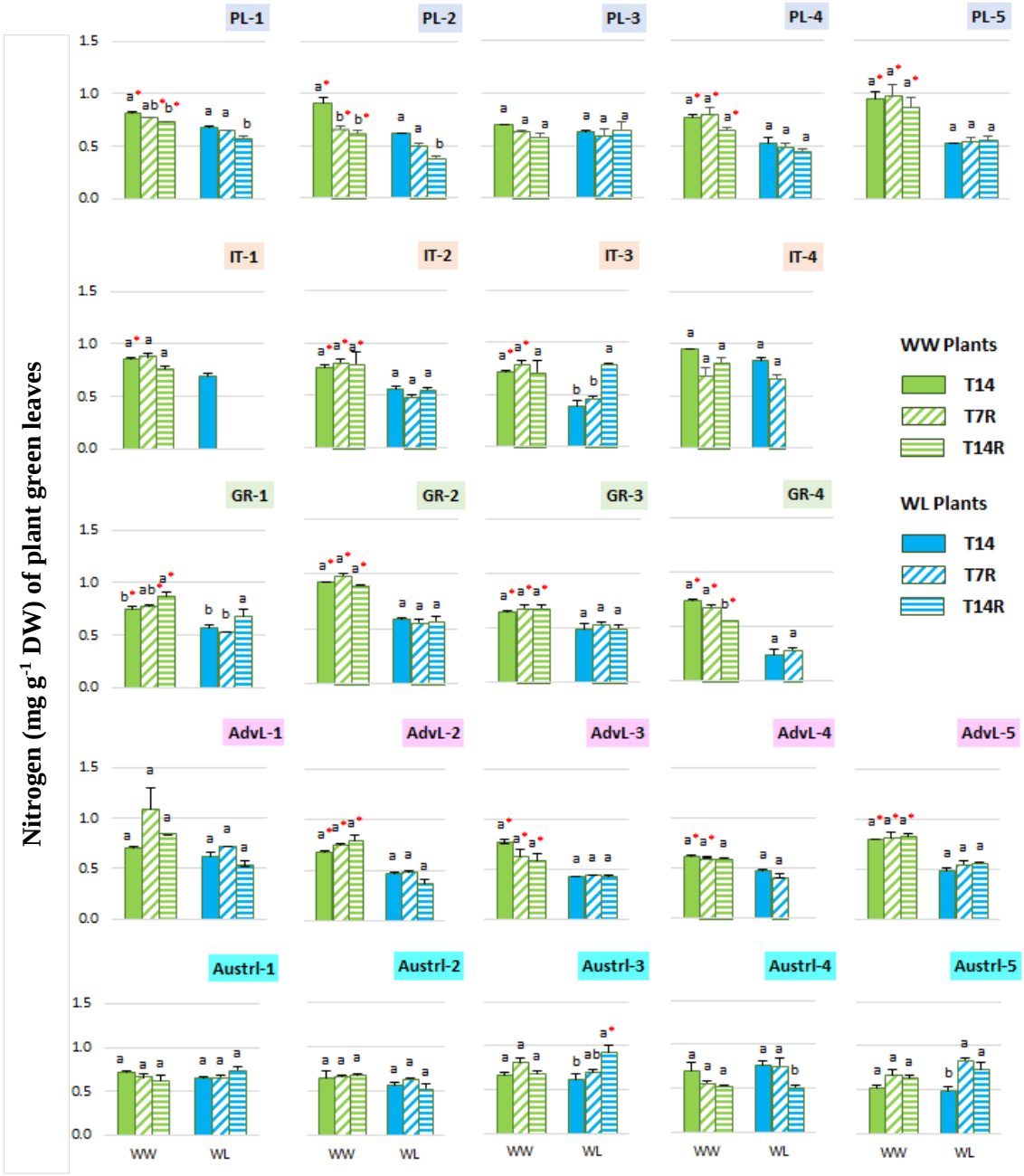


Figure 4.11 – Nitrogen content of the green leaf tissues. Determinations performed, by Kjeldahl method, in *T. aestivum* L. genotypes, representing five distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme (INIAV, I.P.); Australian varieties (Austrl). Analysis was performed at the end of the water stress (T14), and at the 7th (T7R) and 14th (T14R) days of recovery. Comparisons were conducted per genotype, highlighting the differences between WW and WL plants with an * (highest value; ANOVA, n = 3-6, $p<0.05$). Differences between T14, T7R, and T14R, were performed for each water regime and are given by letters **a**, **b**, and **c**, with **a** for the highest value (ANOVA, n = 3-6, $p<0.05$).

The recovery period revealed the strongest impacts, with eleven genotypes denoting similar reductions to those seen at T14. Moreover, IT-1, IT-4, GR-4, and AdvL-4 only had residual nitrogen content (data not shown) due to the absence of green tissues in their leaves. Despite this, and with the exception of GR-4, which exhibited a 66.9% decline at T14, these genotypes did not show the most

pronounced reductions in nitrogen content at T14. In fact, at this stage, IT-1 and IT-4 were unaffected, while AdvL-4 decreased by 23.4%. In these genotypes, the biggest impacts were observed at T7R (IT-1) or T14R (IT-4 and AdvL-4). Conversely, Australian varieties, PL-3 and AdvL-1, stood out by showing no reductions in nitrogen content from T14 to T14R. In addition, Austrl-3 presented a 76.7% increase at T14R (Figure 4.11).

4.3.10.2 Phosphorus.

Regarding phosphorus content, measurements were conducted in both green and senescent leaf tissues (Figures 4.12). Similarly to nitrogen, the absence of green leaf tissues in IT-1, IT-4, GR.4, and AdvL-4 prevented the analysis at T7R and/or T14R.

In senescent leaves, phosphorus values often fell below the 0.05 mg g^{-1} limit of detection (LOD). When present, phosphorus values of WW plants were slightly over LOD (0.06–0.07 mg g^{-1}), while WL plants showed values ranging from 0.09-0.37 mg Kg^{-1}. These levels, however, were still very low compared to the average concentration of 1.2 mg g^{-1} in WW plants and 0.9 mg g^{-1} in WL plants found in the green leaves.

The phosphorus content of WW plants in the green leaf tissues varied from 0.48 to 1.95 mg g^{-1}, with a strong decrease in the lowest values (to 0.55 mg g^{-1}) in WL plants (Figure 4.12).

Waterlogging impacted on genotypes from all germplasm groups. However, the GR group was the only one that exhibited decreases in phosphorus values across all genotypes at T14, T7R, and T14R (Figure 4.12).

Ten genotypes did not display phosphorus reductions at T14 due to water stress, in contrast with the remaining genotypes, which experienced declines ranging from 10.3% to 77.5% (Figure 4.12). Nevertheless, seven of the ten genotypes unaffected by WL exhibited significant declines during the recovery period. This was the case for PL-3, PL-5, IT-3, and AdvL-2, which showed reductions of 47.8%, 21.8%, 43.9% and 43.9%, earlier at T7R, respectively. Furthermore, IT-1, which displayed a 50.0% increase at T14, presented a strong response during recovery, resulting in the senescence of all leaves. Conversely, PL-2 and AdvL-4 were capable of maintaining the phosphorus level until T7R; however, they showed reductions of 92.4% and 42.1% at T14R, respectively (Figure 4.12). Of the 23 genotypes under evaluation, only IT-2, AdvL-3, and Austrl-2 showed similar results between WW and WL plants from T14 to T14R (Figure 4.12).

Figure 4.12 - Waterlogging-induced changes in phosphorus content of the green and senescent leaves obtained by X-ray fluorescence in *T. aestivum* L. genotypes. Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL); Australian varieties (Austrl). Data acquired at the end of the water stress (T14), at the 7th (T7R) and 14th (T14R) days of recovery. Comparisons were conducted per genotype, highlighting the differences between WW and WL plants with an * (highest value; ANOVA, n = 3-6, $p<0.05$). Differences between T14, T7R, and T14R, were performed for each water regime and are given by letters a, b, and c, with a for the highest value (ANOVA, n = 3-6, $p<0.05$). < LOD (Below the Limit of Detection).

4.3.10.3 Potassium.

Measurements of potassium content were conducted in both green and senescent leaf tissues (Figures 4.13).

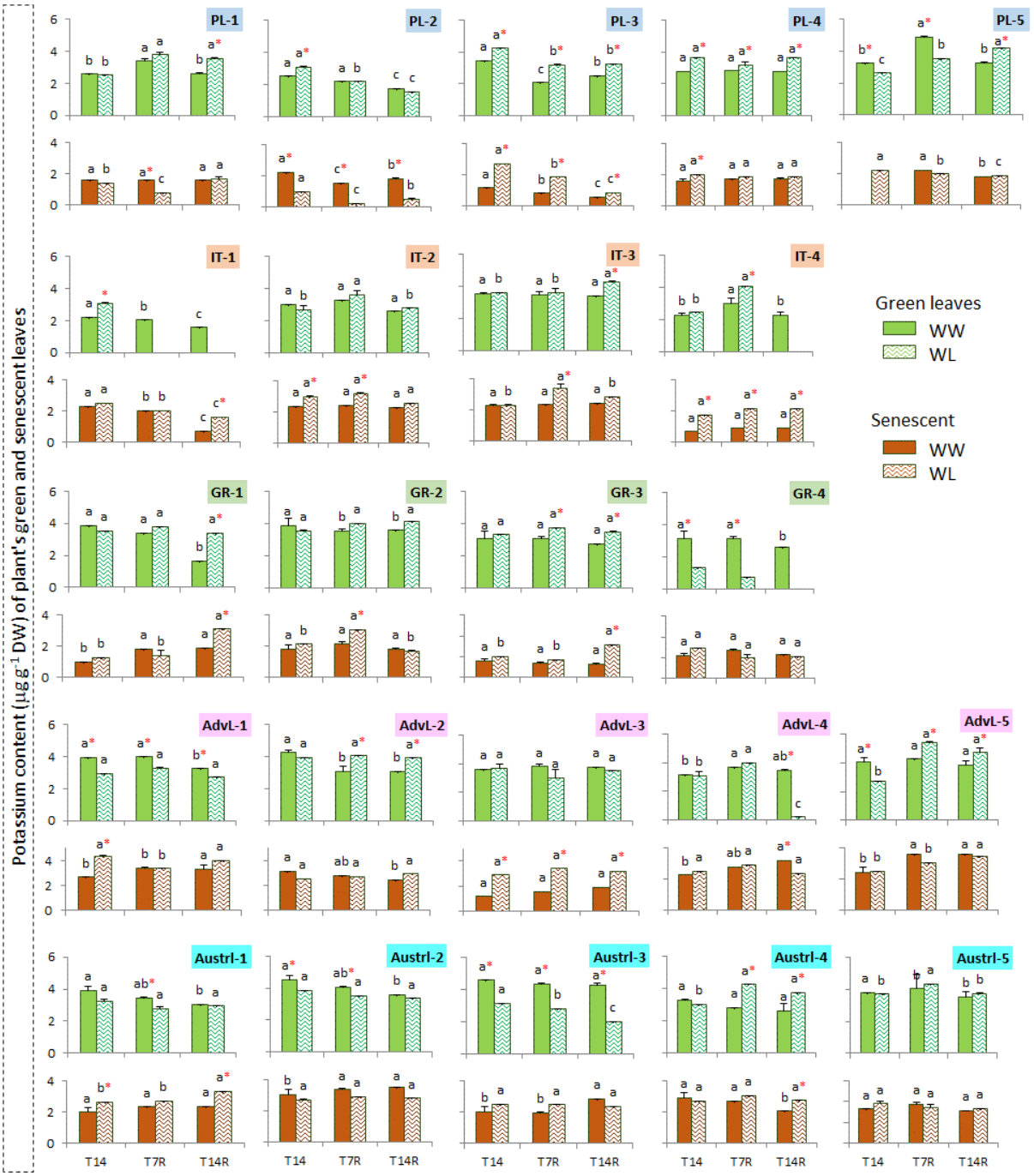


Figure 4.13 – Waterlogging-induced changes in potassium content of the green and senescent leaves obtained by X-ray fluorescence in 23 *T. aestivum* L. genotypes.: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme; Australian varieties (Austrl). Analyses were performed at the end of the water stress (T14) and at the 7th (T7R) and 14th (T14R) days of recovery. Comparisons were conducted per genotype, highlighting the differences between WW and WL plants with an *. Differences between T14, T7R, and T14R, for each water regime are given by letters a, b, and c. Letter a and * for the highest value (ANOVA, n = 3-6, $p<0.05$).

From T14 to T14R, potassium content in WW plants green leaves ranged from 1.56% to 5.39% and in senescent tissues, from 0.54% to 4.01%. Under waterlogging, the highest values for green and senescent tissues remain stable (5.48% and 4.41%). However, the lowest values for the same tissues were reduced to 0.17 and 0.21, respectively (Figure 4.13).

Within the germplasm under study, a significant diversity of responses was observed. During the recovery period, results suggested a tendency for a decline in the number of genotypes that showed unaffected potassium content of the green leaves. In these tissues, at the end of water stress (T14), thirteen genotypes exhibited equal values to those of the WW plants. However, this number changed to nine and seven genotypes on the 7th and 14th days of the recovery period, respectively. In senescent leaves, a similar number (13 to 15) of unaffected genotypes was found throughout T14 to T14R (Figure 4.13). Genotypes that showed changes in potassium amount due to water stress did not always exhibit the same response, with some showing increases and others showing decreases. These results were observed for both green and senescent leaves and influenced by the observation period (T14, T7R, or T14R) (Figure 4.13).

At the end of water stress, PL-2, PL-3, PL-4, and IT-1 exhibited increments (22.6% to 39.7%) of potassium content in their green leaves, while in the senescent leaves, values raised 26.9% to 160.2% (PL-3, PL-4, IT-2, IT-4, AdvL-1, AdvL-3, and Austrl-1). Conversely, reductions were found, affecting the green leaves of PL-5, GR-4, AdvL-1, AdvL-5, Austrl-2, and Austrl-3 (14.8% to 58.0%) and the senescent leaves of PL-2 (57.5%) (Figure 4.13).

During the recovery period, PL-3, PL-4, GR-3, AdvL-2, and AdvL-5 green leaves of WL plants showed an increased potassium level at both T7R and T14R (13.6%-54.7%), while PL-1, PL-5, IT-4, and GR-1 only at T14R (26.6%-105.1%). Furthermore, waterlogging also resulted in decreased potassium amounts. At T7R, PL-5, Austrl-1, and Austrl-2 exhibited declines of 13.3% to 27.5%, while AdvL-4 showed a reduction of 94.4% at T14R. In GR-4, AdvL-1, and Austrl-3, declines were observed at both T7R and T14R. Senescent leaves showed increments (31.7% to 150.6%) during the recovery period at T7R (IT-2, IT-3, and GR-2), at T14R (IT-1, GR-1, Austrl-1, and Austrl-4), and at both T7R and T14R (PL-3, IT-4, and AdvL-3). Similar to that observed in green leaves, reductions were also found, but to a lesser extent. At T7R, PL-1 displayed a 49.3% decline, while AdvL-4 showed a 26.5% reduction at T14R. PL-2 displayed decreased potassium content at both T7R and T14R (85.9% and 73.1%, respectively) (Figure 4.13).

4.3.11 Changes in Iron, Manganese and Aluminium leaf content

Iron, manganese, and aluminium, can accumulate to harmful levels in waterlogged soils, potentially accelerating senescence when present in excess in plant tissues. Table 4.4 presents these elements levels in green and senescent leaves of well-watered (WW) and waterlogged (WL) plants at T14, T7R, and T14R.

Table 4.4 – Waterlogging-induced changes in iron, manganese and aluminium content in green and in senescent leaves. Determinations performed by X-ray fluorescence, at the end of the water stress (T14), and at the 7th (T7R) and 14th (T14R) days of recovery in *T. aestivum* L. genotypes, representing five distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme; Australian varieties (Austrl). Differences between WW and WL plants were conducted per genotype and highlighted by an *****. Differences between T14, T7R, and T14R, for each water regime are given by letters a, b, and c. (ANOVA, n = 3-6, $p<0.05$, with letter a and ***** for the highest values).

		Iron (Fe, μg g^{-1} DW)						**Manganese** (Mn, μg g^{-1} DW)						**Aluminium** (Al, mg g^{-1} DW)					
		Green leaves			Senescent leaves			Green leaves			Senescent leaves			Green leaves			Senescent leaves		
		T14	T7R	T14R	T14	T7R	T14R	T14	T7R	T14R	T14	T7R	T14R	T14	T7R	T14R	T14	T7R	T14R
PL-1	WW	289 *a*	268 *a*	246 *a*	857 *a*	944 *a*	866 *a*	145 *a*	121 *b*	127 *b*	129 *a*	124 *a*	123 *a*	9.3 *a*	7.5 *b*	7.1 *b*	16.7 *b*	19.2 *a*	19.3 *a*
	WL	348 *a*	241 *b*	211 *b*	1332 *a**	1409 *a**	1267 *a**	126 *a*	123 *a*	124 *a*	176 *a*	172 *a*	141 *b*	8.1 *a*	7.9 *a*	9.2 *a**	18.4 *c*	19.9 *a*	20.0 *b*
PL-2	WW	283 *a*	215 *a*	317 *a*	801 *a*	846 *a*	829 *a*	117 *a*	106 *a*	117 *a*	141 *a*	141 *a*	152 *a*	7.9 *a*	6.6 *a*	8.0 *a*	16.1 *c*	18.0 *b*	20.1 *a*
	WL	286 *a*	221 *a*	230 *a*	1026 *b**	1023 *b**	1119 *a**	144 *a*	91 *a*	109 *a*	155 *a*	145 *a*	148 *a*	7.6 *b*	6.6 *b*	10.0 *a**	18.8 *c**	20.4 *b**	22.7 *a**
PL-3	WW	281 *a*	271 *a*	298 *a*	875 *a*	832 *a*	817 *a*	194 *a**	133 *b*	104 *b*	157 *a*	147 *a*	145 *a*	8.6 *a*	6.2 *b*	7.5 *ab*	21.4 *a*	19.1 *a*	17.1 *c*
	WL	299 *a*	244 *a*	260 *a*	1084 *a**	1047 *a**	1018 *a**	121 *a*	140 *a*	87 *a*	173 *a*	198 *a**	188 *a**	7.3 *bc*	6.6 *c*	8.6 *ab*	19.6 *a*	19.7 *a*	20.7 *a**
PL-4	WW	292 *a*	263 *a*	271 *a*	774 *a*	755 *a*	814 *a*	117 *a*	115 *a*	107 *a*	171 *a*	128 *a*	150 *a*	9.0 *a*	6.3 *b*	8.2 *a*	18.2 *a*	18.5 *a*	15.7 *a*
	WL	279 *a*	250 *a*	237 *a*	984 *b**	1377 *a**	1484 *a**	103 *a*	113 *a*	123 *a*	155 *a*	160 *a*	162 *a*	7.6 *a*	6.9 *a*	7.4 *a*	20.9 *a**	20.8 *a**	18.0 *a**
PL-5	WW	278 *a*	258 *a*	235 *a*	-	960 *a*	977 *a*	114 *a*	100 *a*	121 *a*	-	126 *a*	111 *a*	7.9 *a*	6.1 *a*	7.4 *a*	-	18.1 *a*	17.1 *a*
	WL	288 *a*	248 *a*	242 *a*	1387 *a*	1575 *a**	1494 *a**	141 *a*	114 *a*	116 *a*	166	158 *a*	133 *a*	7.9 *a*	7.0 *a*	8.4 *a*	20.8 *a*	19.1 *a*	18.6 *a*
IT-1	WW	270 *a*	317 *a*	238 *a*	1104 *a*	959 *a*	942 *a*	137 *a*	113 *a*	108 *a*	227 *a*	170 *ab*	134 *b*	7.3 *a*	7.2 *a*	7.7 *a*	19.7 *a*	18.1 *b*	19.6 *a*
	WL	248	-	-	2090 *b**	1519 *c**	2447 *a**	128	-	-	262 *a**	195 *ab**	175 *b**	8.4	-	-	19.5 *a*	18.6 *b*	20.4 *a*
IT-2	WW	203 *b*	266 *a*	265 *a*	896 *a*	1455 *a*	1103 *a*	120 *a*	114 *a*	152 *a*	230 *b*	297 *a*	159 *c*	7.7 *a*	5.8 *a*	7.4 *a*	18.1 *a*	18.0 *a*	19.2 *a*
	WL	241 *a*	268 *a*	261 *a*	1008 *a*	1398 *a*	1103 *a*	96 *b*	139 *a*	144 *a*	207 *a*	212 *a*	146 *b*	8.2 *a*	6.9 *a*	7.2 *a*	17.1 *b*	17.9 *ab*	19.1 *a*
IT-3	WW	280 *a*	291 *a*	265 *a*	847 *a*	768 *a*	798 *a*	130 *a*	90 *a*	128 *a*	156 *a*	158 *a*	165 *a*	8.1 *a*	6.6 *a*	8.0 *a*	19.7 *a*	14.9 *b*	15.2 *b*
	WL	256 *a*	228 *a*	244 *a*	1940 *a**	1613 *b**	1971* *a*	127 *b*	89 *a*	103 *a*	273 *a*	291 *a*	165 *a*	7.2 *a*	6.7 *a*	7.4 *a*	18.9 *a*	17.8 *a**	18.8 *a**
IT-4	WW	234 *c*	309 *a*	254 *bc*	308 *b*	489 *a*	447 *a*	106 *a*	129 *a*	129 *a*	77 *b*	106 *a*	115 *a*	7.0 *a*	7.2 *a*	7.5 *a*	17.6 *a*	17.1 *a*	17.1 *a*
	WL	265 *a*	273 *a*	-	932 *c**	1087 *a**	993 *bc**	115 *a*	121 *a*	-	177 *a**	185 *a**	168 *a*	9.1 *a**	8.5 *a**	-	22.1 *a**	19.4 *b**	20.0 *b**

GR-1	WW	276 *a*	281 *a*	266 *a*	1043 *a*	783 *b*	654 *b*	121 *a*	119 *a*	125 *a*	176 *a*	171 *a*	127 *b*	7.0 *a*	6.5 *b*	7.1 *b*	24.9 *a*	16.1 *a*	16.1 *a*
	WL	415 *a**	233 *b*	248 *b*	1479 *a**	1646 *a**	1705 *a**	137 *a*	108 *a*	101 *a*	207 *b*	189 *b*	325 *a**	7.3 *a*	6.1 *b*	7.9 *c*	24.3 *b*	18.1 *ab*	16.3 *a*
GR-2	WW	287 *a*	211 *a*	293 *a*	651 *b*	678 *b*	1203 *a*	103 *a*	106 *a*	134 *a*	163 *a*	170 *a*	171 *b*	6.1 *b*	5.8 *b*	8.3 *a*	14.3 *c*	15.2 *b*	20.9 *a*
	WL	203 *a*	237 *a*	249 *a*	821 *b**	751 *b*	1424 *a**	148 *a*	118 *a*	140 *a*	153 *b*	160 *b*	277 *a**	6.8 *a*	6.7 *a*	7.4 *a*	20.5 *a**	15.9 *b*	21.4 *a*
GR-3	WW	274 *a*	264 *a*	271 *a*	585 *c*	710 *b*	871 *a*	147 *a*	141 *a*	147 *a**	155 *a*	151 *a*	169 *a*	6.4 *a*	5.9 *a*	6.8 *a*	18.7 *a*	20.0 *a*	19.5 *a*
	WL	233 *a*	235 *a*	219 *a*	825 *b**	954 *a**	1011 *a**	138 *a*	144 *a*	100 *b*	168 *a*	160 *a*	196 *a*	8.5 *a**	5.8 *b*	7.6 *ab*	19.1 *a*	19.6 *a*	20.1 *a*
GR-4	WW	279 *a*	265 *a*	242 *a*	457 *c*	564 *b*	771 *a*	128 *a**	95 *b*	108 *b*	113 *a*	102 *a*	118 *a*	7.1 *a*	5.7 *a*	6.7 *a*	19.6 *a*	17.6 *b*	18.2 *ab*
	WL	235 *a*	256 *a*	-	950 *c**	1028 *b**	1858 *a**	92 *a*	95 *a*	-	156 *a**	185 *b**	305 *a**	8.5 *a**	8.9 *b**	-	22.7 *a**	20.8 *a**	21.7 *a**
AdvL-1	WW	277 *a*	272 *a*	282 *a*	1239 *a*	1077 *b*	1059 *b*	127 *a**	127 *b*	118 *a*	130 *a*	151 *a*	141 *a*	7.8 *a*	6.5 *a*	7.9 *a*	17.7 *a*	16.2 *a*	17.0 *a*
	WL	224 *a*	222 *a*	235 *a*	1422 *b**	2020 *a**	1936 *a**	97 *c*	154 *b*	191 *a**	219 *a**	189 *a**	208 *a**	7.4 *a*	6.5 *b*	8.7 *a*	16.7 *a*	15.8 *a*	16.5 *a*
AdvL-2	WW	249 *a*	239 *a*	280 *a*	896 *a*	928 *a*	959 *a*	110 *a*	101 *a*	117 *a*	147 *a*	151 *a*	155 *a*	7.4 *a*	6.5 *a*	8.2 *a*	16.7 *a*	16.6 *a*	16.5 *a*
	WL	336 *a**	224 *b*	265 *b*	1195 *c**	1253 *a**	1111 *a**	135 *a*	97 *a*	126 *a*	149 *a*	176 *a*	183 *a*	7.6 *a*	6.8 *b*	7.2 *a*	20.5 *a**	19.7 *ab**	18.9 *b**
AdvL-3	WW	315 *a*	277 *ab*	249 *b*	895 *a*	846 *a*	829 *a*	146 *a*	124 *a*	127 *a*	143 *a*	161 *a*	136 *a*	8.4 *a*	6.8 *a*	7.6 *a*	18.3 *a*	15.6 *b*	15.7 *b*
	WL	258 *a*	256 *a*	302 *a*	1163 *b**	1069 *a**	1123 *b**	128 *a*	141 *a*	127 *a*	165 *a*	205 *a*	169 *a*	7.4 *a*	7.5 *b*	7.9 *a*	19.3 *a*	15.4 *c*	17.3 *b*
AdvL-4	WW	271 *a*	281 *a*	240 *a*	934 *a*	792 *b*	585 *c*	134 *a*	102 *a*	110 *a*	160 *a*	155 *a*	135 *a*	8.0 *a*	6.0 *b*	7.2 *ab*	21.8 *a*	18.0 *b*	17.5 *b*
	WL	236 *a*	248 *a*	207 *a*	2879 *a**	2404 *b**	1510 *c**	121 *a*	122 *a*	114 *a*	205 *a**	265 *a**	260 *a**	6.5 *b*	6.9 *b*	13.2 *a**	21.8 *b*	16.9 *c*	27.8 *a**
AdvL-5	WW	274 *a*	284 *a*	291 *a*	934 *a*	1011 *a*	931 *a*	125 *a*	116 *a*	103 *a*	111 *a*	163 *a*	148 *a*	7.3 *a*	7.0 *a*	7.9 *a*	18.9 *a*	16.9 *b*	16.5 *b*
	WL	245 *a*	268 *a*	259 *a*	1434 *b**	1739 *a**	1599 *ab**	124 *a*	142 *a*	123 *a*	205 *a**	242 *a**	193 *a*	7.7 *a*	7.8 *a*	8.5 *a*	20.9 *a**	19.0 *b**	20.2 *b**
Austrl-1	WW	265 *a*	296 *a*	288 *a**	635 *b*	707 *b*	806 *a*	129 *a*	115 *a*	116 *a*	144 *a*	175 *a*	177 *a*	8.2 *a*	7.3 *a*	7.4 *a*	16.6 *a*	16.0 *a*	16.5 *a*
	WL	275 *a*	222 *ab*	195 *b*	856 *b**	929 *ab**	1003 *a**	119 *a*	148 *a*	136 *a*	156 *a*	206 *a**	210 *a**	8.5 *a*	6.9 *a*	7.8 *a*	22.3 *a**	17.8 *b*	18.1 *b*
Austrl-2	WW	254 *b*	281 *b*	357 *a**	845 *a*	781 *a*	861 *a*	140 *a*	112 *a*	118 *a*	194 *a*	157 *a*	162 *a*	7.9 *a*	7.0 *a*	7.8 *a*	22.3 *a*	17.3 *b*	19.6 *b*
	WL	251 *a*	288 *a*	254 *a*	799 *b*	901 *b**	1079 *a**	151 *a*	148 *a*	149 *a*	152 *a*	214 *a*	197 *a*	7.4 *a*	6.4 *a*	8.5 *a*	23.0 *a*	16.5 *b*	18.6 *b*
Austrl-3	WW	263 *b*	252 *b*	313 *a**	675 *b*	635 *b*	1067 *a**	148 *a*	114 *a*	117 *a*	143 *a*	144 *a*	183 *a*	6.6 *a*	6.4 *a*	7.8 *a*	19.7 *a*	16.6 *b*	17.8 *b*
	WL	239 *a*	238 *a*	194 *a*	802 *b**	851 *b**	1081 *a*	175 *a*	135 *b*	124 *b*	189 *a*	177 *a*	191 *a*	8.3 *a**	6.1 *a*	8.8 *a*	18.5 *a*	17.2 *b*	19.4 *b*
Austrl-4	WW	249 *a*	236 *a*	231 *a*	712 *a*	857 *a*	793 *a*	139 *a*	122 *a*	114 *a*	134 *a*	156 *a*	133 *a*	7.0 *a*	7.2 *a*	7.6 *a*	18.2 *a*	18.3 *b*	16.8 *b*
	WL	308 *a**	220 *b*	243 *b*	937 *b**	1070 *a**	1072 *a**	169 *a*	127 *a*	129 *a*	160 *a**	145 *a*	199 *a*	6.9 *a*	6.5 *a*	7.8 *a*	18.3 *a*	20.2 *b*	17.6 *b*
Austrl-5	WW	280 *a*	275 *a*	302 *a*	990 *a*	952 *a*	946 *a*	122 *a*	107 *a*	125 *a*	206 *a*	174 *a*	168 *a*	7.7 *a*	7.8 *a*	8.3 *a*	17.7 *b*	21.7 *a*	17.0 *b*
	WL	270 *a*	261 *a*	244 *a*	1435 *a**	1117 *b*	1065 *b*	131 *a*	94 *b*	118 *b*	201 *a*	202 *a*	201 *a*	9.4 *a*	8.5 *a*	8.7 *a*	17.9 *b*	20.4 *a*	18.0 *b*

Overall, the green leaves exhibited some stability regarding iron, manganese, and aluminium content. At the end of the stress period (T14), green leaves of WL plants PL-3, GR-4, and AdvL-1 showed manganese reductions (23.8% to 37.3%). On the other hand, increases (23.5% to 50.2%) of iron in GR-1, AdvL-2, and Austrl-4, as well as of aluminium in IT-4, GR-3, and GR-4 (19.5%-33.0%), were found. On the 7th day of recovery, aluminium content remained higher in IT-4 and GR-4 green WL plants. At T14R, the leaves of these two genotypes were senescent. Additionally, reductions in iron were observed in Austrl-1, Austrl-2, and Austrl-3 (28.8% to 38.0%) and in the manganese content of GR-3 (31.6%). Conversely, AdvL-1 displayed a 61.1% increment of manganese at T14R.

Senescent leaves have a higher content of iron, manganese, and aluminium compared to non-senescent leaves. In WW plants, average values for senescent leaves were 841 μg g^{-1} for iron, 152.4 μg g^{-1} for manganese, and 18 mg g^{-1} for aluminium, while values for non-senescent leaves were 270 μg g^{-1}, 121.7 μg g^{-1}, and 7.3 mg g^{-1} respectively. Senescent leaves in WL plants achieved average values of 1305 μg g^{-1} for iron, 191.6 μg g^{-1} for manganese, and 19.4 mg g^{-1} for aluminium, while non-senescent leaves maintained average values comparable to those of WW plants (Table 4.4).

Waterlogging led to an increased accumulation of iron in senescent leaves across genotypes, from T14 to T14R. However, IT-2 remained unchanged, while Austrl-2 and AdvL-1 maintained their value at T14, showing increases of 25.3% and 82.8%, respectively, by T14R. Conversely, Austrl-5, which displayed an increased iron content at the end of water stress, showed values similar to WW plants at T14R (Table 4.4). The highest iron increments in senescent leaves were observed in PL-4, IT-1, IT-3, IT-4, GR-1, GR-4, AdvL-1, AdvL-4, and AdvL-5, with increases ranging from 27.1% to 202.6% at T14, from 58.4% to 203.5% at T7R, and from 71.8% to 160.7% by the end of the recovery period (T14R).

Regarding manganese content, changes were observed in the senescent leaves of waterlogged plants in eleven genotypes. While twelve genotypes maintained similar values of the WW plants at T14, T7R, and T14R, PL-3 and Austrl-1 showed unchanged values at T14. However, with the end of water stress, values increased by *ca*. 18% in the latter and *ca.* 32% in PL-3 during the recovery period. In addition, GR-1 and GR-2, also with unchanged values at T14, showed increased manganese amounts later at T14R (156.9% and 62.0%, respectively). In IT-1, IT-4, GR-4, AdvL-1, AdvL-4, and AdvL-5, values rose earlier, with genotypes displaying increments (14.5% to 158.1%) for T14, T7R, and T14R (Table 4.4).

The accumulation of aluminium in WL plants was observed at T14 for GR-2 and Austrl-1, but only during the recovery period for PL-3, IT-3, and AdvL-4. Aluminium levels rise between 10.3% and 25.9% in PL-2, PL-4, IT-4, GR-4, AdvL-2, and AdvL-5, with impacts at T14, T7R, and T14R (Table 4.4).

4.3.12 Integrating growth and senescence: a heat map analysis

The responses to 14-day waterlogging initiated at the tillering stage showed great variability, as evidenced by the results addressed in this chapter. Correlations between the analysed traits may contribute to a better understanding of the dynamics induced by waterlogging on wheat plant development and senescence (Figure 4.14).

Figure 4.14 reveals the presence of both positive and negative correlations, predominantly ranging from weak to moderate, reflecting the variability within and between germplasm groups. However, when considering the germplasm as a whole, several correlations (given by the correlation coefficient, *r*) can be identified between variables:

Number of adventitious roots

The number of AR was negatively (*r*, -0.6) linked with the lower manganese content in the senescent leaves.

Plant´s foliar biomass

The plant foliar biomass strongly correlates positively with the leaf green area in both WW (*r*, +0.8 to +1.0) and WL plants (*r*, +0.6 to +0.9). Furthermore, at the end of the recovery period, WL plants with higher biomass also presented a lower percentage of senescent leaves and a higher K-PT/Max.

Senescent foliar biomass

In WW plants, a higher contribution of the senescent leaves to the plant foliar biomass implies a decreased green area, with *r* ranging from -0.6 to -0.7. The same trend was observed in WL plants, where *r* displayed values ranging from -0.7 to -0.8. Moreover, during the recovery period, when senescent biomass increased, the SPAD values decreased, as did the nitrogen content of the green leaves (*r* values of -0.5 to -0.6).

Total green leaf area

In addition to the positive correlation with the plant foliar biomass and the negative correlation with the senescent biomass, plants with a higher green area at T14R tended to have a higher potassium content in their green leaves (*r*, +0.7).

SPAD values

During the recovery period, waterlogged plants showed a negative correlation between SPAD values and senescent biomass.

Nitrogen

In addition to the negative correlation between nitrogen and higher senescent biomass, WL plants exhibited a positive correlation (+0.6) with K-PT/Max during the recovery period.

Phosphorus

In the green leaves of WW plants, a positive correlation (*r*, +0.5) was observed between phosphorus and iron. However, the green leaves of WL plants exhibited an association between elevated levels of phosphorus and larger amounts of potassium (*r*, +0.5 to +0.7) and iron (*r*, +0.6), with the latter occurring only at the end of the recovery period.

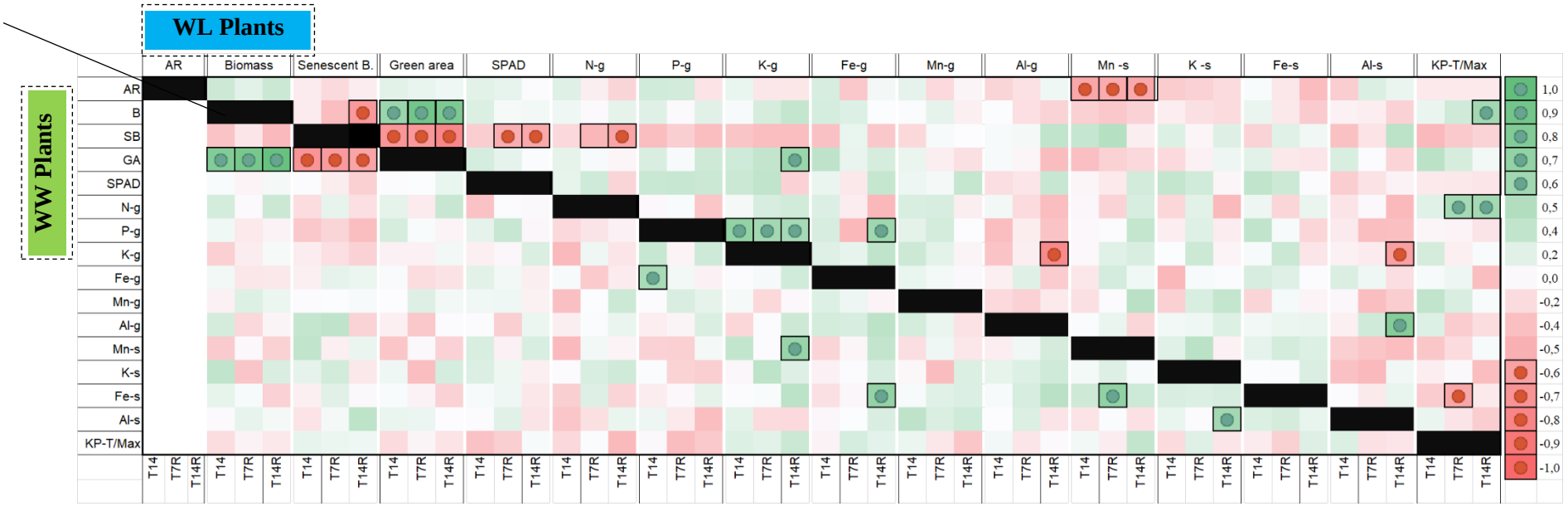


Figure 4.14 - Heat maps for well-watered (WW) and waterlogged (WL) plants, at the end of 14-day waterlogging (T14), and at the 7th (T7R), and the 14th (T14R) of recovery. Correlation matrix developed between the following variables: Number of adventitious roots (AR); plant foliar biomass (B); % of plants senescent foliar biomass (SB); foliar green area (GA); average of plant SPAD values (SPAD); Nitrogen content of the green leaves (N–g); Phosphorus content of the green leaves (P–g); Potassium content of the green leaves (K–g); Iron content of the green leaves (Fe–g); Manganese content of the green leaves (Mn–g); Aluminium content of the green leaves (Al–g); Potassium content of the senescent leaves (K–s); Iron content of the senescent leaves (Fe–s); Manganese content of the senescent leaves (Mn–s); aluminium content of the senescent leaves (Al–s).

Iron

When iron rose in green leaves, it also increased (*r*, +0.7) in senescent leaves in WW plants at T14R. As previously mentioned, in the green leaves of WL plants, higher concentrations of iron are related to more phosphorus.

Manganese

Plants with more adventitious roots had lower manganese values in their senescent leaves.

Aluminium

Higher levels of aluminium in the senescent leaves of WW plants correlate with a higher potassium content. At T14R, an increase in the aluminium content of the green leaves in WL plants coincided (*r*, +0.6) with a lower potassium concentration.

K-PT/Max

Higher biomass (*r*, +0.6) and higher [N] (*r*, +0.6) in WL plants resulted in a higher KP-T/Max ratio. Conversely, a reduced KP-T/Max was found with higher iron levels in the senescent leaves at T7R.

4.4 Discussion

Waterlogging induced several changes in both the soil and the plant. The results showed a strong soil potential redox (Eh) fall from *ca*. 400 mV to 100 mV after the 1st day of waterlogging. This decrease progressed until values close to 0 mV were reached by the end of the water stress period. This is in accordance with other studies (Greenway *et al*., 2006; Robertson *et al*., 2009), who refer to the drop of soil-atmosphere gas exchanges, together with the soil pores filled with water rather than gas, leading to a lower oxygen concentration in flooded soils and consequently reducing the Eh. The observed reduction in Eh to values close to 0 mV, denoting an oxygen concentration closest to 1%, causes roots to switch from aerobic to anaerobic metabolism, as reported in several studies (Husson *et al*., 2013; Søndergaard, 2009). Additionally, impairing root functional capacity leads to detrimental effects on shoots. With the root system being affected by waterlogging, the emission of adventitious roots may help overcome nutrient and water deficiency (Hockett *et al*., 1986; Steffens *et al*., 2016; Manghwar *et al*., 2024) mitigating the negative impacts on the plant above-ground portion (Malik *et al*., 2003).

In response to waterlogging, some genotypes were able to emit at least three adventitious roots per plant. Among these genotypes, a prevalence of PL and Austrl varieties was observed. In the PL group, this may be due to the genetic heterogeneity of regional varieties (Almeida *et al*., 2016), as they are part of a wheat germplasm collection (Vasconcelos, 1933) that has been grown for generations in Portugal. Furthermore, locally adapted cultivars are considered a valuable source of variability for breeding programmes (Leigh *et al*., 2022). Our results showed heterogeneity among varieties in the PL group, in both the number of AR and their emission precocity. Breeding programmes expect this heterogeneity in landrace varieties and consider it an asset (Leigh *et al*., 2022). Australian breeders advances in developing waterlogging-tolerant varieties may account for the higher number of adventitious roots in Austrl group.

Among germplasm, waterlogging induced a tiller number decrease in at least one observation from the beginning of the stress period to harvest. However, Austrl-2 remained unchanged during the stress and recovery phases and showed an increased fertile tiller number at harvest. Other genotypes showed an early and strong tiller growth arrest at T7 that lasted until harvest (IT-1, IT-3, GR-4, and AdvL-4). On the other hand, IT-2 and IT-4 showed a similar pattern, but only from T14 onward. With the end of waterlogging, 20 genotypes displayed decreased tillers number at recovery (T7R and/or T14R), suggesting a deleterious effect when oxygen availability is re-established. This might cause the formation of reactive oxygen species (ROS) due to O_2 re-entry in plant tissues, leading to cell membrane damages as reported by Amri *et al*. (2014). Still, the 18-100% reductions in the number of living tillers among the 23 studied genotypes reflected the great variability within germplasm, as well as the same pattern reported by several authors (Malik *et al*., 2001; Robertson *et al*., 2009; Herzog *et al*., 2016; Amri *et al*., 2014; Dickin and Wright, 2008; Wu *et al*., 2015), which found decreases up to 62%. The wide range of decreases in our experiment may be due to a higher number of genotypes comparing to the 1-6 wheat varieties the referenced authors studied. Results showed that several genotypes were either unaffected during waterlogging and the recovery period (Austrl-2) or, despite reductions, were able to generate new tillers, achieving at the end of the growth cycle comparable values to those of the well-watered plants (PL-1, PL-5, GR-1, GR-2, GR-3, AdvL-2, AdvL-3, and Austrl-1) or even higher (Austrl-5). According to Xie *et al*. (2016) [45], tillering ceases just prior to the elongation stage, and the remaining axillary buds become dormant. However, negative impacts in the main shoot can reverse this dormancy, and tillering can occur during the entire growth cycle (Xie *et al*., 2016; Rameau *et al*., 2015). This may explain the observed results and points to some genotypic variability regarding recovery ability following waterlogging. The decreased tiller number observed in the majority of genotypes in well-watered plants may result from nutrient resource remobilization from late to primary tillers (Fioreze *et al*., 2020). This can occur as tillers can serve as reservoirs of assimilates for the main culm during the growth cycle (Almeida *et al*., 2000). Thus, the number of emitted tillers does not always serve as a reliable indicator of fertile spikes being produced.

For well-watered plants, the K-PT/Max ratio (number of kernel-producing tillers *vs* highest number of emitted tillers) is consistent with the findings of several authors, who reported average mortality rates of 10-80% of all initiated tillers (Xie *et al*., 2016, Berry et al., 2003, Sharma *et al*., 1995). Waterlogging strongly impacted this ratio. Twelve genotypes showed a decreased value as a result of either the death of all emitted tillers or the decline (47.5% to 77.8%) of kernel-producing tillers at harvest, with possible impacts in yield. These results lined up with the findings of Collaku and Harrison (2002), who reported reductions of up to 66% of fertile tillers at maturity when waterlogging was imposed at the tillering stage.

Even in challenging environmental conditions, genotypes with a higher K-PT/Max ratio (PL-1, PL-5, GR-3, Austrl-2, and Austrl-5) or a stable ratio (GR-1, GR-2, AdvL-1, AdvL-2, Austrl-a, and Austrl-3) demonstrated a good capacity to produce fertile tillers. In fact, other studies found that wheat genotypes subjected to waterlogging during the tillering stage reduced the number of initiated tillers

during stress, although at maturity, they presented similar values to WW plants. This is reinforced by Herzog *et al*. (2016), who reported the formation of new tillers when waterlogging ended.

Although Renziehausen *et al*. (2024) suggested a growth retardation strategy upon waterlogging stress to save energy reserves, our results revealed an unchanged or even advanced growth stage at the end of water stress, as evaluated by Zadoks growth scale. Only five out of the 23 genotypes showed a delay in the development stage on the 14th day of recovery, while the remaining genotypes accompanied well-watered plants. Other strategies, such as morphological adaptations (*e.g.*, adventitious roots, aerenchyma formation) as well as physiological and biochemical changes, can contribute to plants tolerance to O_2 reduction at root level. Our results also showed sporadic changes in main culm height in waterlogged plants, with PL-2, PL-4, and IT-4 presenting 16.2–20.2% reductions at harvest and Austrl-4 displaying a 24.5% increase. Jiang *et al*. (2022) reported that waterlogging wheat plants for 14 days started at the third-leaf stage (ZGS 13) lowered plants height (1.1–19.5%) due to the reduced energy levels resulting from the shift to anaerobic metabolism. Other studies have reported that plants can elongate their culms as a strategy to escape submersion (Herzog *et al*., 2016; Loreti *et al*., 2020); however, this strategy has a high energy cost and was not advantageous to AdvL-4 since water was maintained at *ca*. 0.5 cm above the soil surface. The accumulation of ethylene can play a significant role in these findings. Several authors (Jackson, 2008; Loreti *et al*., 2020) have referred to the role of ethylene as a mediator that promotes culm elongation during waterlogging. However, our study did not address ethylene levels.

At the end of waterlogging, only five genotypes showed changes in leaf biomass, including decreases in GR-1, GR-2, and GR-4, as well as increases in PL-5 and AdvL-5. These changes were caused by increases in the main culm foliar biomass (PL-5), on tillers (AdvL-5), or non-significant reductions on both the main culm and tillers (GR-1, GR-2, and GR-4). Despite maintaining a stable green leaf area, the higher leaf biomass in PL-5 coincided with a significant increase in the senescent mass of both the main culm and the tillers. Thus, both the non-significant increase in tillering and the difference between the growth stages of WW (ZGS 35) and WL plants (ZGS 39) likely influenced the unchanged green area. In fact, in WL plants of PL-5, ZGS 39 indicated the emission of the flag leaf and, therefore, an extra leaf in the main culm comparing to WW plants. In AdvL-5, WW and WL plants presented equal ZGS (54-55), with WL plants exhibiting increased foliar biomass (68%) at the end of water stress. This was due to higher tillering observed earlier on the 7th day of waterlogging, resulting in a 189.5% increase in tiller foliar mass. However, at the end of stress, the senescent foliar biomass had increased in both the main culm (133.3%) and tillers (222.8%), and the green area decreased by 51.5% and 21.6%, respectively. Therefore, the strategy to compensate for the potential loss of primary tillers by initiating more secondary tillers doesn't suggest advantages for this genotype. Changes in the plant hormonal balance, namely the levels of ethylene and gibberellins, can alter the tillering process, so despite unfavourable conditions, the plant still emits tillers, but their productivity may not increase, as secondary tillers may be less productive or may result in an unbalanced crop stand. At the end of the recovery period, only PL-5, IT-4, and GR-3 had lower foliar biomass in WL plants. However, the majority of genotypes showed increased senescent foliar biomass and a decreased green area. In addition to

decreasing green area, SPAD values were also lower, intensifying the negative impact on photosynthetic metabolism. Although plant biomass accumulation has been looked at as an intuitive indicator that reflects stress tolerance (Li *et al*., 2018), several studies reported that waterlogging imposed at the tillering stage did not induce changes in wheat shoot biomass or green leaf area (de San Celedonio *et al*., 2017). Other authors, however, reported reductions in wheat shoot biomass as a result of lower tillering, decreased leaf elongation, or even growth arrest (Herzog *et al*., 2016; Feng *et al*., 2022; Tong *et al*., 2021), resulting in lower green area. Additionally, decreased chlorophyll content and, consequently, SPAD values were also reported (Ballesteros *et al*., 2014; Herzog *et al*., 2016).

At the end of the waterlogging period, the reductions in SPAD values ranged from 11.8% to 87.7% and affected twenty of the twenty-three studied genotypes. This revealed the degradation of photosynthetic pigments and premature leaf chlorosis. Furthermore, in waterlogged plants, the majority of genotypes also had lower amounts of the essential macronutrients nitrogen and phosphorus. This may have contributed to chlorosis, suggesting an impairment of the root system and/or decreases in nitrogen and phosphorus bioavailability in the soil. Iron and manganese are part of the essential micronutrients, and plants required smaller amounts than the macronutrients, however they are essential to plant to complete its live cycle. If the concentration is well above the beneficial range, they can be toxic. In fact, iron and manganese, together with aluminium, are common toxic elements in waterlogged soils. In plant tissues, the ideal iron concentration should be up to 0.2 mg g^{-1} leaf dry mass (Strawn *et al*., 2020), with values above 0.5 mg g^{-1} considered toxic (Lapaz *et al*., 2022). Our results displayed an average iron concentration of 0.27 mg g^{-1} for the green leaves in WW plants and sporadic increases in WL plants (GR-1 and AdvL-2), although with values < 0.5 mg g^{-1}. Regarding senescent leaves, iron content was well above the toxic reference in both WW and WL plants; nevertheless, in WL plants, values reach an average of *ca*. 1.3 mg g^{-1} and in WW plants, *ca*. 0.8 mg g^{-1}. Leaf senescence is the final developmental leaf stage, during which leaf cells are dismantled in a coordinated manner to remobilize nutrients for use in younger, growing tissues (Schippers *et al*., 2015). However, certain nutrients, like iron, are either strongly translocated to grains or remain in the leaves due to their lower phloem mobility compared to other nutrients like nitrogen, phosphorus, or potassium. Additionally, the breakdown of cellular structures during senescence might release bound iron, further contributing to its accumulation in the leaves (Schippers *et al*., 2015). Plants primarily take up iron by their roots, but due to the high demand for of this element to build up and operate the photosynthetic apparatus in the chloroplasts of photosynthetically active tissues, the largest proportion is translocated to the leaves. During senescence, iron is liberated from the photosynthetic apparatus, leading to an intracellular reallocation and a remobilization towards new sink tissues (Sági-Kazár *et al*., 2022). Our results, showed adequate iron levels in the green leaves, in both WW and WL plants, which may inhibit its remobilization to the growing parts of the plant and so protecting those tissues from precocious senescence (Schippers *et al*., 2015).

4.5 Conclusions

Results from this study support the existence of adequate genetic variability among germplasm and reinforce that wheat responses to waterlogging are complex and can vary based on several factors, including the genotype. As the root system plays a major role in waterlogging conditions, the good capacity of landraces to emit adventitious roots appears to be a solid option for selecting and crossing parental genotypes depicting beneficial root features, aiming for more adapted ideotypes.

The negative impacts during water stress include lower tiller emission and/or survival, increased senescent foliar mass, decreased green foliar area, and declining SPAD values. The observed changes occurred during the first days of the experiment, and continued waterlogging exacerbated them. Following the recovery phase, the reintroduction of oxygen worsened the adverse effects at the plant level, resulting in substantial decreases in the number of tillers in the majority of genotypes. The decrease in green biomass and its area, along with the rise in senescent biomass, affected both the main culm stem and tillers, with the most substantial impacts on the latter. Overall, stress also led to a decrease in the nitrogen content in green leaves, which occasionally coincided with losses in phosphorus and potassium.

Within the germplasm studied, several genotypes displayed the most significant damage, while others showed higher levels of tolerance. The genotypes IT-1, IT-4, GR-4, AdvL-4, and AdvL-5 exhibited negative results due to tiller death, with impacts on the number of fertile tillers at harvest. Furthermore, an important increase in senescent mass, a decline in green foliar area, reduced SPAD values, and lower nitrogen, phosphorus, and potassium content strongly impacted those genotypes. However, PL-1, GR-3, AdvL-3, and Austrl-5 exhibit potential traits for waterlogging tolerance due to their ability to recover from stress damage, achieving senescent mass values similar to those of unstressed plants. Moreover, in these genotypes, an increased kernel-producing tiller at harvest and an unchanged plant leaf biomass were observed.

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5 WATERLOGGING DURING TILLERING STAGE AFFECTS ECOPHYSIOLOGICAL TRAITS IN BREAD WHEAT GENOTYPES*

Abstract

Waterlogging strongly affects the root system, impacting shoot gas exchanges and key metabolic processes such as photosynthesis and respiration. Membrane integrity is crucial to ensure plant performance, growth, and maintenance of cellular homeostasis, enabling plants to cope with oxidative stress resulting from downregulation of the photosynthetic machinery. In this study, 23 bread wheat genotypes subjected to 14-day waterlogging at tillering were evaluated based on their physiological responses to oxygen deficiency. The work included measurements at different shoot levels, namely leaf water status, gas exchanges, Photosystem II (PSII) photochemical efficiency (F_v/F_m and F_v'/F_m'), lipid composition and lipoperoxidation, pigments, and sugar contents.

Stable photosynthetic rate (P_n) values were observed in AdvL-2, AdvL-3, Austrl-4, and Austrl-5, but most genotypes depict strong decreases. Stomatal conductance (g_s) also decreased considerably in the majority of the genotypes, although PL-4 and Austrl-5 denoted different patterns, with the first exhibiting unchanged g_s, while the latter showing an increment.

Under waterlogging, genotypes exhibited variability in physiological traits, with five genotypes consistently showing the greatest negative impacts on P_n, SPAD values, F_v/F_m and F_v'/F_m', (PL-4, IT-1, IT-4, GR-4, and AdvL-4). Conversely, AdvL-2, AdvL-3, Austrl-4, and Austrl-5 appeared to be more tolerant, as inferred from stable membrane permeability, as well as lipid content and lipoperoxidation, unchanged P_n and increased g_s, and unaffected F_v/F_m, and F_v'/F_m'. This variability is important for identifying genotypes with high tolerance under such conditions.

Key-words: Leaf gas exchanges; Lipoperoxidation; Membrane integrity; Photosynthetic metabolism; Sugar and pigment contents.

*This chapter include some sections of the review paper:
Pais IP, Moreira R, Semedo JN, Ramalho JC, Lidon FC, Coutinho J, Maçãs B, Scotti-Campos P (2023). Wheat Crop under Waterlogging: Potential Soil and Plant Effects. Plants, 12:149. https://doi.org/10.3390/plants12010149.

5.1 Introduction

Maintaining water balance, membrane integrity, and the efficiency of key metabolic processes is essential for efficient plant performance. These mechanisms are pivotal for sustaining growth, maintain cellular homeostasis, and enable plants to acclimate and survive under challenging environmental conditions.

Plants react to waterlogging in many complex ways, with the reduced soil oxygen availability primarily causing the adverse effects observed in several crops (Colmer and Voesenek, 2009; Zheng *et al*., 2009; Bhagat *et al*., 2014; Herzog *et al*., 2016). Adverse conditions in the root zone might impact some fundamental metabolic processes, leading to changes in the physiological performance of the shoot part. In waterlogging susceptible plants, physiological activities are drastically reduced and may cause cell death, whereas tolerant plants depict less severe effects or even an improvement in the response of some traits (Malik *et al*., 2001; Zaidi *et al*., 2004).

Waterlogging, along with other biotic and/or abiotic stresses, can induce changes at the membrane level, affecting permeability, cellular compartmentalisation, and function. Increased electrolyte leakage from cells can reflect these changes (Dias *et al*., 2011; Shabala, 2011; Scotti-Campos *et al*., 2014, 2015), leading to reduced plant survival under these constraints (Blokhina and Fagerstedt, 2010; Yadav *et al*., 2015). The ability to maintain membrane integrity under stress conditions not only ensures cellular compartmentalisation but also the proper functioning of metabolic processes (Duro *et al*., 2016; Scotti-Campos *et al*., 2016), contributing to greater protoplasmic tolerance (Scotti-Campos *et al*., 2011). Thus, membrane stability is frequently used as a valuable indicator of plant tolerance or susceptibility to environmental stresses (Campos *et al*., 2003; Dias *et al*., 2010; Lee and Zhu, 2010; Partelli *et al*., 2011; Demidchik *et al*., 2014; Scotti-Campos *et al*., 2014b; Herzog *et al*., 2016).

Lipids are fundamental plant macromolecules, playing key roles in membrane structure, energy storage, and metabolic signalling (Wang, 2004; Xiao and Chye, 2011; Xu *et al*., 2019; Xie *et al*., 2021). Additionally, membrane integrity is highly dependent on the qualitative and quantitative modifications that may occur in the lipid matrix composition in response to stresses (Dias *et al*., 2010; Scotti-Campos *et al*., 2011). Remodelling plasticity of membrane lipids strongly contributes to maintaining membrane functionality under limiting environmental conditions (Ramalho *et al*., 1998; Blokhina *et al*., 2003; Campos *et al*., 2003; Xu *et al*., 2019). Oxygen deficiency can trigger changes in membrane lipid composition (Xie *et al*., 2015), influencing the fluidity, integrity, and permeability of plant cell membranes. Tolerant plants could increase the degree of unsaturation of membrane lipids (Generosova and Vartapetian, 2005) and also enhance lipid biosynthesis under such conditions (Blokhina and Fagerstedt, 2010; Xie *et al*., 2021). Even so, high levels of polyunsaturated fatty acids (PUFA) in cell membranes enhance their susceptibility to lipoperoxidation (Ayala *et al*., 2014; Duhan *et al*., 2019), since carbon-carbon double bonds in lipids, particularly abundant in chloroplasts, are preferential targets for reactive oxygen species (ROS). Oxidative stress often occurs with both biotic and abiotic stresses as a result of the downregulation of the photosynthetic machinery contributing to excessive ROS generation within the leaf. When accumulated in mesophyll cells, their strong oxidizing activity can lead to lipoperoxidation

and degradation of membrane lipids (Campos *et al*., 2003; Duro *et al*., 2016; Bali and Sidhu, 2019), and cause oxidative damage to proteins and DNA, resulting in severe cells injuries (Baxter *et al*., 2014; Pan *et al*, 2021). Although ROS are a normal product of plant cell metabolism, a rise in their content indicates the occurrence of lipoperoxidation events (Nikolaeva *et al*., 2010) which are often linked to higher concentrations of malondialdehyde (MDA), a compound produced during lipid oxidation (Ayala *et al*., 2014). ROS accumulation also causes a significant disruption to plant ionic homeostasis, directly influencing the functioning of various cations (Shabala *et al*., 2014) and anion channels (Pottosin *et al*., 2018). Wheat plants can overcome oxidative stress through activation of antioxidative defence systems involving enzymatic and non-enzymatic mechanisms to neutralize excessive ROS and reduce the extent of oxidative damage (Bailey-Serres and Voesenek, 2008; Lal *et al*., 2019; Bali and Sidhu, 2019; Gill and Tutela, 2010; Li *et al*., 2012).

Photosynthetic pigments play an important role in plants, mainly in light capture and the production of reducing compounds (Anjum *et al*., 2011). Besides their light harvesting function, carotenoids are crucial for photoprotection. Through their antioxidant action, like quenching singlet oxygen and scavenging free radicals, they contribute to the neutralisation of the negative effects of ROS (Ashraf, 2012). As photosynthetic pigments, carotenoids extend the range of light wavelengths that plants can absorb, complementing the absorption spectra of chlorophylls (Hashimoto *et al*., 2016). They capture light energy and transfer it to chlorophyll molecules. Through the xanthophyll cycle, they also play a role in protecting photosystems I and II in the thylakoid membranes (Logan *et al*., 2007; Latowski *et al*., 2011; Barickman *et al*., 2019) by quenching excited chlorophyll molecules and dissipating excess energy as heat. Regarding chlorophylls, they are indispensable pigments for capturing the light used in photosynthesis, allowing the conversion of light radiation into chemical energy. Chlorophyll *a* is present at the reaction centres of both photosystems (PSI and PSII), whereas chlorophyll *b* is the most important accessory light-absorbing pigment in light-harvesting complexes. Thus, they play a crucial role in the plant photosynthetic efficiency, influencing their growth and ability to adapt to different environments (Anjum *et al*., 2011). In waterlogged plants, the rapid chlorosis of the leaves at the plant base is one of the first visible signs of stress. This yellowing, which precedes premature leaf senescence (Manik *et al*., 2019), is caused by the remobilization of nitrogen into the younger leaves (Herzog *et al*., 2016). In the remaining leaves, the decrease in chlorophyll content is an indicator of oxidative stress and may be a result of photooxidation (Anjum *et al*., 2011). Additionally, waterlogging may have an impact on the content of carotenoids (Avola *et al*., 2008), decreasing these photosynthetic pigments in susceptible wheat genotypes (Collaku and Harrison, 2002; Ploschuk *et al*., 2018; Alizadeh-Vaskasi *et al*., 2018).

In the light-harvesting antenna, light photons are captured by chlorophyll and partially (*ca*. 2%) re-emitted as fluorescence (Cessna *et al*., 2010). Chlorophyll fluorescence is a reliable and sensitive tool to assess light-harvesting efficiency in plants (Lázar, 1999, 2006), which complements information obtained through gas exchanges. Under stress conditions chlorophyll *a* fluorescence can decrease, allowing a quantitative comparison of the stress responses, and indirectly providing information on leaf photosynthetic performance. Maximal quantum efficiency of PSII (F_v/F_m) represents the maximal potential efficiency with which light absorbed by chlorophyll in PSII is used for photochemistry under dark-

adapted conditions, while quantum yield of PSII under light-adapted conditions (F_v'/F_m') indicates the efficiency of energy conversion in PSII when the plant is exposed to light. Reductions in these ratios can indicate downregulation, impairments and/or damage to the photosynthetic apparatus which may result in P_n decreases due to a decreased use-efficiency of captured photon energy (Shao *et al*., 2013).

The high sensitivity of photosynthetic processes to stress conditions often leads to significant changes in both respiration and photosynthetic rates (Fortunato *et al*., 2010; Partelli *et al*., 2011; Scotti-Campos *et al*., 2014a; Ramalho *et al*., 2018; Singh and Thakur, 2018; Singh *et al*., 2019). In response to waterlogging, plant responses include stomatal closure, reduced transpiration, and inhibited photosynthesis (Zheng *et al*., 2009; Bhagat *et al*., 2014; Soleh *et al*., 2018). Stomatal conductance to water (g_s) is a key factor influencing photosynthesis (Medrano *et al*., 2002) and can significantly impact photosynthetic rates under waterlogged conditions (Striker *et al*., 2005). The stomatal closure and the resulting decrease in g_s reduce water loss through down-regulation of leaf transpiration (Herzog *et al*., 2016). However, this also lowers internal CO_2 concentration (C_i), which in turn limits carbon fixation, reducing photosynthesis (Malik *et al*., 2001; Wu *et al*., 2014). However, photosynthetic rates can also decrease due to non-stomatal factors such as chlorophyll degradation and decreased chlorophyll synthesis (Herzog *et al*., 2016; Ploschuk *et al*., 2018; Amri *et al*., 2014), damage to PSII by ROS, decreased photosynthetic enzyme activities, low nitrogen content, and accumulation of sugars in leaves (Herzog *et al*., 2016; Ploschuk *et al*., 2018; Amri *et al*., 2014; Langan *et al*., 2022).

Since plants convert CO_2 and water into sugars through photosynthesis (P_n), and waterlogging can cause changes in P_n and respiration, this water stress condition has consequences on sugar metabolism and therefore on the available energy to plant metabolism (Singh and Thakur, 2018; Fukao *et al*., 2019). The accumulation of sugars in the wheat leaf tissues of plants subjected to waterlogging (Huang and Jonhson, 1995; Malik *et al*., 2002; Ahmed *et al*., 2013; Herzog *et al*., 2016) may result from root hypoxia. This constraint may lead to rapid growth inhibition of both the root and the above-ground part of the plant at the onset of waterlogging. In this situation, although a reduction in P_n also occurs, sugar production in the leaves exceeds its consumption (Setter *et al*., 1987; Malik *et al*., 2001, 2002; Herzog *et al*., 2016). Simultaneously, constraints in the root system reduce the roots phloem transport capacity (Malik *et al*., 2002; Herzog *et al*., 2016), contributing to the accumulation of photoassimilates in the leaves. The resulting sugars accumulation, together with a reduced ability for phloem transport in hypoxic roots, leads to further decrease in P_n as a negative feedback of carbohydrate accumulation (Herzog *et al*., 2016).

5.2 Objective of the study

This study aimed to assess the impact of 14 days of waterlogging on physiological traits in 23 genotypes of bread wheat (*Triticum aestivum* L.) germplasm with a unique genetic background under environmentally controlled conditions. The investigation of physiological plant responses to oxygen deficiency caused by waterlogging encompassed assessing membrane integrity through an electrolyte leakage test, the determination of membrane fatty acids and their degree of unsaturation, as well as the

impacts on leaf gas exchanges, sugar metabolism, chlorophyll fluorescence parameters, and photosynthetic pigments. By understanding the variability among germplasm and identifying traits that contribute to tolerance or susceptibility to this stress, it may contribute to a more accurate screening of germplasm and the development of better-adapted wheat plants to changing climatic conditions.

5.3 Results

5.3.1 Changes in relative water content (RWC)

Six genotypes showed RWC reductions (5.0 to 10.4%) at the end of the waterlogging period. PL-4, IT-1, GR-4, and AdvL-4 showed the most significant decreases (9.5, 9.7, 10.4, and 8.4%, respectively). IT-2 and AdvL-2 followed with a decline of 5.0% and 6%, respectively. On the other hand, IT-4 and Austrl-1 exhibited an opposite response, with an increase of approximately 5% in their RWC in WL plants (Figure 5.1).

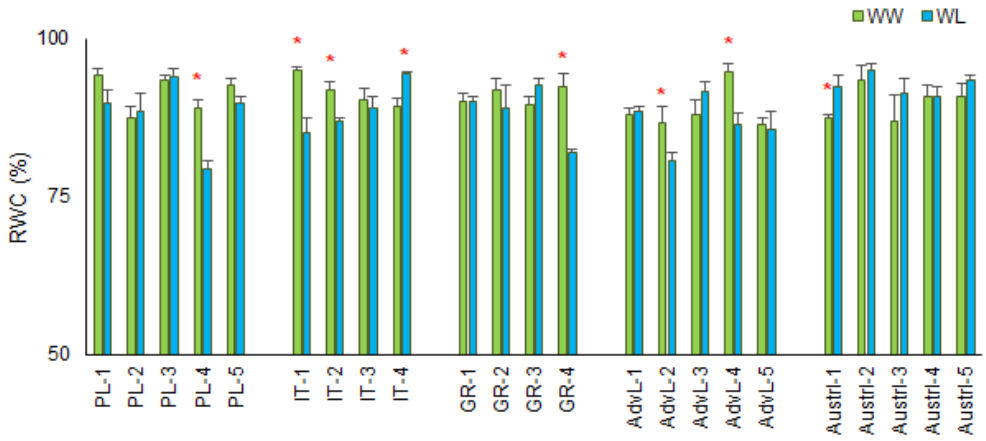


Figure 5.1 - Waterlogging-induced changes in relative water content (%). Evaluations performed on the 14th day of waterlogging in leaves of well-watered (WW), and waterlogged (WL) plants, of *T. aestivum* L. genotypes, from five distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme (INIAV, I.P.); Australian varieties (Austrl). Comparisons were conducted per genotype, highlighting the differences between WW and WL plants with an *. (One-way ANOVA, n = 3, $p<0.05$).

5.3.2 Waterlogging induced changes in membrane stability

5.3.2.1 Membrane permeability (I%)

The injury index in well-watered plants, as determined by the electrolyte leakage test, ranged between 3.3 and 11.8%. Waterlogging resulted in an increase in several genotypes across all germplasm groups, with the exception of the Australian, which remained unaffected (Figure 5.2). Conversely, the I% values of all genotypes in the IT group in WL plants were increased by 1.4- to 2.2-fold when compared to in WW plants. The genotypes PL-4, GR-4, AdvL-1, and AdvL-5 achieved the maximum I% values in waterlogged plants, corresponding to a 2.5- to 4.0-fold increase. Significant increases were also observed in PL-1 (1.9-fold), GR-1 (2.7-fold), and AdvL-4 (3.2-fold) (Figure 5.2).

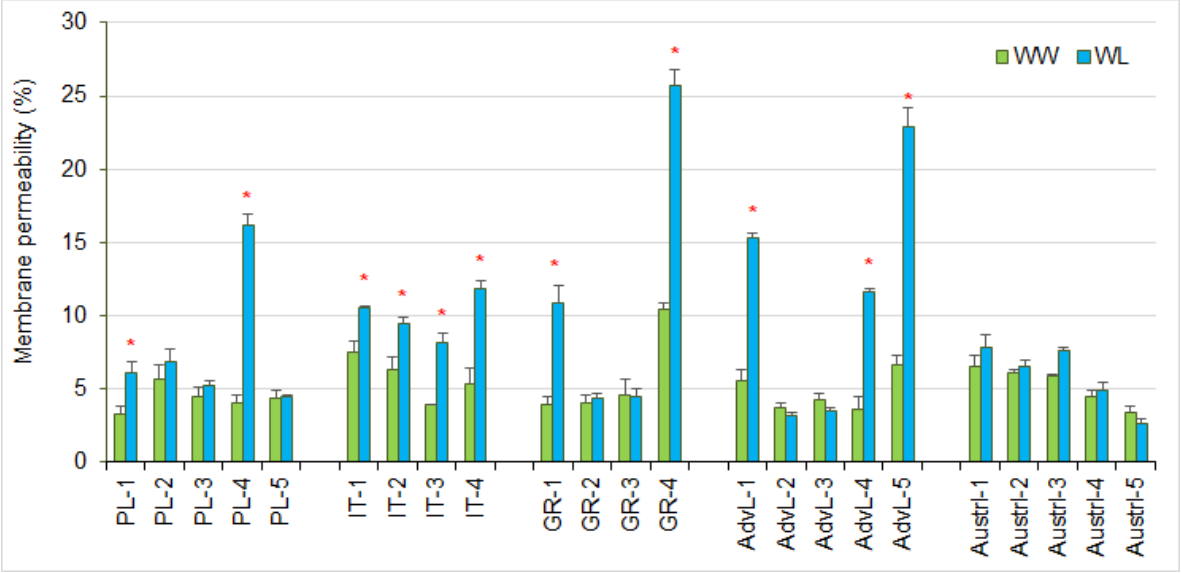


Figure 5.2 - Waterlogging-induced changes in the membrane permeability (%) achieved by the electrolyte leakage test. Evaluations performed on the 14th day of waterlogging in leaves of well-watered (WW), and waterlogged (WL) plants, of *T. aestivum* L. genotypes, from five distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme; Australian varieties (Austrl). Comparisons were conducted per genotype, highlighting the differences between WW and WL plants with an *. (One-way ANOVA, n = 3, $p<0.05$).

5.3.2.2 Membrane lipids

In well-watered plants (WW), the total fatty acid (TFA) content of the leaves varied between 3.5 and 7.3 mg g^{-1} DW (Figure 5.4). Waterlogging caused TFA reductions ranging from 13.7% to 57.2% in twelve genotypes. The strongest decreases were observed in IT-4 (57.2%), AdvL-1 (54.9%), AdvL-5 (54.2%), GR-3 (46.6%), IT-3 (42.5%), Austrl-1 (38.1%), and GR-4 (33.9%). Reductions in the unsaturation index (DBI) accompanied these decreases (except for GR-3), with IT-4, AdvL-4, and Austrl-1 showing the greatest changes, reducing the degree of unsaturation of membrane lipids by 33.7% to 44.1%. The genotypes AdvL-3, Austrl-3, Austrl-4, and Austrl-5 showed unchanged values of TFA with waterlogging; however, there was a change in DBI, being the only genotypes that exhibited an increased DBI.

For the genotypes PL-1, PL-3, PL-5, IT-2, GR-2, and AdvL-2, stable values were found in TFA content as well as in DBI (Figure 5.2). However, this stability may not correspond to constant relative proportions of fatty acids, as indicated by FA (mol%) (Figure 5.3).

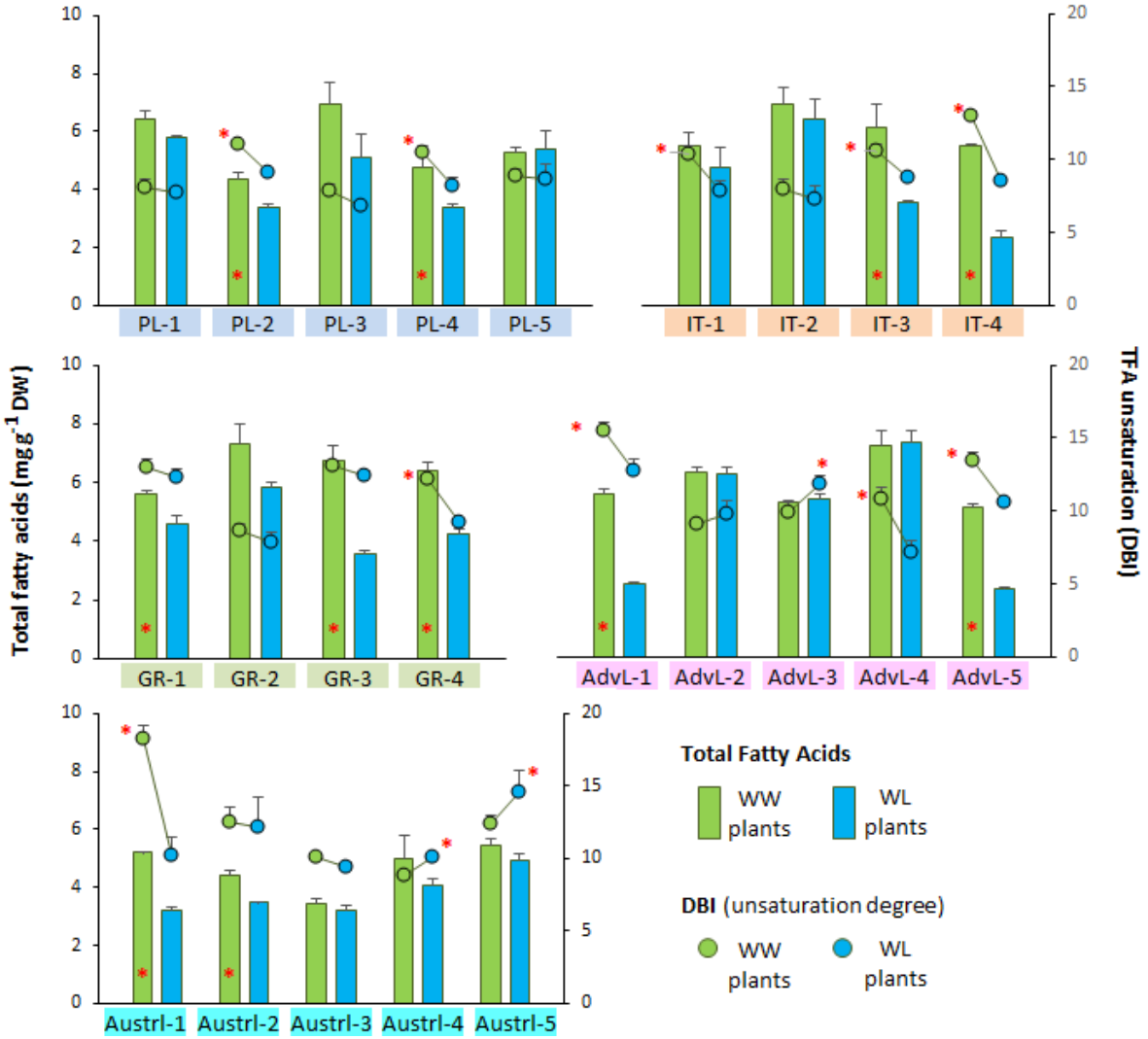


Figure 5.3 - Waterlogging-induced changes in leaf total fatty acids (TFA, mg g^{-1} DW) and their unsaturation degree (given by the double bond index, DBI). Evaluations performed on the 14th day of waterlogging in leaves of well-watered (WW), and waterlogged (WL) plants, of *T. aestivum* L. genotypes, from five distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme (INIAV, I.P.); Australian varieties (Austrl). Comparisons were conducted individually for TFA and DBI, and per genotype, highlighting the differences between WW and WL plants with an * (One-way ANOVA, n = 3, $p<0.05$).

Regarding the fatty acid profile, linolenic acid (C18:3) was the most abundant, accounting for 60-80% of the total fatty acids. Following it was palmitic acid (C16:0), representing 12-21%, linoleic acid (C18:2, 5-11%), stearic acid (C18:0, 1-9%), and oleic acid (C18:1, 1-6%). Palmitoleic acid (C16:1) was the least abundant, with 1-2% of the TFA. Waterlogging caused some changes in the relative percentage of some fatty acids, but it did not alter their abundance order (Figure 5.4).

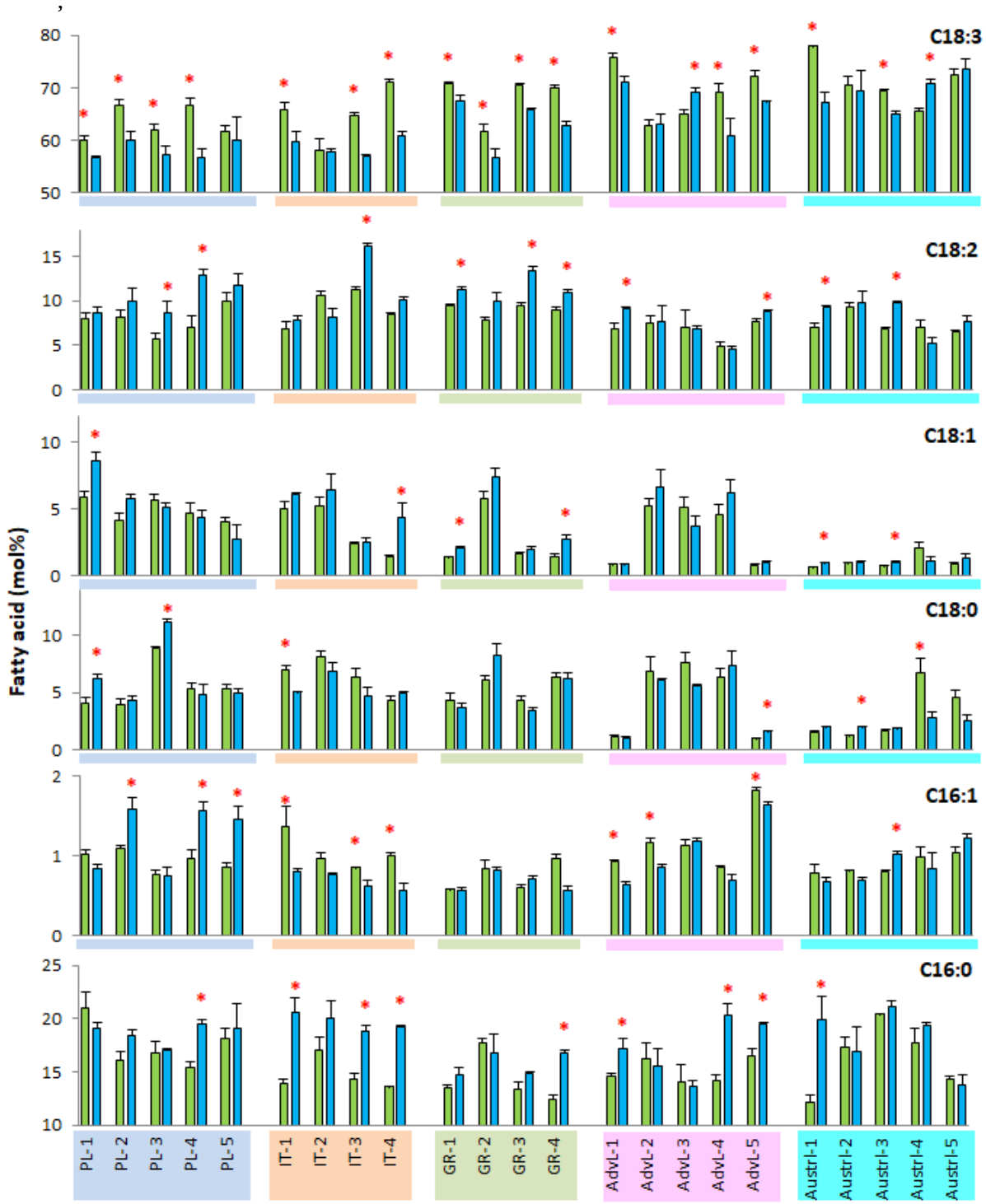


Figure 5.4 - Waterlogging-induced changes in the relative amount (mol%) of the main fatty acids (C16:0, Palmitic acid; C16:1, Palmitoleic acid; C18:0, Stearic acid; C18:1, Oleic acid; C18:2, Linoleic acid, and C18:3, Linolenic acid). Evaluations performed on the 14th day of waterlogging in leaves of well-watered (WW), and waterlogged (WL) plants, of *T. aestivum* L. genotypes, from five distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme (INIAV, I.P.); Australian varieties (Austrl). Comparisons were conducted individually for each FA and per genotype, highlighting the differences between WW and WL plants with an *. (One-way ANOVA, n = 3, $p<0.05$).

Waterlogging increased C16:0 (mol%) in PL-4, IT-1, IT-3, IT-4, GR-4, Austrl-1, AdvL-1, AdvL-4, and AdvL-5. The latter also experienced a strong increase in C18:0. Water stress also enhanced C18:0

in PL-1, PL-3, and Austrl-2, whereas a decrease was observed in IT-1 and Austrl-3. WL plants showed an overall decrease in C18:3, the most abundant FA, ranging from 4.3% to 14.8%; however, PL-5, IT-2, AdvL-2, Austrl-2, and Austrl-5 remained unaffected. Regarding C18:2 and C18:1, waterlogging increased its relative abundance in several genotypes, with the remaining exhibiting stable mol%. The less representative FA, C16:1, showed different responses with waterlogging. In IT-1, IT-3, IT-4, GR-4, AdvL-1, and AdvL-5 decreases were found while PL-2, PL-4, PL-5, and Austrl-3 raise their values. The remaining genotypes were able to keep C16:1 mol% unchanged (Figure 5.4).

The results presented a high variability in responses, with six genotypes maintaining both the TFA and DBI values. However, only one genotype (IT-2) showed no changes in FA mol%. On the other hand, in PL-5 and AdvL-2, changes in mol% of C16:1 did not affect the TFA or the DBI, due to its low abundance. The rise in either C18:1 (PL-1) or C18:2 (PL-3 and GR-2) counterbalanced the reduction in C18:3 in the three remaining genotypes (Figure 5.4).

Regarding the four genotypes that showed an increase in the DBI, the responses were also diverse. The rise in C18:3 was responsible for the DBI increase in AdvL-3, whereas in Austrl-4, an increase in this FA coincided with a decline in C16:0. Austrl-3 showed a decrease in C18:3 that was compensated by the increase in C16:1, C18:1, and C18:3, leading to an increase in unsaturation. Despite the absence of significant differences in any of the FA, Austrl-5 showed a decreased DBI. This was the reflex of the downward trend of C18:0 and the upward trends of C16:1, C18:1, and C18:2, resulting in an increased unsaturation (Figure 5.4).

The observed decline in DBI across ten genotypes was always linked with a drop in C18:3. This C18:3 decrease was accompanied by rises of C16:0 (the two more abundant FA) in eight out of the ten genotypes. In addition, several genotypes exhibited increased mol% of C18:2, C18:1, and C18:0 (Figure 5.4).

5.3.2.3 Lipoperoxidation

An increase in the concentration of malondialdehyde (MDA), a by-product of lipoperoxidation, was observed in waterlogged plants of ten different genotypes. The rises ranged from 42.0% to 117.9%, with the most significant impacts found in IT-4, followed by AdvL-1 (94.5%) and PL-2 (93.9%). GR-4, IT-1, AdvL-4, and PL-3 also showed significant increases (73% to 79%). Similarly, IT-3, AdvL-5, and Austrl-1 suffered an increment between 42% and 57.7% (Figure 5.5).

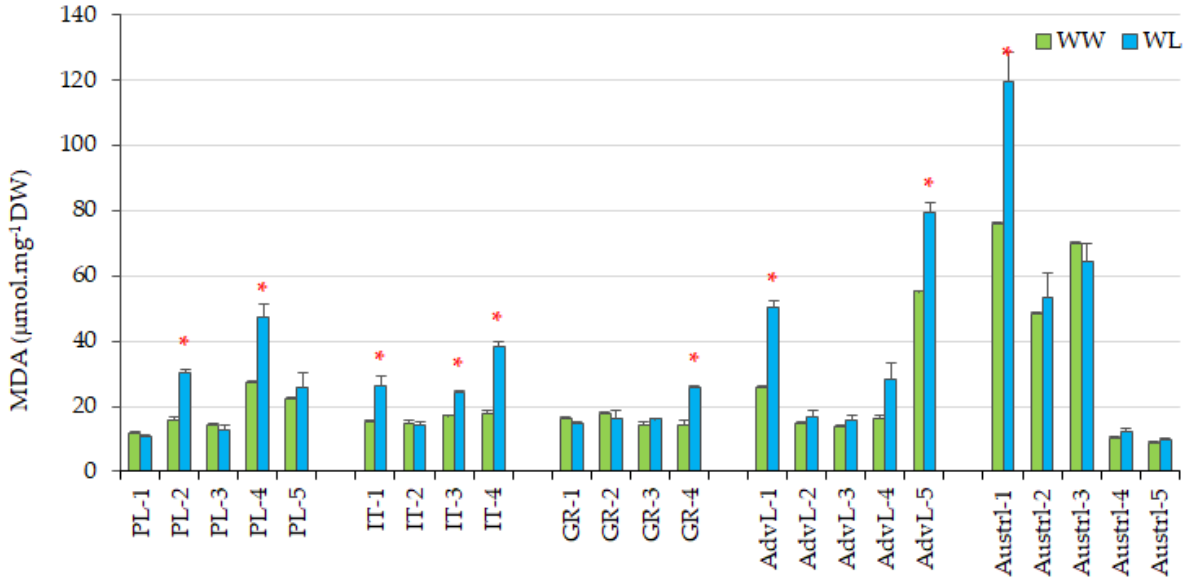


Figure 5.5 - Waterlogging-induced changes in lipoperoxidation level achieved by the content of Malondialdehyde (µmol mg^{-1} DW). Evaluations performed on the 14th day of waterlogging in leaves of well-watered (WW), and waterlogged (WL) plants, of *T. aestivum* L. genotypes, from five distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme (INIAV, I.P.); Australian varieties (Austrl). Comparisons were conducted per genotype, highlighting the differences between WW and WL plants with an *. (One-way ANOVA, n = 3, $p<0.05$).

5.3.3 Waterlogging impacts on foliar gas exchanges

5.3.3.1 Photosynthetic rate

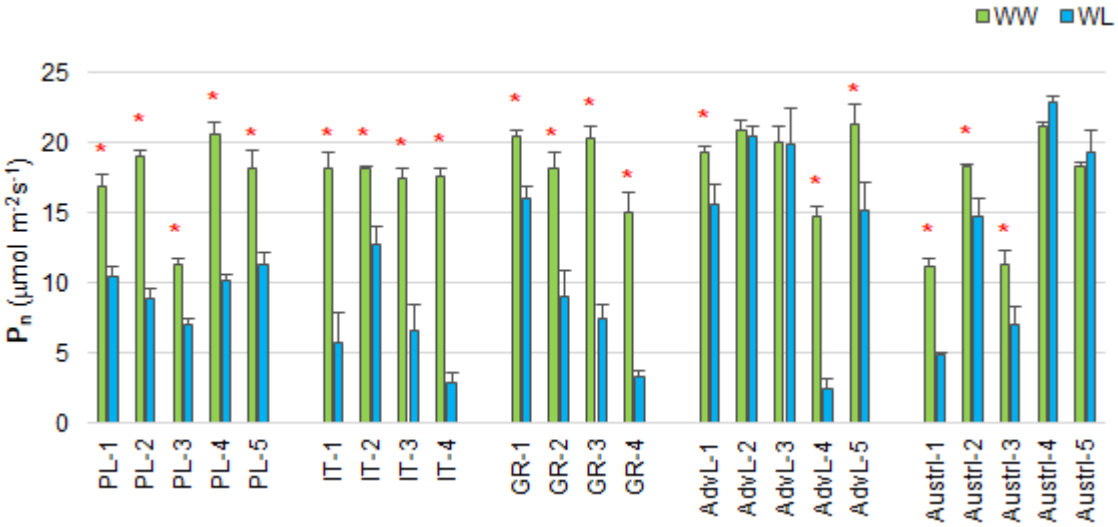


Figure 5.6 - Waterlogging-induced changes in the photosynthetic rate (P_n, µmol m^{-2} s^{-1}). Evaluations performed on the 14th day of waterlogging in leaves of well-watered (WW), and waterlogged (WL) plants, of *T. aestivum* L. genotypes, from five distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme; Australian varieties (Austrl). Comparisons conducted per genotype; * indicate differences between WW and WL plants. (One-way ANOVA, n = 3, $p<0.05$).

The majority of the genotypes tested showed a significant decrease in P_n at the end of the 14-day waterlogging period. The only genotypes not affected were AdvL-2, AdvL-3, Austrl-4, and Austrl-5,

while IT-4, GR-4, and AdvL-4 exhibited the most substantial reductions (83.5, 78.1, and 82.9%, respectively). IT-1, IT-3, and GR-3 also showed significant decreases in photosynthetic activity ranging from 61.9% to 68.3%, and PL-2, PL-4, GR-2, and Austrl-1 from 50% to 56.7%.

5.3.3.2 Stomatal conductance, intercellular CO_2 concentration, and transpiration rate.

Regarding gas exchanges, PL-4 and Austrl-5 stood out, with the first exhibiting unchanged stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (Tr) at the end of waterlogging, while the latter rises in all traits (Figure 5.7).

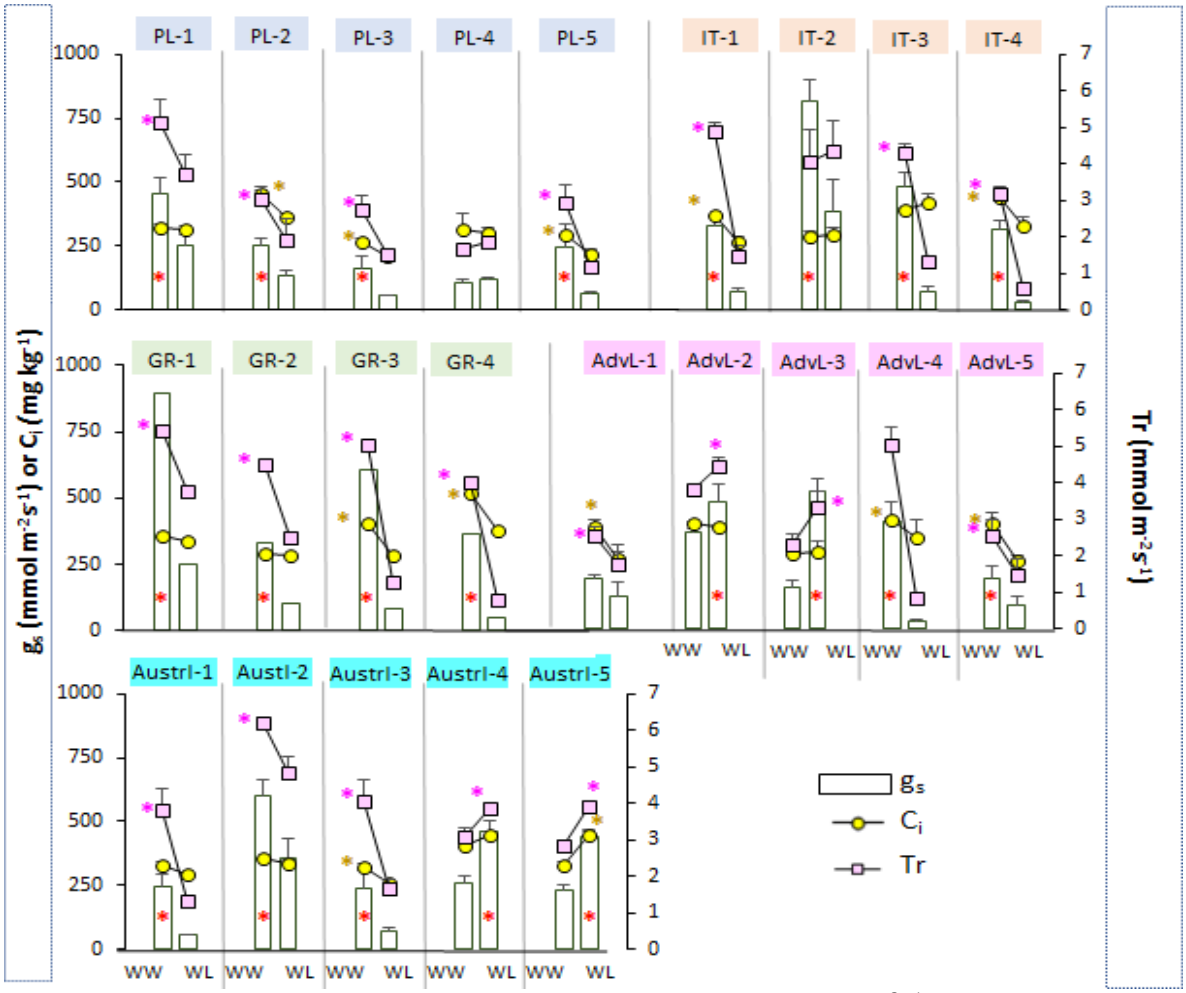


Figure 5.7 - Waterlogging-induced changes in the foliar stomatal conductance (g_s, mmol $m^{-2}s^{-1}$), intercellular CO_2 concentration (C_i, mg kg^{-1}), and transpiration (mmol m^{-2} s^{-1}). Evaluations performed on the 14th day of waterlogging in well-watered (WW), and waterlogged (WL) plants, of *T. aestivum* L. genotypes, from five distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme; Australian varieties (Austrl). Comparisons were conducted individually for g_s, C_i, and Tr as well as per genotype, highlighting the differences between WW and WL plants with an *. (One-way ANOVA, n = 3, $p<0.05$).

Among the remaining germplasm, g_s values decreased from 31.1% to 92.2%, impacting the majority of the genotypes. PL-5, IT-1, IT-3, IT-4, GR-1, GR-3, GR-4, AdvL-4, Austrl-1, and Austrl-3 showed the strongest declines, ranging from 71.2% to 92.2%. Conversely, AdvL-3 presented an

accentuated increase (230%), followed by Austrl-5 (89.1%), Austrl-4 (77.2%), and AdvL-2 (31.1%). Rises were concomitant with higher transpiration rates in these four genotypes (43.8%, 39.2%, 26.3%, and 16.2%, respectively). Furthermore, IT-2 demonstrated a stable value for Tr, whereas the remaining genotypes showed decreases ranging from 22.1% to 83.3%. In addition to the rise found in Austrl-5, the intercellular CO_2 concentration (C_i) dropped (15.2% to 36.0%) in several genotypes, while it stayed unchanged in others.

In sum, PL-2, PL-3, PL-5, IT-1, IT-4, GR-3, GR-4, AdvL-1, AdvL-4, AdvL-5, and Austrl-3 all demonstrated simultaneous reductions in g_s, Ci, and Tr. On the other hand, PL-1, IT-3, GR-1, GR-2, Austrl-1, and Austrl-2 maintained the C_i values and reduced both g_s and Tr. Despite the observed decrease in g_s, the IT-2 genotype maintained both C_i and Tr values. With a different behaviour, AdvL-2, AdvL-3, and Austrl-4 increased the g_s and consequently the Tr while maintaining the values of Ci. Furthermore, PL-4 showed reductions in g_s, Ci, and Tr, while Austrl-5 increases.

5.3.3.3 Leaf dark respiration

As with to P_n, g_s, C_i, and Tr, foliar respiration (R_d) showed variability among germplasm, with reductions in PL-2, GR-1, GR-3, GR-4, and AdvL-4 and increases in AdvL-1, AdvL-5, Austrl-2, Austrl-3, and Austrl-5. In the remaining genotypes, R_d was unaffected by waterlogging (Figure 5.6). Reductions ranged from 43.4% (PL-2) to 76.1% (GR-3) and increases between 41.4% (Austrl-3) and 121.6 (AdvL-1). In the IT group, all genotypes showed an unchanged respiration rate, comparing WW and WL plants.

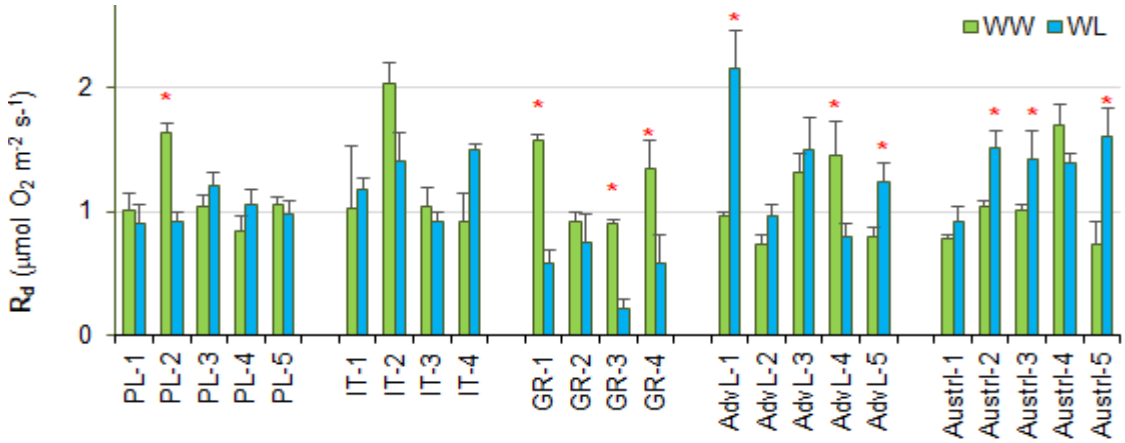


Figure 5.8 - Waterlogging-induced changes in foliar dark respiration (Rd, µmol O_2 $m^{-2}s^{-1}$) were evaluated on the 14th day of waterlogging in well-watered (WW) and waterlogged (WL) plants of *T. aestivum* L. genotypes from distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme; Australian varieties (Austrl). Comparisons were conducted per genotype, highlighting the differences between WW and WL plants with an *. (One-way ANOVA, n = 3, $p<0.05$).

5.3.4 Leaf total chlorophyll and carotenoids

All genotypes in waterlogged plants showed decreased chlorophyll content, with the exception of AdvL-2, AdvL-3, Austrl-3, and Austrl-5, which presented similar values to those in WW plants. GR-4 showed the strongest decline (82.4%), followed by GR-3, IT-3, IT-4, Austrl-1, PL-3, Austrl-2, IT-1, and

PL-4, with declines from 42.1% to 58.8%. In the remaining genotypes, waterlogging lowered the chlorophyll amount by 24.7% to 39.8% (Figure 5.7).

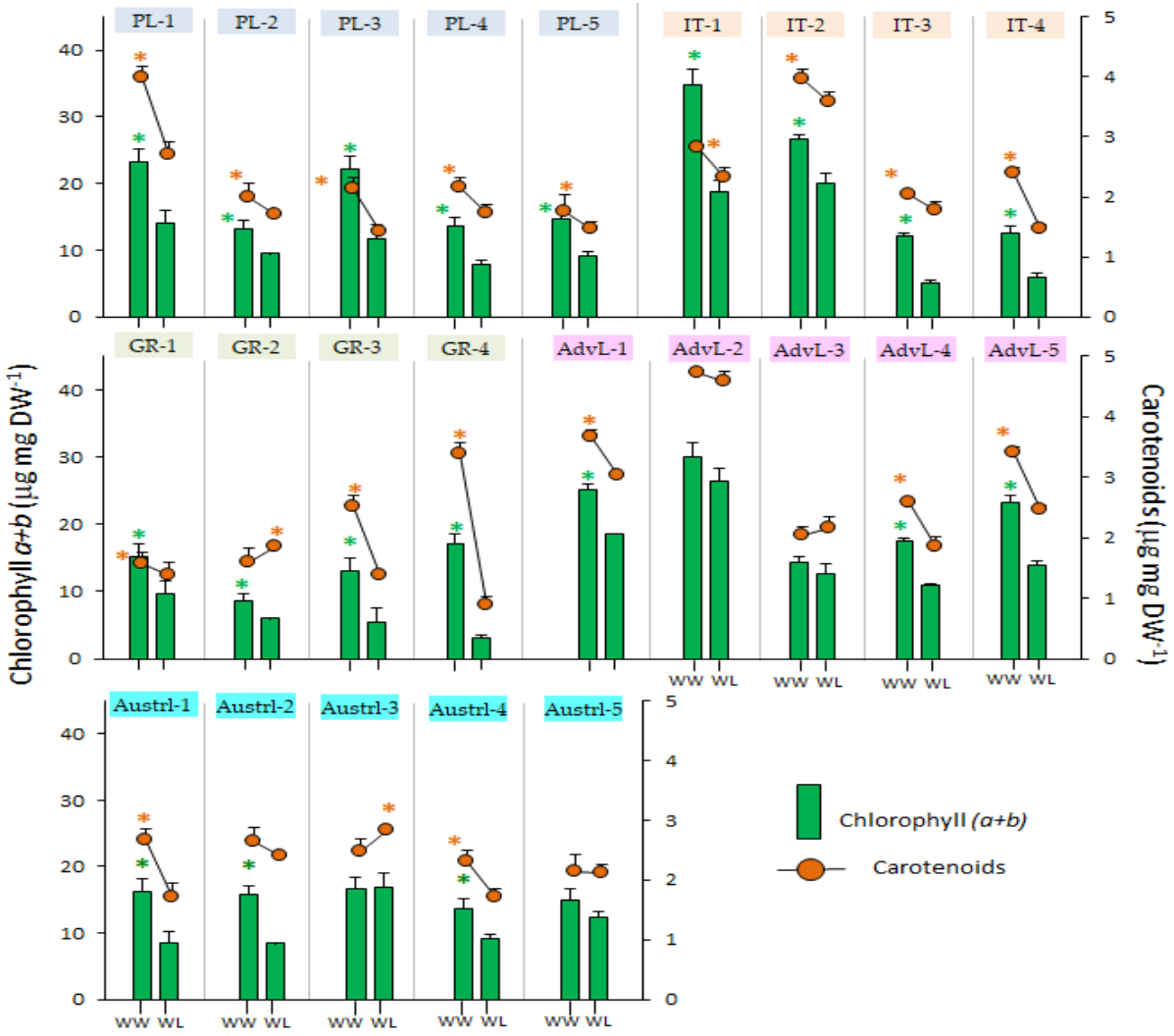


Figure 5.9 - Waterlogging-induced changes in the total chlorophyll and carotenoids content (µg mg DW^{-1}). Evaluations performed on the 14th day of waterlogging in well-watered (WW), and waterlogged (WL) plants, of *T. aestivum* L. genotypes, from five distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme (INIAV, I.P.); Australian varieties (Austrl). Comparisons were conducted per genotype, highlighting the differences between WW and WL plants with an *. (One-way ANOVA, n = 3, $p<0.05$).

Regarding carotenoids, four genotypes (AdvL-2, AdvL-3, Austrl-2, and Austrl-5) were able to maintain equal values between WW and WL plants. Conversely, WL plants in GR-2 and Austrl-3 showed an increment of 17.3% and 13.6%, respectively. GR-4 had the lowest value, showing a reduction of 73.9% compared to its WW plants. The majority of genotypes displayed a simultaneous decrease in chlorophyll and carotenoids content. Nevertheless, Austrl-2 and GR-2 presented lower chlorophyll values, but an unchanged carotenoids content in the first and an increased content in the latter. Furthermore, AdvL-2, AdvL-3, Austrl-3, and Austrl-5, which maintained Chl values, also showed unaffected carotenoid content or even a higher value (Austrl-3) (Figure 5.7)

5.3.5 Photochemical efficiency - Chlorophyll *a* fluorescence

Well-watered plants achieved an average maximal photochemical efficiency of PSII (F_v/F_m) of 0.82 and 0.68 for actual PSII photochemical efficiency of energy conversion under light-adapted conditions (F_v'/F_m'), indicating good conditions for P_n and the absence of stress factors (Figure 5.10).

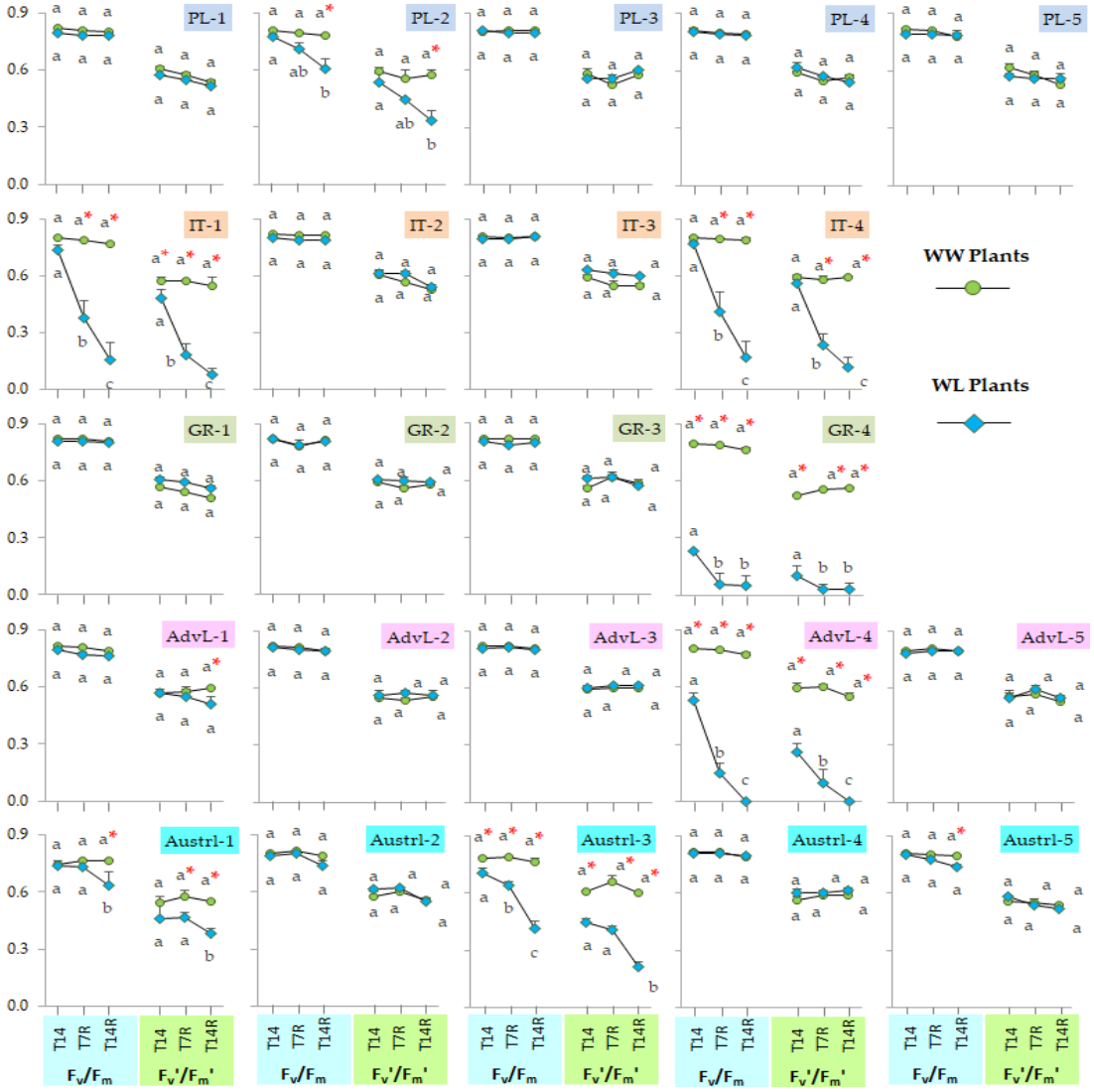


Figure 5.10 – Maximal photochemical efficiency of PSII (F_v/F_m) and the actual PSII photochemical efficiency of energy conversion under light-adapted conditions (F_v'/F_m') evaluated on the main-culm at 14-day waterlogging (T14), and after 7 (T7R) and 14 (T14R) days of recovery, in well-watered (WW), and waterlogged (WL) plants of *T. aestivum* L. genotypes. Germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme; Australian varieties (Austrl). Comparisons were conducted individually for F_v/F_m or F_v'/F_m', and per genotype, highlighting the differences between WW and WL plants with an *. (Two-way ANOVA, n = 3, $p<0.05$).

At the end of waterlogging, notable differences were observed in the F_v/F_m and F_v'/F_m' values in the genotypes GR-4, AdvL-4, and Austrl-3. Of these, GR-4 was the most severely impacted with both parameters showing drastic reductions. The F_v/F_m value decreased sharply from 0.79 to 0.22 while F_v'/F_m' from 0.53 to 0.10. These findings suggest a profound disruption in the photosynthetic efficiency of GR-4 during the stress period.

During the recovery period, when tissue re-oxygenation occurred, the negative effects of waterlogging intensified and extended to PL-2, IT-1, IT-4, AdvL-1, and Austrl-1 suggesting a delayed response in these genotypes. At the T7R, IT-1, IT-4, and AdvL-4 exhibited similarly low F_v/F_m values, ranging from 0.15 to 0.41 while their F_v'/F_m' between 0.10 and 0.18. By the end of the recovery period (T14R), significant and persistent reductions in these parameters were detected across several genotypes (PL-2, IT-1, IT-4, GR-4, AdvL-1, AdvL-4, Austrl-1, and Austrl-3) indicating that the effects of waterlogging had long-lasting consequences on their photosynthetic systems (Figure 5.10).

5.3.6 Soluble sugars content

Sugars in plant leaves are crucial for energy metabolism, structural integrity, signalling, nutrient transport, osmoregulation, defence, and environmental responses, being indispensable for plants growth, development, and survival.

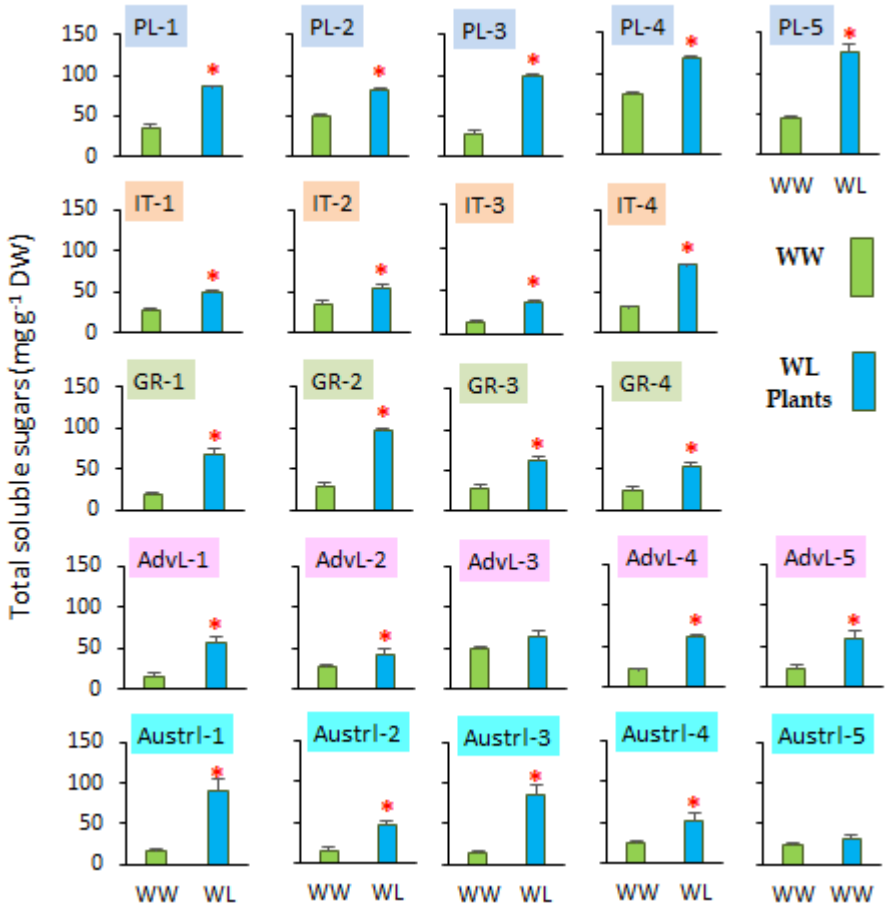


Figure 5.11 – Waterlogging-induced changes in the leaf total soluble sugars (mg g^{-1} DW). Evaluations performed on the 14th day of stress in well-watered (WW) and waterlogged (WL) plants of *T. aestivum* L. genotypes from five germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme; Australian varieties (Austrl). Comparisons were conducted per genotype, highlighting the differences between WW and WL plants with an *. (One-way ANOVA, n = 3, $p<0.05$).

Waterlogging increased total soluble sugars in all genotypes except AdvL-3 and Austrl-5, which remained unchanged (Figure 5.11). Increases ranged from 55.1% (AdvL-2) to 432.4% (Austrl-1),

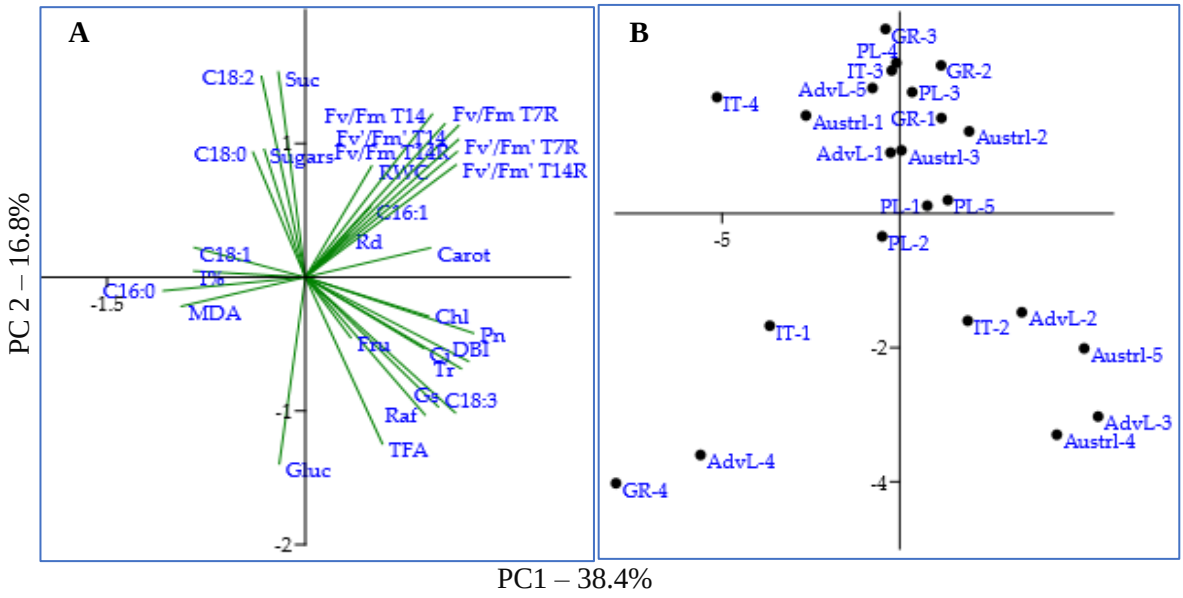
showing, as with other traits, a great variability among germplasm concerning waterlogging responses. In well-watered plants (WW), total soluble sugars ranged from 13.2 to 74.2 mg g^{-1} DW, whereas in waterlogged plants (WL), values increased to 31.0 to 126.8 mg g^{-1} DW. Sucrose and glucose were the most abundant, with concentrations of *ca.* 5.0-35 mg g^{-1} DW in WW plants and *ca.* 7.0-52 mg g^{-1} DW in WL plants (Figure 5.12). Fructose followed with 2.1-18.5 and 6.1-26.7 mg g^{-1} DW for WW and WL plants, respectively. The less representative was raffinose, with lower values of 1.0 (WW) and 1.6 (WL) mg g^{-1} DW and a higher concentration of 5.9 (WW) and 12.2 (WL) mg g^{-1} DW (Figure 5.12).

Figure 5.12 – Waterlogging-induced changes in main individual leaf soluble sugars, raffinose, sucrose, glucose, and fructose (mg g^{-1} DW). Evaluations performed on the 14th day of water stress in well-watered (WW) and waterlogged (WL) plants of *T. aestivum* L. genotypes from five distinct germplasm groups: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Programme; Australian varieties (Austrl). Comparisons were conducted individually per sugar and per genotype, highlighting the differences between WW and WL plants with an *. (One-way ANOVA, n = 3, $p<0.05$).

Waterlogging resulted in increased amounts of sucrose, glucose, and fructose in the majority of genotypes. In fact, only PL-2, IT-2, AdvL-2, Austrl-4, and Austrl-5 displayed unchanged amounts, as well as the latter regarding fructose and glucose (Figure 5.12). In contrast to sucrose, glucose, and fructose, the majority of genotypes showed unchanged values with waterlogging. However, significant rises were observed in PL-1 (48.0%), PL-5 (89.0%), GR-1 (114.5%), GR-2 (141.9%), GR-3 (90.5%), Austrl-2 (197.7%), Austrl-3 (418.8%), Austrl-4 (259.5%), and Austrl-5 (233.6%).

5.3.7 Multivariate analysis of key traits: A PCA approach

Principal component analysis (PCA) was performed with results obtained in this chapter to explore correlations between traits and similarities between genotypes. The first two principal components explain 55.2% of the total variance. PC1 is primarily influenced by photosynthetic rate (P_n), lipid unsaturation (DBI), leaf transpiration (Tr), linolenic acid (C18:3), chlorophyll *a* fluorescence (F_v/F_m and F_v'/F_m'), stomatal conductance (g_s), membrane injury index (I%), lipoperoxidation (MDA), palmitic acid (C16:0), and photosynthetic pigments (Chl and Carot). PC2 captures variations in sucrose (Suc), glucose (Glu), and frutose (Fru) (Figure 5.13 A).



Loadings																
	RWC	I%	TFA	MDA	DBI	C16:0	C18:0	C16:1	C18.1	C18:2	C18:3	Pn	Gs	Ci	Tr	Rd
PC1	0.53	**-0.69**	0.41	**-0.67**	**0.87**	**-0.76**	-0.28	0.36	**-0.60**	-0.23	**0.80**	**0.90**	**0.71**	**0.63**	**0.83**	0.23
PC2	0.52	-0.02	-0.56	-0.10	0.29	-0.05	0.4	0.24	0.10	0.68	-0.46	-0.20	-0.44	-0.24	-0.31	0.14
PC3	0.51	0.08	**-0.58**	0.54	-0.11	0.34	-0.18	0.32	0.38	-0.04	-0.02	0.12	0.28	0.13	0.14	**0.65**

	Total sugars	Raf	Suc	Gluc	Fru	Chl	Carot	Fv/Fm			Fv'/Fm'		
								T14	T7R	T14R	T14	T7R	T14R
PC1	-0.22	0.64	-0.14	-0.14	-0.24	**0.65**	**0.66**	**0.68**	**0.82**	**0.81**	**0.74**	**0.81**	**0.80**
PC2	**0.43**	0.46	**0.69**	**0.63**	**-0.20**	0.13	0.34	0.55	0.51	0.42	0.52	0.46	0.38
PC3	0.03	-0.30	-0.05	-0.09	0.52	-0.09	-0.00	0.20	-0.00	-0.10	0.20	-0.08	-0.15

Figure 5.13 – Principal Component Analysis (PCA) performed with all traits analysed in this chapter. Scatter plot displays the scores for the first two principal components (A)and the distribution of genotypes associated to the analysis (B). Loadings of PC1, PC2, and PC3 are presented. Relative water content (RWC), membrane injury index (I%), total fatty acids (TFA), lipoperoxidation (MDA), lipid unsaturation (DBI), palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16;1), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), photosynthetic rate (P_n), stomatal conductance (g_s), intracellular CO_2, leaf transpiration (Tr), leaf respiration (R_d), total soluble sugars (Sugars), Raffinose (Raf), sucrose (Suc), glucose (Gluc), fructose (Fru), photosynthetic pigments (Chl and Carot), and chlorophyll *a* fluorescence (F_v/F_m and F_v'/F_m').

The scatter plot displays the scores for the first two principal components (PC1 and PC2) and the distribution of genotypes associated to the analysis (Figure 5.13 B).

Strong and positive correlations exist between DBI, C18:3, P_n, g_s, C_i, Tr, Chl, Carot, raffinose (Raf), F_v/F_m, and F_v'/F_m' (PC1), suggesting that as these variables' values increase, genotypes positioned in that direction also tend to display higher values. PCA analysis suggests opposite trends between one set of PC1 traits mentioned above and a second set of traits (C16:0, MDA, and I%) in PC1, indicating that these variables exhibit comparable responses along this component. An increase in one variable leads to an increase in the others, albeit in the opposite direction of PC1's positive trend (*i.e.*, both exhibit higher absolute values). Different loadings show that these variables behave in opposite ways along PC1. Genotypes that showed increases in DBI, C18:3, P_n, g_s, C_i, Tr, Chl, Carot, Raf, F_v/F_m, F_v'/F_m', and PC1 were linked to decreases in C16:0, MDA, and I%, and vice versa. RWC, Total sugars, Suc, Gluc, and Fru are positively correlated, presenting a synchronic response along PC2. Based on the ecophysiological traits evaluated in this chapter, the genotypes were distributed across the four quadrants by the PCA analysis. This demonstrated the diversity of germplasm that are present in all groups, as evidenced by the similarity in response between genotypes from different germplasm groups (Figure 5.13).

5.4 Discussion

All studied traits showed significant variability within the germplasm. This may be due to the genetic background variance; however, the presence of variability within the different germplasm groups suggests that breeding did not reduce genotypic diversity.

Relative water content (RWC) is a measure of the water status in plant tissues and reflects its capacity to maintain turgor pressure, which is crucial for cell expansion, growth, and overall plant vitality. Despite the abundance of water, during waterlogging conditions plants have to maintain adequate water levels in tissues. In contrast to well-watered plants, PL-4, IT-1, IT-2, GR-4, AdvL-2, and AdvL-4 exhibited a decreased leaf RWC (Figure 5.1) when subjected to 14-day waterlogging. This suggests that leaves are losing water at a rate that exceeds their ability to absorb it. Regardless of strong decreases in the transpiration rate (70.3% to 83.3%) that prevent water loss, the genotypes IT-1, GR-4, and AdvL-4 showed the most significant impacts, denoting root impairment (and so reduced water uptake) as a consequence of the lack of oxygen. The observed reduction in leaf RWC due to waterlogging has been reported for mungbean (Kumar *et al*., 2013), sesame (Anee *et al*., 2019), soybean (Sathi *et al*., 2022), and wheat (Cottrozi *et al*., 2021). In the latter, a similar level of leaf hydration was observed on either plants exposed to waterlogging or to drought (Ostrowska and Hura, 2022), implying that even in an environment with excess water, plants may deal with water deficit. Conversely, IT-4 and Austrl-1 denoted a slight but significant RWC rise (5-6%). This increment may be a result of an impaired transpiration rate (81.7% and 65.1% decrease, respectively) due to stomatal closure or a sign of the plant struggle to manage excess water. Given the significant decrease in P_n and other traits such as TFA and DBI, suggesting deterioration in the plant physiological health, RWC increase does not seem in these cases an adaptive or beneficial response.

Findings on leaf gas exchanges (Figures 5.6 to 5.8) denote some variability in wheat responses to waterlogging. Stable P_n values may occur (in AdvL-2, AdvL-3, Austrl-4, and Austrl-5), but most genotypes depict P_n decreases. The stomatal closure played a significant role in these declines, with the most drastic reductions in P_n also accompanied by tremendous g_s decreases. Due to stomatal limitations, intercellular CO_2 (C_i) was also reduced in the majority of genotypes, as was transpiration rate. Stomatal closure, the reduction of transpiration, and the inhibition of photosynthesis are common responses to environmental constraint and are enhanced by poor root functioning (Zheng *et al*., 2009; Bhagat *et al*., 2014; Soleh *et al*., 2018). Under waterlogging, stomatal closure was already observed (Striker *et al*., 2005), strongly affecting photosynthetic C-assimilation (Medrano *et al*., 2002; Herzog *et al*., 2016; Rodrigues *et al*., 2024), as was the case in PL-2, PL-3, PL-5, IT-1, IT-4, GR-3, GR-4, AdvL-1, AdvL-4, AdvL-5, and AdvL-3, where the decline in g_s was accompanied by a lower C_i, thus justifying at least part of the Pn drop.

Some genotypes such as IT-3, GR-1, GR-2, Austrl-1, and Austrl-2, showed P_n reduction together with stable C_i values concomitantly to stomatal closure and a reduced transpiration rate, suggesting the occurance of non-stomatal limitations to P_n (Herzog *et al*., 2016). Additionally, RWC remained stable and frequently I% depicted an increased value, suggesting a significant stress that is impairing cellular compartmentalization and function. This may indicate a strategy to preserve plant hydration through stomatal closure, but inefficient use of available CO_2 inferred by P_n lowering, suggesting that biochemical factors or physiological damages are affecting P_n. Additionally, the plant´s antioxidant defence system and the tissue regeneration mechanisms could not mitigate the negative effects of waterlogging. Regarding genotypes that exhibited stable P_n values, only in Austrl-5 a higher C_i was observed, with AdvL-2, AdvL-3, and Austrl-4 maintaining intracellular CO_2. Moreover, g_s and Tr showed enhanced values. Stomatal opening is expected to result in an increase in transpiration as well as an elevation in the intercellular CO_2 concentration, thus improving P_n. The observed stability of P_n in Austrl-5 despite higher C_i may suggest that plants may be operating closer to the CO_2 saturation point, with increased C_i not providing advantages to photosynthesis. However, the RuBisCo activity or the efficiency of the Calvin-Benson cycle may restrict the plant ability to fix carbon. The accumulation of non-structural carbohydrates in leaves, decreased photosynthetic pigments, or nitrogen deficiency could also be involved in P_n decreases (Herzog *et al*., 2016).

As widely known, the photosynthetic processes are highly sensitive to stress situations, resulting also in changes in respiration rates (Fortunato *et al*., 2010; Partelli *et al*., 2011; Scotti-Campos *et al*., 2014a; Ramalho *et al*., 2018; Singh e Thakur, 2018). Respiration rates were measured after prolonged darkness (R_d) to capturing the plant basal respiration rate, which is typically lower than respiration under light (Figure 5.8). The PCA analysis revealed a moderate and opposite relationship between R_d and total fatty acid content (TFA) in PC3. After a night in the dark, our results showed that R_d increased in AdvL-1, AdvL-5, Austrl-2, Austrl-3, and Austrl-5 while TFA content either decreased or remained stable (even if a decreasing trend was observed). However, PC3 only explains 7.9% of the data variance, suggesting the need for more comprehensive studies.

The physiological state of PSII can be assessed through chlorophyll *a* fluorescence by quantifying the efficiency of photosystem II (PSII) in utilising the light energy absorbed by chlorophyll and the degree to which the efficiency is reduced by stress. The F_v/F_m ratio reflects the maximal photochemical efficiency of PSII (Sharma *et al*., 2015). Waterlogging resulted in negative impacts in F_v/F_m, as well as F_v'/F_m' (Figure 5.10), observed at the end of waterlogging in IT-1, GR-4, and AdvL-4. Reductions in these traits were accompanied by increases in MDA levels and membrane injury index (I%) and strong declines in RWC, P_n, g_s, C_i, Tr, Chl, Carot, and DBI. A decrease in F_v/F_m is usually accepted as being linked to a reduction in the maximal quantum yield of photosynthesis. Therefore, any decrease in F_v/F_m has a detrimental effect on the plant ability to absorb carbon with repercussions on the rest of plant metabolism (Sharma *et al*., 2015). On the other hand, the decay of F_v'/F_m' to extremely low values at the end of water stress, worsening during the recovery period, denotes a tremendously decreased efficiency of the conversion of absorbed light into chemical energy in PSII likely indicating enhanced photoinhibition status of PSII since light energy absorbed by light-harvesting complex II (LHCII) pigments exceeds the capability for its photochemical use. This might increase the probability of ROS overproduction, promoting lipoperoxidation and affecting MDA levels, membrane leakage, selectivity, stability, and integrity, wich in turn will aggravate the impact at the PSII level.

Regarding photosynthetic pigments (Figure 5.9), the strongest chlorophyll decrease was observed in GR-4, while AdvL-2, AdvL-3, Austrl-3, and Austrl-4 were unaffected. Furthermore, carotenoids rose only in GR-2 and Austrl-3, remained stable in AdvL-2, AdvL-3, Austrl_2, and Austrl-5, and suffered reductions from 10% up to 74% among the remaining genotypes. According to Scotti *et al*. (2015), chlorophyll reductions may be used as an indicator of plant senescence, while increasing amounts of carotenoids may reveal a better ability to cope with the excess of light energy, thus preventing or mitigating oxidative stress conditions. Several authors observed a decrease in chlorophyll content in waterlogged wheat. At emergence stage, 10-day stress induced chlorophyll reductions between 15 and 33% (Yadav *et al*., 2015), whereas at tillering stage, reductions ranged from 41% to 61% (Amri *et al*., 2014). Wheat susceptible plants often displayed lower carotenoid content due to waterlogging (Ploschuk *et al*., 2018; Collaku and Harrison, 2002; Alizadeh-Vaskasi *et al*., 2018), with reported reductions of 16-38% with 14-day water stress at the tillering stage (Alizadeh-Vaskasi *et al*., 2018). In sharp contrast, tolerant genotypes total carotenoid amount usually remained stable or even higher (Ozcubukcu and Ergun, 2013). The PCA analysis showed a strong positive correlation between both photosynthetic pigments (chlorophyll and carotenoids) and gas exchanges (except Rd), lipid unsaturation (DBI, mol% of C18:3), and photochemical efficiency (F_v/F_m, F_v'/F_m'). Also, lower photosynthetic pigments are related to higher levels of MDA, injury index, C18:1, and C16:0.

Leaf-soluble sugars are essential components of non-structural carbohydrates. They play a critical role in maintaining an adequate C-partitioning, which is vital for the supply of energy and C-skeletons to support metabolism, growth, development, and reproduction. In addition to their role in stress perception, sugars have also multiple roles, namely by contributing to osmotic adjustment and interacting with hormones, gene expression regulation, and proteomic patterns, including those related to photosynthetic metabolism (Saddhe *et al*., 2021; Rodrigues *et al*., 2024). In addition, sugars are also involved

in membrane and protein stabilisation and protection, as well as in ROS scavenging (Rejšková *et al*., 2007; Keunen *et al*., 2013; Saddhe *et al*., 2021). Although moderate increases in sugar levels are part of the osmotic adjustment, an abrupt rise may indicate a transition to an extended stress response. Waterlogging caused strong rises in the majority of the studied genotypes (Figure 5.11), consistent with other studies that found waterlogging stress to increased leaf sugars despite reductions in P_n (Herzog *et al*., 2016). In this scenario, sugars are not only functioning as osmolytes but are also being accumulated due to the plant reduced capacity to convert them into energy. The levels of sugars in plant cells, their transport, utilisation, and storage are regulated and strongly dependent on cell physiological activity (Jeandet *et al*., 2022). The observed sugar accumulation may be the result of impaired balanced partitioning of sugars within plant cells, causing changes in their transport from source to sink organs. In plants, the raffinose family oligosaccharides (RFOs) provide abiotic stress tolerance, serve as storage compound, maintain osmotic balance, and stabilise membranes (Sanyal *et al*., 2023). Our findings showed the highest increases in raffinose content in genotypes GR-2, AdvL-3, Austrl-4, and Austrl-5. Those genotypes also depicted stable RWC, I%, TFA, C16:0, and C18:0, as well as stable or even higher carotenoid content, Rd, DBI, C16:1, C18:1, and C18:2, suggesting some tolerance to waterlogging.

The electrolyte leakage test was employed to evaluate the genotypes tolerance to waterlogging, using the injury index (I%) as an indicator of membrane integrity (Figure 5.2). Waterlogging may promote electrolyte leakage (Shabala *et al*., 2011), which is a constituent part of plants stress response (Demidchik *et al*., 2014). This test has already been successfully used in several crops (Matos *et al*., 2002; Campos *et al*., 2003; Scotti-Campos *et al*., 2015; Castanheira *et al*., 2017). Maintaining cellular metabolism under stress conditions and preserving the structure, composition, and functionality of membranes is crucial for plant tolerance. Therefore, a significant increase in I% may indicate irreversible membrane injury caused by oxidative processes and lipoperoxidation phenomena. These processes initiate cell and tissue senescence. Our results revealed significant increases in I% in several genotypes under water stress, suggesting loss of membrane selectivity, likely associated with damage. However, RWC and I% are not independent of each other since PL-4, IT-1, IT-2, IT-4, GR-4, and AdvL-4 changes in RWC were concomitant with increases in I%. The preservation of membrane lipids in the presence of limiting environmental conditions significantly enhances the functionality of membranes (Ramalho *et al*., 1998; Blokhina *et al*., 2003; Campos *et al*., 2003). The observed variation in lipid content in waterlogged plants shows diverse genotype responses. Previous research has identified various strategies, including remodelling of chloroplast membrane lipids through *de novo* synthesis or modification of pre-existing FA (Scotti-Campos *et al*., 2019). An investment in maintaining cell membrane integrity, which reveals the ability to synthesise lipids under anoxygenic conditions and thus some stress tolerance, may link to TFA maintenance (Generosova and Vartapetian, 2005; Xie *et al*., 2021). Conversely, waterlogging shift from aerobic to anaerobic respiration may cause an energy shortage, leading to the observed decrease in TFA in twelve genotypes. Given the limited production of just 2 ATP per glucose molecule in glycolosis, there is insufficient energy available to synthesise new lipids and/or repair the existing ones. This leads to a weakening of the membrane structure, ultimately resulting in cellular damage (Blokhina *et al*., 2003; Gibbs and Greenway, 2003; Ploschuk *et al*., 2018).

In addition to the quantitative changes in TFA content, the qualitative changes reflected in the unsaturation degree (DBI), plays a key role in the cell membrane properties and acclimation. In fact, the plasticity required to remodel lipid composition in order to maintain ideal membrane function in response to external stimuli comprises changes in lipid content and FA unsaturation degree (Partelli *et al*., 2011; Scotti-Campos *et al*., 2019). These long-term response changes (usually within days range) have an impact on the fluidity, integrity, and permeability of the cellular membrane in plants (Zheng *et al*., 2011; Xie *et al*., 2021) and are strategies to overcome abiotic stresses impacts (Xu *et al*., 2019). The higher DBI observed in AdvL-3, Austrl-3, Austrl-4, and Austrl-5 indicates more double bonds on the FA chains, thereby increasing membrane fluidity and potentially allowing for better structural integrity. In fact, those genotypes did not show changes in RWC, injury index, MDA, and TFA, suggesting a better physiological condition. Despite the scarcity of studies on the impacts of waterlogging on lipid metabolism in wheat, several authors suggest that under hypoxia, lipid remodelling and lipid dynamics, such as desaturation and the production of lipid-signalling molecules, contribute to modulating hypoxia signalling responses, enhancing plant survival under hypoxic imposed by waterlogging and/or re-oxygenation stress (Xu *et al*., 2019; Xie *et al*., 2021). On the other hand, the preferential synthesis of saturated FAs and the resulting decrease in DBI lead to lower polyunsaturated FA (PUFA) and thereby to a decreased susceptibility to oxidative degradation. This has been referred to as an adaptive feature in anoxic environments in rice (Generosova e Vartapetian, 2005). However, our findings revealed an overall increase in the membrane injury index, as well as a reduced RWC in genotypes that presented reduced TFA content and an increased FA saturation (lower DBI, likely due to a preferential degradation of unsaturated FAs than due to a synthesis of saturated ones, as reflected in lowered TFA), suggesting a weakening of the membrane structure, resulting in cell damage.

Unsaturated FAs are crucial for membrane fluidity and photosynthetic metabolism. Changes in the UFA palmitoleic acid (C16:1) abundance were observed in the present study, reinforcing the existence of adjustments in the lipid metabolism due to waterlogging. Being a minor component of the TFA, C16:1 plays a significant role in chloroplasts, particularly in maintaining the fluidity and functionality of thylakoid membranes where photosynthesis occurs. As mentioned, the fluidity of the membrane is crucial for the mobility and interaction of membrane proteins and for the performance of membrane related processes, such as transfer of electrons during photosynthesis. Our findings revealed a wide range of responses, with genotypes increasing the C16:1 proportion, decreasing it, or even remaining unaffected. In the PCA analysis, the C16:1 proportion aligns with the PSII photochemical efficiency (F_v/F_m, F_v'/F_m'); however, a small representation in both PC1 and PC2 did not support a direct causal relationship between those traits and C16:1 change, likely because analysis was performed at cellular membrane level in which C16:1*t* has a quite low representation among FAs. Also, in several genotypes, waterlogging reduced C18:3 mol% but increased the unsaturated FAs C18:2 and C18:1. The C18 unsaturated FAs have the ability to undergo modifications and transform into bioactive molecules such as jasmonates and nitroalkenes, that play a role in regulating oxidative stress (Sharma et al., 2019). He and Ding (2020) have proposed that the increase in unsaturation of FA could function as a mechanism to counteract the vulnerability to peroxidation and as a strategy to better cope with ROS. These may be the

case with our findings, since PCA analysis revealed a strong but opposite correlation between DBI and MDA (higher unsaturation correlates with lower MDA content). In turn, when MDA increases, the injury index is also higher. In this way, the observed increases in MDA are indicative of lipoperoxidation and subsequent membrane injury, both of which lead to foliar senescence. Numerous crops, including wheat, have shown waterlogging-induced ROS production and subsequent lipid peroxidation (Wei *et al*., 2013; Herzog *et al*., 2016; Ren *et al*., 2016; Ullah *et al*., 2017; Zheng *et al*., 2017). Moreover, some varieties had the ability to maintain MDA content in their tissues, which may indicate a higher tolerance to oxidative stress (Duhan *et al*., 2019).

5.5 Conclusions

The imposition of a 14-day waterlogging period beginning at the tillering stage had significant impacts on all studied traits across all germplasm groups. Some genotypes exhibited strong effects when exposed to these water stress conditions, while others showed favourable performance, preserving several features that can be used to evaluate tolerance or sensitivity to abiotic stress. Quantifying MDA and I% levels confirmed a link between lipoperoxidation and membrane injury. Findings suggest that in watterloged wheat plants, the degree of unsaturation in membrane lipids is inversely related to lipoperoxidation and membrane injury. Moreover, genotypes with the ability to remodel membrane lipids and/or synthesise new lipids appear to have a higher tolerance to excess water conditions, exhibiting also better photosynthetic performance. Among soluble sugars, and despite being present in small amounts, raffinose may have also played an important role in wheat stress tolerance to waterlogging. Five genotypes consistently exhibited the greatest negative impacts (PL-4, IT-1, IT-4, GR-4, and AdvL-4), while AdvL-2, AdvL-3, Austrl-4, and Austrl-5 seem to be the more tolerant. The photochemical efficiency of PSII demonstrated to be a reliable indicator for identifying the genotypes that are most severely affected by water stress. During the recovery phase, when plant tissues were reoxygenated, the observed negative effects were clearly evident being aggravated in several cases. Therefore, waterlogging research should cover that specific time frame considering both the time of stress exposure but also the recovery period thereafter (after stress conditions removal) in order to provide more reliable and robust outcomes.

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6 WATERLOGGING AND WHEAT YIELD: INTEGRATIVE ANALYSIS OF MORPHOLOGICAL AND ECOPHYSIOLOGICAL RESPONSES*

Abstract

Climate change includes severe weather events that threaten bread wheat (*Triticum aestivum* L.) production. Among them, periods of excessive rainfall can lead to soil waterlogging. When plants suffer from this hydric stress at the tillering stage, yield may be impaired; however, several genotype features may counterbalance the adverse impacts. While previous chapters have addressed the impacts on morphological traits, plant development, precocious senescence, and key metabolism, the influence of waterlogging on yield remains crucial for the selection of waterlogging-tolerant genotypes. This chapter assesses the final yield, fertile spikes, kernels per plant, single kernel weight, and yield contributions from both the main culm and tillers of the wheat germplasm. Additionally, an integrative analysis of yield and all traits, including the seminal root system, was performed and revealed variability in waterlogging tolerance. Several genotypes showed promising results with unaltered yield, resulting from stable contribution by both the main culm and tillers (PL-1,GR-1, Austrl-2) , while others exhibited increases in tillers yield (GR-2) or in both the main culm and tillers (AdvL-3). In other cases, decreases in one or more traits (*e.g*, fertile spikes, kernels per plant, single kernel weight) were compensated by increases in different ones, namely, in PL-1, PL-5, GR-1, GR-3, AdvL-2, Austrl-2, and Austrl-4. The best performance was observed in AdvL-3, with 46.6% yield increase. In sharp contrast, GR-4 showed the strongest impact, with an 86.6% yield decline. Results suggest that the growth stage reached during waterlogging negatively correlates with yield, with the majority of genotypes with longer growth cycles showing fewer impacts. SPAD values and photochemical efficiency of PSII, two non-destructive measurements, showed a higher correlation with yield in all observations. Our findings may contribute to a deeper understanding in wheat responses to waterlogging and to develop solutions to mitigate the socio-economic impacts of the worldwide 20-50% wheat yield reductions due to waterlogging.

Keywords: Roots and yield; Screening traits; *Triticum aestivum* L.; Yield components.

*This chapter include some sections of the research papers:

Pais IP, Moreira R, Semedo JN, Reboredo FH, Coutinho J, Lidon FC, Maçãs B, Scotti-Campos P (2023). Water-logging effects in adventitious roots, tillering and yield of bread wheat germplasm. *Agric, Res, Technol, Open Access J*, 27:556383. https://doi.org/10.19080/ARTOAJ.2023.27.556383.

Pais IP, Moreira R, Coelho AR, Semedo JN, Reboredo FH, Coutinho J, Lidon FC, Maçãs B, Scotti-Campos P (2023). Unveiling the impact of growth traits on the yield of bread wheat germplasm subjected to waterlogging. *Agriculture*, 12:241. https://doi.org/10.3390/agriculture4020241.

6.1 Introduction

Wheat, a widely cultivated crop due to its high yields and remarkable nutritional and pro-cessing properties (Wei *et al*., 2021), is closely linked to global food security. The majority of the wheat produced (79%) is used in wheat-based products, consumed by 2.5 billion people, providing vitamins, minerals, and 15-20% of the daily protein and energy requirements (Herzog *et al*., 2016; Wei *et al*., 2021; Nóia Junior *et al*., 2023).

Significant heterogeneity regarding the main culm or tillers contribution to yield may be expected under both optimal and adverse environments, since a variable number of tillers can be found according to the cultivar and environmental conditions. Low-tillering varieties minimise competition among main culm and tillers, ensuring uniform maturity (de Vita *et al*., 2007; Fischer, 2016; Arduini *et al*., 2018). On the other hand, high tillering may allow yield maintenance by compensating for reductions in average kernel weight and/or kernels per spike (Elhani *et al*., 2007). Nonetheless, stable fertile tillers may not assure yield maintenance, as their contribution to the final yield may be affected (Valério *et al*., 2008).

Waterlogging results in soil water oversaturation around plant roots, significantly affecting several traits and hence impacting final yield through decreases in several yield traits. In fact, this water stress may result in decreased average kernel weight, number of kernels per spike, and spike number per plant or unit surface (Araki *et al*., 2012; Ding *et al*., 2020; Ashraf, 2012; Shao *et al*., 2013; Hossain *et al*., 2011: de San Celedonio *et al*., 2018). Depending on their growth stage, wheat plants respond differently to waterlogging. When imposed at the tillering stage, reduced spike and kernel numbers can be found, while at the booting stage, lighter kernels were reported (de San Celedonio *et al*., 2014; Wu *et al*., 2015). Yield reductions under waterlogging have been linked to reduced kernel size, fewer fertile tillers (Malik *et al*., 2001, 2002), and low tiller survival (Condon and Giunta, 2003; Yaduvanshi *et al*., 2012). Furthermore, decreased number of kernels per spike has been reported for both the main culm and tillers (Olgun *et al*., 2008; Alizadeh-Vaskasi *et al*., 2018; Ding *et al*., 2020). Environmental conditions can alter yield components, making it possible to select the most promising ones for yield gain (Ferrante *et al*., 2013; Slafer *et al*., 2014) and develop waterlogging-tolerant varieties as a key to maintaining crop production in areas prone to such climate events.

The impact of waterlogging stress has been assessed when applied at a specific growth stage. However, the evaluation did not consider whether variations in the growth cycle, result in genotypes being exposed to stress in one or more growth phases. Besides the effects on final yield and the changes during the stress period, plant responses in a post stress period, particularly during tissue reoxygenation, may also contribute to a more comprehensive understanding. Gaining better insight into yield losses and the role of different traits as potential indicators for tolerance or susceptibility could lead to a more accurate screening of varieties and the identification of water-tolerant cultivars. Moreover, an early detection of susceptibility or tolerance to water-logging offers significant advantages. Identifying these traits early in the growth cycle may allow management practices to be adjusted or more resilient varieties to be selected to mitigate the adverse effects of excess water. Breeding programmes also benefit from

early detection by accelerating the development of waterlogging-tolerant wheat varieties, contributing to more resilient agricultural systems in the face of climate variability.

6.2 Objective of the study

The aim of this study was to evaluate, under controlled environmentally conditions, the impact of 14-day waterlogging imposed at tillering stage in yield and yield related traits of 23 bread wheat (*Triticum aestivum* L.) genotypes from distinct origins. The effect of stress in final yield, spike number per plant, kernel number per plant and per main culm and total of tillers, single kernel weight (from main culm and tillers) and changes in the main culm and tillers contribution to final yield, were assessed. At the end of waterlogging, changes in the phenotypic development due to waterlogging were also evaluated, along with the correlation of these differences on yield and its components.

Additionally, the objective was also to evaluate possible correlations among the studied traits, including root system, plant growth, senescence, yield, and ecophysiological responses. The ultimate goals were to differentiate between genotypes that are tolerant or sensitive and to identify markers that facilitate a quicker and more effective selection process.

6.3 Results

6.3.1 Yield and Yield-Related Traits under Waterlogging

6.3.1.1 Grain yield

Waterlogging induced significant yield changes in the majority of the genotypes, with negative effects being particularly evident in 11 genotypes. The genotype GR-4 was the most affected, with an 86.6% yield reduction. AdvL-4, IT-4, IT-1, AdvL-5, and PL-4 also exhibited drastic reductions, exceeding 50% yield loss. Water stress impacted all germplasm groups, but its incidence was highest in the IT group, where all genotypes showed yield decreases (21.0–70.8%) (Figure 6.1). Among the 23 genotypes, four showed increased yields. AdvL-3 demonstrated the best performance, increasing plant yield by 46.6% and was followed by Austrl-5, Austrl-3, and GR-2, with yield increases ranging from 16.6 to 32.8%. The genotypes PL-1, PL-3, PL-5, GR-1, GR-3, AdvL-2, Austrl-2, Austrl-4 were able to maintain yield stability (with non-significant changes ranging from -14.8 to +13.6%) demonstrating also a good potential for tolerance to waterlogging, imposed at the tillering stage and under the described environmental conditions (Figure 6.1).

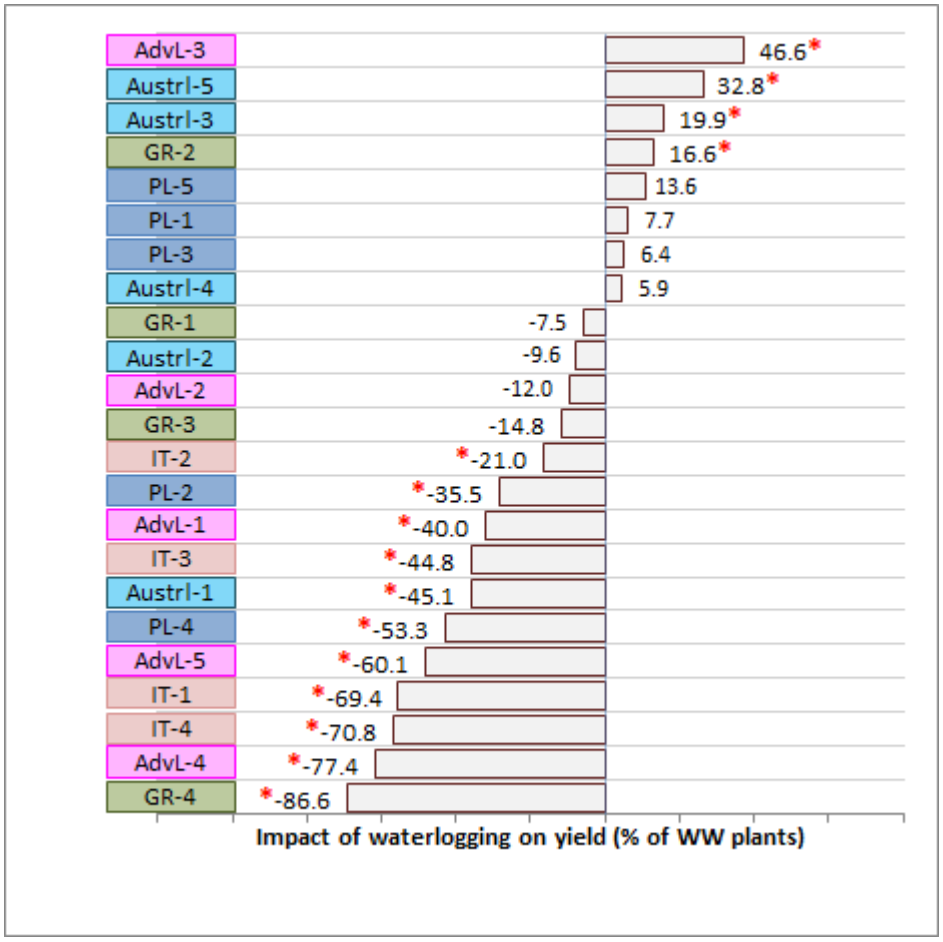


Figure 6.1 - Impact (% of the respective well-watered plants) on yield (g plant^{-1}) in plants subjected to 14-day waterlogging in bread wheat germplasm with distinct genetic background: Portuguese Landraces (PL); Varieties with introduced Italian germplasm (IT); Post-Green revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Program (INIAV); Australian varieties (Austrl).* Statistical differences between WW and WL plants (n=9-18; $p<0.05$).

6.3.1.2 Main culm and tillers contribution to final yield

Regarding the contribution of the main culm and tillers to the final yield (g plant^{-1}), a large variability was observed in all germplasm groups in control and waterlogged plants (Figure 6.2).

In both WW and WL plants, tillers tended to provide a lower input to yield than main culms (less 46.4-74.6% and 43.6-100%, respectively). Even though the same number of genotypes (8 genotypes) showed this trend in both WW and WL conditions, the main culm and tillers final contributions incorporated several changes in stressed plants.

In WL plants, some genotypes were able to maintain yield in both main culm and tillers. With stable values in the main culm, tiller yield increased by 50.1% in GR-2, while PL-4 and AdvL-1 decreased (71.9-100%) in stressed plants. Similar to AdvL-1, WL tillers did not contribute to yield of GR-4, AdvL-4, and AdvL-5 at harvest. In addition, this was concomitant with declines in the main culm yield of GR-4 (69.6%) and AdvL-4 (75.0%). IT-1, IT-3, and IT-4 suffered simultaneous reductions in the main culm (53.2-75.0%) and tillers (43.8-96.4%) yield (Figure 6.2). With opposite behaviour, AdvL-3 increased main-culm (40%) and tillers (69.2%) yield in WL plants.

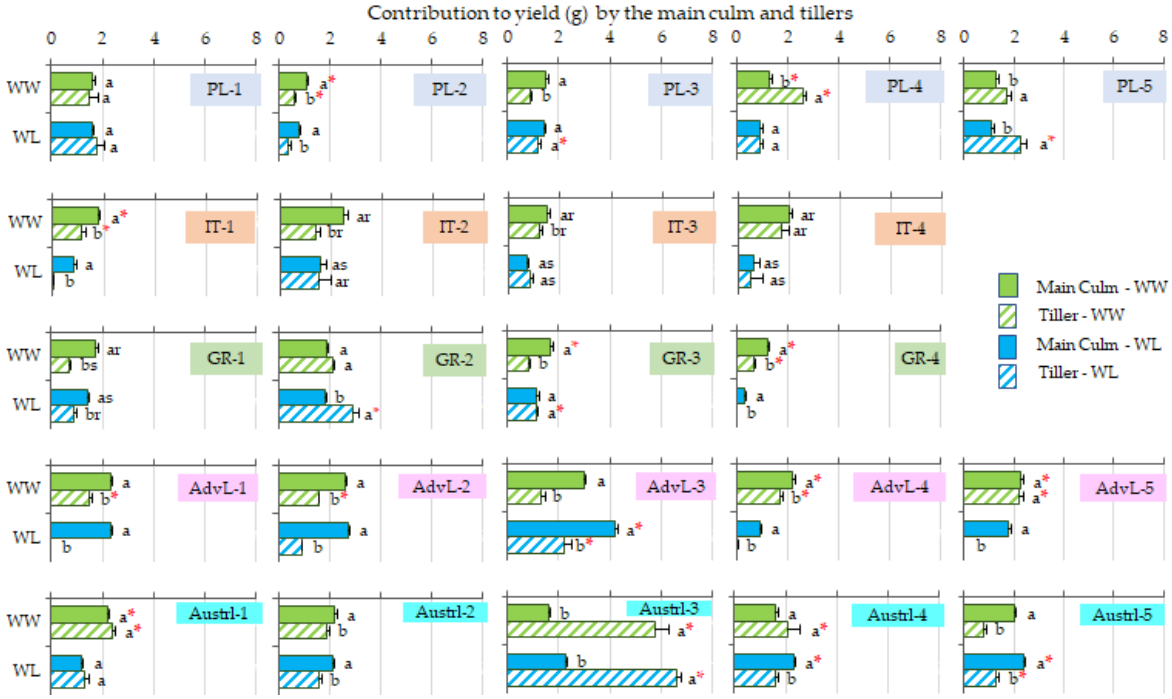


Figure 6.2 - Contribution (mean ± SE) to yield (g plant^{-1}) of the main culm (MC) and total of tillers (T) in 23 *T. aestivum* L. genotypes from five germplasm groups with different genetic backgrounds: Portuguese Landraces (PL); varieties with introduced Italian germplasm (IT); Post-Green Revolution varieties with introduced CIMMYT germplasm (GR); Advanced lines (AdvL) from the Portuguese Cereal Breeding Program; Australian varieties (Austrl). For each genotype, comparisons were performed for the main culm and tillers in well-watered (WW) or waterlogged (WL) plants with different letters (a, b) indicating significant differences between MC and T in the same water regime (WW or WL). Differences between WW and WL for the same tissue (MC, T) are highlighted by an *. (ANOVA, n = 3, $p<0.05$)

6.3.1.3 Number of spikes per plant

Regarding the number of spikes per plant, a wide range of results were observed, with the WW plants reaching harvest with values between 1.5 and 7.8 grain-producing spikes and the WL plants maintaining the maximum value and decreasing the minimum to 1.0 (Figure 6.3). This variability was observed not only between the 23 genotypes but also within each group of germplasm. The minimum value did not differ between groups, ranging from 1.5 to 2.4 for WW plants and 1.0 to 1.9 for WL plants. In contrast, the maximum value for WW plants ranged from 3.6 to 7.8 in WW plants and from 1.8 to 7.7 in WL plants (Figure 6.3).

Waterlogging reduced the number of spikes per plant in 11 genotypes by up to 83% (AdvL-4). In contrast, WL plants of Austrl-2 and Austrl-5 produced 38 and 67% more fertile spikes at harvest, respectively, compared to WW plants.

Except for the IT group, which experienced negative effects on all varieties, the remaining groups exhibited at least two genotypes with an unaltered number of spikes in waterlogged plants. As all genotypes ended the growing cycle with a fertile spike on the main culm, the observed differences resulted from the loss of tillers due to waterlogging.

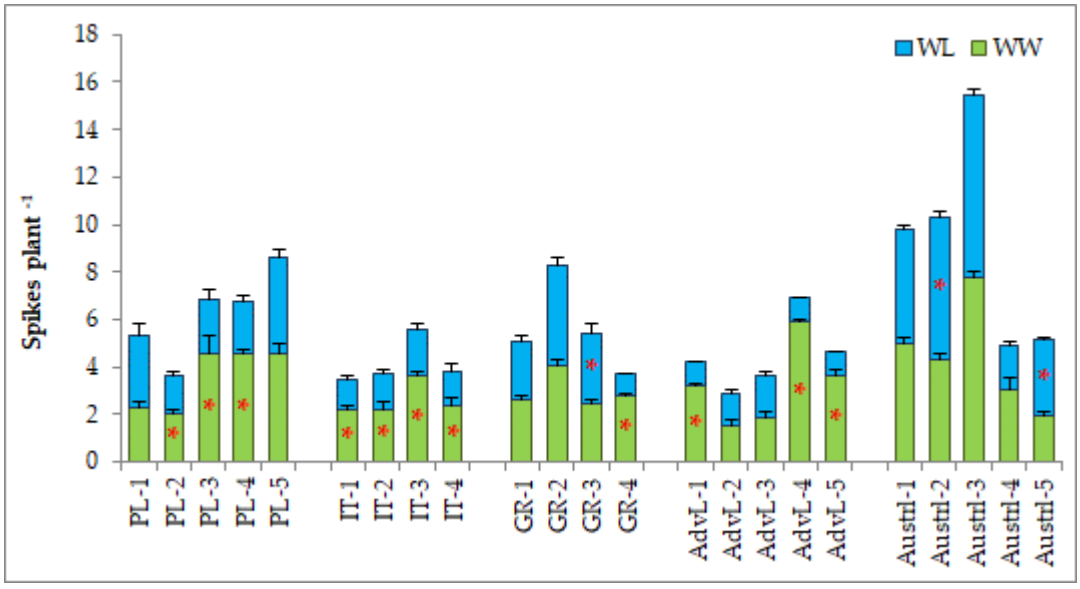


Figure 6.3 - Number of spikes per plant (mean ± SE) in control (WW) and waterlogged (WL) plants of 23 bread wheat genotypes (*T. aestivum* L.) belonging to 5 distinct germplasm groups: (PL) Portuguese Landraces; (IT) Varieties with introduced Italian germplasm; (GR) Post-Green Revolution varieties with introduced CIMMYT germplasm; (AdvL) Advanced lines from the National Cereal Breeding Programme; (Austrl) Australian varieties;. Differences between WW and WL plants, as well as for the highest value are indicated by an *

6.3.1.4 Kernel number per plant

Waterlogging led to significant decreases (16.4% to 61.1%) in the kernel number per plant of 14 genotypes across all studied groups (Figure 6.4, Plant). The IT group experienced the highest impact, showing decreases in the number of kernels per plant across all varieties. On the other hand, an increased kernel number per plant in WL was found in PL-3 (21.9%), GR-2 (29.0%), and AdvL-3 (18.1%), whereas PL-1, PL-5, GR-1, GR-3, Austrl-2, Austrl-3, and Autrl-5 were unaffected. These findings may reflect changes in the main culms kernel number, the total number of tillers, or both.

Waterlogging tended to reduce the kernel number in the main culm and tillers (Figure 6.4). However, these decreases were only significant in the main culm of 5 genotypes (23.4-47.9%) and in tillers of 14 genotypes (31.0% to 100% reduction). Among the latter, eight genotypes showed declines of over 50% in the kernel number produced by tillers. In IT-3, IT-4, and Aus-trl-1, the observed plant declines were the result of a concurrent reduction in both the main culm and tillers. On the other hand, decreases in 9 genotypes (PL-2, PL-4, IT-1, IT-2, GR-4, AdvL-1, AdvL-2; AdvL-4, and AdvL-5) were due to a lower value only in tillers, as the number of main culm kernels was unaffected by waterlogging. Among these genotypes, GR-4, AdvL-1, AdvL-4, and AdvL-5 exhibit a 100% reduction, with tillers not producing any kernels (Figure 3, Tillers). The main culm of Austrl-4 produced a higher kernel number (46.8%), but this was insufficient to compensate for the loss in tillers, resulting in a decrease in plant kernel number (Figure 6.4).

Among the genotypes whose number of kernels per plant was unaffected, this apparent stability was the result of an unchanged grain number in both the main culm and tillers only in PL-1, PL-5, and Austrl-3. An increased kernel number in total tillers of GR-1 (55.4%) and GR-3 (29.9%) has compensated for the losses found in the main culm (23.5 and 31.4%, respectively). Regarding the increase in

kernel number per plant, GR-2, AdvL-3, and Austrl-5 stood out, with gains of 29.0%, 18.1%, and 18.7%, respectively. In these plants, the total number of kernels produced by tillers increased by 58.0% (GR-2), 48.8% (AdvL-3), and 61.6% (Austrl-5) (Figure 6.4).

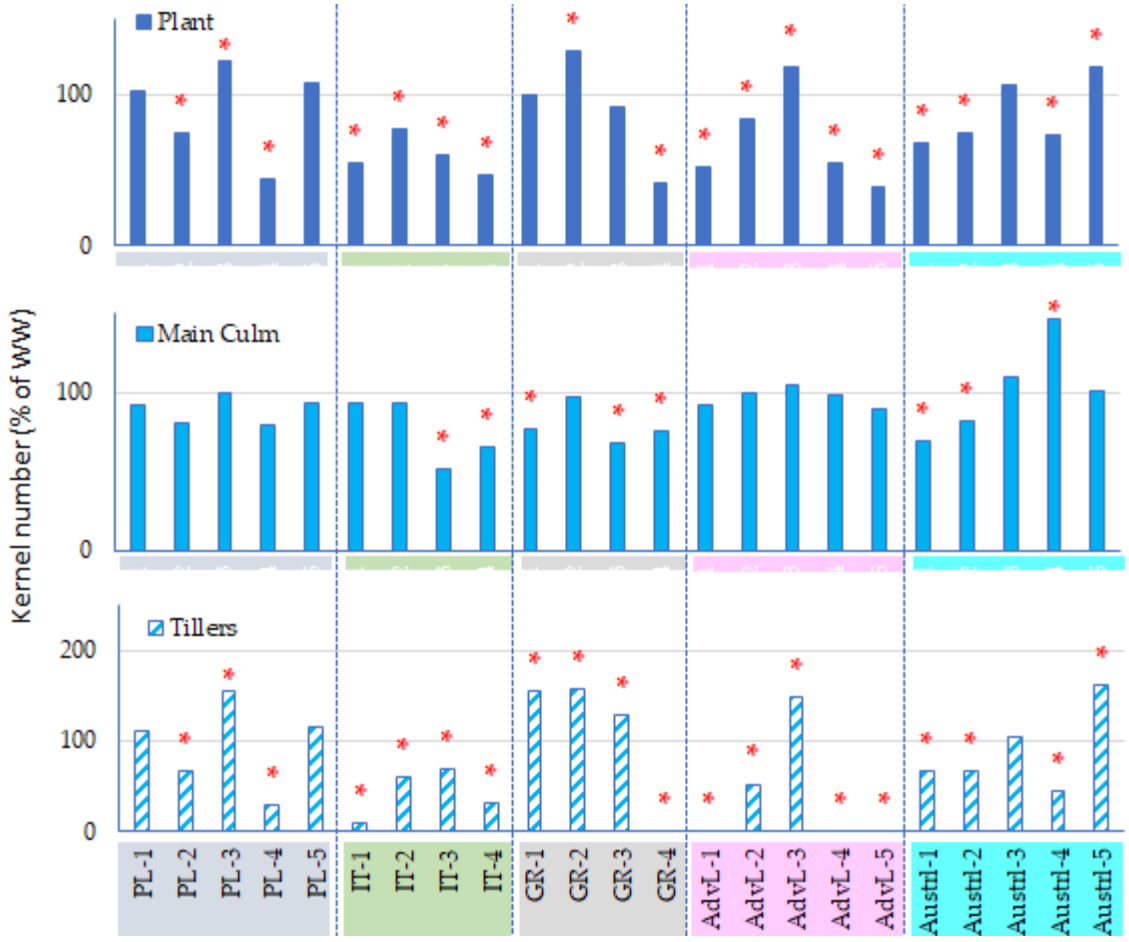


Figure 6.4 - Impact (%) in waterlogged plants (WL) when compared to well-watered plants (WW) of 14-day waterlogging on the final kernel number (Plant, Main culm, and total of tillers) in *T. aestivum* L. genotypes from five germplasm groups with different origins. (PL) Portuguese Landraces; (IT) Varieties with introduced Italian germplasm; (GR) Post-Green Revolution varieties with introduced CIMMYT germplasm; (AdvL) Advanced lines from the National Cereal Breeding Programme; (Austrl) Australian varieties. For each genotype, significant differences (n = 3; $p<0.05$) between WW and WL plants are indicated by an *

6.3.1.5 Kernel number per spike

In 16 genotypes, the main culm produced more kernels per spike than the tillers. On the other hand, in PL-1, PL-2, PL-4, IT-2, IT-4, and AdvL-2, the main culm and tillers produced an equal number of kernels per spike, while in Austrl-4, tillers produced more kernels per spike (43) compared to the main culm (33). Overall, PL genotypes displayed lower values than those of the other groups (Figure 6.4).

The number of kernels per spike on the main culm of IT-3, IT-4, GR-1, GR-3, Austrl-1, and Austrl-2 decreased between 18.1% and 47.0% due to waterlogging. In the tillers of WL plants of GR-4, AdvL-1, AdvL-4, and AdvL-5, no grains were produced, while decreases of 29.6, 62.0, and 56.0% were

observed in PL-1, IT-1, and Austrl-2, respectively (Figure 6.5). However, water stress also increased the number of kernels per spike in the main culm of Austrl-4 (46.8%), as well as in the tillers of PL-3 (332.0%), IT-2 (66.6%), IT-3 (89.5%), GR-1 (82.8%), and AdvL-3 (48.8%). In IT-3 and GR-1, these increases coincided with the aforementioned decreases in the main culm (Figure 6.5).

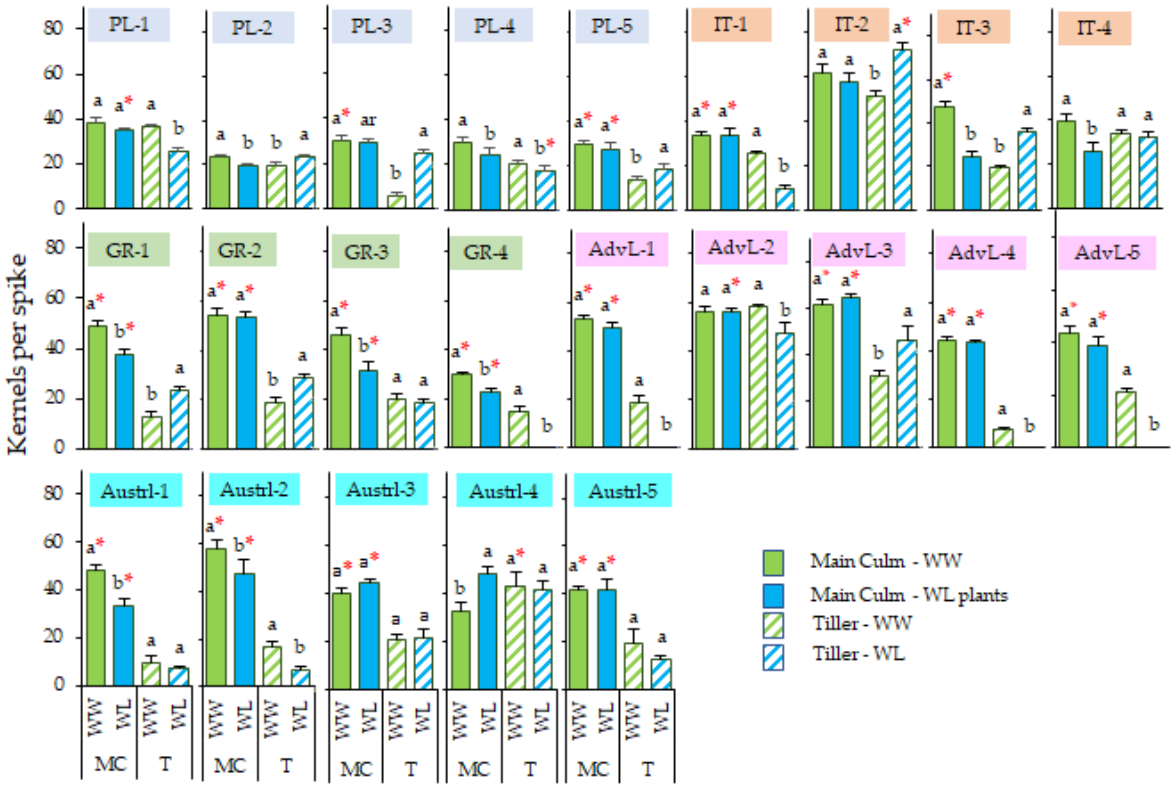


Figure 6.5 – Changes in kernels per spike at harvest (mean ± SE) in the main culm (MC), and per tiller (T) in well-watered (WW) and waterlogged (WL) plants. 14-day waterlogging imposed at the tillering stage on *T. aestivum* L. genotypes from different origins. (PL) Portuguese Landraces; (IT) Varieties with introduced Italian germplasm; (GR) Post-Green Revolution varieties with introduced CIMMYT germplasm; (AdvL) Advanced lines from the National Cereal Breeding Program; (Austrl) Australian varieties. Significant differences (n = 3; $p<0.05$) between WW and WL for the MC or T are indicated by letters (a, b) for each genotype. An * expresses significant differences between MC and T for the same water regime (WW or WL) and for each genotype. The letter a or an * represents the highest values.

6.3.1.6 Single kernel weight (SKW)

Regarding the average single kernel weight (SKW), variability was observed among genotypes and within groups (Figure 6.6).

In control plants, SKW ranged from 32.3 to 53.4 mg in the main culm and 21.8 to 62.4 mg in tillers. There were no differences between SKW values of main culm and tillers in 16 genotypes, but a heavier kernel in the main culm was observed in two Portuguese landraces (PL-1, PL-2), three IT genotypes (IT-2, IT-3, and IT-4), one from the GR group (GR-4), and one advanced line (AdvL-5) (Figure 6.6).

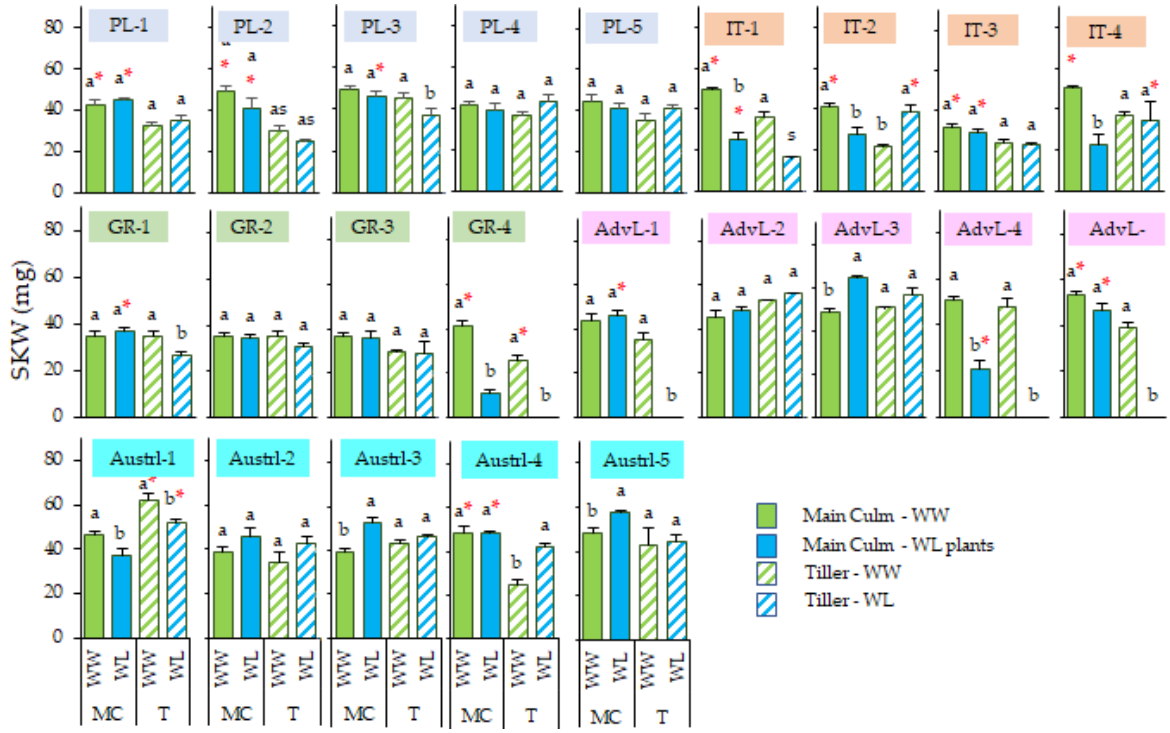


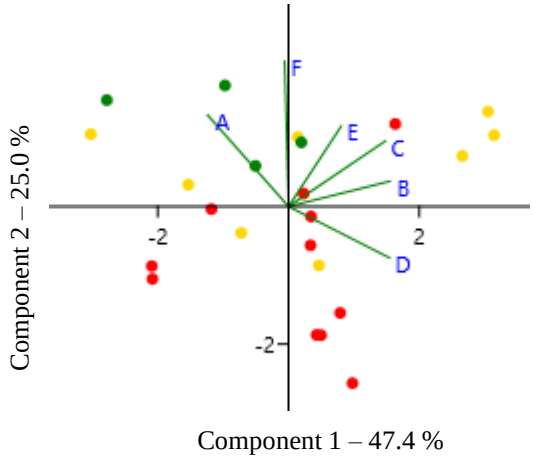
Figure 6.6 - Single kernel weight (SKW, mg, Mean ± SE) of 23 *T. aestivum* L. genotypes from five germplasm groups with different genetic background: Portuguese Landraces (PL); varieties with the introduction of Italian germplasm (IT); Post-Green Revolution varieties with the introduction of CIMMYT germplasm (GR); Advanced lines (AdvL) from the National Cereal Breeding Program; Australian varieties (Austrl). Comparisons of mean SKW (mg) for each genotype: well-watered (WW) or waterlogged (WL) plants, in the main culm (MC) and tillers (T). For each genotype, different letters (a, b) denote significant differences between WW and WL in either MC or T, while letters (r, s) indicate significant differences between MC and T within the same water regime (WW or WL). (ANOVA, n = 9, $p<0.05$).

In WL plants, the SKW fluctuated between 11.0 and 64.5 mg in the main culm and from 16.7 to 56.9 mg in tillers (Figure 6.6), while tillers produced no kernels in GR-4, AdvL-1, AdvL-4, and AdvL-5. In addition, waterlogging did not change the SKW trend in 12 genotypes, with 3 genotypes maintaining their heavier main culm kernels (PL-1, PL-2, IT-3) and 9 preserving the same SKW between main culm and tillers SKW (PL-3, PL-4, PL-5, GR-2, GR-3, AdvL-2, AdvL-3, Austrl-1, Austrl-2, Austrl-3, Austrl-4, and Austrl-5) (Figure 6.6). In IT-4, the lower SKW value observed in tillers of control plants changed in response to stress, with WL plants exhibiting identical main culm and tiller values. This change was due to a decrease in main culm SKW together with an unchanged value in tillers SKW. On the other hand, the decreased SKW of tillers in GR-1 altered the balance between the SKW of the main culm and tillers. Despite IT-1 WL plants maintaining lighter kernels in tillers, stress induced decreases of SKW in both the main culm and tillers (49.5 and 53.5%, respectively). In GR-4 and Advl-4, the absence of kernels in the tillers was concomitant with SKW decreases in main culm (73.3 and 58.3%, respectively).

6.3.2 Integrative analysis of yield impacts with roots, plant growth, senescence, and ecophysiological Responses

6.3.2.1 Yield impacts and root system

The traits related to the seminal root system showed a strong correlation between the values measured on the different days after sowing (Chapter 3). This was observed for the radicle length (RadL), the length of the 1st pair of lateral seminal roots (LatSL), and the number of seminal roots (NSR). Thus, only values obtained 2 day after sowing (DAS) were included for the following analysis, since it emphasizes the seminal root precocity as well as differences between genotypes, as the higher growth rate was observed from 1 to 2DAS (Chapter 3). The remaining studied root traits, namely root growth angle (RGA) and highest number of adventitious roots (AR), were also considered.



Loadings			
		PC 1	PC 2
A	Root growth angle	-0.69	0.54
B	Radicle lenght 2DAS	0.86	0.15
C	Number seminal roots 2DAS	0.82	0.38
D	Lenght 1st pair seminal roots	0.86	-0.30
E	Highest number adventitious roots	0.45	0.49
F	Yield in waterlogged plants	-0.03	0.85

Figure 6.7 – Principal Component Analysis (PCA) performed with some features of the seminal root system: Root growth angle (A), length of the radicle (B) and the 1st pair of lateral seminal roots (C) measure 2 days after sowing, number of adventitious roots on the last day of waterlogging (E) and yield in waterlogged plants (F). Data was standardised prior to analysis, and scatter plot shows the scores of the first to principal components. Loadings of PC1 and PC2 are presented. In the scatter plot, red dots corresponded to genotypes with decreased yield, green dots to genotypes with increased yield, and yellow dots to genotypes with unchanged yield.

The PCA revealed that the first principal component (PC1) accounts for 47.4% of the total variance, while the second principal component (PC2) explains 25% of the variance with a total of 72.4% of the variance in the data. The first principal component (PC1) is strongly influenced by the RadL, NSR, LatSL, which have high positive loadings (0.86, 0.82, and 0.86, respectively). This suggests that PC1 represents a combination of these variables, which contribute significantly to the variability captured by this component. RGA also has a substantial negative loading on PC1 (-0.69), indicating an inverse relationship with the component. The relatively low loading of yield in WL plants on PC1 (-0.03) implies it has minimal influence on this component.

The second principal component (PC2) is primarily driven by yield in WL plants, which has the highest positive loading (0.85). Other variables, such as RGA and AR, also contribute positively to PC2 with loadings of 0.54 and 0.49, respectively (Figure 6.7).

The PCA plot shows similarity between some genotypes that had strong impacts on yield due to waterlogging (red dot); however, some dispersion is also evident regarding the unaffected genotypes (yellow dots) and those that exhibited excellent performance (green dots). This might indicate that despite some contribution to yield responses, the seminal root system and adventitious roots are part of a more complex response to waterlogging tolerance (Figure 6.7).

6.3.2.2 Waterlogging at tillering stage in bread wheat genotypes with distinct phenological cycles may influence yield.

As mentioned in Chapter 4, different growth cycle lengths led to different stages of development being affected during the 14-day waterlogging, even though stress started at the same phenological state. This is highlighted in Figure 6.8 A, while in igure 6.8 B, those results are related to the observed yield impacts.

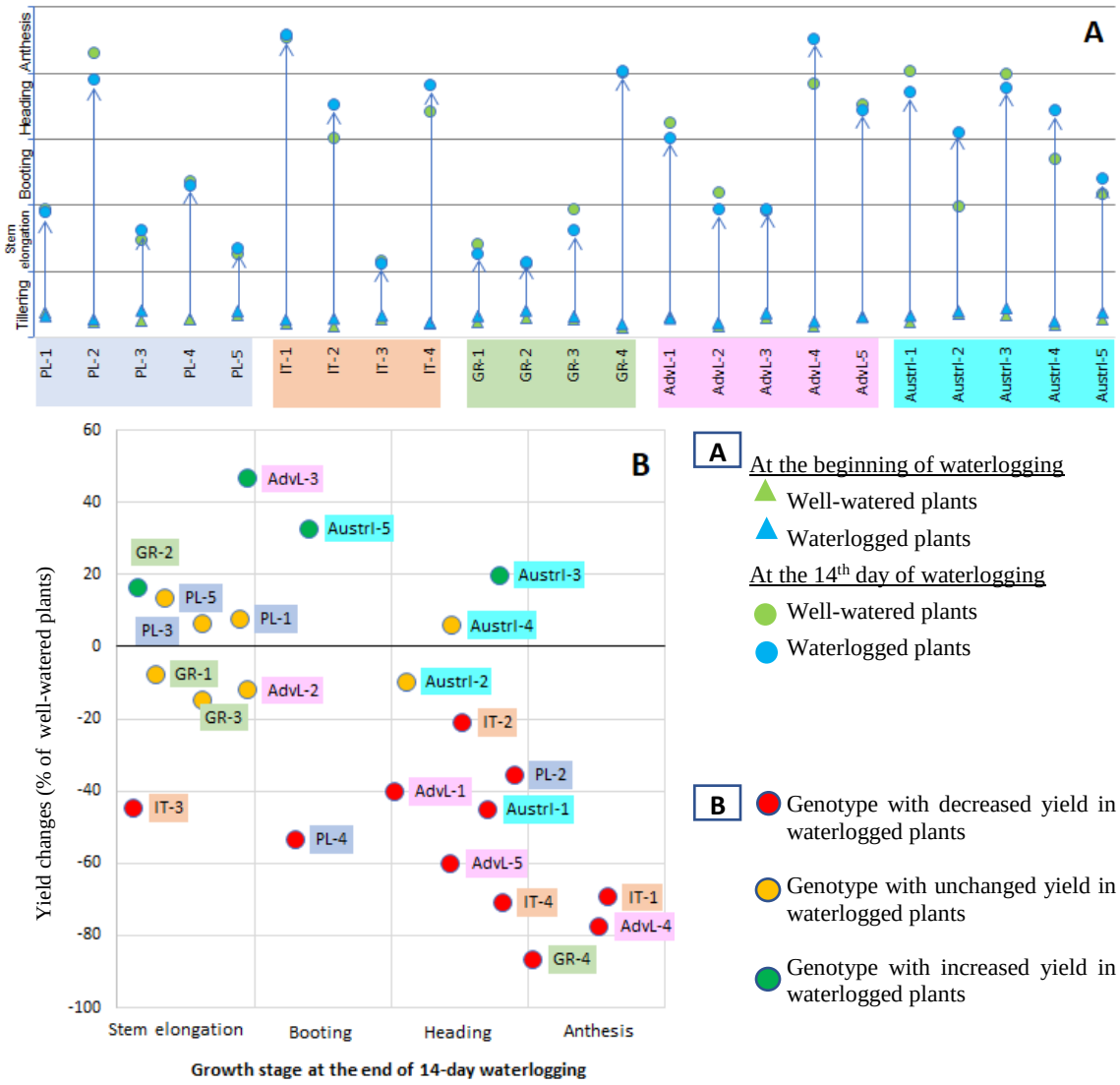


Figure 6.8 - Influence of the growth cycle length on yield changes of wheat plants exposed to 14-day waterlogging, imposed at the tillering stage. **A** – represents the progress, and thus the growth stage, achieved at the last day of water treatment in each genotype, in well-watered and waterlogged plants; **B** – scatter chart showing the yield impact in waterlogged plants compared with well-watered plants and the relation with the growth stage reached after 14-day waterlogging.

At the end of the waterlogging period, nine genotypes reached the stem elongation growth stage. Among these, only IT-3 experienced significant yield reductions (which accounts for 11% relative to the 9 genotypes). Austrl-5 and PL-4 have developed up to the booting stage; however, the latter experienced a significant yield decline. With a longer growth cycle, 9 genotypes reached the heading stage; 67% of these experienced yield losses, often very pronounced, as is the case with AdvL-5 and IT-4. Finally, on the last day of waterlogging, three genotypes were already in anthesis; these were the most affected, along with AdvL-5 and IT-4 (Figure 6.8 B). These results suggest that genotypes with longer growth cycles may be more tolerant to waterlogging at the tillering stage, and under the established environmental conditions. On the other hand, shorter cycles, and thus an advanced phenological stage at the end of stress, may experience stronger yield reductions. The Australian germplasm shows responses that don't completely align with the remaining groups, denoting their genetic background and the ongoing effort in waterlogging tolerance investment.

6.3.2.3 Analysed traits and their relation to yield

This section presents a principal component analysis (PCA) to determine which traits are the most strongly linked to the observed yield response to waterlogging, excluding those related to roots and ZGS, previously addressed in sections 6.3.2.1 and 6.3.2.2. Furthermore, the investigation also focused on identifying the strongest correlations among traits and determining if they change during waterlogging and recovery periods (Figure 6.9). The PC1 and PC2 accounted for 45.9% of the variance. In addition to PC1 and PC2 loadings, those of PC3 are also presented. PC 3 explained 8.1% of the variance, a similar value to PC2.

The traits that made the most significant influences to PC1 were those that were associated with photosynthetic pigments (SPAD, chlorophylls, and carotenoids), leaf gas exchanges (P_n, g_s, C_i, Tr), the photochemical efficiency of PSII (F_v/F_m, and F_v'/F_m'), green leaf area, levels of nitrogen, phosphorus, potassium, and iron in green leaves, as well as iron in senescent leaves, the content of raffinose, levels of C16:0 and C18:3, lipid unsaturation (DBI), lipoperoxidation (MDA), and membrane damage (I%). Regarding PC2, the variables that made the most significant contributions were the number of living tillers, the main culm height, the senescent biomass, and the total leaf biomass. The content of total soluble sugars as well as the sucrose, glucose and fructose were the variables that most contribute to PC3 (Figure 6.9).

Several variables had minimal representation in PC1, PC2, or PC3. These include the plants total leaf biomass at the end of the stress and after 7 days of recovery; the total leaf mass and senescence of the main culm in all observation periods; and the green leaf area of the main culm at the end of the recovery. Furthermore, the RWC and respiration rates, as well as aluminium, iron, and manganese in green leaves, did not significantly contribute to any of the PCs presented in all or the majority of the observations.

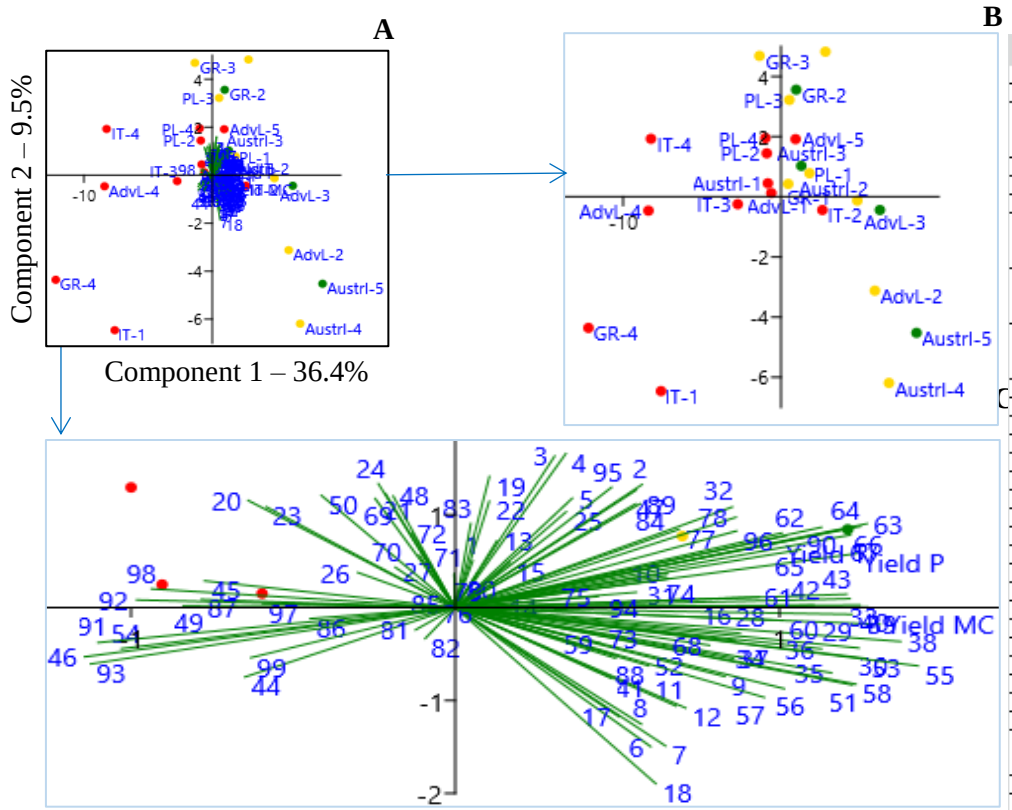


Figure 6.9 - Principal Component Analysis (PCA) was performed with all traits (except root and ZGS traits) analysed from the 7th day of waterlogging until harvest (A). The scatter plot shows the scores of the first to principal components. For a better visualisation, (B) shows the distribution of genotypes, where red dots corresponded to genotypes with decreased yield, green dots to genotypes with increased yield, and yellow dots to genotypes with unchanged yield. In (C), the PCA vectors from PC1 and PC2 are presented. The variables identification (by numbers) can be found in the correlation loadings table. Data was standardised prior to analysis.

PCA Loadings

		7th-day WL			14th-day WL			7th-day Recovery			14th-day Recovery			Harvest		
		PC 1	PC 2	PC 3	PC 1	PC 2	PC 3	PC 1	PC 2	PC 3	PC 1	PC 2	PC 3	PC 1	PC 2	PC 3
Yield	Plant													Yield P	0.8	0.2	0.0
	Main culm													Yield MC	0.8	-0.1	0.2
	Tillers													Yield T	0.7	0.3	-0.1
Tillers number		1	0.0	0.2	0.6	2	0.4	0.5	0.4	3	0.2	0.6	0.1	4	0.2	0.6	-0.1			
Kernel producing tillers																		5	0.2	0.6	-0.1
Main culm heigh						6	0.4	-0.6	0.2	7	0.4	-0.6	0.0	8	0.4	-0.5	0.3	9	0.5	-0.3	0.3
Foliar Biomass (Total)	Plant					10	0.4	0.1	-0.2	11	0.4	-0.4	-0.1	12	0.5	-0.4	0.3				
	Main culm					13	0.1	0.3	-0.3	14	0.1	0.0	0.0	15	0.1	0.2	0.2				
	Tillers					16	0.5	0.0	0.0	17	0.3	-0.5	-0.2	18	0.4	-0.7	0.2				
Foliar Biomass (Senescent)	Plant					19	0.1	0.5	-0.4	20	-0.4	0.5	-0.1	21	-0.1	0.5	-0.1				
	Main culm					22	0.1	0.4	-0.3	23	-0.4	0.4	0.0	24	-0.2	0.4	-0.1				
	Tillers					25	0.2	0.4	-0.4	26	-0.2	0.1	-0.1	27	-0.1	0.2	0.0				
Green foliar area	Plant					28	0.6	-0.1	0.3	29	0.8	0.0	-0.1	30	0.8	-0.2	-0.2				
	Main culm					31	0.4	0.1	0.2	32	0.5	0.4	-0.2	33	0.8	0.0	-0.4				
	Tillers					34	0.6	-0.1	0.4	35	0.7	-0.3	0.0	36	0.7	-0.2	-0.1				
SPAD	Main culm	37	0.6	-0.2	0.2	38	0.9	-0.1	-0.1	39	0.8	0.0	-0.2	40	0.8	0.0	-0.4				
Relative water content (RWC)						44	-0.4	-0.3	-0.4												
Membrane injury index (I%)						45	-0.5	0.1	0.0												
Palmitic acid (C16:0)						46	-0.7	-0.2	0.2												
Palmitoleic acid (C16:1)						47	0.4	0.5	0.0												
Stearic acid (C18:0)						48	-0.1	0.5	0.5												
Oleic acid (C18:1)						49	-0.5	0.0	0.3												
Linoleic acid (C18:2)						50	-0.3	0.5	0.0												
Linolenic acid (C18:3)						51	0.8	-0.3	-0.2												
Total Fatty Acids (TFA)						52	0.5	-0.2	0.0												
Lipids unsaturassion (DBI)						53	0.8	-0.2	-0.3												
Lipoperoxidation (MDA)						54	-0.7	-0.2	0.0												
Photosynthetic rate (Pn)						55	0.9	-0.2	0.0												
Stomatal conductance (Gs)						56	0.7	-0.3	-0.2												
CO_2 intracellular (Ci)						57	0.6	-0.4	0.0												
Transpiration rate (Tr)						58	0.8	-0.3	-0.2												
Respiration rate (Rd)						59	0.3	-0.1	0.3												
Chlorophyll content						60	0.6	-0.1	0.3												
Carotenoids content						61	0.6	0.0	0.1												
Photochemical eficiency of PSII	Fv/Fm					62	0.6	0.3	-0.1	63	0.8	0.3	-0.1	64	0.8	0.3	-0.3				
	Fv'/Fm'					65	0.7	0.2	-0.3	66	0.8	0.3	-0.3	67	0.8	0.3	-0.4				
Raffinose						68	0.6	-0.2	0.4												
Sucrose						69	-0.2	0.4	0.6												
Glucose						70	-0.1	0.2	0.7												
Frutose						71	0.0	0.1	0.7												
Total Soluble Sugars						72	-0.1	0.3	0.7												
Nitrogen	Green leaves					73	0.5	-0.2	0.3	74	0.6	0.1	0.4	75	0.5	0.0	0.3				
Phosphorus	Green leaves					41	0.3	-0.3	0.3	42	0.7	0.0	0.5	43	0.8	0.1	0.3				
Potassium	Green leaves					76	0.0	0.0	-0.4	77	0.5	0.3	-0.1	78	0.6	0.4	-0.1				
	Senescent					79	0.0	0.1	-0.3	80	0.0	0.1	-0.2	81	-0.1	-0.1	-0.5				
Alluminium	Green leaves					82	-0.1	-0.1	-0.1	83	0.0	0.3	0.0	84	0.4	0.4	0.4				
	Senescent					85	0.0	0.1	0.0	86	-0.3	0.0	-0.2	87	-0.5	0.0	0.2				
Iron	Green leaves					88	0.3	-0.2	-0.1	89	0.4	0.4	0.2	90	0.5	0.3	-0.1				
	Senescent					91	-0.7	-0.1	0.0	92	-0.6	0.0	0.0	93	-0.7	-0.2	-0.2				
Manganese	Green leaves					94	0.4	0.1	0.2	95	0.3	0.4	0.4	96	0.4	0.3	0.4				
	Senescent					97	-0.4	0.0	-0.1	98	-0.6	0.1	0.1	99	-0.5	-0.2	-0.1				

The vector analysis revealed a strong correlation between the plant overall yield, the main culm yield, and the total tiller yield. Additionally, strong correlations between RWC, I%, lipoperoxidation, C16:0, and C18:1 levels were also observed. The levels of manganese, iron, and aluminium also exhibit a correlation with these traits, but only during the recovery period. Furthermore, there was a negative correlation between these 8 traits and yield.

Throughout the study, photochemical efficiency of the PSII and SPAD were strongly related with yield. Several ecophysiological traits also showed the same trend at the end of waterlogging. The photosynthetic rate, stomatal conductance, CO_2 intracellular, transpiration rate, lipid unsaturation, linolenic acid, chlorophyll and carotenoids content, and raffinose all showed a positive correlation with yield. During the recovery period, strong positive correlations were observed between yield and the previously mentioned chlorophyll fluorescence (F_v/F_m, F_v'/F_m') and SPAD, as well as with the green leaf area and the levels of phosphorus and potassium in the green leaves.

Although there was variability in the influence of the studied traits on susceptibility or tolerance to waterlogging, this analysis reveals that membrane damage significantly affected most of the genotypes that experienced yield losses. This was evident in the membrane injury index (I%), the production of MDA (indicating lipoperoxidation and oxidative stress), an increase in senescent leaf mass, and the accumulation of sugars (except raffinose) in the leaves. On the other hand, the performance of photosynthetic processes, the preservation of photosystem II, the maintenance or increase of photosynthetic pigment levels, the presence of higher raffinose levels, and the levels of nitrogen and phosphorus in the leaves, strongly influenced most of the genotypes that showed stress tolerance, maintaining or even increasing yield.

6.3.2.4 Pinpointing effective yield indicators for stress and recovery periods

Among the parameters that showed significant correlations (Pearson linear correlation with $p<0.05$), it will be important to identify those that could serve as effective indicators for screening. The table below presents the respective correlation coefficients (r), highlighting their relationship with the observed impacts on yield in response to 14 days of waterlogging initiated at tillering stage. Correlation values (r) will be presented for both the parameters assessed during the stress period and those measured in the recovery phase, aiming to clarify which are more suitable for screening in each phase (Table 6.1).

Among the parameters with significant r values ($p<0.05$) during the waterlogging period, only SPAD showed a positive correlation with yield on the 7th day of stress. This correlation was excluded from the table 6.1, as its value aligns with that of the 14th day of stress ($r = +0.6$). All other values refer to the final day of waterlogging.

During waterlogging period, the strongest correlation was observed between yield and lipid unsaturation, reinforcing the findings from Chapter 5, which indicated that genotypes capable of increasing lipid unsaturation achieved higher yields under stress conditions. Conversely, a lower content (mol%) of palmitic acid (C16:0) strongly correlated with better performance. Similarly, higher levels of photosynthetic pigments, improved photosynthesis rates, higher SPAD values, lower lipid peroxidation, and reduced membrane permeability were associated with less impact on yield (Table 6.1).

During the recovery period, no measurements were taken for gas exchanges, membrane damage, lipid and sugar metabolism, or laboratory quantification of photosynthetic pigments. The photochemical efficiency of PSII showed strong values (r = +0.8) on both the 7th and 14th days after the stress ended. Additionally, SPAD values and yield exhibited a correlation of r = +0.7, while leaf phosphorus (P) and nitrogen (N), as well as the amount of green leaf area were positively correlated (r = +0.6) with yield (Table 6.1). Conversely, genotypes that showed the greatest increase in iron content in senescent leaves exhibited a negative correlation (r = -0.7) with yield, indicating reductions in yield (Table 6.1).

Table 6.1 - Pearson correlation coefficients (r) of the significant values (p<0.05) between the evaluated traits and yield under 14 days of waterlogging initiated at tillering stage. The table includes correlation values for both the stress period and the recovery phase, highlighting the parameters that may serve as potential indicators for effective screening in each stage.

Waterlogging period	
Yield *versus*	*r*
Lipids unsaturation	0.8
Chlorophyll content	0.7
Carotenoids content	0.7
Photosynthesis rate	0.7
Linolenic acid	0.7
SPAD	0.6
Transpiration rate	0.6
Stomatal conductance	0.6
Raffinose	0.6
Total fatty acids	0.6
Green area -Main culm	0.6
Palmitoleic acid	0.6
Intracellular CO_2	0.5
F_v/F_m	0.5
F_v'/F_m'	0.5
Living Tillers	0.5
Senescent biomass - Tillers	-0.4
Mn in senescent leaves	-0.4
Fe in senescent leaves	-0.6
Membrane permeability	-0.6
Lipoperoxidation	-0.7
Palmitic acid	-0.8

Recovery period		
Yield *versus*		*r*
F_v/F_m	T7R	0.8
F_v/F_m	T14R	0.8
F_v'/F_m'	T7R	0.8
F_v'/F_m'	T14R	0.8
SPAD	T14R	0.7
SPAD	T7R	0.7
Green area - Plant	T7R	0.6
P in green leaves	T14R	0.6
N in green leaves	T7R	0.6
P in green leaves	T7R	0.6
Green area - Tillers	T7R	0.6
Fe in green leaves	T14R	0.6
Green area - Plant	T14R	0.5
Green area - Tillers	T14R	0.5
Green area - Main culm	T14R	0.5
K in green leaves	T14R	0.5
Green area - Main culm	T7R	0.4
Al in senescent leaves	T14R	-0.4
Mn in senescent leaves	T7R	-0.4
Fe in senescent leaves	T7R	-0.6
Fe in senescent leaves	T14R	-0.7

6.4 Discussion

Significant yield losses in waterlogged plants ranged from 21.0% to 86.6%. Several studies reported similar decreases, ranging from *ca*. 30% to 90% (Collaku and Harrison, 2002; Olgun *et al*., 2008; Amri *et al*., 2014; de San Celedonio *et al*., 2014; Ploschuk *et al*., 2018). The average size of single kernels, as determined by their dry mass or weight, and the number of kernels per plant or unit area are the two primary factors that influence wheat yield (Nóia Junior *et al*., 2023). In turn, the number of spikes per plant and kernels per spike condition the total number of plant kernels. Even so, the spike

number is strongly dependent on the fertile tiller amount. This study observed a large variability in the number of fertile spikes in both WW and WL plants (Chapter 4, Section 4.3.3). Yield decreases were observed in 11 genotypes and appear to be closely linked to all tillers death (GR-4, AdvL-1, AdvL-4, and AdvL-5) or decreased tillers survival (PL-3, PL-4, Austrl-4; IT-1, IT-2, IT-3, and IT-4). Poor growth or/and an increased abortion cause a decreased tiller survival, which in turn led to a reduced spike number. This reduction in spikes has already been linked with yield reductions due to waterlogging (Malik *et al*., 2001, 2002). Still, tiller number is the yield component that exhibits the greatest degree of plasticity (Slafer *et al*., 2014), and environmental constraints can affect its emergence, development, senescence, and fertility (Robertson *et al*., 2009; Hossain *et al*., 2011; Shao *et al*., 2013; de San Celedonio *et al*., 2016; Malik *et al*., 2022). Moreover, cereals often counterbalance yield components (Sharma *et al*., 1995), and the observed rise in spikes per plant in GR-3, Austrl-2, and Austrl-5 could be a result of the observed enhanced tillering, acting as a compensatory mechanism for adverse climatic conditions, similar to the traits exhibited by some cultivars highly acclimated to the Mediterranean climate (Elhani *et al*., 2007; Acevedo, 2002). Waterlogging led to substantial reductions in kernel number per plant in 13 genotypes across all examined groups. In 10 of these genotypes, these declines were consistent with the drop in the number of spikes, which can be attributed to a fall in tiller number (Chapter 4). Several authors referred that kernel number per plant can be the yield attribute most affected by waterlogging (Collaku and Harrison, 2002; Malik *et al*., 2002; Hossain *et al*., 2011; Marashi *et al*., 2010). Despite an increase in spike number in Austrl-2 and stability in Austrl-1, waterlogging led to reductions in the number of kernels per plant in these genotypes, suggesting a decrease in spikelet and/or floret fertility. Conversely, an increased kernel number per plant in WL plants of GR-2, AdvL-3, and Austrl-5 did not necessarily results from increased spikes per plant, as the end of the growing cycle revealed a stable number in the first two genotypes and an increased number in the latter. While tillering reduction is a major factor limiting yield, other factors may also affect yield in waterlogged plants (Collaku and Harrison, 2002; Malik *et al*., 2002). In our study, some genotypes showed reduced, increased, or unaffected kernel number per spike. These results support several authors findings that waterlogging decreases kernel number per spike (Robertson *et al*., 2009; Marashi *et al*., 2010; Amri *et al*., 2014; Martin *et al*., 2015; de San Celedonio *et al*., 2018) or wheat plants compensation capacity to increase it (Slafer *et al*., 2014). Some genotypes can cope with waterlogging, displaying no changes in this trait in stressed plants (Araki *et al*., 2012; Amri *et al*., 2014). We may also link our findings on waterlogging tolerance to genotype differences in the growth cycle duration. Even though all genotypes were at the same phenological stage at the beginning of the treatment (tillering, between ZGS 22 and ZGS 25), plant development may affect the number of days subjected to waterlogging during the period in which the number of kernels per spike is established (20-30 days prior and 10 days after anthesis) (Fisher, 1975; Hawkesford *et al*., 2013). During this time, floret primordia differentiate, some degenerate and die, and the number of surviving florets determines the kernels per spike (Hawkesford *et al*., 2013; de San Celedonio *et al*., 2018). Previous research found that waterlogging during stem elongation (ZGS 30-39) reduced spike growth before anthesis (ZGS 60-69) with a concurrent reduction in the number of fertile florets (Hossain *et al*., 2011; Marti *et al*., 2015). In our study, genotypes with a shorter growth cycle and, as a

result, suffering stress in more advanced phenological stages showed decreased kernel per plant, with the majority depicting reductions in both the main culm and tillers kernel number per spike.

Several authors studies in wheat, reported that single kernel weight (SKW) had less influence of has on yield (Collaku and Harrison, 2002; de San Celedonio *et al*., 2014) and changes due to waterlogging were less severe than those observed in the remaining yield traits (Collaku and Harrison, 2002). However, our findings suggested that 14 days of waterlogging beginning at the tillering stage significantly influenced some genotypes single kernel weight, with decreases and/or increases in the single kernel weight of the main culms spikes as well as from tillers. Declines in this trait due to waterlogging were previously reported (Zhang *et al*., 2006; Olgun *et al*., 2008; Shao *et al*., 2013; de San Celedonio *et al*., 2018), although the effects were not distinguished between tillers and main culm. As waterlogging can lead to nutrient deficiency (Ashraf, 2012; Herzog *et al*., 2016), sensitive genotypes may lack resources for the spike growth and kernel filling (Gonzaléz *et al*., 2011; Marti *et al*., 2015), lowering SKW.

In this study, changes in main-culm and tiller yield were caused by variations in one or more yield traits. This is consistent with previous findings that lower tillering or increased tiller mortality reduced spike number per plant (Collaku and Harrison, 2002; Condon and Giunta, 2003; Robertson *et al*., 2009; Amri *et al*., 2014; Wu *et al*., 2015; de San Celedonio *et al*.,2016; Langan *et al*., 2022), and increased floret abortion and/or infertility reduced kernels per spike (Collaku and Harrison, 2002; Olgun *et al*., 2008; Robertson *et al*., 2009; Marashi *et al*., 2010; Amri *et al*., 2014; de San Celedonio *et al*., 2014; Ding *et al*., 2020). In addition, lighter kernels were also found (Olgun *et al*., 2008), mostly due to reduced size or/and inadequate filling related to reduced stored assimilates and poor culm-to-kernel carbohydrates remobilization (Collaku and Harrison, 2002; Zhang *et al*., 2006; Olgun *et al*., 2008; Araki *et al*., 2012). Our results point to decreases in the main-culm yield of 7 genotypes, due to a reduction in the kernel number per spike (IT-3, GR-1, GR-3, and Austrl-2) or in SKW (IT-1, T-2, and AdvL-4) or both (IT-4). In the dynamics of total tiller participation to yield, the final tiller yield was significantly affected by the number of fertile ones. Seven of the nine genotypes with declining tiller yield had fewer spikes per plant, denoting a reduction in tiller number. However, decreases found in tiller yield were also due to a conjugation of less spikes per plant with lower kernels per spike (PL-4), or the conjugation with lower SKW (IT-1).

Among genotypes with incremented tiller yield under waterlogging, GR-2 and Advl-3 had an enhanced kernel number per spike, which is consistent with the wheats capacity to increase kernel number per spike (de San Celedonio *et al*., 2014) since spikes can differentiate up to 9-10 floret primordia per spikelet (González *et al*., 2003). The increased main-culm yield in AdvL-3 was due to single kernel weight.

Our findings pointed to ten genotypes that showed great potential. Among them, eight did not show changes in the yield of the main culm and tillers (PL-1, PL-5, GR-1, GR-3, AdvL-2, Austrl-2, Austrl-4, and Austrl-5). GR-2 exhibited a rise in tiller yield, and AdvL-3 showed an increase in both the main culm and tiller yield. Overall, results also suggested that a lower growth rate seems to have a beneficial effect, as all of the promising genotypes reached stem elongation or booting by the end of

waterlogging period. Moreover, genotypes with a faster rate of development showed the most significant negative impacts, with some cases achieving anthesis at the end of stress.

Photosynthesis is the basis of primary production, directly linked with plant biomass production, including grains. Waterlogging decreases photosynthetic performance, reducing the synthesis of assimilates required for plants metabolic functions and for grain filling. Despite the P_n decline in the majority of genotypes, our findings showed an accumulation of sugars in the leaves of all of them (except in AdvL-3 and Austrl-5), suggesting a potential constraint in the use and translocation of these assimilates to the productive organs. On the other hand, the pronounced accumulation of sucrose, glucose, and fructose may be also a sign of leaf senescence (Li *et al*., 2019). The protective function of raffinose in stabilizing proteins and membranes and its increased content in waterlogged plants may justify the positive correlation to yield; however, it may incur a metabolic cost, as the allocation of this resource for protective mechanisms detracts from growth or grain filling. The robust correlation between chlorophyll *a* fluorescence traits (F_v/F_m and F_v'/F_m') and yield emphasises its connection with photosynthetic activity. Impairments to PSII, which is critical for photosynthesis, lowers photosynthetic capacity, resulting in a decrease in primary production and, ultimately, in the final grain yield. In short, impaired PSII, prevents the plant from efficiently utilizing light, resulting in a decreased growth rate and productive potential. The strong correlation between grain yield and photosynthetic pigments reflects their indispensable role in driving light energy into photoassimilates.

Waterlogging may reduce chlorophylls and carotenoids, decreasing plants ability to capture light energy, which subsequently may result also in decreased photosynthetic activity. And lower photoassimilate production, ultimately, contributing to reduce grain yield.

The significant link between grain yield in waterlogged plants and the membrane damage indicators underscores the importance of membrane integrity and plasticity in resilience to environmental stresses. Membrane integrity is crucial for sustaining cellular metabolism and photosystem function. Damage to the membranes, particularly the thylakoid membranes of the chloroplast, undermines photosynthesis and explains the negative correlation between grain yield, the membrane damage index (I%), and the MDA content, a by-product of lipid peroxidation. These correlations highlight the importance of photosynthetic processes for agricultural productivity and that the use of photosynthetic related parameters can be crucial for selecting stress tolerant varieties.

6.5 Conclusions

Bread wheat genotypes exhibited a range of yield-related responses to a 14-day waterlogging during the tillering stage. The stress impacted several yield traits including the number of spikes and kernel number per plant, the main culm and tillers kernels per spike and, the weight of individual kernels. The number of spikes per plant changed significantly due to tillering decreases or increases. While variability in these traits was observed both within and between germplasm groups, the Italian group was the most adversely affected. Among the remaining groups, different responses were found, with some genotypes exhibiting negative or positive effects, while others were unaffected.

Despite the stress imposed at the same growth stage, the variability in developmental rates, as a consequence of different growth cycles, resulted in diverse impacts. The results indicated that for most genotypes, fewer stress-covered developmental stages (longer cycles) are associated with stable or improved yields. Our study identified six out of eight genotypes that reached the stem elongation stage, and four out of six that reached the booting stage as particularly promising. Additionally, strong correlations between several traits, such as SPAD and chlorophyll fluorescence, were observed during both the stress and the recovery periods. The seminal root system, specifically the root growth angle, exhibited a moderate correlation with yield, suggesting that it may not be a reliable tool alone for assessing waterlogging tolerance in bread wheat.

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7 FINAL REMARKS AND FUTURE PERSPECTIVES

This study revealed that bread wheat tolerance to waterlogging conditions, initiated during the tillering stage, involves a variety of complex plant impacts and responses influenced by several factors.

The analysis of the seminal root system uncovered significant variability among the germplasm under investigation, highliting its complexity.

The selection of tolerant or sensitive genotypes cannot solely rely on the root growth angle, given the observed moderate correlation between RGA and yield. However, clustering analysis of seminal root system revealed some degree of similarity between the majority of genotypes that experienced adverse yield effects due to waterlogging and all the seminal root characteristics analysed (Chapter 3; Chapter 6). Despite this, other traits must also be involved, as cluster 1 and 3 gouped genotypes based on their similarity in the seminal root system, yet they showed differences in yield responses.

This study also highlights the profound impact of waterlogging on both soil conditions and plant responses. The rapid decrease in soil redox potential triggered a shift from aerobic to anaerobic conditions in the rhizosphere, likely affecting root system metabolism. These changes impaired nutrient uptake and can led to morphological alterations, such as adventitious roots emission. The ability to produce these roots, particularly in the PL and Austrl germplasm groups, suggests potential for mitigating some of the negative effects of waterlogging, as these roots are known to promote gas exchange, as well as nutrient and water uptake. Three of the five genotypes in the PL group (PL-1, PL-3, and PL-5) and four of the five genotypes in the Austrl group (AdvL-2, Austrl-3, Austrl-4, and Austrl-5) were able to reach maturity with the same or even higher yield. However, the impacts of waterlogging were not confined to the root system. The above-ground organs also showed significant changes. For instance, the majority of genotypes presenting yield losses also exhibited significant decreases in the number of living tillers at the end of waterlogging period and/or during recovery, along with reduced tiller fertility.

The poor root function and inefficient water and nutrient transport likely accelerated the natural ageing process of leaves and other plant organs. Senescence was evident throughout the entire plant, beginning with the basal leaves, which showed early signs of yellowing associated with chlorophyll degradation. The greatest impact of waterlogging, however, was observed in the increased senescent leaf mass, with tillers contributing more significantly than the main culm. This suggests that tillers, which are often more vulnerable to environmental stress, exhibit a more pronounced senescence impact, likely due to their secondary role in nutrient allocation compared to the main culm. The reduced oxygen availability to the roots compromised cellular respiration, while the photosynthetic activity (P_n) declined further limited energy supply, restricting the overall metabolic activity. Additionally, waterlogged plants showed reductions in green leaf area, which is crucial for photosynthetic activity. In most genotypes, this decrease in green area was accompanied by a decline in SPAD values (a measure of chlorophyll content) in tissues that remain visually green. This suggests that even the leaves that appear healthy may be experiencing a decline in chlorophyll levels, which might negatively impact the performance of the

photosynthetic apparatus.

The lack of oxygen in the roots may result in oxidative stress in the plant above-portion, triggering excessive production of highly reactive species of oxygen (ROS) and chlorophyll. These reactive molecules, particullarly ROS, can damage cellular components, most notably through lipoperoxidation which primarly affects the polyunsaturated fatty acids in cell membranes. Nevertheless, these fatty acids are also involved in the production of signalling molecules that help regulate stress responses, including those related to environmental and oxidative nature. In plants that performed better under waterlogging, this study observed increased lipid unsaturation, which could potentially contribute to improved tolerance at the leaf level. Notably, a positive correlation between yield and the degree of unsaturation (DBI) of leaf membrane lipids supports the idea that greater lipid unsaturation could be beneficial under waterlogging conditions.

In addition to lipoperoxidation, waterlogged conditions lead to alterations in nutrient uptake, particularly nitrogen, which is critical for maintaining chlorophyll content and delaying leaf senescence. A reduction in nitrogen availability, coupled with imbalances in other essential nutrients like phosphorus and potassium, can hasten chlorophyll degradation and impair photosynthesis, further exacerbating senescence. The majority of negative impacts on production were also accompanied by decreases in nitrogen content in the green leaves, alongside with reductions in SPAD values, especially at the end of the waterlogging period or during the recovery phase. Therefore, such decrease in nitrogen content, chlorophyll levels and overall leaf health, further contributing to the accelerated senescence observed.

Other useful parameters for evaluating waterlogging impacts and tolerance include SPAD values and chlorophyll *a* fluorescence, which showed strong positive correlations with wheat yield during the waterlogging period and afterwards, along the recovery period, thus highlighting them as reliable, non-destructive, and efficient methods for assessing plant health and productivity. Moreover, these findings were consistent with biochemical analyses such as MDA (malondialdehyde) content and the membrane injury index, which also showed strong (but negative) correlations with yield. However, these methods require laboratory procedures and destructive leaf analyses. These relationships with yield reinforce the notion that leaf membrane stability is a key factor in maintaining productivity under the imposed water stress conditions. It is important to note that these significant relationships, along with studies on gas exchanges, lipid remodelling, leaf sugar content, chlorophyll, and carotenoid content, among others, were obtained even when analysis were conducted only at the end of waterlogging period. Thus, their use should also be extended to the recovery period to confirm their accuracy throughout the post-stress period. Additionally, gas exchanges were measured only in the main culm.

Some genotypes (Austrl-3, AdvL-2, AdvL-3, and Austrl-5) have shown an increase in yield, which could potentially be due to factors beyond the traits measured at the end of waterlogging in the main culm. For example, Austrl-3 showed lower P_n, F_v/F_m and F_v'/F_m' values but it kept the same number of fertile tillers, foliar biomass, kernels per plant and per spike, and nitrogen content. It also showed a trend towards higher single kernel weight, which supported its higher yield. AdvL-2 and AdvL-3 maintained P_n, increased g_s, C_i, Tr, and nitrogen content, while Austrl-5 showed increased yield through increased g_s and C_i despite decreases in P_n and chlorophyll content. This suggests that the ability to

maintain or even enhance production under waterlogging may involve a combination of physiological impacts and responses, including the maintenance of productive tillers, biomass, and nutrient status, which help offset the impacts of stress on photosynthetic performance.

As regards mineral nutrients, increased iron levels were observed in the senescent leaves of waterlogged plants, a pattern noted across almost all genotypes. This accumulation of iron could be a consequence of disrupted nutrient uptake and transport under waterlogged conditions, potentially contributing to the advanced senescence in these plants. Sporadic increases in manganese and aluminium levels were observed, though their role in senescence remains unclear and less significant in this study.

Overall, the findings from this study offer significant insights into the morphological and ecophysiological responses of wheat genotypes under waterlogging stress. However, several areas warrant further investigation to enhance our understanding and improve agricultural practices. In this context, among the future perspectives, one promising direction will be the incorporation of advanced molecular tools, such as molecular markers, to identify and select genotypes with superior tolerance to waterlogging. Such tools would help to breed more resilient wheat varieties by targeting specific stress-related traits/genes/proteins more precisely. Moreover, expanding the scope of morphological and physiological analyses will be crucial. Future studies should aim to monitor the antioxidant defence system of plants in more detail. Quantifying the activity of key antioxidant enzymes, such as superoxide dismutase, catalase, and peroxidases, can provide a clearer picture of how plants mitigate oxidative damage during both stress and recovery periods. This could lead to the development of new strategies to enhance plant resilience. In addition, hormonal regulation plays a central role in plant responses to waterlogging. Future research should explore the roles of both abscisic acid (ABA) and ethylene in stress tolerance. ABA and ethylene are involved in plant responses to water stress, as well as the formation of root aerenchyma. Investigating the interactions and balance between these two hormones during waterlogging and recovery phases could reveal how different wheat genotypes trigger these signalling pathways to enhance survival and recovery. Thus, the study of potential aerenchyma development in wheat root systems could be of key importance, since this tissue facilitates internal oxygen transport under waterlogging conditions and may play a critical role in genotype-specific tolerance.

It would also be beneficial to extend the monitoring of gas exchange and the biochemical analyses beyond the stress phase to include the recovery period. Such data would help clarify the dynamic of photosynthetic recovery and assess whether certain genotypes are capable of returning to optimal performance more efficiently than others after the stress ending. This would require a more continuous and longitudinal approach to data collection, enabling a better understanding of the transition from stress to recovery.

Beyond waterlogging, future research should also investigate the interaction between waterlogging stress and other emerging environmental stresses, such as elevated CO_2 levels and rising temperatures. The ongoing effects of climate change are likely to result in more complex environmental stresses on crops. Studying the combined impact of these stresses could provide critical insights into

how wheat might perform under future climate conditions.

Molecular approaches, physiological measurements during stress and recovery periods, advanced phenotyping technologies, as well as the use of machine learning and Intelligent Data-Driven Support for Agricultural Systems, will all need to bring knowledge to integrated platforms that could boost the development of wheat varieties that perform better in waterlogged and other stressful environments. Therefore, such integration of different levels of knowledge in this future research could provide valuable insights into broader plant stress physiology, helping to establish new crop management strategies that can mitigate the current and future impacts of climate change on agricultural productivity.

EXPLORING BREAD WHEAT DIVERSITY IN WATERLOGGING RESPONSES: ROOT SYSTEM, PLANT DEVELOPMENT, ECO-PHYSIOLOGICAL TRAITS AND YIELD IMPLICATIONS

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2024