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DEGLI STUDI DELLA
TUSCIA

Università degli Studi della Tuscia di Viterbo

Dipartimento di Scienze Agrarie e Forestali

**PhD course in Plant and Animal Science
XXXV cycle**

Analysis of the response to heat and water-deficit stress
in durum wheat introgression lines
with segmental transfers from wild *Thinopyrum* species

s.s.d. AGR/07

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Academic Year 2021/2022

*Dedicato a voi,
che credevate nelle mie capacità
molto più di quanto ci abbia mai creduto io.*

*Alle mie nonne,
esempio di pazienza,
dolcezza ed amore incondizionato.
Nel mio cuore, per sempre.*

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Chapter 1: General Introduction

Chapter 1: General Introduction

1.1. An overall perspective of durum wheat cultivation

1.1.1. Wheat production – a focus on the Mediterranean basin

Cereals, including wheat, rice and maize, play a fundamental role in the human diet, providing roughly 50% of the caloric intake via feed grains (Fischer et al., 2010). Above all wheat is a major staple crop worldwide and its production is continuously increasing, reaching a total of 760.93 million tonnes (t) in 2020 (**Figure 1.1**) The European area, with a total production of 255.05 million t, is one of the main wheat top producers in the world. Durum wheat (DW, *Triticum durum* L., $2n = 4x = 28$) represents about 8% of the global wheat production (about 38 million t over almost 17 million hectares in 2019), with the European Union as the leading global producer, followed by Canada, Turkey, United States, Algeria, Mexico, Kazakhstan, Syria, and India (Xynias et al., 2020). DW is mainly grown in the regions around the Mediterranean basin, providing important cultural and commercial benefits to the whole area (Zampieri et al., 2020). The Mediterranean zone provides about 60% of world DW production, with major contributions of Italy, Spain, France and Greece (Lidon et al., 2014). Among these countries, accounting for an average production of 4.26 million t in the last decade, grown in about 1.2 million hectares, Italy is considered the leader of DW production and consumption, the end products being deeply connected with the local tradition (Fontana et al., 2015; Xynias et al., 2020).

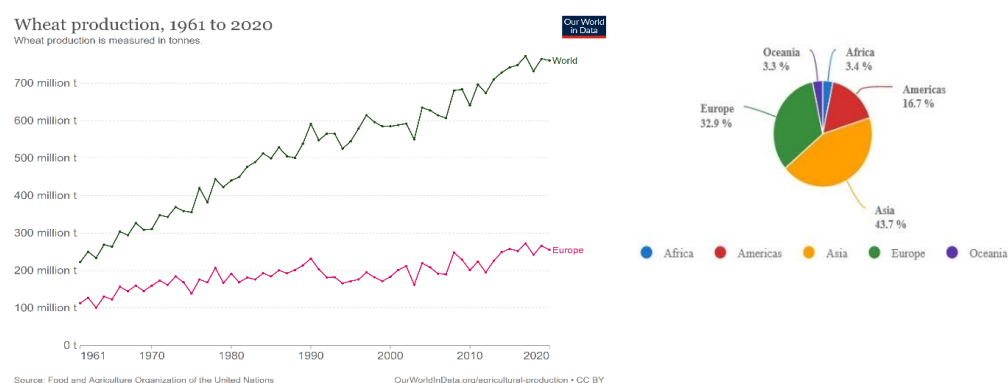


Figure 1.1. Overall wheat production. On the left a line chart representing the wheat production in the period between 1961 and 2020 in the world (green line) and in Europe (violet line) (<https://ourworldindata.org>); on the right a pie chart representing the portioning of wheat production among the main world's areas (<https://www.fao.org/faostat/en/>).

The major end products of DW consist of pasta, noodles, couscous and bread, all of them being historically key foods and a fundamental part of Mediterranean cuisine (Martínez-Moreno et al., 2020). DW semolina is characterized by higher protein content, stronger gluten, lower glycemic index and longer durability than bread wheat flour, and these represent crucial properties to make a good quality pasta (Zampieri et al., 2020). In fact, DW success among the countries of Mediterranean basin is not only due to its better adaptability to the semi-arid local climate than bread wheat, but also to its gluten protein fraction, which confers the dough with the essential viscoelastic properties for making of the above mentioned end products (Shewry, 2009).

1.1.2. Durum wheat way: from the onset until modern era

Wheat, in the form of allotetraploid wild emmer, *T. turgidum* subsp. *dicoccoides* (Körn. ex Asch. and Graebner) Thell, originated approximately 800,000 years ago and was domesticated about 10,000 years ago in the Fertile Crescent, an area located in Western Asia that nowadays includes Israel, Jordan, Lebanon, Syria, south-eastern Turkey, northern Iraq and western Iran (Marcussen et al., 2014; Levy and Feldman, 2022). Wheat was the main staple crop that accompanied the birth of Western civilization (Zampieri et al., 2020), being a central part of the ‘Neolithic Revolution’, i.e. the transition from hunting and collecting of food to settled agriculture (Shewry, 2009). The primitive form of wheat domestication was carried out via the first selections from populations of wild emmer. The elements of selection were based primarily on yield, but also on other characteristics, such as non-brittle rachis and free-threshing naked seeds (Lidon et al., 2014; Xynias et al., 2020; Martínez-Moreno et al., 2020). This first form of selection gave rise to cultivated emmer (*T. turgidum* ssp. *dicoccon* (Schrank) Schübl), which was the main cereal of the Neolithic period until it was replaced by free-threshing and naked forms of DW, originated from a selection of hulled tetraploid wheats similar to emmer (Martínez-Moreno et al., 2020; Xynias et al., 2020). Starting from the Fertile Crescent, DW spread out in all directions: through the Mediterranean Sea, thanks to Phoenicians, Greeks, and above all Romans, and through the Balkans, thanks to the early Arab Empire, that expanded DW cultivation and promoted food types made with semolina (Martínez-Moreno et al., 2020). Heterogeneous landraces of wheat emerged until the 18th century, as a result of DW adaptation to new environmental conditions in various geographical areas and human selection based on favourable phenotypic traits. However, a significant increase in the DW production occurred

in the last decades of the 19th century when a few breeding programs started in Europe following the rediscovered of Mendel's laws. These set up the practice of scientific breeding, based on the crossing of two or more varieties with contrasting characteristic and selection of the best individuals from the segregating populations. During these years the main DW breeding program was carried out by Nazareno Strampelli ("CREA-Centro di Ricerca Cerealicoltura e Colture Industriali", Italy), with the initial release (1915) of the cultivar Senatore Cappelli, obtained through genealogical selection within the North-African landrace Jeanh Rhetifah and widely used in crosses. Strampelli is considered the pioneer of the modern DW breeding and his work gave rise to the so-called "Green Revolution", that was then engineered at the International Maize and Wheat Improvement Center (CIMMYT), where new varieties were released for developing countries from the 1960s onward (Martínez-Moreno et al., 2020; Xynias et al., 2020; Martínez-Moreno et al., 2022). The Green Revolution was one of the most important events in the history of agriculture, since a new generation of semi-dwarf, high yielding and disease resistant wheat cultivars has been released. In this way, a remarkable yield increase was achieved in many national breeding programs during the second half of the 20th century. This was mainly attributable to the introduction of semi-dwarf genes into bread wheat, which resulted in the replacement of tall cultivars with shorter ones, characterized by resistance to lodging and responsiveness to high inputs (Xynias et al., 2020). Another key event in the evolution of wheat breeding was the foundation of the International Centre for Agricultural Research in the Dry Areas (ICARDA) in 1977 in Syria, which focused on DW breeding, providing innovative, science-based solutions for countries across non-tropical dry areas (<https://www.icarda.org/>). Thanks to all the activities above mentioned, between 1961 and 2014 world grain production was increased by 227% (of which + 161% due to yield increase and +31% due to increased crop surface; Fischer et al., 2018). However, in many cases the overall increased performance of the cultivated varieties was attributable to the application of high inputs (e.g. pesticides, fertilizers, irrigation); moreover, in the last decades a limited number of traits has been exploited in new crop selection. Unfortunately, both domestication and Green Revolution activities caused substantial genetic erosion of the wheat genetic basis, with loss of several traits of interest that could be useful in the perspective of the new breeding objectives. A recent work by Maccaferri et al. (2019) confirmed how thousands of years of empirical selection and breeding imposed several modifications and restrictions in DW genome, the strongest reduction in DW genetic diversity having occurred since the domestication of wild emmer wheat. Since then, the reduction of diversity continued more moderately, but spread all over the genome, during the evolution of DW landraces and, more recently, as a consequence

of intense breeding activity from the onset of Green Revolution onward. As a consequence, nowadays the wheat breeding strategies are aimed at not only maintaining but widening the crop genetic basis in order to better cope with the new global agricultural challenges.

1.1.3. New market demands to cope with food security goals

In 2015, the United Nations General Assembly (UNGA) formulated, as part of the Post-2015 Development Agenda, 17 Sustainable Development Goals (SDGs; **Figure 1.2**), aiming to create a future global development framework. The 17 SDGs were formally adopted in a UNGA resolution called “2030 Agenda” and in 2017 were made more actionable through the identification of specific targets and indicators to measure the progress of each SDG. Most targets are planned to be achieved by 2030, although some have no end date.



Figure 1.2. The 17 Global Goals for a sustainable development (<https://www.globalgoals.org/>).

Among the SDGs, the ZERO HUNGER objective (No. 2) consists of the achievement of food security, the improvement of nutrition and the promotion of sustainable agriculture. Main targets of this key global goals are to guarantee the access to safe and nutritious food, to end all forms of malnutrition, to double the productivity of small-scale food producers, to ensure sustainable food production systems and to maintain the existing genetic diversity. It is evident how the ZERO HUNGER objective is closely connected with the achievement of other relevant SDGs, such as the RESPONSIBLE CONSUMPTION AND PRODUCTION (No. 12) and the

CLIMATE ACTION (No. 13), this underlining the need for a synergistic and multi-approach action. As reported above (see ahead, §1.1.1.), wheat is the leading source of plant protein and calories in the human diet and its proper production and quality is strictly connected with the achievement of food security goals. However, many future challenges need to be overcome in order to increase both wheat yield and nutritional quality.

First of all, currently there is a pressing need to increase overall crop production so as to feed a continuously growing world population (**Figure 1.3**). In fact, it is predicted that about 198 million t of additional wheat grain (corresponding to a yield increase of 60-70%) will be required to feed a world population of 9.8 billion people by 2050 (Ray et al., 2013; Jacott and Boden, 2020; Yadav et al., 2022). This yield increment is needed both to feed huge national populations and to provide the export of staple grains that support the food-deficit regions of the world (Fischer et al., 2018).

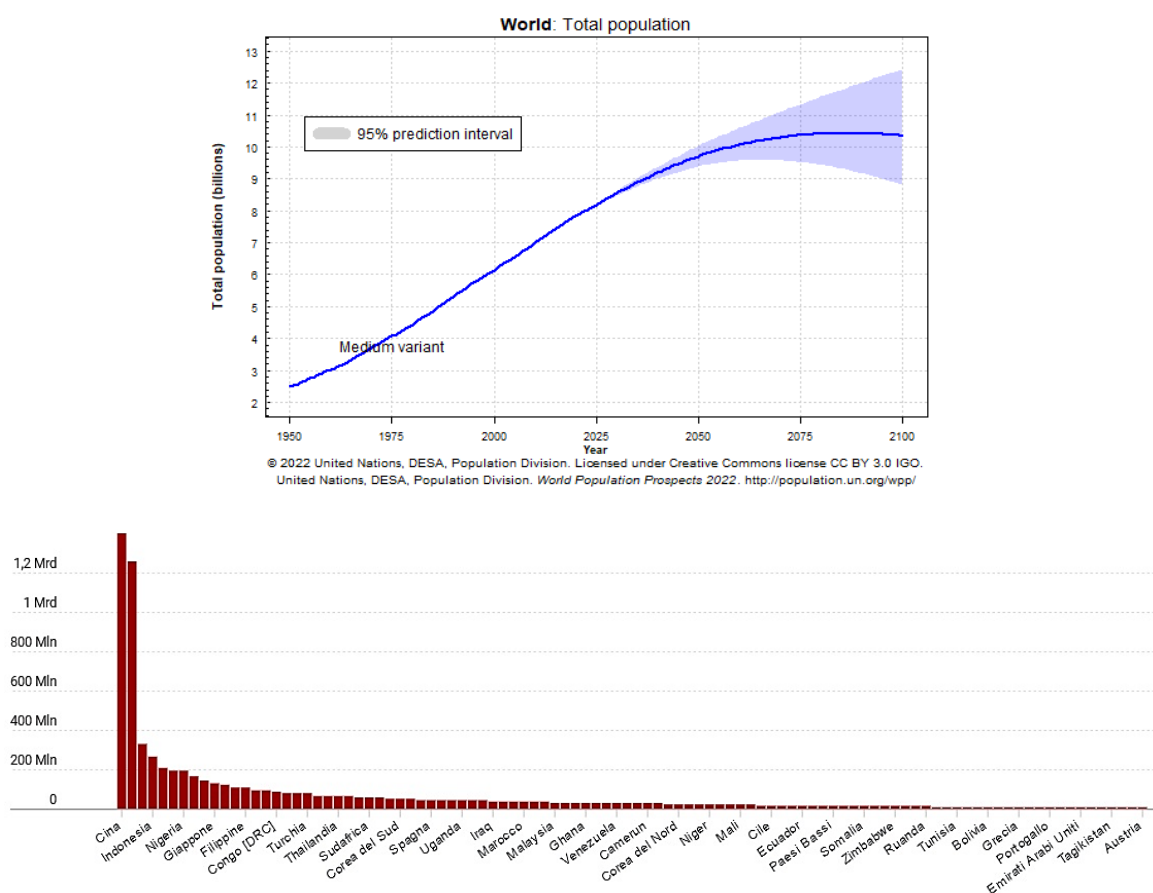


Figure 1.3. World population increase: current status and projections. At the top the projected human population increment between 1950 and 2100 (Source: <https://population.un.org/wpp/Graphs/DemographicProfiles/Line/900>); at the bottom the ranking of the most populous countries in the world (Source: https://datacommons.org/ranking/Count_Person/Country/?h=Earth&hl=it).

Nevertheless, the required increase in wheat production is limited by many factors, primarily the changing climate conditions and the scarcity of further arable land. In fact, the intensification of crops production via increased exploitation of arable areas is largely restricted. The arable land suitable for annual cropping currently stands roughly at 1400 million of ha and, even if there is suitable new land for arable cropping mainly in Sub-Saharan Africa, Latin America and northern parts of Asia, its exploitation will be minimized. The main reasons concern the huge environmental costs, the biodiversity loss and the increasing greenhouse gas emissions. Furthermore, around 2.5 million of ha of arable land is irreversibly lost annually due to urbanization and 6.5 million ha to other causes, like severe soil degradation or the presence of forestry and parks (Fischer et al., 2018). All these considerations underlined how there is a pressing need to increase crop productivity without making use of further arable land. If on one hand the global demand for more high-quality wheat (including DW)-based food has been increasing, on the other this poses a challenge in the face of climate changes, since wheat production is highly dependent on strength and frequency of occurrence of abiotic and biotic stresses (Ceglar et al., 2021; Yadav et al., 2022). Climate models predict that cereals will be exposed to higher average temperatures and more frequent extreme heat stress in the future (Jacott e Boden, 2020). How much food security and market stability in the areas of Mediterranean basin and Middle East, both highly depending on wheat production and its interannual variability, are going to be compromised by global warming is certainly a leading question (Zampieri et al., 2020). Current breeding programs do not sufficiently take into account the climatic uncertainty and variability, although food security mostly relies on the climate resilience of staple food crops. Crops climate resilience has become essential to stabilise food supply and avoid price spikes, hence the availability of a set of cultivars with diverse responses to critical weather conditions is certainly of primary importance (Kahiluoto et al., 2018). Considering the above mentioned global objectives and limitations, the main targets for an efficient wheat breeding program should focus on grain yield improvement, yield stability across different environmental conditions and a better understanding of adaptation mechanisms, responsiveness to inputs and the efficiency in the use of resources, resistance to biotic stresses and tolerance to abiotic stresses, and finally improvement of grain quality (Xynias et al., 2020).

1.2. Wheat genetic variability

1.2.1. Origin and phylogeny of wheat genomes

Both bread wheat (*Triticum aestivum* L.; $2n = 6x = AABBDD$) and durum wheat (*Triticum durum*; $2n = 4x = AABB$) belong to the *Triticum* genus, sub-tribe *Triticinae*, tribe *Triticeae*, family *Poaceae* (grasses). Other important staple crops belong to this family, one of the most species-rich and economically important families in the Angiosperms, namely rice, barley, maize and rye. Among them, wheat is an outstanding model organism to understand the evolutionary theory of allopolyploid speciation, adaptation and domestication in plants (Peng et al., 2011). In the first decades of 20th century Kihara (1919) and Sax (1922) revealed that all the members of *Triticinae* sub-tribe share the same basic chromosome number $x = 7$. The origin of this chromosome set can be found in a common ancestor of the *Poaceae* family that possessed a genome with $n = 5$ chromosomes. In order to clarify the proposed model for the evolution of grass genomes from a common ancestor, more recent works based on comparative genomic studies showed that this primitive genome underwent an event of paleo-duplication ($2n = 10$) to originate an intermediate ancestor with $n = 12$ chromosomes (Bolot et al., 2009; Devos, 2010). The differentiation of the wheat lineage (*Triticum* and *Aegilops*) from this common ancestor began approximately 6.5 million years ago (**Figure 1.4**). However, the first event of hybridization occurred between the A and B genomes about 5.5 million years ago, when *Triticum urartu* ($2n = 2x = 14$, AA) hybridized with a species related to *Aegilops speltoides* ($2n = 2x = 14$, BB) and, via a polyploidization event, originated the allotetraploid emmer wheat (*T. turgidum*; AABB). Bread wheat originated instead from two events of allopolyploidization: the first leading to the previously mentioned allo-tetraploid *T. turgidum*, the second involving the latter and *Ae. tauschii* as the donor of the D sub-genome (Shewry, 2009; Marcussen et al., 2014; Rasheed et al., 2018; Levy and Feldman, 2022). In summary, wheat historical evolution was driven by polyploidization and segmental duplication events that finally gave rise to the three groups of *Triticinae* species, each one presenting a different level of ploidy: diploids ($2n = 2x = 14$), tetraploids ($2n = 4x = 28$) and hexaploids ($2n = 6x = 42$). The particularity of allopolyploid wheats is that each chromosome of a specific genome (e.g. A) has its corresponding chromosome in the other genomes (e.g. B, D), defined as “homoeologous” or partially homologous. Since the basic chromosome number of wheats is 7, there are 7 different groups of homoeology (e.g. group 1: homoeologous chromosomes 1A, 1B, 1D). No doubt, the allopolyploid nature of bread and durum wheats is a significant source of variability *per se*

which allows them to tolerate extensive cytogenetic, genetic and epigenetic changes and made them more adaptable and competitive than their diploid ancestors (Feldman and Levy, 2009).

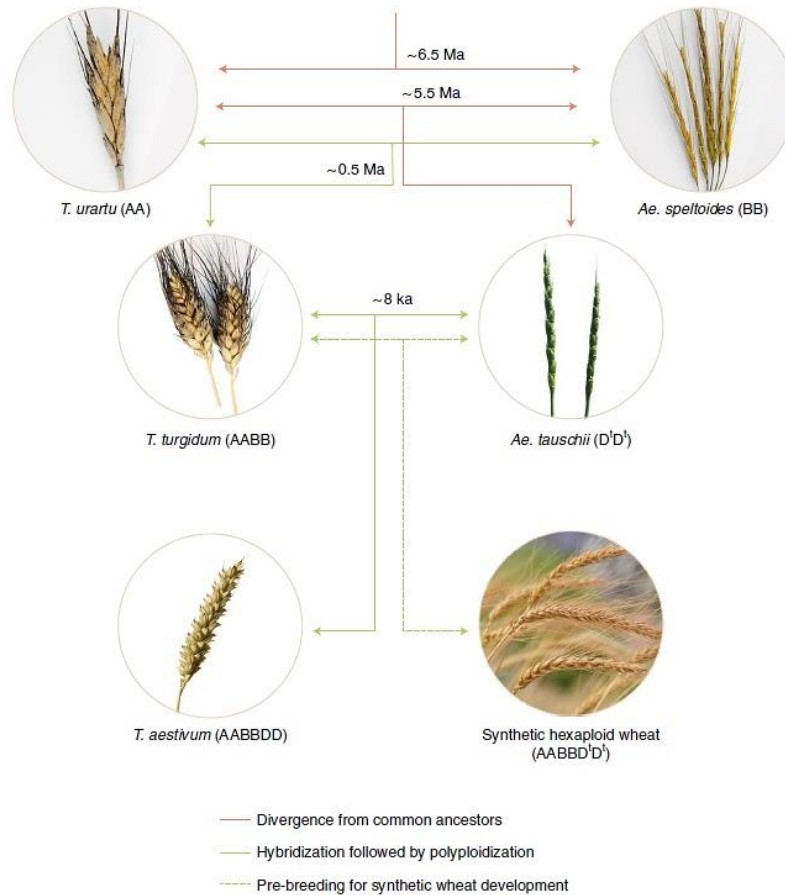


Figure 1.4. Evolutionary history of durum and bread wheat. Approximate dates for divergence are indicated (Ma: million years ago) (from Rasheed et al., 2018).

Polyloid wheats present a regular disomic transmission of traits, thanks to the establishment of a meiotic behaviour similar to that of diploid lines (Feldman et al., 1995). This, together with other relevant modifications, led to genome stabilization and a full-fertility fixation, corresponding to a self-pollination type of reproduction. Another remarkable advantage possessed by allopolyploid wheats is the buffer capacity of their genomes, that allow them to tolerate conspicuous alterations. E.R. Sears (1952, 1954, 1966) carried on an extensive cytogenetic characterization of these species showing how in nulli-tetrasomic lines, the lack a single chromosome pair is fully compensated by an extra dose of another pair of the same group. These observations, together with the analysis of addition and substitution wheat lines, allowed to demonstrate the relationship of homoeology existing between the chromosomes of different

sub-genomes of cultivated allopolyploids and others *Triticinae* (Feldman and Sears, 1981). Finally, cytogenetic results, together with the exploitation of new tools for the establishment of genetic and physical maps, allowed to confirm the high level of synteny (preserved gene content between related species originating from the same ancestor) and colinearity (same gene order) existing among homoeologous chromosomes of various *Triticinae* (Devos, 2010).

1.2.2. The significance of broadening wheat genetic basis

Crop domestication represents a process of successive rounds of selection that finally results in the obtainment of the genetic diversity nowadays available to agricultural purpose, or rather in the crops that feed the world today. Both the domestication process (started in the Fertile Crescent thousands years ago) and the more recent Green Revolution contributed to replace the cultivation of wild relatives and landraces with that of new selected cultivars exhibiting traits of agronomic interest. The cost of this process is the reduction of the existing genetic variation, resulting in an inevitable loss of allelic diversity and potentially useful genetic variants. This phenomenon is called “domestication bottleneck” and it is continuously exacerbated by the demand for highly productive and uniform crops by the field and the global market (Dempewolf et al., 2017; Martínez-Moreno et al., 2020). In support of this evidence, Haudry et al. (2007) analysed nucleotide diversity at 21 loci in wild and domesticated durum and bread wheats, and revealed that genetic diversity was reduced in cultivated forms during domestication by 69% in bread wheat and 84% in DW. Unfortunately, an indirect consequence of crop domestication and of the resulting genetic erosion, is the increased susceptibility and vulnerability to environmental stresses, pests and diseases. The domesticated varieties exhibit an improved fitness for human purpose, but often at the expense of their survival in natural environments (Peng et al., 2011). On the other hand, the conservation of DW genetic diversity is fundamental to improve the ability to overcome the new agricultural challenges due to climate uncertainty. In fact, the required genetic diversity for meaningful interventions is very limited in the extant crop gene pool, thus resulting as insufficient to adequately respond to current and future challenges. A promising strategy for wheat improvement is to utilize the adaptive genetic resources of the wild progenitors and other wheat relatives, together with the creation of new gene banks, where the germplasm can be collected and characterized for the identification of alleles to introduce into modern breeding programs (Martínez-Moreno et al., 2020).

1.2.3. Wild wheat relatives: a valuable source of genetic diversity

In recent years, the “smart breeding” concept has inspired new breeding approaches (McCouch, 2004). It supports an intelligent use of the natural resources, considering current or emerging/projected needs and the efficient exploitation of new technologies. In this way, crop genetic variability can be increased by resorting to wild and cultivated relatives as donors of useful traits. In the case of wheat, its taxonomic family is extremely rich in species with more or less related genomes, from which traits of interest can be transferred to cultivated wheat varieties by recombination, following the “smart breeding” approach (Gennaro et al., 2007). The main genetic resources available to enlarge the wheat genetic basis are: wild wheat relatives (WWRs), landraces, genetic and cytogenetic stocks, obsolete cultivars, modern cultivars in current use and breeding lines (Bramel, 2017). In particular, WWRs can serve as a source of valuable genetic diversity for various traits (e.g. disease and pest resistance, nutritional quality, increase of the adaptive capacity) to potentially use in wheat breeding programs (Dempewolf et al., 2017; Maccaferri et al., 2019; Rampino et al., 2019). WWRs are widely distributed among Mediterranean regions and central Asia throughout a wide area characterized by huge variability of pedoclimatic conditions; therefore, they have also evolved mechanisms of abiotic stress tolerance absent in cultivated species (Rampino et al., 2019). The presence of some incompatibility barriers (e.g. poor pollen growth, problems in the embryo formation, hybrid viability and fertility) can limit the use of WWRs because of the evolutionary distance between wild and cultivated species. For this reason, identification and characterization of the alien donor genomes and individual chromosomes of them, together with the establishment of their cytogenetic relationship with those of the recipient species, are the first essential steps to undertake. The available genetic resources potentially available to breeders can be divided into three gene pools (primary, secondary, tertiary), based on formal taxonomy and genetic relatedness, hence by the crossing ability between related species (**Figure 1.5**; Guzzon et al., 2022). Species in the primary gene pool typically share full homology with wheat genomes and have no or only limited crossing barriers with the latter, so gene transfer in this group is considered to be easy (Bramel, 2017; Dempewolf et al., 2017). Species in more distant genepools (secondary, tertiary) commonly encounter cross- incompatibility and hybrid sterility; and inter-specific gene transfer is possible with increasing difficulty (Dempewolf et al., 2017). Polyploid species in the secondary gene pool have one or more shared genomes but overall reduced recombination rates with wheat; however, gene transfer is possible between

homologous chromosomes. The tertiary gene pool includes diploid and polyploid species that do not share any homologous genome with wheat and gene transfer cannot occur by homologous recombination. In the latter case, a cytogenetic approach, named “chromosome engineering” (see §1.2.4.), can be applied. Basically, the primary gene pool of wheat comprises the domesticated and wild wheats belonging to *Triticum* genus. The secondary gene pool mainly includes species of the genus *Aegilops*, naturally growing in the Fertile Crescent, Turkey, the southern Caucasus, as well as the shores of the Aegean Sea. In these areas spontaneous hybridizations between *Aegilops* species and cultivated wheats have also been observed. The tertiary gene pool includes grass species of the genera *Agropyron*, *Elymus*, *Leymus* and *Thinopyrum*. Given the sexual barrier between cultivated wheats and their tertiary gene pool, transfer of their traits to wheat can be performed using physical and genetic methods, inducing random chromosome breaks and translocations, in vitro culture, irradiation, and induced homoeologous recombination (Guzzon et al., 2022).

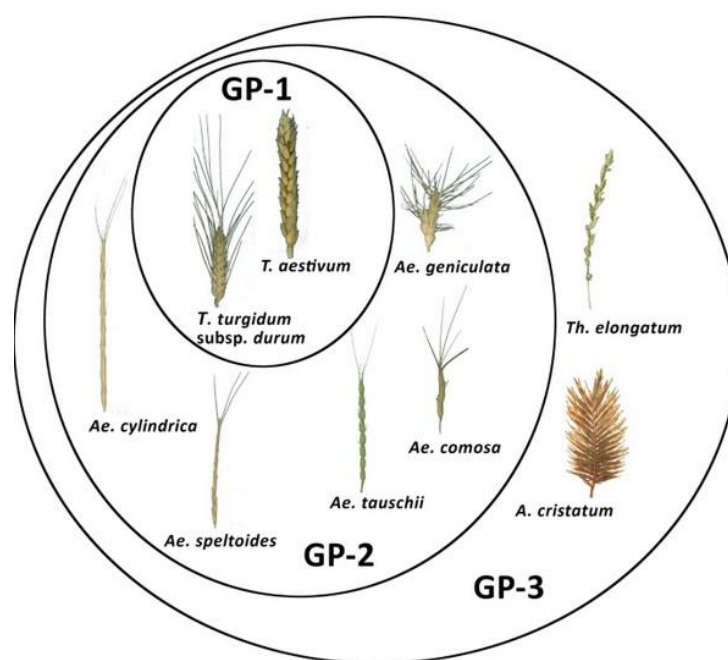


Figure 1.5. Schematic representation of wheat gene pools (only some species are shown). GP-1: Primary gene pool; GP-2: Secondary gene pool; GP-3: Tertiary gene pool (from Guzzon et al., 2022).

In the last decades many WWRs have been appreciated as donors of useful traits for wheat enhancement. For example, Placido et al. (2013, 2020) elucidated at the physiological and molecular level the consequences of the introgression of an alien chromosome segment derived from the wild species *Agropyron elongatum* (syn. *Thinopyrum ponticum*) into *Triticum*

aestivum. The analyses performed reveal that the translocation line possesses improved root adaptive traits, attributable to a reduced transcript level of *LATERAL ROOT DENSITY (LRD)* gene, hypothesized to favour better performance under water limited conditions. Suneja et al. (2019) identified accessions of *Ae. tauschii* and *T. dicoccoides* as potential donors of water stress adaptive plasticity. Pradhan and Prasad (2015) analysed some wheat chromosomal translocation lines presenting small segments of chromosome from *Ae. speltoides* into bread wheat cv. Chinese Spring background, observing a greater heat tolerance during the grain filling phase. Pour-Aboughadareh et al. (2020) investigated the behaviour of wild wheat relatives under water deficit and found *Ae. tauschii* and *Ae. crassa* accessions to display good tolerance.

1.2.4. Chromosome engineering strategy for the development of DW-wild introgressions

In spite of the great potential utility of WWRs to enhance wheat agronomic and tolerance performance, the effective exploitation of such a rich gene reservoir can be still considered at its onset. Firstly, this is due to the above mentioned crossing barriers existing among different gene pools. A wide range of techniques can be adopted to overcome the issue, such as the creation of “bridge crosses” (consisting in the use of an intermediary species crossable with both the wild and the cultivated parents). If the resulting interspecific hybrids manifests fertility problems, the “embryo rescue” technique can be adopted. Furthermore, in some cases, a chromosome doubling via the utilization of chemicals (e.g. colchicine) has to be performed of the inter-specific hybrid to cytologically stabilize it through development of the corresponding amphiploid. However, synthetic amphiploids were generally proved to be inferior to established crops. In fact, wild species possess many undesired genes associated with poor agronomic performance (low yield, seed shattering) and these genes may be linked to the ones of interest. This phenomenon, called “linkage drag”, is a major deterrent to using wild relatives in crop breeding programs, even if molecular marker-assisted selection (MAS) can be used to facilitate the selection of target genes (Dempewolf et al., 2017; Guzzon et al., 2022). In spite of their inferior agronomic characteristics, amphiploids can be useful bridging materials for the transfer of specific wild genes and also starting points for the production of alien chromosome addition and substitution lines into wheat backgrounds (Feldman and Sears, 1981). These lines, in turn, can serve as materials for the introduction of sub-chromosomal alien segments, leading to a progressive reduction of undesired genetic contribution from the wild parent. The DW genome, compared to that of bread wheat, exhibits a much lower degree of tolerance toward genic and

chromosomal alterations, which, in turn, cause lower cytological stability and plant fertility (Ceoloni et al., 2005b; Gennaro et al., 2007). For this reason, the achievement of a progressive shortening in the amount of alien chromatin flanking the desired genes is fundamental in the case of DW (Ceoloni et al., 2005a). The main cytogenetic procedures that permit to successfully engineer the wheat genome with small alien chromosome introductions were originally delineated by E.R. Sears (1972), who coined the term “chromosome engineering”. This strategy enables the “...transfer of segments of alien chromosomes carrying particular desired genes to wheat chromosomes” (Sears, 1972), thus giving access to the wide gene pools of more or less closely related *Triticeae* species. Chromosome engineering proved to be particularly beneficial in the case of transfers from the tertiary gene pool, because of the low cytogenetic affinity and hence limited pairing and recombination between the wheat and alien homoeologous chromosomes. This is also the result of the fact that, under normal conditions, a complex wheat pairing-control system, in which the *Ph1* gene, located on the 5BL wheat arm, exerts the strongest effect, restricts pairing and recombination to homologous chromosomes only (Sears, 1976). While this system allows maintenance of meiotic regularity and therefore the integrity and stability of the wheat genome, it represents an obstacle to wheat-alien introgressions via homoeologous recombination. However, in the absence of the *Ph1* gene, such stringent control is relaxed and the pairing and exchange of genetic material between homoeologous chromosomes or chromosome segments can be realised (Ceoloni et al., 2005a). The development of chromosome engineering was boosted by the isolation of mutant lines at the *Ph1* locus in bread (Sears, 1977) and durum wheat (Giorgi, 1983), which allow the access to homoeologous genomes of alien species from the *Triticeae* tribe in complete or partial hybrid combinations. The advantages of a homoeologous recombination-based strategy include the production of well-compensated wheat-alien products and also the possibility to minimize the size of the transferred alien segment, which can significantly reduce the possibility of linkage drag phenomena. Indeed, through successive cycles of induced homoeologous recombination, or combining suitable primary recombinants, the amount of introgressed alien chromatin can be shortened (Sears, 1983; Ceoloni et al., 2005a). Introgressions of minimal size but still containing the target gene(s) can be selected by both genetic and physical mapping. The first involves the use of molecular markers, mostly PCR-based, the second the use of molecular cytogenetic approaches, such as fluorescence in situ hybridization (FISH, GISH), used to detect and locate specific DNA sequences or portions of genomic DNA onto fixed chromosomes on slides, through suitable probes tagged with fluorescent dyes (Ceoloni et al., 2005a; Ceoloni et al., 2005b). Several are the examples of the successful use of chromosome engineering strategy

for the transfer of useful traits (resistance to biotic and abiotic stresses, quality attributes, yield-related traits) from wild relatives to both cultivated bread and durum wheat (e.g. Ceoloni et al., 2014; Ceoloni et al., 2017b).

1.2.5. Exploitation of species belonging to *Thinopyrum* genus and traits of interest successfully transferred into wheat

The *Thinopyrum* genus, belonging to the *Triticeae* tribe, is part of the wheat tertiary gene pool. The genus encompasses a large number of perennial species, several native of southern and eastern Europe, western Asia and northern Africa (**Figure 1.6**; Monsen et al., 2004; Wang, 2011), having a wide range of ploidy levels, from diploidy (2x) up to decaploidy (10x), and containing a wide array of useful traits for wheat improvement.

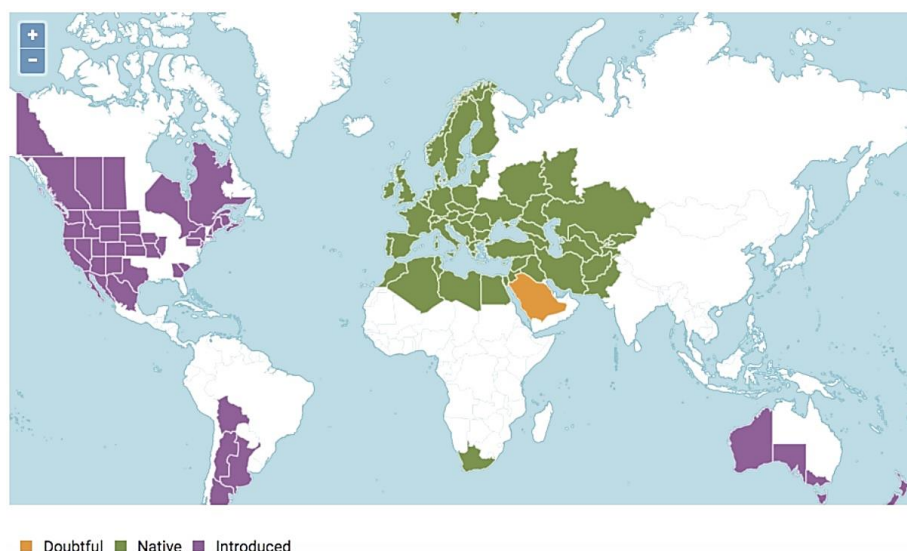


Figure 1.6. Geographical distribution of the species belonging to the *Thinopyrum* genus (from: Kew Science - Plants of the World online: *Thinopyrum* Á.Löve, <https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:1190729-2>).

Both the diploids *Th. elongatum* and *Th. bessarabicum*, (carriers of a similar E genome, differentiated into E^e and E^b, respectively) and many polyploids, particularly the hexaploid *Th. intermedium* and the decaploid *Th. ponticum* (auto-allopolyploids with controversial genome formulas, see Ceoloni et al., 2015), have been among the most extensively exploited in wheat breeding, not only among perennial Triticeae, but among wheat relatives as a whole (reviewed in Ceoloni et al., 2015). Presence of valuable genes/QTLs for biotic and abiotic stress resistance,

quality traits and yield performance, prompted the development of an array of addition, substitution, translocation and recombinant wheat-*Thinopyrum* spp. lines, many involving 2x *Th. elongatum* and 10x *Th. ponticum* (Ceoloni et al., 2015). The former has been targeted to improve tolerance towards several abiotic stresses, such as drought, waterlogging and salinity (Shu et al., 2015 and references therein). In fact, a superior drought resistance was observed in its chromosome 3E addition line (Liu et al., 2015a) and tolerance genes, affecting stress signaling, reduction in oxidative damage, synthesis of protectants and root development, emerged from analysis of the *Th. elongatum* transcriptome under water deficit conditions (Shu et al., 2015). Regarding salt tolerance, most chromosomes of *Th. elongatum* were found to be involved (Dvorak et al., 1988; Omielan et al., 1991; Zhong and Dvorak, 1995; Kasai et al., 1998; Deal et al., 1999; Mullan et al., 2007), altogether determining improved regulation of leaf Na⁺ concentrations. As for resistance to biotic stresses, it is noteworthy that spike inoculation with *Fusarium graminearum* of a series of bread wheat cv. Chinese Spring-*Th. elongatum* addition, substitution and ditelosomic lines showed chromosome 7E to possess on its long arm (7EL) a major locus conferring very high resistance to the extremely harmful fungal disease Fusarium Head Blight (FHB) (Ceoloni et al., 2017a and references therein).

Also the decaploid tall wheatgrass, *Th. ponticum*, is characterized by superior levels of tolerance versus abiotic stresses, of which the most studied is salt stress (Suiyun et al., 2004; Colmer et al., 2006; Wang et al., 2008), as well as superior levels of resistance versus various diseases (e.g. Li and Wang, 2009; Ceoloni et al., 2015). In order to obtain wheat-*Thinopyrum* spp. lines characterized by ample genetic variability and superior performance under stressful (abiotic and biotic) conditions, chromosome engineering is undoubtedly a promising strategy (see above, § 1.2.4.). In a pioneering experiment, Sears (1973) obtained a primary bread wheat-*Th. ponticum* recombinant line (called “Transfer No. 12” = Tr#12) resulting from recombination between 7A and 7Ag (from *Agropyron*, now renamed 7el₁) chromosomes. On the 7AgL arm (= 7el₁L) of Tr#12, *Th. ponticum* genes of great interest for the DW improvement were present, including *Lr19*, conferring resistance to leaf rust and the closely linked *Sr25* (resistance to stem rust) and *Yp* (high yellow pigment in the endosperm) genes. However, the amount of the 7el₁L chromatin turned out to be excessively large to be tolerated by the DW genome, almost completely preventing transmission of the primary recombinant chromosome (same of Tr#12 introduced into DW) through the male germline (Ceoloni et al., 1996). Therefore, substantial efforts were made to produce recombinant lines with smaller amounts of 7el₁L onto DW 7AL. By use of a *ph1c* mutant, several secondary recombinant lines were obtained (Ceoloni et al.,

2005a), among which desirable ones, containing the target genes within 7el₁L segment of limited length, were identified (**Figure 1.7**).

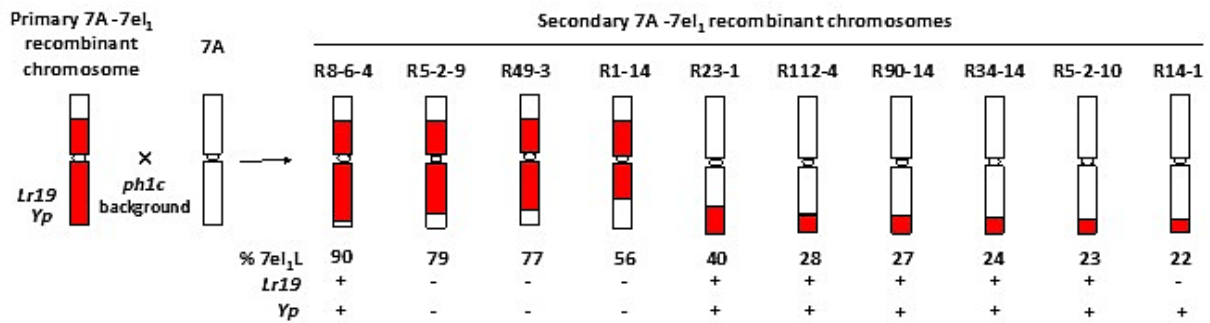


Figure 1.7. Recombinant chromosomes obtained via chromosome engineering, leading to segmental introgression of *Th. ponticum* 7el₁ into DW chromosome 7A. Presence (+) and absence (-) of *Lr19* and *Yp* target genes into the alien chromatin of each recombinant chromosome is indicated (modified from Ceoloni et al., 2005a).

Among them, the best DW-*Th. ponticum* recombinant types, also in view of use in breeding, possessed the telomeric region of 7AL replaced by relatively small amounts (from 23 to 40% of the arm length, see recombinant types in **Figure 1.7**) of the wild 7el₁L chromosome segment, all containing the *Lr19* and *Sr25* rust resistance genes, and the *Yp* gene, positively impacting on quality of semolina and agronomic performance (Ceoloni et al., 2014; Kuzmanovic et al., 2014, 2016). The 7A/7el₁ recombinant lines, however, were not equipped with resistance against FHB, a disease more traditionally associated with bread wheat, yet becoming a threat for the DW crop as well (Giancaspro et al., 2016; Prat et al., 2017). In order to incorporate resistance to FHB into 7A-7el₁ recombinants, an effective QTL (named *Fhb7*), proved to be located in the distal end of the chromosome arm 7el₂L, derived from another *Th. ponticum* accession, was firstly targeted. In this case, the transfer was facilitated by the close homology existing between the 7el₁L and 7el₂L arms, which made pyramiding of the corresponding genes of interest (*Lr19*+*Sr25*+*Yp* and *Fhb7*, respectively) achievable in bread (Forte et al., 2014; Ceoloni et al., 2017a) and DW (Forte et al., 2014) without resorting to the use of *ph1* mutants. Furthermore, an additional and even more effective *Thinopyrum* source of resistance against FHB, originating from *Th. elongatum* (long arm of 7E chromosome, i.e. 7EL), was targeted. The major locus, *Fhb-7EL*, was successfully pyramided with the previously mentioned *Th. ponticum* 7el₁L genes through a fine chromosome engineering strategy, leading to “nested” 7EL segments into 7el₁L ones, firstly in bread wheat (Ceoloni et al., 2017a), and, from these, into

DW as well (Kuzmanović et al., 2019). Also in this case, recombinant lines carrying 7e_lL+7EL segments were obtained by spontaneous recombination between close, albeit homoeologous, 7e_lL+7EL chromosome arms (belonging to closely related *Thinopyrum* species), albeit at a relatively low frequency. As anticipated, some of the 6x recombinant types containing the composite *Thinopyrum* segments (see R69-9 and R74-10 in **Figure 1.8**) were used as donors of the 7e_lL+7EL gene package to DW, giving rise to R69-9/R5, R69-9/R112 and similar R74-10 derivatives (**Figure 1.8**).

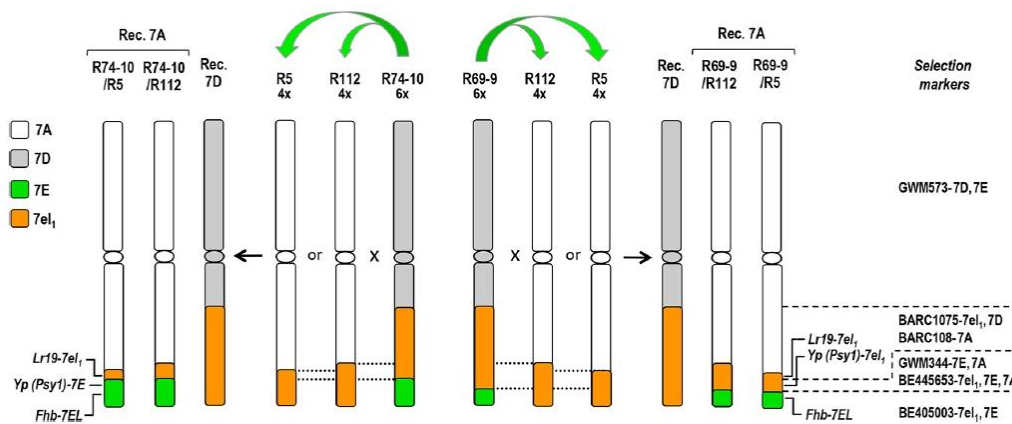


Figure 1.8. Development and cytogenetic maps of DW-*Thinopyrum* spp. chromosomes carriers of 7e_lL+7EL segments (from Kuzmanovic et al., 2019).

A few years ago, the DW-*Th. ponticum* recombinant lines have been tested for the first time in a multi-location field trials (including locations of Italy, Morocco and South Australia) to evaluate the environmental-dependent effects of the alien introgressions (Kuzmanović et al., 2018). Overall, the presence of 7e_lL chromosome segments did not negatively affect the yield of the recombinant lines. On the contrary, they showed a superior performance for some yield traits, indicating a stable expression of such traits across different environments. Since some of the tested environments were characterized by abiotic stress conditions, this study stimulated further investigations of possible associations between presence of the wild segments and tolerance to one or more abiotic stresses. More recently, some derivatives of the 7A-7e_lL DW-*Th. ponticum* recombinant lines, containing a 7EL “nested” segment from *Th. elongatum* into the 7e_lL one (R69-9 and R74-10 types, see **Figure 1.8**) have also been assessed for their breeding potential over two seasons under different water regimes (rainfed and irrigated; Kuzmanović et al., 2021). As well as confirming their genetic stability and the absence of relevant yield penalties, an extremely good performance of R69-9 types (R69-9/R5 and R69-

9/R112, see **Figure 1.8**) was observed under both irrigation conditions. As a whole, these observations on DW-*Thinopyrum* spp. recombinants are in line with that of Placido et al. (2013), who found improved tolerance to applied water stress to a bread wheat line containing a small 7elL *Thinopyrum* chromosome segment onto its 7DL arm. The superior tolerance was tentatively associated to the presence of the *LRD* gene, a major gene that confers the ability to sustain and maintain root growth and biomass under limited water conditions (Placido et al., 2020). So far there is no other evidence of the possible (positive) correlation between presence of 7el₁/7E specific regions and the tolerant behaviour, besides that specific underlying mechanisms and eventually genes.

1.3. Impact of climate change on crop production

1.3.1. The issue of heat and drought incoming – A global challenge

Abiotic factors are main components of the environment, defining plant distribution and productivity; for this reason the understanding of the relationships existing between these factors and plant adaptation is essential to achieve global food security targets (Zhang et al., 2022). In the Mediterranean environments, both yield and quality of its main grain crop, i.e. DW, are greatly affected by weather variability, which is one of the main reasons for year-to-year yield instability, especially under rain-fed conditions (Ferrise et al., 2015). Climate changes are extremely dangerous for crop production not only per se but also because they are strictly connected with reduction in arable surface and spread of pathogens. Indeed, if the global temperature will continue to increase, in the next future arable areas climatically suitable for DW are expected to shift towards regions of central and western Europe and in some parts of Russia; this causing a considerably overall decrease of DW cultivation surface. Climate change may decrease the suitable area by 19% at mid-century and by 48% at the end of the century, causing a widespread land loss especially in the Mediterranean regions and northern America (Ceglar et al., 2021). Moreover, numerous fungal species and strains are extremely susceptible to environmental factors (e.g. temperature, humidity) and fluctuations of these factors may predispose wheat to more severe epidemics of even unfamiliar diseases, such as FHB on DW (Kuzmanović et al., 2019 and references therein).

Focusing on the most direct consequences of climate change, tightening of heat and drought conditions is of primary concern. In general, DW is more sensitive to cold than bread wheat, being, in turn, better adapted to high temperatures and semi-arid climates (Lidon et al., 2014).

This major tolerance seems to be related to a more resistant photosynthetic metabolism, more stable cellular membranes and a less affected plant transpiration mechanism under hot conditions (Lidon et al., 2014; Fontana et al., 2015). Since DW is mainly grown under rain-fed conditions in the semi-arid area of the Mediterranean basin, its yield is heavily influenced by rainfall amount and abiotic stresses (heat, cold, sunlight, etc.) (Xynias et al., 2020; Sinha et al., 2021). The Mediterranean area is considered a hot spot of climate change: here heat waves accompanied by drought phenomena are foreseen to be more and more severe and frequent (Cramer et al., 2018), with expected conspicuous loss of crop production and increased yield volatility. In fact, if the global mean temperature has increased of roughly 1°C from 1960 to today, the Mediterranean atmospheric warming has exceeded the global average rates (**Figure 1.9**). Moreover, according to the reports of the Intergovernmental Panel on Climate Change (IPCC), future annual warming rates in the Mediterranean area are projected to be 20% higher than the global annual average (Hilmi et al., 2022), which in turn is expected to increase by 1.5 °C within the next two decades (IPCC, 2021).



Figure 1.9. Global mean temperature increase between 1961 and 2021
(<https://www.fao.org/faostat/en/#data/ET/visualize>).

As for heat stress (HS), it can be defined as the temperature rise above a given threshold for a sufficient period of time to cause a permanent damage to plant growth and development. The persistent overcome of this threshold can also be lethal. HS events are composed by different factors: “intensity”, that is the maximum value of temperature reached, “frequency”, which indicates how often the events occur and “duration”, that indicates how long the events last (Janni et al., 2020). Moreover, although the term “heat stress” is widely used in the scientific

community, it encompasses some sub-categories such as “heat shock”, “heat waves” and “warming experiments”, which differ from each other in some points (Jagadish et al., 2021):

- Heat shock (or acute HS) indicates a short-term exposure to severe high temperature. In this case the stress duration lasts from minutes to hours and the air temperature increase is often 20 °C or more over the optimal temperature;
- Heat waves (or chronic HS) indicate the exposure to high temperatures (generally 5-10°C over the optimum) for a duration from hours to days. Often, but not always, the stress treatment is repeated after a recovery period, to better mimic naturally occurring heat waves;
- Warming experiments indicate the exposing to elevated growth temperatures for long periods, such as weeks or years. In this case the increase in temperature is low, about 2-6°C over the optimum.

Under both natural and controlled conditions, different levels of HS intensity, frequency and duration can have a wide range of effects on plants (longer exposure times and higher temperatures being more damaging). Although HS can affect plants throughout the growth cycle, some developmental phases are more heat-sensitive than others. Optimum temperatures for wheat are 16°C at the terminal spikelet stage, 23°C at anthesis and 26°C at grain filling (Yadav et al., 2022). Two of the most susceptible phases are those of plant early establishment (early seedling stage) and flowering. As to the first stage, the poorly developed root system limits the ability to uptake water and hence to relieve the HS effects. Regarding flowering, the overall reproductive system is heavily affected by high temperatures, resulting in yield loss (Jagadish et al., 2021). Although the main physiological and molecular effects of HS on plants will be further detailed (see § 1.3.2.), it is important to emphasize how HS phenomena cause a remarkable reduction in wheat yield of about 4-6% for every 1°C increase in temperature (Jacott and Boden, 2020; Dhakal et al., 2021). Future projections about the effects of climate change in the Mediterranean area predict that, if no adaptation will take place, wheat production reliability will be threatened already at 1.5°C global warming; thus the limitation of global warming levels well below 2°C is of fundamental importance (Zampieri et al., 2020; Ceglar et al., 2021). In fact, higher temperatures as well as lower rainfall rate have been projected in the Mediterranean region especially during the summer season and the simulated impacts of these occurrences on DW cultivation include grain yield reduction, a slightly increase in kernel weight and earlier anthesis dates. Since grain size is both a yield component and a quality indicator (affecting semolina milling properties), climate change can also lead to a reduction in

DW technological value with negative impact also for the entire European food industry (Dettori et al., 2017).

As well as HS phenomena, also water deficit condition is one of the main factors limiting DW yield, and the simultaneous occurrence of high temperature and water limitation in field conditions of the Mediterranean area is not uncommon. The term “drought” generally indicates an extended period of abnormal below average natural water availability, for example a long period of no or unusual levels of rain (Naumann et al., 2018; Tyagi and Pandey, 2022). These periods of water scarcity could be extremely detrimental to crop production, in fact roughly 9-10% of cereal losses are due to drought and extreme weather conditions (Hasanuzzaman et al., 2018 and references therein). Drought phenomena are expected to continuously increase in both frequency and severity in the next future (**Figure 1.10**), mainly as a consequence of the decrease in precipitations, but also because of the increasing evaporation driven by global warming (Hasanuzzaman et al., 2018; Naumann et al., 2018). This would be particularly true for the Mediterranean basin, besides Africa, West and Southern Asia, Central America and Oceania; on the contrary other regions like North America and North-West Australia have experienced lower drought episodes in the second half of the 20th century (Naumann et al., 2018).

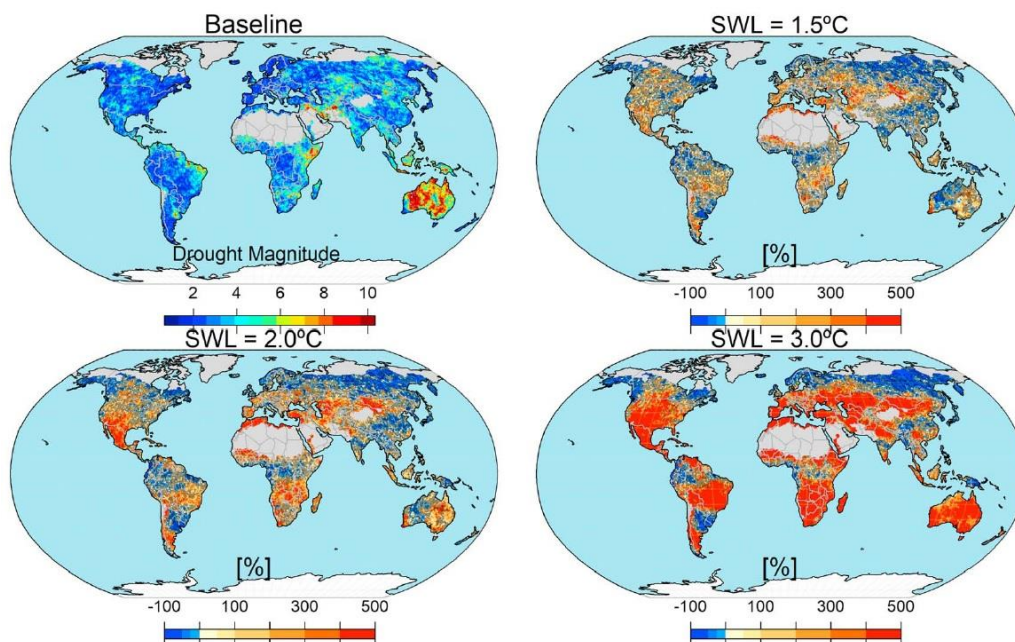


Figure 1.10. Drought magnitude and the three relative changes in drought magnitude (%) with respect to the baseline for the three specific warming levels (SWL) of 1.5, 2 and 3 °C. Areas in grey are excluded from the analysis (from Naumann et al., 2018).

1.3.2. Main effects of high temperature and water deficit on cellular and plant functions

All abiotic stress conditions affect several aspects of plant morphology and physiology, causing widespread changes in cellular processes. Some of these changes reflect the damage inflicted by the stressor (e.g. changes in membrane fluidity and protein denaturation), whereas others are part of the plant response system (e.g. accumulation of antioxidants and osmoprotectants). As illustrated in **Figure 1.11**, heat and drought stresses often cause similar consequences, having a great negative impact on plants growth, development and vitality and ultimately its productivity. Indeed, upstream of the yield decline, several physiological/biochemical/metabolic perturbations of the plant system occur due to high temperatures and/or water scarcity.

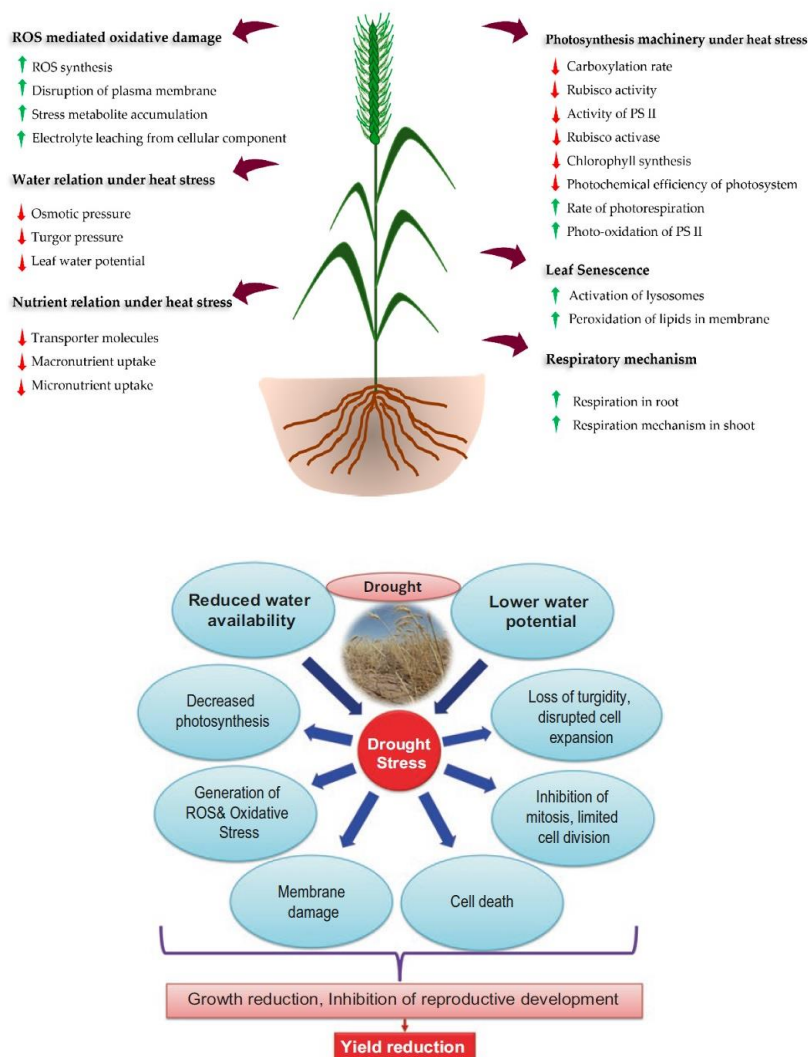


Figure 1.11. Main effects of heat (above) and drought (below) stresses on plants (from Yadav et al., 2022 and Hasanuzzaman et al., 2018).

Among the developmental and morphological perturbations that occur in wheat under HS there are: inhibition of seed germination and emergence, decline of plant growth by causing leaf senescence, reduction of leaf development, plant height and of productive tiller formation, decrease of seed mass, by accelerating seed growth rate and shortening the grain-filling period (Kaushal et al., 2016; Akter and Islam, 2017). Leaf senescence is a typical symptom following HS, accompanied by chloroplast structural changes, vacuolar collapse, loss of plasma membrane integrity and interference with cellular homeostasis. In wheat, temperatures superior to 34°C accelerate leaf senescence because of the inhibition of chlorophyll production (Akter and Islam, 2017; Tyagi and Pandey, 2022). An important aspect to be considered when plants are exposed to high temperatures is the possible change in the overall water status. In fact, plants maintain nearly stable water relations when moisture is adequate, but high temperature causes a rapid loss of water from the plant surface resulting in dehydration. This indirect effect is mainly due to the enhanced leaf transpiration under HS conditions: the more intense the transpiration process, the higher the leaf cooling, but also water loss from leaves via evaporation. In turn, a rapid decrease of the plant water potential and leaf relative water content (RWC) is observed, causing a drop in cell osmotic potential (Kaushal et al., 2016; Akter and Islam, 2017; Yadav et al., 2022). Osmotic stress can be a direct consequence of both drought and heat stress and consists of a rapid withdrawal of water from the cell, leading to a loss of cell turgor and even cell collapse. HS also affects mitochondrial activity and hence the process of respiration. Initially, the rate of respiration increases exponentially with increasing temperature, but beyond threshold levels respiration decreases, due to damage to the respiratory apparatus (Kaushal et al., 2016). The increased respiration occurring in mitochondria increases free electron accumulation, which can reduce O₂, resulting in production of toxic reactive oxygen species (ROS) (Medina et al., 2021).

A morphological symptom indicative of a drought condition is the presence of waxes and pubescences on the leaf surface and/or leaf rolling. In fact, leaf rolling could be a clear symptom of a water deficiency, since it is either a mechanism to reduce canopy light interception and/or a plant response to water stress (Pietragalla et al., 2012). In general, drought has a huge impact on wheat vitality and the severity of the damages is dependent on both plant stage and magnitude and duration of the stress. Uptake and transportation of water and nutrients is restricted under drought conditions, these having a great impact on photosynthesis, respiration, transpiration and translocation of assimilates. Another typical aspect that has to be considered under water-limiting conditions is the occurrence of stomata closure. Stomata, determining the rate of CO₂ uptake in leaves and, consequently, the amount available at the Rubisco site through

their opening/closing mechanism, are key players in photosynthetic efficiency and plant transpiration (Faralli et al., 2019). However, when water shortage affects the root system, several signals (mainly ABA-mediated) are sent from roots to leaves in order to limit stomatal conductance and hence reduce plant water loss. In turn, the closure of stomata reduces the rate of transpiration and CO₂ entry. If internal CO₂ concentration decreases, a reduced amount of CO₂ is available for the Calvin Cycle, thus reducing the overall photosynthetic efficiency and decreasing the regeneration of NADP⁺ provoking an excess reduction of the photosynthetic electron transport chain. As a result, the chloroplasts get exposed to excessive excited energy from reaction centres (RCs) and enhance ROS production (Hasanuzzaman et al., 2018).

As in all green plants, photosynthesis is a vital process that is severely hampered in wheat by both heat and drought conditions, mainly due to disruption of chloroplast structure, reduction of chlorophyll content and inactivation of chloroplast enzymes (Akter and Islam, 2017; Ni et al., 2018; Dhakal et al., 2021). Both the ribulose1,5-bisphosphate carboxylase/oxygenase (RuBisCo) and the photosystem II (PSII) are extremely thermolabile components of the photosynthetic process (Kaushal et al., 2016). At high temperatures, the key regulatory enzyme of RuBisCo, i.e. RuBisCo activase, is dissociated and the photosynthetic efficiency of leaves inevitably inhibited (Akter e Islam, 2017). Likewise, PSII is very sensitive to HS, due to leakiness and swelling of thylakoid membranes, in turn leading to physical separation of PSII light harvesting complex from the PSII core complex and disruption of PSII-mediated electron transfer (Ni et al., 2018). The inactivation of PSII functionality, together with damage to its structure due to protein denaturation, is a well-known negative effect of HS. Curiously, however, PSII thermostability seems to be enhanced under a combination of heat and drought stress. Indeed, if high temperatures cause a complete and irreversible inhibition of PSII under well-watered conditions, under water-limiting conditions the reduction of PSII efficiency is lower and reversible (Bussotti et al., 2012 and references therein). Also the increase in ROS concentration is a common consequence of several abiotic and biotic stresses, included those of heat and drought. Hydrogen peroxide (H₂O₂), superoxide (O₂^{•-}), singlet oxygen (¹O₂), and hydroxyl radicals (OH[•]) are the most commonly generated species. ROS are normally produced in limited amounts by different cell organelles (mitochondria, chloroplast, peroxisome and the cell membrane) and are needed in multiple metabolic pathways, such as lignin formation, leaf and flower abscission, cell senescence, and fruit ripening. Nevertheless, under stress conditions there is an overproduction of ROS, which leads to the occurrence of an oxidative stress (Yadav et al., 2022). As previously mentioned, the major sites of ROS production under stress are the RCs of chloroplast, together with plasma membrane, mitochondria, peroxisomes, apoplasts and

endoplasmic reticulum (Kaushal et al., 2016). All ROS have a huge potential toxic effect: they may induce protein oxidation, DNA damage, lipid peroxidation in membranes, destruction of pigments and even cell death (Kaushal et al., 2016; Yadav et al., 2022). Lipids, in particular, are among the first targets of ROS activity. Since lipids are major components of cell and organelle membranes, oxidation of these molecules can be extremely deleterious for maintenance of cell functionality. In particular, the process of “lipid peroxidation” consists in the stealing of electrons from the lipids in cell membranes by free radicals. This process mostly affects polyunsaturated fatty acids, since they contain multiple double bonds in between which lie methylene bridges (-CH₂-) that possess especially reactive hydrogen atoms. Lipid peroxidation is enhanced when plants are exposed to several abiotic stresses but especially under HS conditions, and it can lead to membrane damage, electrolyte leakage and cell death (Janni et al., 2020). Furthermore, high temperatures cause protein denaturation and an increment in unsaturated fatty acids, which in turn increase the fluidity and permeability of the membranes. This phenomenon is related to the upset movement of water, ions and organic solutes across the membrane (Kaushal et al., 2016; Ni et al., 2018).

All the negative effects reported above (morphological alterations, photosynthetic reduction, ROS damages) due to the exposure to heat and drought stress can affect the plant system at each developmental stage, leading to an unavoidable drop in final yield. However, some growth stages are more vulnerable than others and, if they are affected by the stress, the resulting yield reduction becomes more significant. Among these extremely sensitive growth stages, anthesis is the most important and closely associated with final wheat production. Both heat and drought conditions have a huge impact on reproductive processes. Major negative effects of high temperatures on potential yield occur at meiosis (coinciding with the early booting stage in wheat), as demonstrated by meiotic abnormalities (presence of micronuclei, absence of metaphase plate, abnormal tetrads, laggard chromosomes, abnormal cytokinesis, pyknosis and cytomixis) observed in pollen mother cells, as well as development of abnormal ovaries and ovules (no or small embryo sacs and reduced nucelli) and stigmas, altogether leading to reduced seed set (Kaushal et al., 2016; Tyagi and Pandey, 2022; Ullah et al., 2022). Post-meiosis stages (coinciding with heading and anthesis in wheat) are also critical: short episodes of HS at heading resulted in substantial damage to floret fertility and even a total reproductive failure (Janni et al., 2020). Although grain number in wheat is generally determined in pre-anthesis phases, it is strongly and negatively affected by abiotic stresses also at anthesis, in particular during anther dehiscence (Dolferus et al., 2011; Fernie et al., 2022): the failure to release pollen from anthers and a reduced pollen viability limit the successful fertilization and hence the final

seed formation (Dolferus et al., 2011; Fernie et al., 2022; Ullah et al., 2022). A major impact on final yield, with reduction of grain number, but also seed weight and quality, is caused by exposure to high ambient temperatures during the grain filling stage (Jacott and Boden, 2020; Ullah et al., 2022).

Not only high temperatures but also a water-limiting condition is extremely dangerous for the success of a proper reproduction and hence for yield maintenance. When drought stress occurs around the reproductive phase, it consistently reduces the supply of carbohydrates and the number of endosperm cells and amyloplast in the grains, resulting in a final abortion of kernels (Mahrookashani et al., 2017). This in turn affects key traits related to DW spike and plant fertility, such as fertile florets per spikelet and grain number per spike and per plant (Liu et al., 2015b; Vahamidis et al., 2019). The great influence of drought during the flowering and grain formation phases on total production is confirmed by the remarkable drop (up to 92%) observed in wheat yield (Raza et al., 2019). Summarizing, from the agronomic point of view, both heat and drought stresses occurring around the reproductive process significantly reduce wheat grain number and hence the final yield.

Moreover, seed quality is impaired by abiotic stress occurrence as well. In fact, heat and drought impact the normal source-sink relationships by reducing the rate of carbon assimilation and the portioning of carbon and nitrogen within the plant (Mahrookashani et al., 2017). As a consequence, sugar and protein metabolism in leaves and seeds is altered. Under optimal environmental conditions, photosynthetic products (sucrose and glutamine) are translocated from leaves/spikes to seeds; but when photosynthesis is inhibited, the plant needs to search for other sources of carbon in order to support seed development. Due to this, stem reserves become extremely important, since water-soluble carbohydrates are translocated from the stem to reproductive organs (Akter and Islam, 2017). Together with changes in source-sink relationships, which can affect seed quality, also the starch content of kernels is affected by abiotic stresses. For example, HS significantly limits starch biosynthesis in wheat seeds (about -30% at temperatures between 30-40 °C) and in contrast increases the protein and soluble sugar content (Akter and Islam, 2017; Ni et al., 2018; Yadav et al., 2022). An increment in the protein content of wheat grains, and in particular an over-accumulation of gliadins, is associated with a reduction in the sedimentation index, which affects the bread-making quality of the flour (Yadav et al., 2022 and references therein).

Although responses to either drought or heat stress have been extensively analysed in plants, the combination of both environmental stresses has only recently become a matter for research. In fact, even if the plant responses to the single stresses are largely known, the effects of a stress

combination is unique and cannot be directly extrapolated from the effects of the individual stresses (Mittler, 2006). In fact, the plant reaction to multiple stress combinations is hardly predictable, and cannot be directly extrapolated from the response to individual stresses. In natural environments heat and drought stress often occur simultaneously, especially under the Mediterranean climate, but in some cases they can lead to the advent of conflicting responses. For example, as previously detailed, wheat plants increase their stomatal conductance in order to cool the leaves under HS; however if HS is accompanied by a condition of water scarcity, the plant will not open the stomata in order to limit water loss and this will imply a higher increase of inner temperature (Barnabas et al., 2008). In different studies, the combined effects of heat and drought are reported as synergistic (the combination is more severe than either stress alone or added), antagonistic (the combination is less severe than either stress alone or added) or hypo-additive (the effect of combined stress is higher than the individual effects but lower than their sum) (Mahrookashani et al., 2017 and references therein). Regarding crop phenology and physiology, the combination of heat and drought stress anticipates the flowering stage (reducing the microgametogenesis duration) and shortens the grain filling period, besides having a negative and additive impact on overall biomass, photosynthesis and chlorophyll content (Barnabas et al., 2008; Tricker et al., 2018; Sinha et al., 2021). Simultaneous occurrence of the two stresses also exacerbates the mentioned negative impacts on reproductive processes, causing a magnified yield drop (Tricker et al., 2018; Sinha et al., 2021). In fact, all the traits related to plant growth and production showed a synergistic response to combined heat and drought stress, except for straw yield, which showed a hypo-additive response (Mahrookashani et al., 2017). Episodes of drought and heat stress occurring around wheat's anthesis generally reduce the final seed set in a more severe way than the heat stress alone does (Tricker et al., 2018). In the case of a prolonged water limitation from anthesis to grain filling, an early senescence is induced and hence a shortening of the grain-filling period. This can concomitantly increase the remobilization of assimilates from leaves and stems to the grains, raising the grain filling rate (Barnabas et al., 2008).

1.3.3. Plant stress tolerance: main mechanisms and genes involved

Plants are sessile organisms; this means they are permanently restricted to their site of germination and cannot physically escape from unfavourable conditions. To compensate for lack of mobility, plants evolved unique mechanisms enabling to rapidly react and flexibly adapt

to changes in the surrounding environment. Strategies carried out to overcome a stress condition are multiple and can be interpreted in terms of “escape”, “avoidance” and “tolerance”.

- “Escape” mechanisms involved the alteration of plant growth cycle, often anticipating the reproductive phase or shedding their vegetative structures (Janni et al., 2020). For example, a main mechanism of drought escape consists in the production of seeds before the harsh conditions come. This is made possible by shortening the vegetative growth period, resulting in an early flowering (Abobatta, 2019).
- “Avoidance” includes any mechanism that allows the plant to be non-susceptible to the stressor. Many avoidance mechanisms are short-term processes and consist of changing leaf orientation, reflecting solar radiation, leaf shading, extensive rooting and altering membrane lipid composition (Kaushal et al., 2016; Yadav et al., 2022).
- “Tolerance” is surely the most interesting response from the agronomic point of view. Tolerance does not involve substantial modifications in plant growth or development, which can be potentially deleterious for final yield. A tolerant plant is inclined to normally continue its growth cycle quite independently of the stress (Janni et al., 2020). Thus, “heat tolerance” is defined as the plant ability to grow and produce economic yield under HS (Tyagi and Pandey, 2022); similarly, “drought tolerance” is defined as the plant capacity to survive, grow and reproduce under sub-optimal water supply (Abobatta, 2019).

All the molecular mechanisms involved in the plant response to abiotic stresses show a multilevel nature, from stress sensing and signalling to genome-wide reprogramming. The multifaceted plant responses to heat and drought are governed by several genes, which regulate a variety of biochemical and physiological traits (**Figure 1.12**). Initially, the stress is sensed in various cellular compartments, mainly on membranes, that can detect even mild perturbations in the surrounding environment. For example, under HS conditions, membrane fluidity increases and sensors here located detect the physical transition phase. Signalling events involve messengers and regulatory proteins, such as ROS, Ca^{2+} and protein kinases. The activation of calcium channels induces Ca^{2+} influx across the plasma membrane, hence Ca^{2+} concentration increases in cytoplasm and combines with calcium-dependent kinases, which in turn activates multiple transcription factors. The mechanisms of stress signalling trigger changes in gene transcription, RNA processing, protein translation and modifications, which result in the synthesis or alteration of proteins involved in stress response. Some of them are

proteins that are involved in osmotic adjustment and detoxification of ROS (Kaushal et al., 2016; Zhang et al., 2022).

Growing evidence highlights how hormones, such as salicylic acid (SA), polyamines (PA), brassinosteroids (BRs), ethylene (Eth) and mainly abscisic acid (ABA), play a fundamental role in the tolerance regulation in wheat. The latter is a main chemical signal between root and shoot tissues and is involved in both drought and heat stress tolerance acquisition (Kaushal et al., 2016; Tyagi and Pandey, 2022). Indeed, the increase in ABA concentration under abiotic stresses is linked to the up- or down- regulation of several key genes, coding for beneficial proteins able to alleviate the negative effects caused by the stress condition.

In the last decades several genetic regions containing interesting genes/QTLs involved in heat, drought and combined stress tolerance have been identified in wheat. For example, El Hassouni et al., (2019) found that in durum wheat the most critical loci associated with heat tolerance at the reproductive stage are localized on chromosome 1AL, 5BL and 6BS, even if only the combination of all three positive alleles seems to generate a true yield advantage. Sharma et al. (2017) searched for QTLs and candidate genes linked to F_v/F_m , a main chlorophyll fluorescence trait, for improved photosynthesis in wheat under heat stress and identified two significant genomic regions on chromosome 3B and one on chromosome 1D. Some candidate genes, that potentially have a direct impact on maintaining high F_v/F_m during stress, were found to be involved in the biogenesis, assembly, stabilization, and repair of PSII complex. Lu et al. (2020) showed a major QTL on the 7AL arm to be responsible for heat stress tolerance in wheat, located in a genomic region rich in genes related to stress response. The candidate genes here detected mainly code for proteins involved in the ABA-dependent signaling pathway, but also protein binding, zinc finger, F-box domain, galactose oxidase, peroxidase, sugar transporter family protein, and Heat Shock Proteins (HSPs). When two DW cultivars, differing in their water use efficiency (Cappelli and Ofanto, with a higher and lower water use efficiency, respectively), have been compared for their heat and drought stress response, several differences in the activation of stress-responsive genes were detected (Aprile et al., 2013). Under HS, Cappelli showed a remarkable induction of genes coding for enzymes involved in fatty acids β -oxidation and in the glyoxylate cycle; on the contrary, a minimal gene activation was observed in response to drought. Generally, a number of genes involved in drought tolerance in wheat code for signal transduction factors, including protein kinases and transcription factors, but also proteins involved in metabolism, osmotic regulation, protein turnover, protein modification and ROS scavenging and transportation (e.g. DREB: dehydration-responsive element binding protein, CBF: C-repeat-binding factor and MYB:

myeloblastosis oncogene; Lv et al., 2018; Wang et al., 2021). Interestingly, a combined (heat+drought) stress imposition activates a pool of genes that are not induced by the application of single stresses, highlighting how a stress combination is not a mere sum of drought and heat stress, but is rather perceived as a new stress condition (Rampino et al., 2012; Aprile et al., 2013). Genes that in DW turned out to be regulated by the combined stress include the *Td3ITM2* and *Td15ITM6* genes, coding for proteins belonging to the ABC transporter super-family (acting as ion channel regulators and regulating the ion balance), the *Td1ITF1* gene, coding for receptor proteins homologous to putative receptor protein kinase, the *Td4TF1* gene, homologous to a putative t-complex protein 1 theta chain and the lipid remodelling gene named *Td11ITM2* (Rampino et al., 2012).

Among genes that are promptly activated under various abiotic stresses, especially HS, are those coding for HSPs. HS, in fact, causes protein miss-folding and denaturation, which severely impact on their functionality. Misfolded proteins are the targets of HSPs, acting as molecular chaperones and contributing to prevent protein misfolding and to recover a proper folding of misfolded or aggregated proteins (Al-Whaibi, 2011). HSPs, whose expression is positively correlate with thermo-tolerance acquisition in plants (Janni et al., 2020), are water-soluble and consequently imparts HS tolerance in plants probably via hydrating cellular structures.

In addition to the involvement of specific protein types, like HSPs, to cope with a variety of stress conditions, both biotic and abiotic, plants generally enhance the antioxidant defence system, which is essential to prevent oxidative damage to cellular components. The antioxidant system consists of both enzymatic and non-enzymatic components. The first group includes enzymes such as: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX) and glutathione-S-transferase (GST); the non-enzymatic molecules include, among others, ascorbic acid (AsA), glutathione (GSH), phenolic compounds, alkaloids, non-protein amino acids and tocopherols. All these substances co-ordinately work to maintain a balanced ROS concentration, so to prevent the occurrence of an oxidative stress (Kusvuran et al., 2016; Ighodaro and Akinloye, 2018). Among the antioxidant enzymes, SOD is at the forefront in the defence against ROS, catalysing the dismutation of superoxide anion (O_2^-) into molecular hydrogen peroxide (H_2O_2) and oxygen (O_2). SOD is found in many isoforms, classified into three groups accordingly to their active site metal: Cu/ZnSOD, copper-zinc containing SOD, that is the most common, MnSOD, containing manganese and FeSOD, containing iron (Kusvuran et al., 2016; Ighodaro and

Akinloye, 2018; Chen et al., 2022). Both CAT and POD are able to remove the extremely toxic H_2O_2 produced by SOD, converting it into harmless H_2O and O_2 . In general, POD, CAT and APX together play an essential protective role in the scavenging process, coordinated with SOD activity. CAT is found mostly in glyoxysomes of lipid-storing tissues in plants and contains a tetrameric haeme that catalyses the conversion of H_2O_2 . In the ascorbate–glutathione cycle, APX reduces H_2O_2 into H_2O by oxidising ascorbate into monodehydroascorbate (MDHA), which in turn is converted into ascorbate via the MDHA reductase enzyme (Kusvuran et al., 2016). Commonly, an overall increase in the activities of key antioxidant enzymes is correlated with a superior tolerance in DW plants subjected to heat and drought stresses. An enhanced SOD, CAT and APX activity in heat stressed DW genotypes has been positively correlated with chlorophyll content and negatively correlated with membrane injury in field conditions (Almeselmani et al., 2006). Similarly, higher heat tolerance was found to be directly linked with an increase in SOD, CAT and GPX activity in wheat seedlings (Gupta et al., 2013). The same observations were made for water stressed wheat plants, with drought tolerant genotypes showing higher levels of both SOD and POD activities (Dong et al., 2017). However, both in wheat and other species, it is not unusual to find an opposite trend between SOD and CAT/APX activities after an HS occurrence (Lei et al., 2005; Gur et al., 2010) or a drought stress application (Yasar et al., 2014). Moreover, an extremely intense stress treatment could result in a consistent decrease in the overall antioxidant activity, even if an increased activity was previously observed under a moderately intense stress treatment (Nayyar et al., 2014). For all these reasons, a variable modulation of the antioxidant enzyme activities could be observed after a stress application and several aspects, such as the stress timing and intensity, but also the overall plant reaction, should be considered.

Under stressful conditions, together with the above described antioxidant molecules, a series of versatile metabolites are over-produced in order to increase overall plant tolerance and overcome the stress state. Metabolites such as amino acids, phenolics, hormones, polyamines, solutes, antioxidants and others are essential for plant growth and resistance (Chen et al., 2022). In particular, a group of small organic metabolites called “osmolytes” or “compatible solutes” are involved in plant response to counteract mainly drought and salt stress, but also other types of abiotic stress (e.g. cold, heat, heavy metal, nutrient stress). After the occurrence of an osmotic drop in cell water potential, a net and rapid accumulation of these solutes is fundamental to attract water from the outside to the inside and hence maintain the correct cellular pressure turgor (Tyagi and Pandey, 2022). The most common osmolytes are betaines, polyols, amino acids (such as proline), sugars (like glucose, mannitol, trehalose and sorbitol) and polyamines.

They are characterized by a low molecular weight, a high water solubility and non-toxicity even at elevated concentrations, thus not interfering with the normal cellular processes. Among the numerous osmolytes, proline (Pro), a non-essential amino acid, is one of the most studied and extensively reported. Although the exact role of Pro in stress response is still debated (Zhang et al., 2022), numerous studies suggest a positive relationship between Pro accumulation and plant stress tolerance acquisition (Kishor et al., 2005; Siddique et al., 2018). Besides the maintenance of cellular turgor, Pro acts also as a stabilizer of sub-cellular structures, buffer of cellular redox potential, ROS scavenger, signal molecule and protein folding chaperone (Hayat et al., 2012; Siddique et al., 2018; Chen et al., 2022). Not least, Pro degradation is also beneficial for the overall cell recovery after stress, because of the releasing of a high amount of energy, nitrogen and reducing power. Considering this, surely there is a pressing need to produce more detailed studies focused on stress recovery phase, especially when the stress applied is transient (Jagadish et al., 2021), regarding not only Pro degradation, but also the progress of other delayed tolerance mechanisms. In wheat, a substantial Pro increase in leaves of heat (Mustafa et al., 2021; Ahmed and Hasan, 2011), drought (Mwadzingeni et al., 2016; Barunawati et al., 2016) and combined heat + drought (Qaseem et al., 2019; Sattar et al., 2020; Ru et al., 2023) stressed plants has been consistently reported, correlating the activation of this defence mechanism with a higher stress tolerance. However, less is known about Pro accumulation in fundamental sink organs, such as spikes, directly related to yield preservation (Li et al., 2020), and about its role in plants subjected to heat stress and water-deficit simultaneously. In fact, the rate and direction of Pro accumulation in spikes under combined stresses are not yet clear and are probably connected with the intensity and timing of stress application. Rizhsky et al. (2004) reported that *Arabidopsis* plants subjected to a combination of heat and drought accumulate sucrose instead of proline, suggesting that sucrose may replace proline as the major osmoprotectant, probably to safeguard the overactive and sensitive mitochondria from the potentially toxic pyrroline-5-carboxylate, an intermediate in Pro biosynthesis and degradation. These findings suggest that also in other plants during a stress combination or even under an intense stress treatment, sucrose or other osmolytes could play the osmoprotecting role instead of or in addition to Pro. As previously reported, high temperatures at anthesis cause the acceleration of leaf senescence in wheat, this reducing the overall sugar reserves accumulation of the plant. Despite that, tolerant plants exhibit at the cellular level a positive osmoregulation through the synthesis of soluble sugars and Pro in order to improve membrane stability. In fact, increased levels of total soluble sugars, together with those of Pro, showed to play a significant role in osmotic adjustment and ROS scavenging under HS conditions (Dwivedi et al., 2019).

Also in case of water scarcity at anthesis, the metabolic response of wheat plants involved an accumulation of antioxidant- and drought tolerance-related sugars, particularly in spike tissues. In fact, the increase of sugars especially in the glumes may prevent oxidative damage and contribute to the acquisition of a high spike performance under water stress (Vergara-Diaz et al., 2020). Soluble sugars accumulate within cells also under heat and drought stress combination, even exhibiting a superior accumulation rate from those of single stresses, efficiently reducing the cellular osmotic potential (Ru et al., 2023).

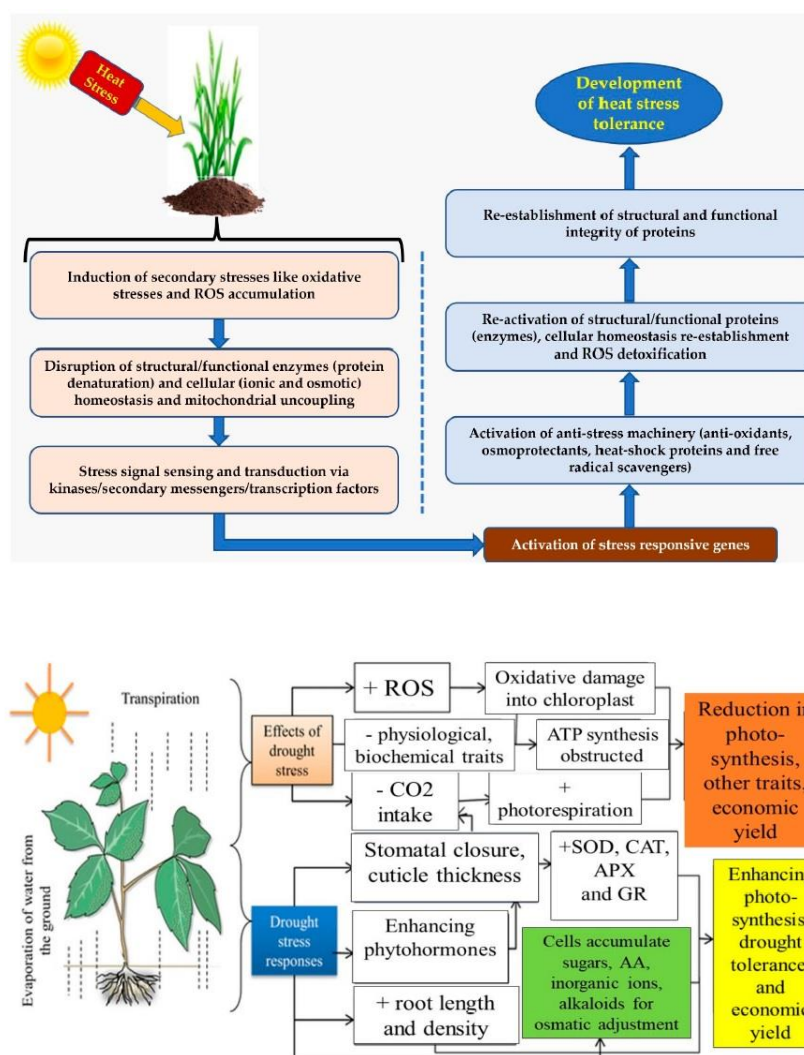


Figure 1.12. Mechanisms of plant tolerance in response to heat (above) and drought (below) stress (from Yadav et al., 2022 and Seleiman et al., 2021).

1.4 Objectives

The DW-*Thinopyrum* spp. near isogenic recombinant lines (NIRLs) developed by Ceoloni et al., 2005a and Kuzmanovic et al., 2019 (see Introduction and **Figure 1.7** and **1.8**) through the chromosome engineering strategy are valuable genetic materials to carry out a number of basic research investigations and also for breeding exploitation. The availability of NIRLs, carriers (+) and non-carriers (–) of a given wild segment in a common DW genetic background, permits to perform accurate comparative analyses and hence attribute traits of interest to the presence of specific alien chromosome segments, where candidate genes can ultimately be identified. The genetic enrichment carried out by transferring small amounts of wild *Thinopyrum* spp. chromatin into DW was initially aimed at improving disease resistance traits (§ 1.2.5). However, the native and wide distribution areas of *Thinopyrum* species, the still limited knowledge of the behaviour to abiotic stress associated with wheat-*Thinopyrum* lines (see § 1.2.5.), and the preliminary evidence of good performance of the DW-*Thinopyrum* spp. recombinants above recalled even in unfavourable field conditions (Kuzmanović et al., 2018, 2021), all represented stimulating factors to more deeply explore the NIRLs' response to abiotic stresses, performing experiments under controlled conditions. Positive results from this kind of investigations would represent a valuable addition to the already useful genetic makeup of these materials, particularly in view of the evolving climatic conditions in the Mediterranean area, where their exploitation could be foreseen. Apart from possible breeding advantage, knowledge on their mechanisms of reaction against abiotic stress application would represent an important achievement.

Based on these premises, the main objectives of this thesis have been:

- The analysis of the response mechanisms exerted by the DW-*Th. ponticum* NIRLs (carriers vs. non carriers of the respective alien segment) to overcome the imposition of a heat and a combined heat+drought stress at a juvenile growth stage (seedlings at two-leaf stage). One aim of this first analysis was the identification of early selection criteria for tolerance traits and the comparison of the early response with that exhibited at later stages (e.g. the reproductive phase), similarly to what recently performed in bread wheat (Lu et al., 2022). The analyses performed on juvenile plants are reported in “[Chapter 2](#)”.
- The analysis of physiological, biochemical and agronomic traits/mechanisms characterizing the response to heat and combined heat+drought stress, applied around the particularly stress sensitive phase of anthesis, exerted by contrasting NIRLs and eventual

attribution of the genetic determinants of such traits to the respective wild chromosome segments. The description of this objective and the results obtained, which have been the subject of a journal publication (Giovenali et al., 2023), represent “Chapter 3” of this thesis.

- The analysis of the NIRLs’ behaviour, in terms of physiological and yield performance vs. control lines, when subjected to a very intense heat stress at the anthesis stage, in order to hopefully identify candidate genotypes for highly stress tolerant performance, for both mechanisms’ exploration and breeding use. Analyses and results concerning this assay are reported in “Chapter 4”.
- The evaluation of the physiological response and yield performance under rainfed and irrigated field conditions of a set of selected genotypes, comprising the *DW-Thinopyrum* spp. NIRLs and some DW varieties originating from Italy, Algeria and Tunisia. The comparison between the response exhibited by the genotypes under controlled conditions and in a field environment could be extremely useful as a validation assay of yield stability under favourable vs. stressed conditions. The analyses performed and the observed results are described in “Chapter 5”.

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Chapter 2: Morpho-physiological and biochemical response to heat and combined heat+drought stress applied to seedlings of chromosomally engineered DW-*Thinopyrum* spp. recombinant lines

Chapter 2: Morpho-physiological and biochemical response to heat and combined heat+drought stress applied to seedlings of chromosomally engineered DW-*Thinopyrum* spp. recombinant lines

Abstract

Global warming is one of the most threatening challenges for the enhancement and stability of durum wheat (DW) production in the Mediterranean basin. In this area, heat waves and high temperature peaks together with drought phenomena heavily affect DW fertility and final yield. Introgression of novel genes/QTLs from wild relatives represents a suitable and sustainable strategy to enlarge the crop genetic basis, and hence its ability to tolerate abiotic stresses. Here, we tested the response to heat (H) and combined H + water-limited (WH) conditions of DW-*Thinopyrum* spp. near-isogenic recombinant lines (NIRLs), carriers (+) and non-carriers (–) of differently sized alien chromosome segments transferred from the wild species on their 7AL arms. In order to identify early tolerance criteria and promptly select the best performing genotypes under the single H and combined WH stresses, the reaction of 14 days-old seedlings was evaluated. Comparative analyses highlighted morpho-physiological, biochemical and molecular differences in stress responses associated with the presence of the wild *Thinopyrum* segments. In particular, the 23%-long *Th. ponticum* segment, common to R5+ and R69-9/R5+ genotypes, provided substantial advantages under stressful conditions, mainly involving the maintenance of activity of the antioxidant enzyme SOD under H, and of RWC under WH. This work substantiates the advantage of enriching the DW genetic basis via exploitation of wild gene pools in view of improving DW tolerance to abiotic stresses, also appreciable at juvenile phenological phases.

2.1. Introduction

Wheat (*Triticum* spp.), with a total yield of 760.93 million tonnes produced in 2020, is one of the main staple crop worldwide. Durum wheat (*Triticum turgidum* subsp. *durum* Desf., DW) represents about 8% of the total wheat production and is mainly grown in the area of the Mediterranean basin (Xynias et al., 2020), where it is particularly appreciated for its typical end products (pasta, noodles, couscous and bread), which are at the basis of the human diet and of the economy in many countries there located.

Nowadays, due to the continuous rise in human population and the consequent need to provide higher amounts of quality food, a pressing need to increase the overall wheat production is emerging. Nevertheless, the achievement of this objective is prevented by the increasingly frequent exposures of DW to high temperatures extremes and drought phenomena, especially in the semi-arid area of the Mediterranean basin, considered a hot spot of climate change (Cramer et al., 2018).

A promising strategy to overcome the issue is the enlargement of DW genetic basis via the exploitation of new and still little explored genetic source of wild relatives, which have the potential to significantly improve wheat tolerance to abiotic stresses (Dempewolf et al., 2017; Rampino et al., 2019). In particular, the *Thinopyrum* genus, which is part of the wheat tertiary gene pool, includes several perennial species native and naturally adapted to the harsh environments of southern and eastern Europe, western Asia and northern Africa (Monsen et al., 2004; Wang, 2011). In the recent years, a number of DW-*Thinopyrum* spp. recombinant lines, have been developed through the “chromosome engineering” strategy (Ceoloni et al., 2005; Ceoloni et al., 2014). This cytogenetic approach, consisting of the transfer of segments of alien chromosomes carrying particular desired genes to wheat chromosomes (Sears, 1972), allows to incorporate into the crop genome only small amounts of the wild chromatin, thus substantially reducing probability of linkage drag phenomena. The DW-*Thinopyrum* spp. recombinant lines obtained, already proved to possess disease resistance attributes and some yield and quality advantages (see general introduction §1.2.5.; Kuzmanovic et al., 2018; Kuzmanovic et al., 2019; Ceoloni et al., 2014), have been recently tested for their tolerance to drought stress in the field (Kuzmanovic et al., 2021) and to heat and combined heat+drought stresses applied under controlled conditions at the critical stage of anthesis (Giovenali et al., 2023).

Even if wheat plants rarely experience heat or drought conditions at the seedling phases, especially in the areas of the Mediterranean basin, an early selection performed during juvenile stages could provide useful predictive information. In order to accelerate the breeding programs for wheat tolerance, and hence saving time for the selection of the best performing genotypes, an early selection strategy on laboratory scale would be extremely advantageous. The screening of tolerant wheat genotypes on the basis of seedling traits is easy and cheap, and can provide predictive evidences about the response performed by the plant at the reproductive stage (Lu et al., 2022). In fact, several reports indicate that some QTLs for heat tolerance at seedling and reproductive stage are collocated on the same chromosomes (Li et al., 2012; Maulana et al., 2018), this suggesting that some tolerance QTLs are effective both at seedling and reproductive phases in wheat.

For seedling development, an optimal temperature of 20°C is generally required (Khaeim et al., 2022) and temperatures above the optimum can lead to a number of negative effects, like reduction of photosynthesis, chlorophyll content, respiration rate, and finally seedlings death, due to excessive leaves dehydration (Maulana et al., 2018). Moreover, both heat and drought stresses applied during wheat seedling stage remarkably reduce all morphological traits, as measured by fresh and dry tissue weight and length, and increase membranes lipid peroxidation (Almaghrabi, 2012; Hameed et al., 2012; Gupta et al., 2013; Altaf et al., 2021). In order to tolerate heat and drought stresses, and hence continue to grow, wheat plantlets exploit some adaptive mechanisms, including the accumulation of compatible solutes, such as proline, betaine, fructose and sucrose, and the modulation in the activity of some key antioxidant enzymes able to prevent a consequential oxidative stress (Ahmed and Hassan, 2011; Gupta et al., 2013; Guo et al., 2018). Furthermore, in the case of a water-limiting conditions, the seedlings rooting ability becomes essential to reach a deeper source of water (Ahmed et al., 2019).

In the present work, an overall evaluation of some DW-*Thinopyrum* spp. recombinant lines' performance under heat and a combination of heat and drought stresses was carried out at the seedling (two leaves) stage. Morphological, physiological and biochemical parameters have been measured/assayed on both shoot and root tissues, with the aim to select the best-performing genotypes and to associate given response mechanisms to the presence of specific wild chromatin segments.

2.2. Materials and methods

2.2.1. Plant materials

In both the experiments here reported, the first focused on the application of heat stress alone (H) and the second on the application of combined heat and water-deficit stress (WH), different DW-*Thinopyrum* spp. recombinant lines were used as main plant targets. In the H experiment, 3 DW-*Th. ponticum* NIRLs were employed, named R5-2-10, R112-4 and R23-1 and here referred to as R5+, R112+ and R23+, respectively. The NIRLs were previously obtained via chromosome engineering strategy (Ceoloni et al., 2005) and developed in the background of the Italian DW cv. Simeto by repeated back-crossing (BC₅ for R5+ and R112+ and BC₄ for R23+), followed by several self-generations. The NIRLs possess different portions of the tall wheatgrass species *Th. ponticum* 7e1L chromosome arm replacing 23%, 28% and 40% of the

distal end of the recipient DW 7AL arm, respectively (**Figure 2.1**). The symbol “+” indicates homozygous carriers of the respective 7el₁L segment, while the symbol “–” indicates homozygous sib lines non-carrier of the given 7el₁L segment. Besides these, two secondary “nested” NIRLs were also employed, named R69-9/R5+ and R69-9/R112+, deriving from the primary types R5+ and R112+, respectively (Kuzmanovic et al., 2019). The nested NIRLs present a *Th. elongatum* 7EL chromosome segment distally inserted into the 7el₁L ones (**Figure 2.1**).

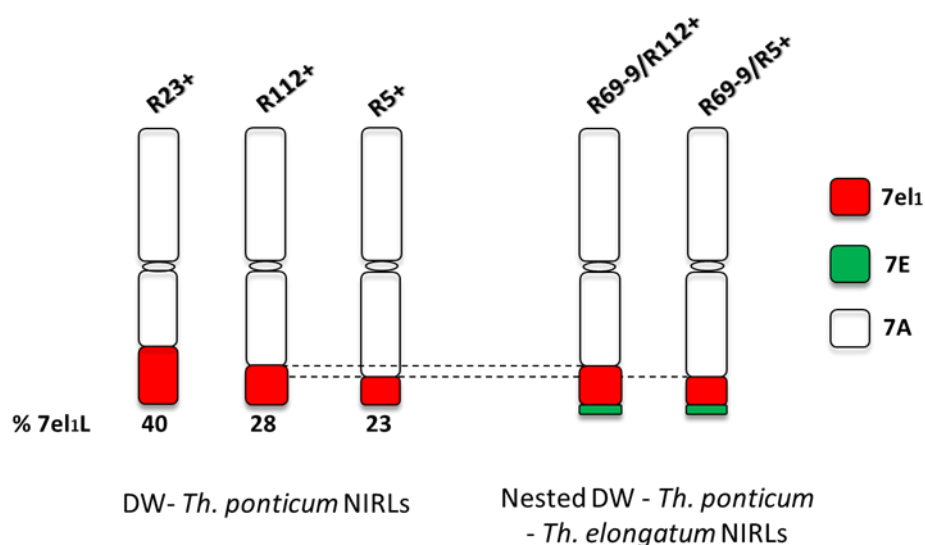


Figure 2.1. DW-*Thinopyrum* spp. recombinant lines tested. **7el₁L**: chromosome segment derived from *Th. ponticum*; **7E**: chromosome segment derived from *Th. elongatum*; **7A**: DW recipient chromosome.

In the combined WH experiment, all the previously described NIRLs were employed except for R23+ (**Table 2.1**). The latter was excluded on the basis of previous experiments, that in which, although showing some characteristics of interest, had exhibited relatively low tolerance even under single and milder stress conditions.

The DW ICARDA cv. Margherita has been introduced in both the experiments as a positive tolerant control, since it expressed a remarkable heat tolerance ability in a range of stressful environments, such as the Senegal River Basin, characterized by temperature extremes during most of the growth cycle (Sall et al., 2018a, b).

Genotypes	Stress condition applied	
	H	WH
R5+	X	X
R5–	X	X
R112+	X	X
R112–	X	X
R23+	X	
R23–	X	
R69-9/R5+	X	X
R69-9/R5–	X	X
R69-9/R112+	X	X
R69-9/R112–	X	X
Margherita	X	X

Table 2.1. Plant materials evaluated for each experimental activity performed.

X: genotype tested at the corresponding stress condition.

2.2.2. Methods

2.2.2.1. Seedling growing conditions and stress treatments performed

All the plant materials were grown under controlled conditions into a cabinet growth chamber. Seed sterilization was carried out for 5 min in a sodium hypochlorite solution (80% distilled water, 20% bleach) followed by two rinses and two hours of soaking in distilled water. Seeds were placed into Petri dishes sealed with parafilm, on a double layer of soaked filter papers, and placed at 4°C for two days to stimulate and synchronize germination. On the third day, seeds were moved into see-through boxes and placed in the growth cabinet (Seed Germination Chamber SG4, LabCo) at 20°C, 16 hr of photoperiod and constant air flux. Petri dishes containing the growing seedlings were regularly watered with sterile and distilled water in order to avoid water deficit and fungal contaminations. The H treatment was applied 14 days post germination (dpg; two-leaf stage, Zadoks scale 12; Zadoks et al., 1974), setting the temperature inside the cabinet at 42°C and maintaining it for 2 consecutive hours. After the H stress, the temperature was re-set at 20°C. The WH treatment, consisting of a combination of osmotic and H stress, started 7 dpg (one leaf stage, Zadoks scale 11), by transferring the seedlings into new Petri dishes containing a double layer of filter paper soaked with PEG solution (17.8 g PEG6000 in 100 ml of distilled water, corresponding to $\Psi = -0.4$), which permits to simulate a controlled water-deficit stress by modulating the osmotic potential of the solution. Seedlings were

maintained in the PEG solution until 14 dpg, when also H stress was applied with the same procedure reported above for the single H imposition. Features of experimental plant growth conditions and of stress treatments application are illustrated in **Figure 2.2**. In both experiments (H and WH stress application), a set of control (C; non-stressed) seedlings were maintained in well-watered conditions and at constant 20°C temperature.

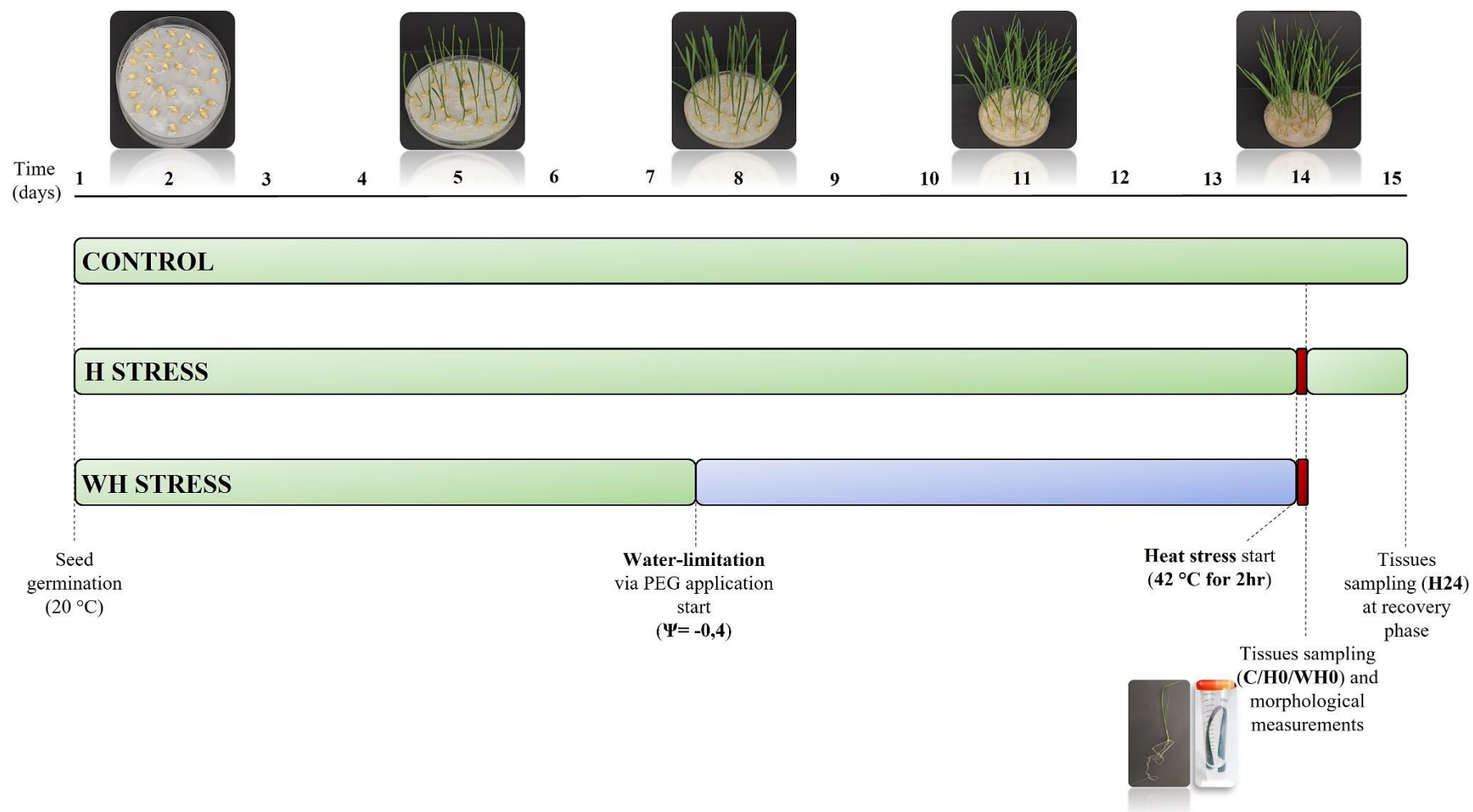


Figure 2.2. Timeline and features of experimental plantlet growth conditions, stress treatments procedure and data recording.

2.2.2.2. Morphological and physiological traits measured after stress imposition

All the morphological and physiological, as well as biochemical and molecular analyses (described in §2.2.2.3. and 2.2.2.4) performed on the plantlets are schematized in **Table 2.2**.

Morphological analyses were carried out 14 dpg for both control C0) and stressed (H0, WH0) seedlings. Morphological parameters measured on both shoot and root tissues were: shoot length (SL), root and shoot fresh weight (RFW, SFW), root and shoot dry weight (RDW, SDW) and root/shoot ratio (R/S). Only in the case of the WH experiment, root number (RN) and root total length (RTL) have also been measured. Dry weights (RDW, SDW) were obtained placing tissues in an oven at 65 °C for at least two days, until the tissues reached a stable and constant weight. Leaf RWC was calculated using the formula: $RWC = (FW - DW) / (TW - DW)$, where FW, DW and TW stand for fresh, dry and turgid leaf weight, respectively. Briefly, for RWC determination, a 2 cm-long section was cut from the middle portion of the leaf, its FW immediately recorded before putting it in tubes with cut ends immersed into distilled water. Tubes were placed at 4°C overnight for TW measurement the next day. Finally, turgid leaf segments were oven-dried for two days at 65 °C to assess the corresponding DW. At least 5 plants/genotype/treatment condition were used for RWC calculation and 8 plants/genotype/treatment for morphological measurements.

Stress applied	Genotypes	Morphological analyses	Leaf RWC	Proline content in shoot	Proline content in roots	TBARS in shoot	TBARS in roots	Enzymatic assays	miRNA expression
H	R5+	X	X	X	X	X	X	X	
	R5-	X	X	X	X	X	X	X	
	R112+	X	X	X	X	X	X	X	X
	R112-	X	X	X	X	X	X	X	X
	R23+	X	X	X	X	X	X	X	
	R23-	X	X	X	X	X	X	X	
	R69-9/R5+	X	X	X	X	X	X		
	R69-9/R5-	X	X	X	X	X	X		
	R69-9/R112+	X	X	X	X	X	X		
	R69-9/R112-	X	X	X	X	X	X		
	Margherita	X	X	X	X	X	X	X	
WH	R5+	X	X	X	X	X	X		
	R5-	X	X	X	X	X	X		
	R112+	X	X	X	X	X	X		
	R112-	X	X	X	X	X	X		
	R69-9/R5+	X	X	X	X	X	X		
	R69-9/R5-	X	X	X	X	X	X		
	R69-9/R112+	X	X	X	X	X	X		
	R69-9/R112-	X	X	X	X	X	X		
	Margherita	X	X	X	X	X	X		

Table 2.2. Analyses performed in the present work for each genotype and stress condition. TBARS: Thiobarbituric acid assay. **X**: genotype tested in the corresponding analysis.

2.2.2.3. Biochemical analyses performed on control and stressed seedlings

2.2.2.3.1. Proline quantification

Sampling of seedling shoots and roots was performed immediately after the H stress application in both H and WH experiments, namely 14 dpg for both control (C) and stressed (H0, WH0) seedlings. Only in the case of the H experiment seedling tissues were collected also 24 hr after the H application (H24), in order to evaluate plantlets' recovery activity after a temporally limited stress. Each plant tissue was cut, immediately suspended in liquid nitrogen, and stored at -80°C until further use. Proline (Pro) content was quantified following a standardized ninhydrin-based colorimetric assay (Abraham et al., 2010). In the assay, when ninhydrin interacts with Pro forms a red product which can be used for estimation of the concentration of this amino acid. One hundred mg of tissues were grinded in cool mortars with liquid nitrogen until a fine powder was obtained. The powder was then mixed with 100 μl of 3% sulfosalicylic acid and samples were centrifuged for 5 min at room temperature (RT). One hundred μl from the supernatant of plant extract was added in a reaction mixture containing 100 μl of sulfosalicylic acid, 200 μl of acidic ninhydrin (1.25 g ninhydrin in 30 ml of glacial acetic acid and 20 ml of 6M orthophosphoric acid) and 200 μl of glacial acetic acid. The reaction started after tubes incubation at 96°C for 60 min and was stopped by putting the tubes on ice. To each tube, 1 ml of toluene was added, vortexed for 20 sec and kept at RT for 5 min to allow separation of the coloured (proline extract) and the water phases. Two hundred μl of the Pro extracts were transferred into a 96-well plate and read by a microplate reader at 520 nm, using toluene as blank (**Figure 2.3**). Pro concentration of samples was determined in technical duplicates using a standard concentration curve and expressed as $\mu\text{mol/g}$ FW. The standard curve was prepared with 5 different concentrations of Pro, starting from a 10 mM solution of pure Pro (L-Proline, ReagentPlus(R) $\geq 99\%$, SIGMA-ALDRICH). At least 3 plants/genotype/treatment condition were used for proline quantification.

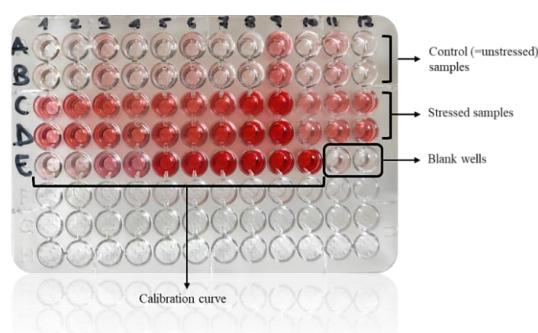


Figure 2.3. Example of a 96-well plate prepared with some proline extracts.

2.2.2.3.2. The assay of lipid peroxidation (Thiobarbituric acid reactive substances -TBARS)

The assay of lipid peroxidation was performed on shoots and roots sampled immediately after H stress application in both H and WH experiments, namely 14 dpg for both control (C) and stressed (H0, WH0) seedlings. As for Pro quantification, in the case of the H experiment also seedling tissues were collected 24 hr after H application (H24). The tissues were put in tubes, immediately suspended in liquid nitrogen, and stored at -80°C until further use. The TBARS assay (Jambunathan, 2010), the most widely used to determine lipid peroxidation, was performed. During the process of lipid peroxidation, the decomposition of polyunsaturated fatty acids produce malondialdehyde (MDA), which reacts with thiobarbituric acid, making a coloured compound that can be used for estimation of the MDA concentration (**Figure 2.4**). One hundred mg of frozen tissues were grinded in cool mortars and homogenized with 2 ml of 0.1% trichloroacetic acid (TCA). The extracts were centrifuged at 10,000 rpm for 15 min and the supernatants were collected. 1 ml of supernatant was mixed with 2 ml of 20% TCA and 2 ml of 0.5% thiobarbituric acid (TBA) and finally the mixture was heated at 95°C for 30 min in a fume hood. After cooling the samples on ice, 200 μl of the compound were transferred into a 96-well plate and read by a microplate reader at 532 nm and 600 nm. The absorbance at 600 nm is the non-specific absorbance and has to be subtracted from the values obtained for the absorbance at 532 nm. The concentration of MDA was calculated using Beer–Lambert's equation (extinction coefficient of MDA: $155\text{ mM}^{-1}\text{ cm}^{-1}$). At least 3 plants/genotype/treatment condition were used for MDA quantification.

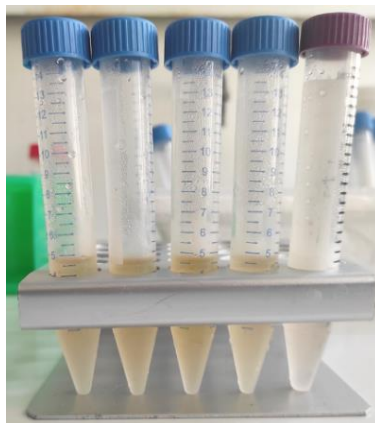


Figure 2.4. Colorimetric reaction in the TBARS assay.

2.2.2.3.4. *Antioxidant enzymes activities*

2.2.2.3.4.1. *Enzymatic extraction protocol*

Enzymatic extraction was performed from leaves of control (C) and H stressed (H0, H24) seedlings. The sampled tissues were put in tubes, immediately suspended in liquid nitrogen, and stored at -80°C until further use. Crude leaf extracts for antioxidant enzymes assays were obtained through the protocol of Elavarthi and Martin (2010), reported by Sunkar (2010), with minor modifications. Briefly, 100 mg of leaf tissue was ground in a cold mortar to a fine powder using liquid nitrogen. The obtained powder was homogenized in 1.2 ml of 0.2 M potassium phosphate buffer (pH 7.0 with 0.1 mM EDTA). The samples were centrifuged at 13000 rpm for 25 min at 4°C . The supernatant was removed, the pellet resuspended in 0.8 ml of the prior buffer and finally the suspension centrifuged for another 20 min at 13000 rpm. The combined supernatants were stored on ice and used to determine different antioxidant enzyme activities. At least 3 samples/genotype/treatment condition were used for enzymatic extraction.

2.2.2.3.4.2. *Protein quantification via Bradford analysis*

The Bradford method (Bradford, 1976) is a colorimetric assay for determination of total protein concentration and is based on the formation of a spectrophotometrically detectable chromophore. The use of Bradford method presents many advantages, since it is simple, fast, economic and sensitive. It is based on the interaction between Coomassie brilliant blue G-250 (CBBG) and arginine, tyrosine, histidine and phenylalanine residuals on proteins producing a detectable blue coloration. Bradford analysis has been performed on all the enzymatic extracts obtained (§ 2.2.2.3.4.1.) and stored at -80°C . After samples thawing, 2 and 4 μl of enzymatic extract was removed and H_2O was added until reaching 0.8 ml. Two hundred μl of Coomassie were added to reach 1 ml of total volume. Finally, the absorbance was measured at 595 nm. Protein concentration of the samples was compared to a calibration line prepared with a standard 0.2 mg/ml bovine serum albumin (BSA) solution. Three different volumes of BSA (5, 15 and 30 μl) was used and H_2O was added until reaching 0.8 ml. 200 μl of Coomassie was added to reach 1 ml of total volume and absorbance was read at 595 nm. As blank, a solution containing H_2O and Coomassie was used.

2.2.2.3.4.3. Superoxide dismutase (SOD) activity

SOD (EC 1.15.1.1) activity was evaluated on seedlings' leaves using a SOD assay kit (19160 SOD determination kit, Sigma-Aldrich). The kit utilizes the highly water soluble tetrazolium salt WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of reduction with O_2 is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD, as shown by **Figure 2.5**. Following the developer's instructions, 20 μ l of each enzymatic extract were added in a 96-well microplate. Two hundred μ l of WST Working Solution (previously obtained diluting 1 ml of WST Solution with 19 ml of Buffer Solution) were added to each well and the solutions were mixed. Finally, 20 μ l of Enzyme Working Solution (previously obtained diluting 15 μ l of Enzyme Solution with 2.5 ml of Dilution Buffer) were added to each sample and the solution was thoroughly mixed. The blank well contained 20 μ l of ultrapure water, 200 μ l of WST Working Solution and 20 μ l of Dilution Buffer. The microplate was incubated at 37°C for 20 min and the absorbance read at 450 nm. SOD activity was calculated as International Unit of enzyme (IU)/mg protein. One IU is defined as a point where a sample gives 50% inhibition of a colorimetric reaction between reactive dye and superoxide anion, so if the absorbance of a sample that does not contain any SOD (blank) is 1.0, another sample that gives 0.5 absorbance is defined as having 1 unit of SOD activity.

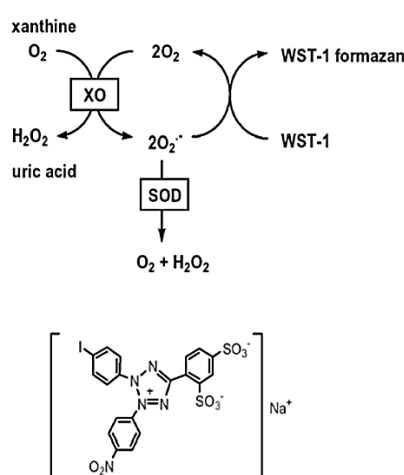


Figure 2.5. Chemical reactions on which the SOD assay kit is based.

2.2.2.3.4.4. Peroxidase (POD) activity

POD (EC 1.11.1.7) activity was evaluated on seedlings' leaves using the Oxiselect Hydrogen Peroxide/Peroxidase Assay Kit (Cell Biolabs, inc.; Catalog Number STA-844). The kit can measure POD activity in biological samples through a sensitive and quantitative colorimetric method: a colorimetric probe reacts with H_2O_2 and horseradish peroxidase (HRP) enzyme to produce a bright pink coloured product. The absorbance values detected using the kit are proportional to POD levels within the samples. Following the manual's instructions, 50 μl of samples' enzymatic extracts were placed into the wells and 50 μl of Peroxidase Working Solution added. The wells' contents were thoroughly mixed and the plate incubated for 30 min at RT in the dark. Finally, the absorbance was read at 540 nm and the concentration calculated comparing the samples absorbance to those of a standard curve ranging from 0 to 10 mU/ml. As reported in the kit, one U is defined as the amount of enzyme that will form 1.0 mg purpurogallin from pyrogallol in 20 sec at pH 6.0 and 20°C.

2.2.2.3.4.5. Catalase (CAT) activity

CAT (EC 1.11.1.6) activity was evaluated on seedlings' leaves following the method reported by Huseynova (2012). Briefly, the decomposition of H_2O_2 was measured spectrophotometrically by following the decrease in absorbance at 240 nm for 1 min. The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 0.1 mM H_2O_2 , and the reaction was initiated by adding 25 μl of enzyme extract. Blank contained the phosphate buffer and H_2O_2 only, without the enzyme. The variation in absorbance ($\Delta\text{Abs}/\text{min}$) was monitored at 240 nm and then converted in UI/mg protein with the following formula: $(\Delta\text{Abs}/\text{min} * V_{\text{tot}})/(\epsilon * l * V_e)$; where V_{tot} stands for the total volume of solution in the well, ϵ is CAT molar extinction coefficient ($\epsilon = 39.4 \text{ mM}^{-1} \text{ cm}^{-1}$), l stands for the length of optical path and V_e stands for the volume of enzyme in the solution.

2.2.2.3.4.6. Ascorbate peroxidase (APX) activity

APX (EC 1.11.1.11) activity was evaluated on seedlings' leaves following the method of Elavarthi and Martin (2010), reported by Sunkar (2010). APX activity was determined spectrophotometrically by monitoring the decrease in absorbance at 290 nm for 3 min due to

the ascorbate oxidation. The 1 mL assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 10 μ L of enzymatic extract and lastly 0.5 mM H₂O₂, to initiate the reaction. Blank contained the phosphate buffer, H₂O₂ and ascorbate only, without the enzyme. The variation in absorbance (Δ Abs/min) was monitored and then converted in UI/mg protein with the same procedure reported in 2.2.2.3.4.5. and considering the APX molar extinction coefficient of 2.8 mM⁻¹ cm⁻¹.

2.2.2.4. microRNA expression analysis on H stressed seedlings

The expression of a selected panel of micro-RNAs (miRNAs) was analysed in R112+ and R112- seedlings subjected and non-subjected (C) to the H stress. Critical time points for miRNAs analysis were two: immediately after H imposition (H0) and 2h after the end of H stress (H2). The main purpose of this analysis was to tentatively associate the heat tolerance showed by R112+ to the differential expression of some gene expression regulators, like miRNAs. The 7 microRNAs analysed (**Table 2.3**) were selected on the basis of a literature survey, indicating them as involved in abiotic stress events. Being this activity part of a joint project with the University of Ferrara (funded by the Italian CIB, Consorzio Interuniversitario Biotecnologie), after samples preparation (made by the author), the expression analysis of the selected miRNAs was performed by the Department of Life Sciences and Biotechnology of the University of Ferrara. Fifty mg of frozen leaf tissues were used for total RNA extraction with Plant/Fungi Total RNA Purification Kit (Norgen), according to the manufacturer's instructions. The obtained RNA was quantified and 300 ng were reverse transcribed using TaqMan microRNA Reverse Transcription Kit (Thermo Fisher Scientific) and miRNA-specific reverse transcription primers. cDNAs from all samples were analysed by reverse transcription droplet digital PCR (RT-ddPCR) and the number of copies for each miRNA was determined using the QX200 Droplet Digital PCR system.

2.2.2.5. Statistical analysis

For all the datasets, one-way analysis of variance (ANOVA) was performed in order to estimate differences ascribable to the genotype (G) effect, while two-factor ANOVA was applied to analyse the effect of the G $\times$ treatment (T) interaction. Whenever a significant F value was obtained for single factors or their interaction, Tukey HSD test was performed at P < 0.05 level.

Before performing the ANOVAs, obvious outliers were eliminated. All statistical analyses were performed by JASP software (JASP Team, 2022; JASP Version 0.16.1). For all parameters, two statistical comparisons (ANOVAs) were performed: in the first one, the datasets of the individual NIRLs (+) and of the corresponding non-carrier sib lines (–) were compared, to associate possible differences in stress behaviour with presence of a specific alien segment; in the second one, the three NIRLs’ datasets were compared to that of the heat tolerant cv. Margherita, to evaluate their performance with respect to a known heat tolerant and productive cultivar.

miRNA name	Species considered	Stress applied	miRNA target	miRNA modulation under stress (see Ref.)	Reference
miR159a	Wheat	Osmotic and salt stresses	MYB TFs, 1-aminocyclopropane-1-carboxylate synthase, oligopeptide transport gene, saur family protein	UP	Gupta et al. 2014
		Cold		DOWN	
	Wheat	WS	Serine/arginine repetitive matrix 1	UP	Ma et al. 2015
	Wheat	HS	WRKY TF	DOWN	Kumar et al., 2015
	Durum wheat	WS	-	UP	Liu et al. 2015
	Durum wheat	WS	MYB TFs	UP	Giusti et al., 2017
		HS		DOWN	
miR160	Barley	HS	ARFs TF	UP	Kruszka et al., 2014
	Wheat	HS	ARFs TF, TPR, HSP70	DOWN	Kumar et al., 2015
	Durum wheat	WS+HS	ARF8, ARF18	UP	Liu et al., 2019
miR166	Barley	HS	HD-Zip TF	UP	Kruszka et al. 2014
	<i>Triticum dicoccoides</i>	WS	HD-ZipIII TF	DOWN	Kantar et al., 2011
	Durum wheat	HS	-	DOWN	Giusti et al., 2017
		WS	-	UP	
miR393	Durum wheat	WS	Auxin signalling F-box (AFB), Transport Inhibitor Response 1 (TIR1)	UP	Liu et al., 2015
	Durum wheat	WS		UP	Giusti et al., 2017
	Wheat	Osmotic and salt stresses	bHLH TF, transport inhibitor response 1/auxin F-box	UP	Gupta et al., 2014
		Cold		DOWN	
miR396	Triticeae	-	Copper SOD, Cu–Zn SOD	-	Alptekin et al., 2017
	<i>Triticum dicoccoides</i>	WS	Growth regulating factor-like (GRL), ceramidase genes, Heat-shock protein containing, GRF-1 homologue thioredoxin and DnaJ domains	DOWN	Kantar et al., 2011
	Durum wheat	WS+HS	HSP90	UP	Liu et al., 2019
miR398	Wheat	HS	Cu/Zn-SOD	DOWN	Kumar et al. 2015
	<i>Triticum dicoccoides</i>	WS	Cu–Zn SOD	UP	Kantar et al., 2011
miR1137	Wheat	HS	Anthranilate synthase	UP	Ravichandran et al., 2019

Table 2.3. Some features of the selected miRNAs for expression analysis of the R112 recombinant subjected to heat stress.
HS = heat stress; WS = water stress; TF = transcription factor.

2.3. Results

2.3.1. Traits showing statistically significant differences for genotype (G), treatment (T) and G × T interaction in the H experiment

All the parameters measured/analysed during the H experiment (**Table 2.4**) showed to be significant for the G factor, indicating that they were highly affected by the wild segments presence (**Table 2.5**).

Acronyms	Measurement/Analysis performed
SL	Shoot Length (cm)
SFW	Shoot Fresh Weight (g)
SDW	Shoot Dry Weight (g)
RWC	Leaf Relative Water Content
RN	Root Number
RL	Total Root Length (cm)
RFW	Root Fresh Weight (g)
RDW	Root Dry Weight (g)
R/S	Root/Shoot
Pro-S	Shoot Proline Content (μmol/g FW)
Pro-R	Root Proline Content (μmol/g FW)
MDA-S	Shoot Malondialdehyde Content (μmol/g FW)
MDA-R	Root Malondialdehyde Content (μmol/g FW)
SOD	Superoxide Dismutase Activity (IU/mg protein)
POD	Peroxidase Activity (mU/mg protein)
CAT	Catalase Activity (IU/mg protein)
APX	Ascorbate Peroxidase Activity (IU/mg protein)

Table 2.4. Trait acronyms used in text and figures, with corresponding trait/parameter.

Considering the morpho-physiological traits, all of them, except for SL and R/S ratio, were significant for the T factor, confirming that the applied stress was effective in modulating several parameters related to plantlet morphology. Among the biochemical parameters, both proline and malondialdehyde content in shoot (Pro-S, MDA-S) and proline content in roots (Pro-R) were highly significant for the T factor. As to the activities of the antioxidant enzymes, the two statistical analyses performed showed two different situations. In the statistical comparison between NIRLs+ and the corresponding sib – lines (see Materials and Methods, § 2.2.2.5.) only the activities of CAT and APX showed a significant modulation due to H imposition. On the other hand, in the statistical comparison between NIRLs+ and the heat

tolerant cv. Margherita all the enzymatic activities showed a significant modulation due to the treatment. When the $G \times T$ interaction was considered, significant differences were observed for leaf RWC and all the traits related to root morphology in both the statistical comparisons performed (**Table 2.5**), suggesting that the genotypes activated a variety of responses toward the H stress. Whereas the statistical comparison between NIRLs+ and the corresponding sibs—highlighted a significant modulation of all biochemical parameters and hence a strong difference among the responses of the NIRLs (and their alien segments) towards the heat stress (**Table 2.5A**), the comparison between NIRLs+ and Margherita (**Table 2.5B**) showed significant differences only for the enzymatic activities and the shoot proline content (Pro-S).

A					B				
Factors	G	T	G x T	Residuals	Factors	G	T	G x T	Residuals
df	9	1	9		df	5	1	5	
SL	43.29 ***	0.11	1.71	2.48	SL	30.36 ***	1.01	2.58	2.25
SFW	0.007 ***	0.03 ***	0.0008	0.0008	SFW	0.008 ***	0.02 ***	0.001	0.0007
SDW	0.00009 ***	0.00006 *	0.00001	0.00001	SDW	0.00005 **	0.0001 **	0.00002	0.00001
RWC	0.09 ***	1.01 ***	0.13 ***	0.01	RWC	0.15 ***	0.63 ***	0.13 ***	0.01
RFW	0.004 ***	0.01 **	0.004 **	0.001	RFW	0.006 ***	0.004 *	0.002 *	0.0009
RDW	0.00003 ***	0.00004 **	0.00002 ***	0.000004	RDW	0.00004 ***	0.00001 *	0.00002 ***	0.000003
R/S	0.10 ***	0.003	0.04 ***	0.01	R/S	0.11 ***	0.004	0.03 ***	0.01
df	9	2	18		df	5	2	10	
Pro-S	118.58 ***	884.38 ***	26.16 **	9.90	Pro-S	92.67 ***	467.72 ***	26.15 ***	6.44
Pro-R	37.42 ***	50.07 ***	17.97 ***	1.58	Pro-R	18.27 ***	15.56 ***	1.95	1.55
MDA-S	12.62 ***	35.43 ***	1.52 *	0.78	MDA-S	12.69 ***	33.35 ***	1.17	0.85
MDA-R	2.20 ***	0.13 *	0.07 **	0.03	MDA-R	2.63 ***	0.12	0.08	0.04
df	5	2	10		df	3	2	6	
SOD	0.14 ***	0.02	0.03 ***	0.01	SOD	0.24 ***	0.02 *	0.02 *	0.01
POD	179.43 ***	20.89	53.29 **	17.63	POD	289.34 ***	81.14 **	53.44 **	14.19
CAT	0.49 ***	0.47 ***	0.17 ***	0.02	CAT	0.40 ***	0.82 ***	0.12 ***	0.02
APX	25.42 ***	7.89 *	16.58 ***	2.06	APX	10.58 **	10.13 **	7.66 **	1.77

Table 2.5. ANOVA mean squares for the traits measured under control and H treatment at seedling stage. **A:** comparison between alien segment carrier (NIRLs+) and non-carrier (NIRLs–) lines; **B:** comparison between NIRLs+ and cv. Margherita. *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively (df, degrees of freedom; G, genotype; T, treatment).

2.3.2. Morpho-physiological evaluation of heat stressed seedlings

Even if all the morphological traits measured after the H imposition showed significant differences for the G factor (**Table 2.5**), when individual alien segments were considered a significant and negative effect was only detected on shoot weights (SFW, SDW) in R23+ NIRL and on RWC in R69-9/R112+ (**Table 2.6A**). All the other NIRLs+ did not exhibited significant differences vs the corresponding sibs–, which indicated no effect due to the wild segment

presence. On the other hand, several significant differences between the NIRLs+ and Margherita were observed, probably ascribable to inter-variatal differences, i.e. between Margherita and the NIRLs' genetic background (cv. Simeto). Margherita exhibited the highest values for most of the measured morphological traits among all the genotypes; however, the DW-*Th. ponticum* recombinant line R5+ and, to some extent, R112+, showed very similar values to those of Margherita, the former exhibiting the highest RWC. Moreover, the highest values for root traits were expressed by the R5-derived “nested” recombinant R69-9/R5+, showing, in particular, a significant increment of the R/S ratio vs. all other genotypes, Margherita included (**Table 2.6B**).

A									B								
Trait		SL	SFW	SDW	RWC	RFW	RDW	R/S	Trait		SL	SFW	SDW	RWC	RFW	RDW	R/S
R5+	Mean	17.65	0.16	0.022	0.817	0.134	0.011	0.5	R5+	Mean	17.65	0.16	0.022	0.817	0.134	0.011	0.5
	SE	0.568	0.01	0.001	0.031	0.008	0.0007	0.017		SE	0.568	0.01	0.001	0.031	0.008	0.0007	0.017
	Tukey	ab	abc	bc	a	abc	ab	bcd		Tukey	ab	ab	abc	a	ab	a	bc
R5-	Mean	17.35	0.171	0.022	0.767	0.135	0.011	0.488	R112+	Mean	15.6	0.154	0.021	0.699	0.13	0.011	0.507
	SE	0.506	0.009	0.0009	0.03	0.01	0.0006	0.022		SE	0.25	0.007	0.0009	0.033	0.007	0.0005	0.021
	Tukey	abc	ab	bc	ab	abc	ab	cd		Tukey	c	bc	abc	ab	ab	a	bc
R112+	Mean	15.6	0.154	0.021	0.699	0.13	0.011	0.507	R23+	Mean	18.807	0.147	0.019	0.755	0.108	0.008	0.437
	SE	0.25	0.007	0.0009	0.033	0.007	0.0005	0.021		SE	0.378	0.005	0.001	0.044	0.008	0.0006	0.02
	Tukey	cde	abcd	bc	ab	abc	ab	bcd		Tukey	a	bc	c	a	b	b	cd
R112-	Mean	16.857	0.163	0.023	0.65	0.136	0.012	0.535	R69-9/R5+	Mean	16.433	0.13	0.02	0.623	0.158	0.012	0.624
	SE	0.299	0.009	0.001	0.047	0.01	0.0004	0.025		SE	0.502	0.009	0.001	0.054	0.008	0.0005	0.029
	Tukey	bcd	abc	ab	bc	abc	a	abc		Tukey	bc	bc	bc	bc	a	a	a
R23+	Mean	18.807	0.147	0.019	0.755	0.108	0.008	0.437	R69-9/R112+	Mean	15.25	0.126	0.023	0.53	0.12	0.008	0.371
	SE	0.378	0.005	0.0010	0.044	0.008	0.0006	0.02		SE	0.289	0.007	0.0009	0.058	0.007	0.0004	0.019
	Tukey	a	bcd	bc	ab	bc	bc	cde		Tukey	c	c	ab	c	b	b	d
R23-	Mean	20.1	0.183	0.027	0.721	0.115	0.011	0.41	Margherita	Mean	18.173	0.191	0.024	0.757	0.157	0.012	0.52
	SE	0.458	0.012	0.0009	0.042	0.018	0.0009	0.031		SE	0.276	0.011	0.0009	0.05	0.01	0.0004	0.019
	Tukey	a	a	a	ab	bc	ab	dc		Tukey	a	a	a	a	a	a	b
R69-9/R5+	Mean	16.433	0.13	0.02	0.623	0.158	0.012	0.624									
	SE	0.502	0.009	0.001	0.054	0.008	0.0005	0.029									
	Tukey	bcd	cde	bc	bc	a	a	a									
R69-9/R5-	Mean	16.281	0.125	0.019	0.631	0.143	0.011	0.597									
	SE	0.323	0.008	0.0008	0.053	0.01	0.0005	0.035									
	Tukey	bcd	de	c	bc	ab	ab	ab									
R69-9/R112+	Mean	15.25	0.126	0.023	0.53	0.12	0.008	0.371									
	SE	0.289	0.007	0.0009	0.058	0.007	0.0004	0.019									
	Tukey	de	de	ab	c	abc	bc	e									
R69-9/R112-	Mean	14.281	0.116	0.019	0.705	0.101	0.008	0.424									
	SE	0.385	0.007	0.001	0.044	0.008	0.0006	0.027									
	Tukey	e	e	bc	ab	c	c	de									

Table 2.6. Means, standard error (SE) and ranking of Tukey test at $P < 0.05$ level for morpho-physiological traits in the H experiment. **A:** Statistical comparison for G factor performed between NIRLs + vs. –; **B:** Statistical comparison for G factor performed between NIRLs + and cv. Margherita.

When the $G \times T$ interactions were considered, no significant differences among the NIRLs+ and the respective sibs– were observed, with the only exception of R69-9/R112+ exhibiting a lower RWC than R69-9/R112– under the H condition (**Figure 2.6A; Table 2.7A**). Interestingly, a significant reduction in RWC values following H imposition was only observed for the R69-9 types (see C vs. H0 in **Figure 2.6A**), which was not detected in the primary NIRLs (**Table 2.7A**). The statistical comparison between NIRLs + and Margherita (**Table 2.7B**) showed major

differences: R5+ and R112+ confirmed highly similar values for morphological traits to those of Margherita, whereas R23+ had significantly lower values for RFW, RDW and R/S traits than Margherita under H stress. This observation highlighted how negatively the high temperature treatment impacted on the root apparatus of R23+. A major difference involved the R69-9/R5+ genotype, whose R/S value showed a significant increase (+27%) after the H stress, more than double that of Margherita (+13%), altogether supporting a major involvement of the root apparatus in this line's response to heat application (**Figure 2.6B; Table 2.7B**).

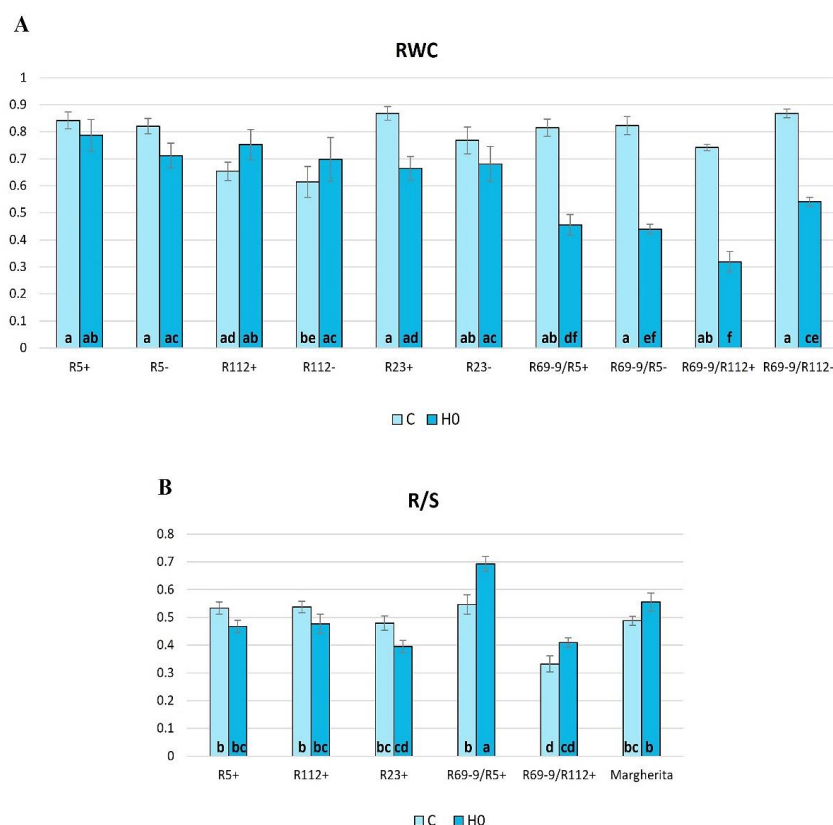


Figure 2.6. Significant $G \times T$ effects on morpho-physiological parameters in the H stress experiment.

A: Statistical comparison performed between NIRLs + vs. -; **B:** Statistical comparison performed between NIRLs + and cv. Margherita. Error bars represent standard errors of the means, while letters into the histograms correspond to ranking of Tukey HSD test at $p < 0.05$ significance level.

A

Trait		SL	SFW	SDW	RWC	RFW	RDW	R/S
R5+	Mean	18.329	0.17	0.024	0.842	0.152	0.013	0.533
	C SE	0.976	0.011	0.001	0.031	0.009	0.0006	0.022
	Tukey	ns	ns	ns	a	ab	a	abcd
	Mean	16.971	0.151	0.02	0.787	0.117	0.009	0.468
	H0 SE	0.538	0.016	0.001	0.058	0.008	0.0010	0.022
	Tukey	ns	ns	ns	ab	abc	ab	bcde
R5-	Mean	17.457	0.179	0.023	0.821	0.165	0.012	0.532
	C SE	0.707	0.01	0.001	0.029	0.011	0.0006	0.025
	Tukey	ns	ns	ns	a	a	a	abcd
	Mean	17.243	0.162	0.021	0.712	0.109	0.009	0.445
	H0 SE	0.777	0.015	0.001	0.046	0.007	0.0007	0.03
	Tukey	ns	ns	ns	abc	abc	ab	cde
R112+	Mean	15.357	0.163	0.021	0.654	0.147	0.011	0.537
	C SE	0.322	0.012	0.001	0.034	0.009	0.0007	0.021
	Tukey	ns	ns	ns	abcd	ab	a	abcd
	Mean	15.843	0.144	0.021	0.752	0.113	0.01	0.477
	H0 SE	0.385	0.006	0.001	0.056	0.007	0.0006	0.035
	Tukey	ns	ns	ns	ab	abc	ab	bcde
R112-	Mean	17.1	0.177	0.024	0.615	0.153	0.012	0.529
	C SE	0.241	0.011	0.001	0.057	0.008	0.0005	0.031
	Tukey	ns	ns	ns	bcde	ab	a	bcd
	Mean	16.614	0.149	0.022	0.698	0.119	0.012	0.54
	H0 SE	0.557	0.011	0.001	0.081	0.015	0.0007	0.042
	Tukey	ns	ns	ns	abc	abc	a	abcd
R23+	Mean	18.7	0.153	0.021	0.868	0.124	0.01	0.479
	C SE	0.573	0.008	0.001	0.025	0.011	0.0008	0.026
	Tukey	ns	ns	ns	a	abc	ab	bcde
	Mean	18.914	0.14	0.018	0.665	0.092	0.007	0.395
	H0 SE	0.536	0.005	0.001	0.044	0.007	0.0006	0.022
	Tukey	ns	ns	ns	abcd	bc	b	de
R23-	Mean	19.529	0.194	0.026	0.768	0.136	0.012	0.477
	C SE	0.795	0.021	0.002	0.05	0.026	0.001	0.041
	Tukey	ns	ns	ns	ab	abc	a	bcde
	Mean	20.671	0.171	0.027	0.681	0.094	0.009	0.343
	H0 SE	0.409	0.013	0.001	0.065	0.024	0.0009	0.032
	Tukey	ns	ns	ns	abc	bc	ab	e
R69-9/R5+	Mean	16.5	0.156	0.022	0.815	0.152	0.012	0.546
	C SE	0.636	0.007	0.001	0.032	0.009	0.0006	0.035
	Tukey	ns	ns	ns	ab	ab	a	abcd
	Mean	16.375	0.107	0.018	0.456	0.164	0.012	0.693
	H0 SE	0.8	0.01	0.001	0.038	0.014	0.0008	0.026
	Tukey	ns	ns	ns	def	a	a	a
R69-9/R5-	Mean	16.375	0.153	0.019	0.823	0.149	0.012	0.627
	C SE	0.557	0.007	0.001	0.033	0.014	0.0006	0.045
	Tukey	ns	ns	ns	a	ab	a	ab
	Mean	16.188	0.098	0.018	0.44	0.137	0.01	0.566
	H0 SE	0.365	0.005	0.0010	0.018	0.015	0.0009	0.055
	Tukey	ns	ns	ns	ef	abc	ab	abc
R69-9/R112+	Mean	14.938	0.145	0.023	0.742	0.111	0.007	0.332
	C SE	0.406	0.008	0.002	0.012	0.012	0.0006	0.029
	Tukey	ns	ns	ns	ab	abc	b	e
	Mean	15.563	0.106	0.023	0.319	0.128	0.01	0.409
	H0 SE	0.406	0.007	0.0009	0.038	0.007	0.0004	0.017
	Tukey	ns	ns	ns	f	abc	ab	de
R69-9/R112-	Mean	14.063	0.127	0.019	0.868	0.078	0.006	0.343
	C SE	0.538	0.011	0.002	0.016	0.01	0.0007	0.019
	Tukey	ns	ns	ns	a	c	b	e
	Mean	14.5	0.105	0.019	0.541	0.125	0.009	0.506
	H0 SE	0.575	0.006	0.001	0.016	0.004	0.0006	0.03
	Tukey	ns	ns	ns	cde	abc	ab	bcd

B

Trait		SL	SFW	SDW	RWC	RFW	RDW	R/S
R5+	Mean	18.329	0.17	0.024	0.842	0.152	0.013	0.533
	C SE	0.976	0.011	0.001	0.031	0.009	0.0006	0.022
	Tukey	ns	ns	ns	ab	ab	a	b
	Mean	16.971	0.151	0.02	0.787	0.117	0.009	0.468
	H0 SE	0.538	0.016	0.001	0.058	0.008	0.001	0.022
	Tukey	ns	ns	ns	ab	abc	ab	bc
R112+	Mean	15.357	0.163	0.021	0.654	0.147	0.011	0.537
	C SE	0.322	0.012	0.001	0.034	0.009	0.0007	0.021
	Tukey	ns	ns	ns	b	ab	a	b
	Mean	15.843	0.144	0.021	0.752	0.113	0.01	0.477
	H0 SE	0.385	0.006	0.001	0.056	0.007	0.0006	0.035
	Tukey	ns	ns	ns	ab	abc	ab	bc
R23+	Mean	18.7	0.153	0.021	0.868	0.124	0.01	0.479
	C SE	0.573	0.008	0.001	0.025	0.011	0.0008	0.026
	Tukey	ns	ns	ns	ab	abc	ab	bc
	Mean	18.914	0.14	0.018	0.665	0.092	0.007	0.395
	H0 SE	0.536	0.005	0.001	0.044	0.007	0.0006	0.022
	Tukey	ns	ns	ns	ab	c	b	cd
R69-9/R5+	Mean	16.5	0.156	0.022	0.815	0.152	0.012	0.546
	C SE	0.636	0.007	0.001	0.032	0.009	0.0006	0.035
	Tukey	ns	ns	ns	ab	ab	a	b
	Mean	16.375	0.107	0.018	0.456	0.164	0.012	0.693
	H0 SE	0.8	0.01	0.001	0.038	0.014	0.0008	0.026
	Tukey	ns	ns	ns	c	a	a	a
R69-9/R112+	Mean	14.938	0.145	0.023	0.742	0.111	0.007	0.332
	C SE	0.406	0.008	0.002	0.012	0.012	0.0006	0.029
	Tukey	ns	ns	ns	ab	bc	b	d
	Mean	15.563	0.106	0.023	0.319	0.128	0.01	0.409
	H0 SE	0.406	0.007	0.0009	0.038	0.007	0.0004	0.017
	Tukey	ns	ns	ns	c	abc	ab	cd
Margherita	Mean	18.7	0.217	0.025	0.879	0.16	0.012	0.488
	C SE	0.377	0.012	0.001	0.04	0.015	0.0007	0.016
	Tukey	ns	ns	ns	a	ab	a	bc
	Mean	17.571	0.16	0.022	0.688	0.153	0.012	0.555
	H0 SE	0.28	0.012	0.001	0.061	0.015	0.0006	0.033
	Tukey	ns	ns	ns	ab	ab	a	b

Table 2.7. Means, standard error (SE) and ranking of Tukey test at $P < 0.05$ level for morpho-physiological traits in the H experiment. **A:** Statistical comparison for $G \times T$ factor performed between NIRLs + vs. –; **B:** Statistical comparison for $G \times T$ factor performed between NIRLs + and cv. Margherita.

2.3.3. Proline and malondialdehyde modulation and activity of key antioxidant enzymes under H stress

Among the tested NIRLs, differences in proline content due to the wild segment introgression were only exhibited by R23+ and R69-9/R112+. R23+ possessed a lower amount of Pro-S and a higher amount of Pro-R than its sib– (**Table 2.8A**), whereas R69-9/R112+ possessed a lower amount of Pro-R than the corresponding sib–. Rather surprisingly, although R23+ showed a major amount of the osmolyte in the roots, a higher MDA content was also detected, which is indicative of a strong damage in the R23+ root apparatus. An opposite trend (lower amount of MDA and Pro than R23–) was observed in R23+ shoot. Regarding the antioxidant activity, only R5+ exhibited a superior SOD activity compared with its sib – and hence a positive antioxidant reaction activated in the presence of its 23%-long 7e₁L chromosome segment, whereas R112+ and R23+ showed an opposite behaviour (**Table 2.8A**). Despite this, R112+ and R23+ had a higher CAT and POD activity, respectively, than their sibs –. When the NIRLs+ were compared with Margherita, the G effect revealed a number of differences, mostly between R5+ and the control cultivar (**Table 2.8B**). In fact, Margherita (similarly to the other two NIRLs+) showed a higher osmolyte accumulation in the shoot (Pro-S) and a lower MDA content, hence a minor membrane damage, in both shoot and roots than R5+. On the other hand, R5+ exhibited a statistically higher SOD, CAT and APX activities than Margherita.

A

Trait	Pro-S	Pro-R	MDA-S	MDA-R	SOD	POD	CAT	APX
R5+	Mean 10.347	5.65	7.745	2.141	0.545	15.279	0.934	5.326
	SE 1.522	0.665	0.384	0.114	0.029	0.97	0.096	0.656
	Tukey bcde	bc	a	a	a	c	a	ab
R5-	Mean 15.189	6.381	6.631	1.659	0.444	19.246	0.848	6.394
	SE 1.939	1.193	0.272	0.06	0.044	1.265	0.062	0.788
	Tukey b	ab	ab	bc	b	bc	a	a
R112+	Mean 9.875	2.732	6.221	1.529	0.246	24.69	0.693	4.723
	SE 1.276	0.428	0.583	0.063	0.031	2.014	0.086	0.425
	Tukey bcde	d	bc	c	c	ab	b	ab
R112-	Mean 7.83	3.499	5.912	1.563	0.433	21.694	0.467	6.544
	SE 1.395	0.44	0.409	0.058	0.023	1.243	0.046	0.924
	Tukey cde	d	bc	c	b	ab	c	a
R23+	Mean 12.934	5.901	5.806	1.897	0.253	27.168	0.509	3.637
	SE 1.332	0.491	0.566	0.055	0.024	1.998	0.107	0.364
	Tukey bc	bc	bc	ab	c	a	bc	bc
R23-	Mean 19.078	2.778	7.67	1.568	0.387	20.837	0.399	2.567
	SE 2.5	0.485	0.522	0.054	0.019	1.722	0.055	0.494
	Tukey a	d	a	c	b	b	c	c
R69-9/R5+	Mean 6.83	3.511	4.193	0.793	NA	NA	NA	NA
	SE 1.087	0.318	0.365	0.054	NA	NA	NA	NA
	Tukey e	d	d	d	NA	NA	NA	NA
R69-9/R5-	Mean 7.555	4.12	5.124	0.849	NA	NA	NA	NA
	SE 1.576	0.639	0.444	0.047	NA	NA	NA	NA
	Tukey de	cd	cd	d	NA	NA	NA	NA
R69-9/R112+	Mean 10.009	3.554	5.708	0.87	NA	NA	NA	NA
	SE 1.044	0.391	0.303	0.062	NA	NA	NA	NA
	Tukey bcde	d	bc	d	NA	NA	NA	NA
R69-9/R112-	Mean 12.316	8.623	6.809	0.84	NA	NA	NA	NA
	SE 1.744	1.73	0.268	0.069	NA	NA	NA	NA
	Tukey bcd	a	ab	d	NA	NA	NA	NA

B

Trait	Pro-S	Pro-R	MDA-S	MDA-R	SOD	POD	CAT	APX
R5+	Mean 10.347	5.65	7.745	2.141	0.545	15.279	0.934	5.326
	SE 1.522	0.665	0.384	0.114	0.029	0.97	0.096	0.656
	Tukey bc	a	a	a	a	c	a	a
R112+	Mean 9.875	2.732	6.221	1.529	0.246	24.69	0.693	4.723
	SE 1.276	0.428	0.583	0.063	0.031	2.014	0.086	0.425
	Tukey bc	b	b	b	c	ab	b	ab
R23+	Mean 12.934	5.901	5.806	1.897	0.253	27.168	0.509	3.637
	SE 1.332	0.491	0.566	0.055	0.024	1.998	0.107	0.364
	Tukey b	a	b	a	c	a	b	b
R69-9/R5+	Mean 6.83	3.511	4.193	0.793	NA	NA	NA	NA
	SE 1.087	0.318	0.365	0.054	NA	NA	NA	NA
	Tukey c	b	c	c	NA	NA	NA	NA
R69-9/R112+	Mean 10.009	3.554	5.708	0.87	NA	NA	NA	NA
	SE 1.044	0.391	0.303	0.062	NA	NA	NA	NA
	Tukey bc	b	b	c	NA	NA	NA	NA
Margherita	Mean 16.256	5.887	5.113	1.599	0.457	21.306	0.574	3.453
	SE 2.996	0.418	0.654	0.092	0.023	1.156	0.052	0.522
	Tukey a	a	bc	b	b	b	b	b

Table 2.8. Means, standard error (SE) and ranking of Tukey test at P < 0.05 level for biochemical traits in the H experiment. NA: data not available. **A:** Statistical comparison for G factor performed between NIRLs + vs. –; **B:** Statistical comparison for G factor performed between NIRLs + and cv. Margherita.

When the $G \times T$ interactions were considered, an overall significant increase in Pro-S was detected in almost all the NIRLs— immediately after the end of H stress imposition (H0). Curiously, among the NIRLs+, only R112+ showed the same behaviour, i.e. a significant increment in Pro-S in H0 samples (**Figure 2.7; Table 2.9A**). Indeed, a general increase in Pro-S H0 was revealed by all the genotypes (**Figure 2.7**), the highest value being shown by the heat tolerant cv. Margherita (**Table 2.9B**). During the recovery phase (H24 samples), an overall decrease in Pro-S was observed vs H0, due to the return to an optimal temperature regime, even if R112+ and R23+, but also R23— and R69-9/R112—, had superior values in Pro-S H24 vs Pro-S C (**Figure 2.7**). When proline content in roots was considered, a significant increase was detected at the end of the treatment (H0) in both R5— and R69-9/R112—, the latter maintaining the superior value also in the recovery phase (**Figure 2.7; Table 2.9A**). Among the values of the MDA-S trait, R23+ revealed a significant lower amount than R23— at the H0 time point, which revealed a possible involvement of the 40%-long 7el₁L segment in the reduction of membrane damage in shoots under high temperature (**Figure 2.7; Table 2.9A**). The same analysis performed at the roots level highlighted a clear difference of the overall MDA-R values between the primary NIRLs (*Th. ponticum*) and the R69-9 types (*Th. ponticum* + *Th. elongatum*), the former having constitutively higher values than the latter (**Figure 2.7**). In particular, R5+ expressed the highest MDA-R content in the control condition and was the only genotype that significantly decreased it after H stress (**Table 2.9A**). Regarding the activities of the antioxidant enzymes, both R112+ and R23+ revealed constitutively lower SOD values than their corresponding sibs— under control conditions (**Figure 2.7; Table 2.9A**). The same was not observed for R5+, implying a negative influence on SOD activity restricted to the *Th. ponticum* 7el₁L chromatin beyond the R5+ breakpoint (see **Figure 2.1**). Moreover, R5+ had higher SOD activity than R5— both at H0 and H24, suggesting a positive influence of the 23%-long 7el₁L segment in SOD modulation under the H stress condition. The involvement of the wild chromosome segments in antioxidant enzyme activities was also detected for CAT activity; in fact, all the three DW-*Thinopyrum ponticum* NIRLs+ had higher values under control conditions than their sibs—, even if the H stress imposition caused a general decrement (from –50% to –60%) in CAT activity (**Figure 2.7**). Whereas CAT does not seem to represent a main enzyme involved in the H stress response of the NIRLs+, a major contribute seems to be due to APX. In fact, both R5+ and R112+ increased their APX activity immediately after stress: the first exhibiting a significant increment (+100%) at H0 and a fast return to control values at H24, the second exhibiting a somewhat lower increase (+70%) at H0, but maintaining higher APX activity also at H24 (**Figure 2.7**). R23+ did not exhibit a significant APX

modulation under the various conditions, altogether indicating no enzymatic activation (of the tested enzymes) brought about by this genotype following the H stress. When the antioxidant enzyme activities of the NIRLs+ were compared with those of Margherita, the constitutively lower SOD activity of R112+ and R23+ was confirmed, whereas R5+ had higher, though not significantly different, SOD activity than Margherita at both H0 and H24 (**Table 2.9B**). As to CAT activity, while all three NIRLs+ significantly decreased at H0 and H24, Margherita proved to be able to maintain almost unchanged CAT values across conditions. Finally, the superior APX activity of R5+ at H0 was again confirmed, being even higher than that of Margherita (**Table 2.9B**).

A

Trait		Pro-S	Pro-R	MDA-S	MDA-R	SOD	POD	CAT	APX
R5+	Mean	7.874	4.767	7.176	2.512	0.494	16.067	1.27	4.117
	C SE	1.245	0.75	0.321	0.131	0.044	1.168	0.12	0.285
	Tukey	cdef	defgh	abcdef	a	ab	cd	a	de
	Mean	15.167	7.817	7.331	1.826	0.581	16.312	0.657	8.177
	H0 SE	1.677	0.679	0.654	0.088	0.038	1.963	0.074	0.424
	Tukey	bcd	cd	abcde	bc	a	cd	bcd	abc
	Mean	6.394	3.939	8.729	2.086	0.556	13.2	0.853	4.087
	H24 SE	1.095	0.968	0.715	0.107	0.063	1.534	0.009	0.551
	Tukey	def	efgh	ab	ab	a	d	bc	de
	Mean	11.38	6.659	6.067	1.832	0.568	17.41	0.948	6.058
R5-	C SE	1.39	0.053	0.123	0.07	0.068	2.366	0.005	0.799
	Tukey	cdef	cdef	bcddefghil	bc	a	cd	ab	bcd
	Mean	20.753	11.657	6.593	1.628	0.346	21.457	0.593	8.87
	H0 SE	2.176	0.884	0.717	0.062	0.03	1.119	0.043	0.941
	Tukey	b	b	bcdefgh	bc	bcd	abcd	bcd	ab
	Mean	7.284	3.048	7.232	1.508	0.376	20.097	0.97	3.54
	H24 SE	0.177	0.793	0.131	0.023	0.036	1.946	0.098	0.3
	Tukey	cdef	gh	abcdef	cde	abcde	bcd	ab	de
	Mean	5.247	3.028	4.68	1.609	0.226	29.707	0.966	3.515
	C SE	0.56	0.862	0.244	0.07	0.038	2.643	0.067	0.33
R112+	Tukey	ef	gh	efghil	bc	de	ab	ab	de
	Mean	15.837	3.511	5.77	1.4	0.372	24.227	0.45	5.927
	H0 SE	0.584	0.569	0.275	0.095	0.009	0.737	0.05	0.494
	Tukey	bcd	efgh	cdefghil	cdef	abcde	abcd	de	bcd
	Mean	9.207	1.656	8.213	1.579	0.155	18.463	0.48	5.027
	H24 SE	1.118	0.454	0.817	0.143	0.005	3.167	0.069	0.739
	Tukey	cdef	efgh	abc	bc	e	bcd	cde	cde
	Mean	4.019	3.524	5.864	1.525	0.446	20.107	0.36	5.558
	C SE	0.643	0.693	0.193	0.125	0.038	1.717	0.023	0.647
	Tukey	f	gh	cdefghil	cd	abc	bcd	de	bcd
R112-	Mean	14.636	4.58	4.555	1.566	0.485	19.343	0.61	4.095
	H0 SE	0.597	0.778	0.164	0.131	0.03	1.041	0.052	0.993
	Tukey	bcd	defgh	efghil	bc	ab	bcd	bcd	de
	Mean	6.034	2.392	7.318	1.6	0.365	25.633	0.383	9.98
	H24 SE	0.737	0.208	0.169	0.079	0.015	1.649	0.049	1.314
	Tukey	ef	gh	abcde	bc	abcde	abc	de	a
	Mean	8.673	4.968	5.163	1.92	0.194	33.3	0.883	3.805
	C SE	0.916	0.822	0.298	0.086	0.032	3.366	0.069	0.618
	Tukey	cdef	cdefgh	defghil	bc	de	a	b	de
	Mean	15.99	7.324	4.305	1.856	0.251	24.957	0.3	4.073
R23+	H0 SE	1.756	0.755	0.708	0.125	0.036	2.156	0.052	0.869
	Tukey	bc	cde	ghil	bc	cde	abcd	de	de
	Mean	13.074	5.411	7.413	1.935	0.334	23.247	0.22	2.977
	H24 SE	2.261	0.271	0.764	0.01	0.027	1.732	0.05	0.366
	Tukey	cdef	cdefgh	abcd	bc	bcd	abcd	e	de
	Mean	8.526	2.399	6.453	1.725	0.435	17.423	0.3	3.52
	C SE	1.357	0.176	0.552	0.049	0.009	0.263	0.035	1.01
	Tukey	cdef	gh	bcddefgh	bc	abc	abcd	de	de
	Mean	27.59	4.114	7.475	1.402	0.407	23.155	0.413	2.193
	H0 SE	2.603	0.787	0.841	0.068	0.001	3.341	0.118	0.603
R23-	Tukey	a	efgh	abcd	cdef	abcd	cd	de	de
	Mean	16.863	1.375	9.553	1.578	0.317	21.16	0.483	1.67
	H24 SE	1.013	0.068	0.442	0.019	0.019	3.556	0.11	0.416
	Tukey	bc	h	a	bc	bcd	abcd	cde	e
	Mean	4.305	3.535	3.426	0.703	NA	NA	NA	NA
	C SE	0.673	0.732	0.35	0.074	NA	NA	NA	NA
	Tukey	f	efgh	i	g	NA	NA	NA	NA
	Mean	10.92	3.651	3.731	0.966	NA	NA	NA	NA
	H0 SE	0.8	0.326	0.439	0.066	NA	NA	NA	NA
	Tukey	cdef	efgh	il	fg	NA	NA	NA	NA
R69-9/R5+	Mean	5.266	3.346	5.424	0.712	NA	NA	NA	NA
	H24 SE	0.562	0.74	0.355	0.057	NA	NA	NA	NA
	Tukey	ef	efgh	cdefghil	g	NA	NA	NA	NA
	Mean	3.956	1.912	3.873	0.834	NA	NA	NA	NA
	C SE	0.754	0.104	0.134	0.03	NA	NA	NA	NA
	Tukey	f	gh	hil	g	NA	NA	NA	NA
	Mean	13.593	5.123	4.452	0.731	NA	NA	NA	NA
	H0 SE	1.033	0.851	0.121	0.04	NA	NA	NA	NA
	Tukey	bcd	cdefgh	efghil	g	NA	NA	NA	NA
	Mean	5.116	5.325	6.566	0.98	NA	NA	NA	NA
R69-9/R112+	H24 SE	0.693	0.706	0.511	0.094	NA	NA	NA	NA
	Tukey	ef	cdefgh	bcddefgh	efg	NA	NA	NA	NA
	Mean	8.09	2.947	5.514	0.989	NA	NA	NA	NA
	C SE	0.922	0.452	0.421	0.159	NA	NA	NA	NA
	Tukey	cdef	gh	cdefghil	defg	NA	NA	NA	NA
	Mean	13.666	4.617	4.961	0.839	NA	NA	NA	NA
	H0 SE	0.859	0.529	0.045	0.056	NA	NA	NA	NA
	Tukey	bcd	defgh	defghil	g	NA	NA	NA	NA
	Mean	8.27	3.097	6.649	0.781	NA	NA	NA	NA
	H24 SE	1.2	0.703	0.425	0.075	NA	NA	NA	NA
R69-9/R112-	Tukey	cdef	efgh	bcddefgh	g	NA	NA	NA	NA
	Mean	5.777	2.792	7.516	0.74	NA	NA	NA	NA
	C SE	0.37	0.57	0.352	0.038	NA	NA	NA	NA
	Tukey	ef	gh	abcd	g	NA	NA	NA	NA
	Mean	16.967	8.782	6.241	0.86	NA	NA	NA	NA
	H0 SE	1.157	1.146	0.396	0.076	NA	NA	NA	NA
	Tukey	bc	bc	bcdefghi	g	NA	NA	NA	NA
	Mean	13.732	14.297	6.858	0.901	NA	NA	NA	NA
	H24 SE	2.304	1.08	0.414	0.167	NA	NA	NA	NA
	Tukey	bcd	a	abcdefg	fg	NA	NA	NA	NA

B

Trait		Pro-S	Pro-R	MDA-S	MDA-R	SOD	POD	CAT	APX	
R5+	C	Mean	7.874	4.767	7.176	2.512	0.494	16.067	1.27	4.117
		SE	1.245	0.75	0.321	0.131	0.044	1.168	0.12	0.285
		Tukey	ef	ns	ns	ns	abc	cd	a	b
	H0	Mean	15.167	7.817	7.331	1.826	0.581	16.312	0.657	8.177
		SE	1.677	0.679	0.654	0.088	0.038	1.963	0.074	0.424
		Tukey	bcd	ns	ns	ns	a	cd	bcd	a
	H24	Mean	6.394	3.939	8.729	2.086	0.556	13.2	0.853	4.087
		SE	1.095	0.968	0.715	0.107	0.063	1.534	0.009	0.551
		Tukey	ef	ns	ns	ns	ab	d	bcd	b
	R112+	C	Mean	5.247	3.028	4.68	1.609	0.226	29.707	0.966
SE			0.56	0.862	0.244	0.07	0.038	2.643	0.067	0.33
Tukey			f	ns	ns	ns	d	ab	ab	b
H0		Mean	15.837	3.511	5.77	1.4	0.372	24.227	0.45	5.927
		SE	0.584	0.569	0.275	0.095	0.009	0.737	0.05	0.494
		Tukey	bc	ns	ns	ns	bcd	abc	efg	ab
H24		Mean	9.207	1.656	8.213	1.579	0.155	18.463	0.48	5.027
		SE	1.118	0.454	0.817	0.143	0.005	3.167	0.069	0.739
		Tukey	cdef	ns	ns	ns	d	cd	defg	ab
R23+		C	Mean	8.673	4.968	5.163	1.92	0.194	33.3	0.883
	SE		0.916	0.822	0.298	0.086	0.032	3.366	0.069	0.618
	Tukey		cdef	ns	ns	ns	d	a	bc	b
	H0	Mean	15.99	7.324	4.305	1.856	0.251	24.957	0.3	4.073
		SE	1.756	0.755	0.708	0.125	0.036	2.156	0.052	0.869
		Tukey	b	ns	ns	ns	d	abc	fg	b
	H24	Mean	13.074	5.411	7.413	1.935	0.334	23.247	0.22	2.977
		SE	2.261	0.271	0.764	0.01	0.027	1.732	0.05	0.366
		Tukey	bcd	ns	ns	ns	cd	abcd	g	b
	R69-9/R5+	C	Mean	4.305	3.535	3.426	0.703	NA	NA	NA
SE			0.673	0.732	0.35	0.074	NA	NA	NA	NA
Tukey			f	ns	ns	ns	NA	NA	NA	NA
H0		Mean	10.92	3.651	3.731	0.966	NA	NA	NA	NA
		SE	0.8	0.326	0.439	0.066	NA	NA	NA	NA
		Tukey	bcd	ns	ns	ns	NA	NA	NA	NA
H24		Mean	5.266	3.346	5.424	0.712	NA	NA	NA	NA
		SE	0.562	0.74	0.355	0.057	NA	NA	NA	NA
		Tukey	f	ns	ns	ns	NA	NA	NA	NA
R69-9/R112+		C	Mean	8.09	2.947	5.514	0.989	NA	NA	NA
	SE		0.922	0.452	0.421	0.159	NA	NA	NA	NA
	Tukey		def	ns	ns	ns	NA	NA	NA	NA
	H0	Mean	13.666	4.617	4.961	0.839	NA	NA	NA	NA
		SE	0.859	0.529	0.045	0.056	NA	NA	NA	NA
		Tukey	bcd	ns	ns	ns	NA	NA	NA	NA
	H24	Mean	8.27	3.097	6.649	0.781	NA	NA	NA	NA
		SE	1.2	0.703	0.425	0.075	NA	NA	NA	NA
		Tukey	cdef	ns	ns	ns	NA	NA	NA	NA
	Margherita	C	Mean	10.439	6.051	4.224	1.658	0.491	19.8	0.563
SE			0.594	0.078	0.631	0.176	0.019	0.766	0.055	0.203
Tukey			bcd	ns	ns	ns	abc	bcd	cdefg	b
H0		Mean	27.778	6.22	3.641	1.566	0.496	18.973	0.476	2.666
		SE	2.368	0.93	0.412	0.171	0.042	1.542	0.084	0.604
		Tukey	a	ns	ns	ns	abc	cd	efg	b
H24		Mean	10.552	5.28	7.475	1.552	0.373	25.143	0.747	4.812
		SE	1.475	0.784	0.539	0.176	0.017	1.357	0.018	1.169
		Tukey	bcd	ns	ns	ns	bcd	abc	bcd	ab

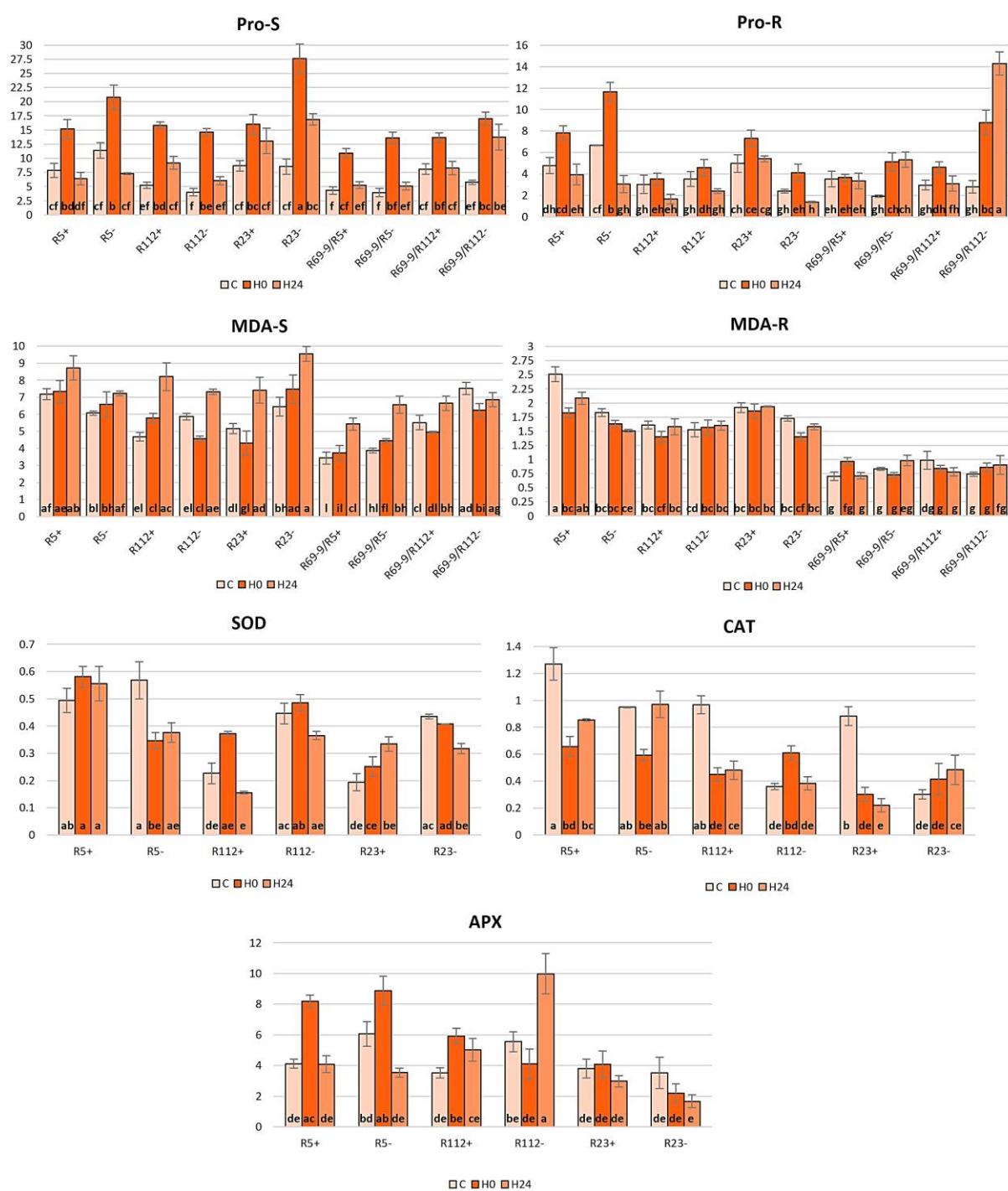


Figure 2.7. Significant $G \times T$ effects on biochemical parameters in the H stress experiment. Statistical comparison performed between the NIRLs + vs. -. Error bars represent standard errors of the means, while letters into the histograms correspond to ranking of Tukey HSD test at $p < 0.05$ significance level.

2.3.4. miRNAs modulation after the H stress imposition: a focus on R112+

On the basis of the promising results obtained for R112+ regarding the morpho-physiological analyses performed at the seedling stage (see above), but also regarding the H tolerance exhibited by R112+ at anthesis stage (see Chapter 3), the expression of a selected panel of micro-RNAs (miRNAs), found to be involved in the response to heat and other abiotic stresses (Table 3), was analysed in R112+ and R112– seedlings subjected and non-subjected (C) to the H stress. As reported in **Figure 2.8**, all the selected miRNAs were modulated by the H imposition. In detail, six of the seven miRNAs were upregulated, while miR-396 was downregulated by H. For both genotypes (“+” vs. “–”), the modulation of all miRNAs was clearly appreciable, even if R112+ demonstrated a lower H-induced variation and a rapid restoration of the physiological (baseline) miRNAs levels during the 2-hours recovery after the H treatment. In general, R112+ appeared to be less sensitive after the stress imposition for most of the miRNA analyzed and to be able to restore baseline expression levels in a shorter time than R112–.

microRNA	R112–		R112+	
	H0	H2	H0	H2
miR-159a	1.50	1.30	1.44	0.64
miR-160	1.18	1.51	1.34	1.09
miR-166	1.71	1.97	1.59	1.15
miR-393	1.93	2.22	1.53	0.81
miR-396	0.70	0.25	0.36	0.99
miR-398	2.05	2.26	1.07	0.28
miR-1137	1.99	1.27	1.43	0.75

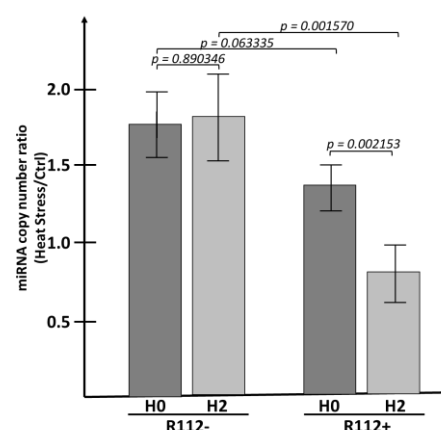


Figure 2.8. miRNAs modulation in R112+ and R112– seedlings subjected to H stress. On the left, variation in miRNAs expression (green: upregulated; red: downregulated), calculated as the ratio between miRNAs copies in H stressed seedlings vs the respective control (unstressed) seedlings. On the right, histograms of the mean ratio for all the 6 upregulated miRNA (miR-396 excluded).

2.3.5. Traits showing significant statistical differences for genotype (G), treatment (T) and G × T interaction in the WH experiment

As reported in the ANOVAs performed (**Table 2.10**), the significance of the genotype effect alone (G) was observed for all the traits assayed in NIRLs+ vs. NIRLs– analysis and, for most

traits, in NIRLs+ vs. Margherita comparison. In addition, essentially all parameters, with the only exception of root length (RL), were also affected by the treatment applied (T). The two statistical comparisons highlighted that a high variation of almost all the traits among genotypes also when the $G \times T$ factor was considered. In fact, only SDW, RN and MDA-R did not differ between NIRLs+ and – (Table 12A), and only SL and MDA-S did not vary between NIRLs+ and Margherita.

A					B				
Factors	G	T	G x T	Residuals	Factors	G	T	G x T	Residuals
df	7	1	7		df	4	1	4	
SL	12.28 ***	84.14 ***	3.81 **	1.17	SL	12.04 ***	77.19 ***	2.66	1.51
SFW	0.002 ***	0.12 ***	0.003 ***	0.0003	SFW	0.002 **	0.12 ***	0.003 ***	0.0004
SDW	0.00005 ***	0.0001 **	0.00001	0.00001	SDW	0.00001	0.00007 *	0.00002 **	0.00001
RWC	0.11 ***	1.60 ***	0.06 ***	0.009	RWC	0.03 **	1.51 ***	0.10 ***	0.008
RN	2.46 **	8.56 ***	0.97	0.65	RN	2.17 *	14.13 ***	1.60 *	0.61
RL	1778.24 ***	175.65	872.54 ***	97.32	RL	1290.73 ***	69.75	476.82 **	119.80
RFW	0.002 ***	0.02 ***	0.002 ***	0.0004	RFW	0.0006	0.02 ***	0.001 *	0.0004
RDW	0.00005 ***	0.003 ***	0.00005 ***	0.00001	RDW	0.00003 *	0.001 ***	0.00003 *	0.00001
R/S	0.08 *	9.40 ***	0.16 ***	0.03	R/S	0.07 **	3.86 ***	0.05 *	0.02
Pro-S	20503.10 ***	124082.27 ***	19974.19 ***	136.49	Pro-S	17875.90 ***	79435.53 ***	17147.86 ***	528.26
Pro-R	1259.73 ***	3997.03 ***	1304.58 ***	18.78	Pro-R	322.31 ***	797.04 ***	325.37 ***	6.21
MDA-S	12.24 ***	96.22 ***	6.24 ***	1.11	MDA-S	17.95 ***	88.10 ***	2.11	1.01
MDA-R	0.59 *	72.40 ***	0.40	0.23	MDA-R	2.05 *	59.38 ***	2.27 *	0.63

Table 2.10. ANOVA mean squares for the traits measured under control and WH treatment at seedling stage: **A:** comparison between alien segment carrier (NIRLs+) and non-carrier (NIRLs–) lines; **B:** comparison between NIRLs+ and cv. Margherita. *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively (df, degrees of freedom; G, genotype; T, treatment).

2.3.6. Morpho-physiological evaluation of combined WH stressed seedlings

Confirming the significant effect due to the G factor (Table 12), presence of specific *Thinopyrum* spp. chromosome segments was found to positively impact on several morphological parameters. Among these, the 23%-long 7e₁L segment present in R5+ determined a significantly higher RWC and RL than its absence (R5–). In R112+ the incremental effect vs. R112– involved the majority of the traits assayed (SL, SFW, SDW, RL and RFW; **Table 2.11A**). The same was also observed for R69-9/R5+, which exhibited a better SFW, RWC and RN than its sib–. On the contrary, the presence of the *Th. elongatum* R69-9 segment into the *Th. ponticum* R112 segment somewhat “flattened” the individual segments’ effects, leading to absence of significant differences between R69-9/R112+ and its corresponding sib–. As previously reported in the H stress experiment, from the statistical

comparison between the NIRLs+ and Margherita, a substantial similarity between R5+ and the heat tolerant cultivar emerged (**Table 2.11B**). In this frame, R5+ was even superior to the control cultivar for the RL trait. Instead, the other NIRLs+ possessed a statistically lower SL than Margherita and, as for R69-9/R5+ and R69-9/R112+, also a minor RL and RN, respectively (**Table 2.11B**).

A										
Trait		SL	SFW	SDW	RWC	RN	RL	RFW	RDW	R/S
R5+	Mean	16.219	0.119	0.022	0.695	6.563	71	0.094	0.017	0.801
	SE	0.494	0.006	0.0008	0.024	0.203	3.554	0.008	0.002	0.071
	Tukey	a	abc	ab	bcd	a	a	a	a	ab
R5-	Mean	15.25	0.105	0.02	0.558	6.125	54.438	0.076	0.015	0.771
	SE	0.411	0.007	0.0009	0.033	0.202	2.459	0.006	0.002	0.104
	Tukey	ab	bc	abc	e	ab	b	abc	ab	ab
R112+	Mean	15.219	0.132	0.022	0.754	6.25	54.763	0.093	0.016	0.718
	SE	0.522	0.014	0.001	0.051	0.194	3.525	0.009	0.0009	0.046
	Tukey	ab	a	a	abc	ab	b	ab	ab	ab
R112-	Mean	13.594	0.102	0.017	0.799	6.313	38.281	0.064	0.012	0.757
	SE	0.238	0.008	0.0008	0.03	0.198	2.661	0.005	0.001	0.083
	Tukey	c	c	c	ab	a	c	c	b	ab
R69-9/R5+	Mean	14.667	0.12	0.02	0.811	6.8	45.833	0.081	0.013	0.652
	SE	0.354	0.012	0.0007	0.035	0.296	1.897	0.007	0.001	0.047
	Tukey	bc	ab	abc	a	a	bc	abc	b	b
R69-9/R5-	Mean	13.5	0.1	0.018	0.63	5.3	36.14	0.069	0.013	0.692
	SE	0.236	0.007	0.0008	0.051	0.153	1.376	0.006	0.002	0.116
	Tukey	c	c	bc	de	b	c	bc	b	ab
R69-9/R112+	Mean	15.031	0.107	0.02	0.736	6	54.656	0.081	0.016	0.805
	SE	0.279	0.012	0.0006	0.052	0.224	2.776	0.005	0.002	0.086
	Tukey	b	bc	abc	abcd	ab	b	abc	ab	ab
R69-9/R112-	Mean	14.156	0.104	0.019	0.704	6	47.256	0.061	0.015	0.868
	SE	0.273	0.012	0.0008	0.054	0.242	4.371	0.004	0.002	0.135
	Tukey	bc	c	bc	cd	ab	bc	c	ab	a

B										
Trait		SL	SFW	SDW	RWC	RN	RL	RFW	RDW	R/S
R5+	Mean	16.219	0.119	0.022	0.695	6.563	71	0.094	0.017	0.801
	SE	0.494	0.006	0.0008	0.024	0.203	3.554	0.008	0.002	0.071
	Tukey	ab	ab	ns	b	ab	a	ns	a	a
R112+	Mean	15.219	0.132	0.022	0.754	6.25	54.763	0.093	0.016	0.718
	SE	0.522	0.014	0.001	0.051	0.194	3.525	0.009	0.0009	0.046
	Tukey	bc	a	ns	ab	ab	bc	ns	ab	ab
R69-9/R5+	Mean	14.667	0.12	0.02	0.811	6.8	45.833	0.081	0.013	0.652
	SE	0.354	0.012	0.0007	0.035	0.296	1.897	0.007	0.001	0.047
	Tukey	c	ab	ns	a	a	c	ns	b	b
R69-9/R112+	Mean	15.031	0.107	0.02	0.736	6	54.656	0.081	0.016	0.805
	SE	0.279	0.012	0.0006	0.052	0.224	2.776	0.005	0.002	0.086
	Tukey	bc	b	ns	ab	b	bc	ns	ab	a
Margherita	Mean	16.781	0.131	0.021	0.784	6.875	57.219	0.084	0.015	0.711
	SE	0.316	0.012	0.001	0.064	0.239	2.603	0.005	0.001	0.071
	Tukey	a	a	ns	ab	a	b	ns	ab	ab

Table 2.11. Means, standard error (SE) and ranking of Tukey test at $P < 0.05$ level for morpho-physiological traits in the WH experiment. **A:** Statistical comparison for G factor performed between NIRLs + vs. –; **B:** Statistical comparison for G factor performed between NIRLs + and cv. Margherita.

The results of Tukey test for significant $G \times T$ interactions (**Table 2.12**) showed, as a whole, a considerable variation of several morphological traits after the WH stress imposition. R5+, and so its corresponding sib–, significantly reduced SL (-15%) and SFW (-25%) but increased RDW (**Figure 2.9A**) and R/S in the WH condition (**Table 2.12A**). This last observation was also true for almost all other genotypes (with the only exception of R112+) and is clearly indicative of a major root involvement and development in a serious water-limited condition. Despite the similar behaviour between R5+ and its genetic control (R5–), R5+ confirmed to possess a higher RWC than R5– under WH (0.67 and 0.49, respectively; **Table 2.12A**, **Figure 2.9A**), suggesting a beneficial effect of the 23%-long 7el₁L segment in water-limited conditions. Despite the results of the G effect alone (see above) had highlighted a superior performance of R112+ vs. R112–, when the $G \times T$ effect was considered it became clear that

the better performance of the former was true for the unstressed condition only. In fact, with the WH stress imposition, R112+ significantly reduced the majority of morphological parameters, whereas R112- showed a reduction of the SFW trait only, with an overall result of no major difference ascribable to presence/absence of the alien segment (**Table 2.12A**). Regarding the R69-9 “nested” recombinant types, both R69-9/R5+ and R69-9/R112+ exhibited a similar behaviour to their corresponding sibs- in both control (C, unstressed) and combined stress (WH) conditions. However, while R69-9/R5- reduced its RWC in WH vs. control conditions by 36%, R69-9/R5+ had a 25% decrease, reaching a statistically higher value than its sib- (**Table 2.12A, Figure 2.9A**). Since the same difference was observed between R5+ vs. R5-, it seems likely to hypothesize that, irrespective of the presence of the *Th. elongatum* segment in R69-9/R5+ (see Materials and Methods, § 2.2.1.), the ability to maintain RWC under WH stress is controlled by genes contained in the *Th. ponticum* portion in common between R5+ and R69-9/R5+ (see Figure 2.1). When the NIRLs+ were compared with Margherita, the positive behaviour based on morphological parameters of R5+ and R69-9/R5+ was confirmed. In fact, better values under WH stress were exhibited by R5+ for RL and by R69-9/R5+ for RWC (**Figure 2.9B; Table 2.12B**). The combined stress application caused a significant (about 50%) decrement of SFW and RWC and a 20% reduction of RN vs control conditions in Margherita, but also an increase in RDW and R/S (as previously reported in this same paragraph for the NIRLs; **Table 2.12B**). The same variation for these parameters also occurred in the NIRLs+, indicating a common response induced by the combined stress.

A

Trait		SL	SFW	SDW	RWC	RN	RL	RFW	RDW	R/S	
R5+	C	Mean	17.625	0.136	0.023	0.715	7.25	66.5	0.119	0.012	0.557
		SE	0.409	0.007	0.001	0.035	0.164	3.162	0.007	0.001	0.048
		Tukey	a	bc	ns	bcde	ns	ab	ab	cde	de
	WH	Mean	14.813	0.102	0.021	0.674	5.875	75.5	0.068	0.022	1.045
		SE	0.559	0.005	0.001	0.033	0.125	6.192	0.005	0.002	0.047
		Tukey	bcde	def	ns	def	ns	a	cd	a	abc
R5-	C	Mean	16.625	0.122	0.022	0.622	6.625	48.688	0.085	0.009	0.414
		SE	0.337	0.006	0.001	0.02	0.183	2.057	0.009	0.000622	0.033
		Tukey	ab	cd	ns	defg	ns	cdef	bcd	e	e
	WH	Mean	13.875	0.088	0.019	0.495	5.625	60.188	0.066	0.021	1.129
		SE	0.263	0.008	0.001	0.056	0.263	3.499	0.006	0.001	0.093
		Tukey	de	efg	ns	g	ns	abcd	cd	ab	ab
R112+	C	Mean	16.625	0.183	0.024	0.935	6.375	64.75	0.122	0.013	0.555
		SE	0.68	0.01	0.002	0.007	0.263	3.723	0.009	0.000871	0.018
		Tukey	ab	a	ns	a	ns	abc	a	cde	de
	WH	Mean	13.813	0.082	0.02	0.574	6.125	44.775	0.064	0.018	0.881
		SE	0.377	0.005	0.001	0.04	0.295	3.3	0.006	0.001	0.033
		Tukey	de	fg	ns	defg	ns	def	cd	abc	bc
R112-	C	Mean	14.188	0.131	0.019	0.871	6.375	44.625	0.073	0.009	0.501
		SE	0.266	0.007	0.001	0.018	0.324	2.962	0.008	0.00064	0.043
		Tukey	cde	bcd	ns	abc	ns	def	cd	e	e
	WH	Mean	13	0.073	0.015	0.728	6.25	31.938	0.056	0.015	1.012
		SE	0.267	0.002	0.000516	0.046	0.25	3.174	0.004	0.001	0.093
		Tukey	e	fg	ns	bcde	ns	f	d	bcd	bc
R69-9/R5+	C	Mean	15.143	0.162	0.02	0.93	6.857	45.929	0.092	0.01	0.477
		SE	0.614	0.01	0.001	0.031	0.261	3.681	0.012	0.000722	0.022
		Tukey	bcd	ab	ns	a	ns	def	abc	de	e
	WH	Mean	14.25	0.084	0.021	0.706	6.75	45.75	0.069	0.017	0.805
		SE	0.366	0.004	0.000832	0.022	0.526	1.825	0.005	0.000742	0.025
		Tukey	cde	efg	ns	cde	ns	def	cd	abc	cd
R69-9/R5-	C	Mean	13.4	0.118	0.019	0.767	5.4	36	0.072	0.007	0.373
		SE	0.43	0.004	0.000944	0.031	0.245	1.718	0.008	0.000702	0.024
		Tukey	de	cde	ns	abcd	ns	ef	cd	e	e
	WH	Mean	13.6	0.083	0.018	0.493	5.2	36.28	0.066	0.018	1.012
		SE	0.245	0.009	0.001	0.034	0.2	2.358	0.009	0.003	0.095
		Tukey	de	efg	ns	g	ns	ef	cd	abc	bc
R69-9/R112+	C	Mean	15.875	0.152	0.02	0.9	6.5	52.938	0.09	0.01	0.519
		SE	0.263	0.005	0.000929	0.017	0.267	3.521	0.008	0.000636	0.025
		Tukey	abc	abc	ns	a	ns	bcde	abcd	de	de
	WH	Mean	14.188	0.063	0.02	0.572	5.5	56.375	0.072	0.022	1.091
		SE	0.249	0.004	0.00077	0.06	0.267	4.449	0.005	0.002	0.089
		Tukey	cde	g	ns	efg	ns	bcd	cd	a	abc
R69-9/R112-	C	Mean	14.938	0.145	0.02	0.878	6.125	33.188	0.06	0.008	0.4
		SE	0.175	0.005	0.00076	0.025	0.398	1.235	0.007	0.000602	0.032
		Tukey	bcd	bc	ns	ab	ns	f	cd	e	e
	WH	Mean	13.375	0.057	0.017	0.505	5.875	61.325	0.062	0.022	1.336
		SE	0.337	0.005	0.001	0.036	0.295	4.88	0.005	0.002	0.12
		Tukey	de	g	ns	fg	ns	abcd	cd	a	a

B

Trait		SL	SFW	SDW	RWC	RN	RL	RFW	RDW	R/S	
R5+	C	Mean	17.625	0.136	0.023	0.715	7.25	66.5	0.119	0.012	0.557
		SE	0.409	0.007	0.001	0.035	0.164	3.162	0.007	0.001	0.048
		Tukey	ns	b	ns	b	ab	ab	a	cd	d
	WH	Mean	14.813	0.102	0.021	0.674	5.875	75.5	0.068	0.022	1.045
		SE	0.559	0.005	0.001	0.033	0.125	6.192	0.005	0.002	0.047
		Tukey	ns	c	ns	bc	cd	a	b	a	ab
R112+	C	Mean	16.625	0.183	0.024	0.935	6.375	64.75	0.122	0.013	0.555
		SE	0.68	0.01	0.002	0.007	0.263	3.723	0.009	0.000871	0.018
		Tukey	ns	a	ns	a	abcd	ab	a	bcd	d
	WH	Mean	13.813	0.082	0.02	0.574	6.125	44.775	0.064	0.018	0.881
		SE	0.377	0.005	0.001	0.04	0.295	3.3	0.006	0.001	0.033
		Tukey	ns	cd	ns	bc	bcd	c	b	ab	bc
R69-9/R5+	C	Mean	15.143	0.162	0.02	0.93	6.857	45.929	0.092	0.01	0.477
		SE	0.614	0.01	0.001	0.031	0.261	3.681	0.012	0.000722	0.022
		Tukey	ns	ab	ns	a	abc	a	ab	d	d
	WH	Mean	14.25	0.084	0.021	0.706	6.75	45.75	0.069	0.017	0.805
		SE	0.366	0.004	0.000832	0.022	0.526	1.825	0.005	0.000742	0.025
		Tukey	ns	cd	ns	b	abcd	c	b	abc	c
R69-9/R112+	C	Mean	15.875	0.152	0.02	0.9	6.5	52.938	0.09	0.01	0.519
		SE	0.263	0.005	0.000929	0.017	0.267	3.521	0.008	0.000636	0.025
		Tukey	ns	ab	ns	a	abcd	bc	ab	d	d
	WH	Mean	14.188	0.063	0.02	0.572	5.5	56.375	0.072	0.022	1.091
		SE	0.249	0.004	0.00077	0.06	0.267	4.449	0.005	0.002	0.089
		Tukey	ns	d	ns	bc	d	bc	b	a	a
Margherita	C	Mean	17.625	0.172	0.024	0.978	7.625	58.063	0.094	0.011	0.461
		SE	0.441	0.01	0.001	0.007	0.263	2.086	0.007	0.000619	0.026
		Tukey	ns	a	ns	a	a	abc	ab	d	d
	WH	Mean	15.938	0.089	0.019	0.524	6.125	56.375	0.075	0.018	0.96
		SE	0.175	0.005	0.000889	0.031	0.125	4.949	0.006	0.001	0.058
		Tukey	ns	cd	ns	c	bcd	bc	b	ab	abc

Table 2.12. Means, standard error (SE) and ranking of Tukey test at $P < 0.05$ level for morpho-physiological traits in the WH experiment. **A:** Statistical comparison for $G \times T$ factor performed between NIRLs + vs. –; **B:** Statistical comparison for $G \times T$ factor performed between NIRLs + and cv. Margherita.

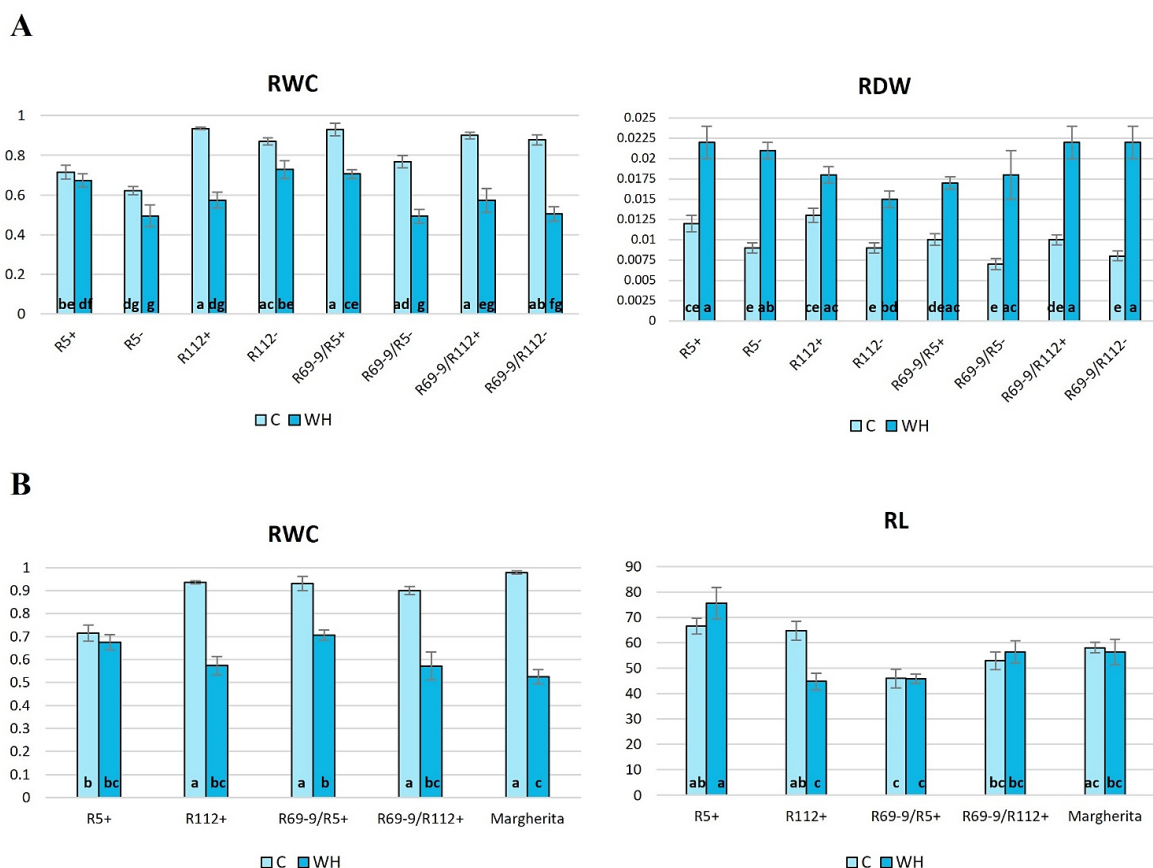


Figure 2.9. Significant $G \times T$ effects on morpho-physiological parameters in the WH stress experiment. **A:** Statistical comparison performed between NIRLs + vs. –; **B:** Statistical comparison performed between NIRLs + and cv. Margherita. Error bars represent standard errors of the means, while letters into the histograms correspond to ranking of Tukey HSD test at $p < 0.05$ significance level.

2.3.7. Biochemical traits modulation in the combined WH experiment

The G effect on the biochemical parameters assayed in the WH experiment highlighted differences mainly for R5+, which presented a lower Pro-S and a higher MDA-S than R5– (**Table 2.13A**). Moreover, R69-9/R112+ confirmed to possess a lower Pro-R content than R69-9/R112–, as previously observed in the H stress experiment. Whether due to the background or other, yet unknown, reasons, compared to the other genotypes, Margherita included, R69-9/R112+ had a much higher amount of the osmolyte in both shoot and roots (**Table 2.13B**). Margherita, in turn, and to some extent all the others NIRLs+, exhibited higher values of MDA-R than R69-9/R112+, which thus appeared as the genotype with the minimum membrane damage in root tissues.

A

Trait		Pro-S	Pro-R	MDA-S	MDA-R
R5+	Mean	18.706	3.547	10.319	2.633
	SE	6.126	0.524	0.522	0.614
	Tukey	c	c	a	ab
R5-	Mean	71.981	9.468	7.755	2.587
	SE	23.836	3.135	0.515	0.752
	Tukey	b	c	bc	a
R112+	Mean	17.487	3.648	7.322	2.099
	SE	6.035	0.93	0.981	0.473
	Tukey	c	c	bcd	ab
R112-	Mean	17.012	5.198	6.695	1.992
	SE	5.269	0.835	0.551	0.587
	Tukey	c	c	bcd	ab
R69-9/R5+	Mean	23.62	5.261	6.442	1.909
	SE	5.899	1.07	0.637	0.487
	Tukey	c	c	cd	ab
R69-9/R5-	Mean	13.341	4.512	5.843	1.848
	SE	3.473	0.79	0.572	0.478
	Tukey	c	c	d	b
R69-9/R112+	Mean	139.788	20.81	7.135	1.803
	SE	60.019	7.961	0.92	0.418
	Tukey	a	b	bcd	ab
R69-9/R112-	Mean	163.483	45.015	8.406	2.091
	SE	55.403	19.408	1.42	0.544
	Tukey	a	a	ab	ab

B

Trait		Pro-S	Pro-R	MDA-S	MDA-R
R5+	Mean	18.706	3.547	10.319	2.633
	SE	6.126	0.524	0.522	0.614
	Tukey	c	b	a	ab
R112+	Mean	17.487	3.648	7.322	2.099
	SE	6.035	0.93	0.981	0.473
	Tukey	c	b	b	ab
R69-9/R5+	Mean	23.62	5.261	6.442	1.909
	SE	5.899	1.07	0.637	0.487
	Tukey	c	b	b	ab
R69-9/R112+	Mean	139.788	20.81	7.135	1.803
	SE	60.019	7.961	0.92	0.418
	Tukey	a	a	b	b
Margherita	Mean	79.583	6.844	6.074	3.68
	SE	25.739	2.22	0.971	0.942
	Tukey	b	b	b	a

Table 2.13. Means, standard error (SE) and ranking of Tukey test at $P < 0.05$ level for biochemical traits in the WH experiment. **A:** Statistical comparison for G factor performed between NIRLs + vs. –; **B:** Statistical comparison for G factor performed between NIRLs + and cv. Margherita.

When the $G \times T$ interactions were considered, the above observations about Pro-S in R5+ were confirmed, since R5+ exhibited a lower Pro-S and a superior MDA-S than its sib– under WH conditions (**Table 2.14A; Figure 2.10A**). Again, the overall Pro content significantly and remarkably increased in both R69-9/R112+ and R69-9/R112–, but with a higher rate in the root of the latter (**Figure 2.10A**). On the other hand, R112+ and R69-9/R5+ did not manifest relevant differences in the overall biochemical modulations in comparison with their corresponding sibs–. Only in the case of the MDA-S content, R112+ showed a significant increment (not detected in R112–) under WH (**Figure 2.10A**), which could be indicative of a major damage at the shoot level (in line with the drop detected for several morphological traits of R112+ shoot; **Table 2.12**). The biochemical response to the combined stress of the heat tolerant Margherita seemed to involve an overall increase in Pro content in both shoot and roots (**Figure 2.10B**), with R69-9/R112+ remaining the top Pro accumulator among all genotypes. Margherita, however, showed clear symptoms of a root tissue damage, since its MDA-R content significantly increased (+560%) in WH vs. control conditions (**Table 2.14B; Figure 2.10B**), whereas all NIRLs+ showed a lower MDA-R increment (between +210 and +290%) under stress.

A

Trait		Pro-S	Pro-R	MDA-S	MDA-R
R5+	C	Mean	5.865	2.673	9.452
		SE	0.968	0.28	0.581
		Tukey	c	d	ab
	WH	Mean	35.827	4.42	11.187
		SE	2.088	0.731	0.521
		Tukey	c	cd	a
R5-	C	Mean	8.407	2.983	7.923
		SE	1.214	0.787	0.863
		Tukey	c	cd	bc
	WH	Mean	119.662	15.953	7.531
		SE	14.816	2.543	0.571
		Tukey	b	c	bcd
R112+	C	Mean	4.43	1.623	4.837
		SE	1.517	0.232	0.441
		Tukey	c	d	d
	WH	Mean	30.543	5.673	9.185
		SE	3.057	0.415	0.753
		Tukey	c	cd	ab
R112-	C	Mean	5.787	4.127	5.499
		SE	0.481	1.075	0.176
		Tukey	c	cd	cd
	WH	Mean	28.237	6.27	7.89
		SE	3.543	1.086	0.234
		Tukey	c	cd	bcd
R69-9/R5+	C	Mean	4.557	5.13	5.021
		SE	0.15	1.645	0.399
		Tukey	c	cd	cd
	WH	Mean	35.058	5.36	7.508
		SE	3.196	1.627	0.678
		Tukey	c	cd	bcd
R69-9/R5-	C	Mean	6.19	2.853	4.774
		SE	1.196	0.518	0.413
		Tukey	c	d	d
	WH	Mean	22.877	6.17	6.555
		SE	1.42	0.323	0.633
		Tukey	c	cd	bcd
R69-9/R112+	C	Mean	6.47	3.03	5.138
		SE	1.419	0.687	0.165
		Tukey	c	cd	cd
	WH	Mean	273.107	38.59	9.133
		SE	15.344	0.572	0.462
		Tukey	a	b	ab
R69-9/R112-	C	Mean	7.207	2.593	5.342
		SE	1.372	0.46	0.722
		Tukey	c	d	cd
	WH	Mean	280.69	87.437	11.471
		SE	7.583	9.145	0.413
		Tukey	a	a	a

B

Trait		Pro-S	Pro-R	MDA-S	MDA-R
R5+	C	Mean	5.865	2.673	9.452
		SE	0.968	0.28	0.581
		Tukey	c	c	ns
	WH	Mean	35.827	4.42	11.187
		SE	2.088	0.731	0.521
		Tukey	c	bc	ns
R112+	C	Mean	4.43	1.623	4.837
		SE	1.517	0.232	0.441
		Tukey	c	c	ns
	WH	Mean	30.543	5.673	9.185
		SE	3.057	0.415	0.753
		Tukey	c	bc	ns
R69-9/R5+	C	Mean	4.557	5.13	5.021
		SE	0.15	1.645	0.399
		Tukey	c	bc	ns
	WH	Mean	35.058	5.36	7.508
		SE	3.196	1.627	0.678
		Tukey	c	bc	ns
R69-9/R112+	C	Mean	6.47	3.03	5.138
		SE	1.419	0.687	0.165
		Tukey	c	c	ns
	WH	Mean	273.107	38.59	9.133
		SE	15.344	0.572	0.462
		Tukey	a	a	ns
Margherita	C	Mean	9.168	1.9	4.006
		SE	0.976	0.183	0.474
		Tukey	c	c	ns
	WH	Mean	135.916	10.553	8.142
		SE	24.482	2.558	0.46
		Tukey	b	b	ns

Table 2.14. Means, standard error (SE) and ranking of Tukey test at $P < 0.05$ level for biochemical traits in the WH experiment. **A:** Statistical comparison for $G \times T$ factor performed between NIRLs + vs. –; **B:** Statistical comparison for $G \times T$ factor performed between NIRLs + and cv. Margherita.

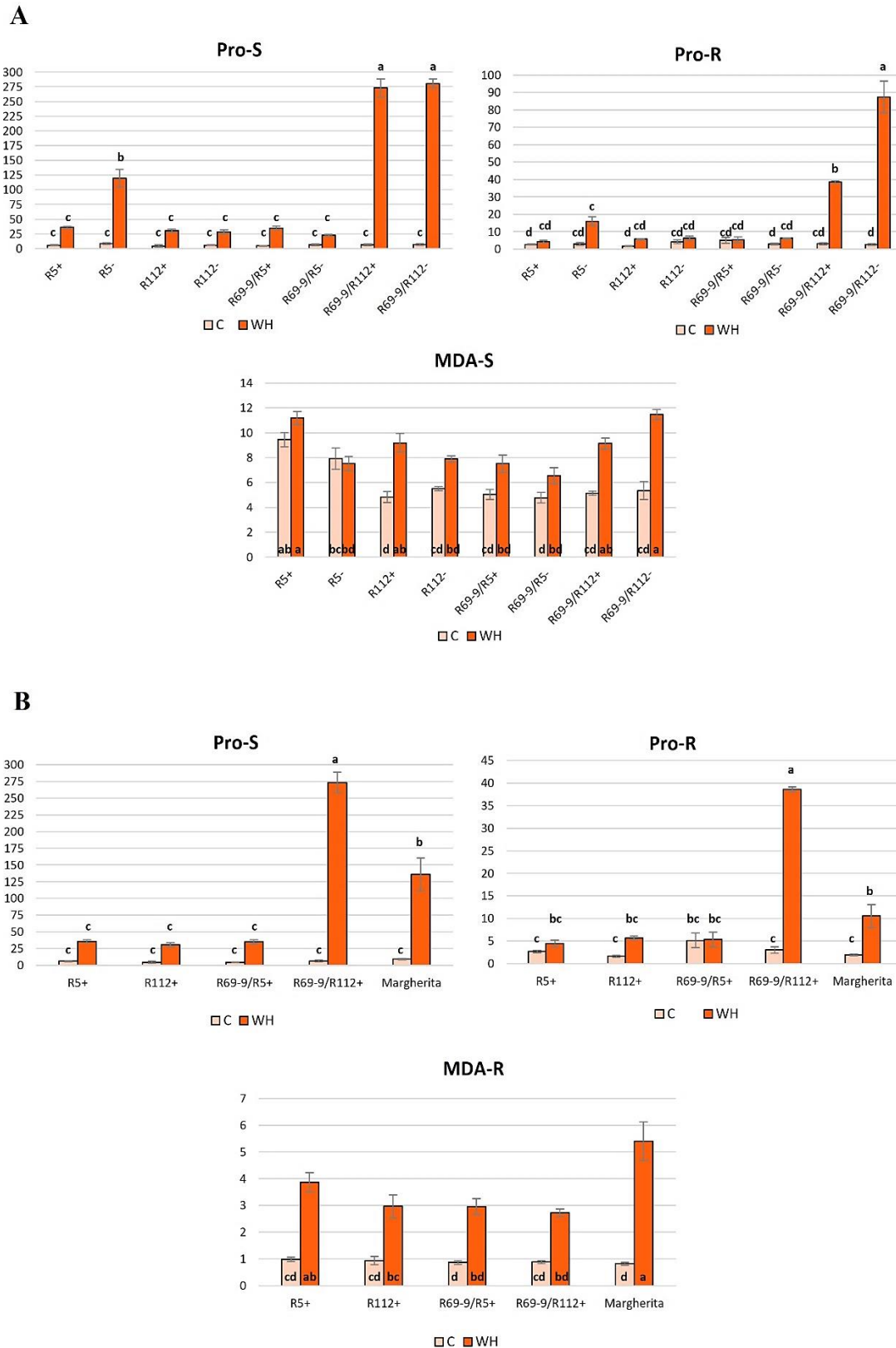


Figure 2.10. Significant $G \times T$ effects on biochemical parameters in the WH stress experiment. **A:**

Statistical comparison performed between NIRLs + vs. –; **B:** Statistical comparison performed between NIRLs + and cv. Margherita. Error bars represent standard errors of the means, while letters into or above the histograms correspond to ranking of Tukey HSD test at $p < 0.05$ significance level.

2.4. Discussion

In the present work, the morpho-physiological and biochemical performance of primary DW-*Th. ponticum* NIRLs+ and secondary DW-*Th. ponticum*/*Th. elongatum* NIRLs+, was evaluated, compared with control lines (respective NIRLs– and cv. Margherita), following the application of heat stress (H) alone and in combination with water-deficit (WH) at seedling stage. In both the H and WH experiments, the stress application procedure resulted appropriate for highlighting differences in traits modulation by the assayed genotypes (**Tables 2.5** and **2.10**). In fact, a number of differences in the genotypes' response after the single and combined stress application could be observed, some of them clearly ascribable to the presence of given wild chromatin introgressions. Moreover, the results here obtained at the seedling stage substantially confirmed the positive or negative response to H and WH conditions brought about by the NIRLs+ at the reproductive stage (Giovenali et al., 2023; see Chapter 3). Thus, this research not only confirmed the possibility of carrying out an early selection for better stress-responsive genotypes (Lu et al., 2022), but also enabled identification of more suitable marker traits to this purpose.

Phenotypic seedling screenings, which include assessment of fresh and dry weight, besides physiological and biochemical assays, were reported as very efficient for the selection of heat tolerant genotypes (Hameed et al., 2012; Lu et al., 2022). In wheat, high temperature treatments were found to significantly reduce seedling shoot and root dry mass and length, besides leaf RWC (Hameed et al., 2012; Gupta et al., 2013), with tolerant genotypes showing comparatively lower decline of these traits. From the present H stress experiment, a positive response of both R5+ and R112+ emerged from the analysis of morpho-physiological traits. These two NIRLs, not only showed no significant reduction of such traits in the H0 vs. C conditions, but also exhibited very similar values to those of the heat tolerant cv. Margherita (**Table 2.7B**). Furthermore, R69-9/R5+, despite reducing its RWC in H0 vs. C condition (similar trend for all R69-9 types; **Figure 2.6A**), showed a positive modulation of root traits, particularly the R/S parameter, under H stress, reaching a statistically higher value than Margherita (**Figure 2.6B**). In line with the observations of Lu et al. (2022) on heat-, but also drought-, stressed wheat plantlets, a minor drop in all root-related parameters was observable in tolerant genotypes, this characterized the response to heat of the R69-9/R5+ recombinant. The biochemical analyses performed partially confirmed the above reported observations regarding recombinant lines R5+ and R112+. In fact, R5+, besides being the only between the NIRLs+ not showing an

inferior SOD activity vs. its sib[−] under C conditions (**Figure 2.7**), emerged as the best-performing genotype for SOD activity, since it possessed the highest values in both H0 and H24 conditions. These observations indicate a positive involvement of the 23%-long 7el₁L segment in SOD modulation under H stress. On the other hand, although all NIRLs⁺ had a higher CAT activity than the corresponding NIRLs[−] in C conditions, a common decrease in CAT was detected in all NIRLs⁺ after H imposition, R5⁺ included. The latter recombinant exhibited a significant increase in APX activity in H0 vs. C conditions (**Figure 2.7**), reaching an absolute value superior to those of all other NIRLs⁺ and Margherita. Thus, the overall antioxidant system in R5⁺ seedlings seems to be more efficient than that of all genotypes tested. This represents a promising behaviour and confirms the conclusions of previous studies on DW plants subjected to heat and drought stresses, that reported an overall increase in the activities of antioxidant enzymes with the stress imposition (Almeselmani et al., 2006; Gupta et al., 2013). Nonetheless, the fact that an extremely intense stress could result in a consistent decrease in the overall antioxidant activity (Nayyar et al., 2014) should not be overlooked. Moreover, specific enzyme response may differ depending on genotypes and treatments. In fact, antioxidants variation mainly depends on stress severity and duration and also on the plant's species considered. For example, in wheat SOD activity can increase or remain stable in the early phase of drought, but prolonged periods of water-deficit can lead to SOD decrease (Weng et al., 2015 and references therein). Furthermore, Hameed et al. (2012) reported that CAT activity of 11-days-old wheat seedlings decreased under H stress, while POD and APX activities concomitantly increased, thus probably compensating for the CAT drop. An interesting biochemical response modulation was implemented towards the H treatment also by R112⁺ plantlets. In fact, R112⁺ was the only NIRLs⁺ to exhibit a significant increase of Pro-S in H0 compared with C conditions (**Table 2.9A; Figure 2.7**). This is likely indicative of heat tolerance of R112⁺, as Pro accumulation and its defensive role under diverse stress condition in plants (Siddique et al., 2018) and seedlings (Ahmed and Hassan, 2011) is widely acknowledged. In general, H stress causes cell membrane injury in seedlings, as indicated by an increase lipid peroxidation (Hameed et al., 2012), and this is inversely correlated with Pro content (Gupta et al., 2013). Both assays, carried out on seedlings, provided with coinciding, hence predictive, responses of R5⁺ and R112⁺ towards H stress applied at adult plant stage (see Chapter 3).

The combined WH seedling treatment similarly confirmed the stress tolerant behaviour of R5⁺, also resulting from combined stress application at the reproductive stage, where it turned out to be the best performing genotype (Giovenali et al., 2023; see Chapter 3). In the seedling

experiment here performed, both R5+ and its derived recombinant R69-9/R5+ showed a significantly higher RWC than the corresponding sibs– in the WH condition (**Figure 2.9A**), suggesting the involvement of genes present in the shared 7e₁L portion in RWC preservation. In several studies on wheat seedlings subjected to drought stress, the RWC trait resulted highly responsive to the stress and was associated with tolerance acquisition (e.g. Ahmed et al., 2019; Altaf et al., 2021). In fact, wheat plants having a higher RWC can better adapt to water-limited environments, since they better sustain leaf wilting. R5+ also exhibited a superior RL than the tolerant cv. Margherita under WH (**Table 2.12B**; **Figure 2.9B**). Ahmed et al. (2019) and Almaghrabi (2012) highlighted that drought stress remarkably reduces plantlets' root length, due to impairment of cell division and elongation, and genotypes with higher values of this trait should be selected as drought tolerant.

In contrast with a consistent tolerance of R5+ towards heat stress alone and in combination with water deficit, in the latter condition, while not under the former, R112+ significantly decreased almost all the morphological traits measured compared with R112– (**Table 2.12A**), indicating a negative impact of the 7e₁L segment on its ability to withstand the combined stress. The same was observed when the combined stress was imposed at anthesis (Giovenali et al., 2023; see also Chapter 3). The MDA-S parameter confirmed the negative effects of heat+drought combination on R112+ shoots, R112+ having a significant increase of MDA content in WH vs. C conditions (the same was not observed in R112–). MDA content, as an indicator of membrane lipid peroxidation, reflects the degree of damage due to stressful conditions and a strong increase of this trait is a symptom of stress susceptibility (Weng et al., 2015). In fact, it represents a confirmation of the susceptibility to water shortage by R112+, in contrast to its heat tolerance.

An unexpected observation performed in the WH experiment regards the Pro content detected in shoots and roots of R69-9/R112+ and also R69-9/R112–. In fact, a substantial increase in the osmolyte content was detected in the WH stressed seedlings of these two genotypes (**Figure 2.10A**). Drought stressed wheat seedlings normally accumulate Pro and sugars (e.g. sucrose, fructose, mannose) and this accumulation is generally recognized as an indicator of stress tolerance (Guo et al., 2018; Altaf et al., 2021). However, it is not clear if such a high Pro increment (much stronger of that showed by Margherita; **Figure 2.10B**) should be considered as a tolerance trait or only a physiological perturbation induced by stress (Zhang et al., 2022). In fact, R69-9/R112+ did not manifest penalty symptoms of morphological traits after the WH stress (as, instead, R112+ did), but also no clear indication of a tolerant behaviour.

As a whole, the results of the present work showed that, among the chromosomally engineered DW-*Thinopyrum* spp. recombinant lines, the one possessing the smallest amount of alien 7el₁L introgression, namely R5+, showed an increased tolerance to both the stress conditions applied (H and WH) at the seedling stage. Presence of its 23%-long wild segment seems to be associated with an enhanced SOD activity under H conditions and a superior RWC under the combined WH stress. These results are in line with the observations performed on the same heat and heat + drought stressed genotypes at anthesis stage, confirming the predictiveness of an early selection analysis at a juvenile stage. The same is true for root traits enhanced in R69-9/R5+ after the H stress treatment, that could be predictive of an improved heat stress tolerance also in advanced phenological stages. In fact, R69-9/R5+ emerged as one of the best performing genotypes after an intense heat treatment applied at the flowering phase (see Chapter 4), presenting a stable RWC, an increased Pro accumulation in flag leaves and a high yield stability across heat and control conditions. In conclusion, the enrichment of DW genetic basis via the chromosome engineering strategy proves to be an effective approach to improve DW abiotic stress tolerance, displayed already at very early phenological phases, besides that at much later stages.

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**Chapter 3: The Response of Chromosomally Engineered Durum
Wheat- *Thinopyrum ponticum* Recombinant Lines to the
Application of Heat and Water-Deficit Stresses: Effects on
Physiological, Biochemical and Yield-Related Traits**

Article

The Response of Chromosomally Engineered Durum Wheat-*Thinopyrum ponticum* Recombinant Lines to the Application of Heat and Water-Deficit Stresses: Effects on Physiological, Biochemical and Yield-Related Traits

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Abstract: Abiotic stress occurrence and magnitude are alarmingly intensifying worldwide. In the Mediterranean basin, heat waves and precipitation scarcity heavily affect major crops such as durum wheat (DW). In the search for tolerant genotypes, the identification of genes/QTL in wild wheat relatives, naturally adapted to harsh environments, represents a useful strategy. We tested three DW-*Thinopyrum ponticum* recombinant lines (R5+, R112+, R23+), their control sibs lacking any alien introgression, and the heat-tolerant cv. Margherita for their physiological, biochemical and yield response to heat stress (HS) application at anthesis, also in combination with water-deficit stress applied from booting until maturity. Under HS, R5+ and R112+ (23%- and 28%-long 7eL *Th. ponticum* chromosome segment distally inserted on DW 7AL, respectively) showed remarkable stability of the yield-related traits; in turn, R23+ (40%-long 7eL *L* segment), despite a decreased grain yield, exhibited a greater spike fertility index and proline content in spike than its control sib. Under water-deficit + HS, R5+ showed the highest increment in water use efficiency and in flag leaf proline content, accompanied by the lowest yield penalty even vs. Margherita. This research confirms the value of harnessing wild gene pools to enhance DW stress tolerance and represents a starting point for elucidating the mechanisms of *Thinopyrum* spp. contribution to this relevant breeding target.

Keywords: wild wheat relatives; abiotic stresses; stress combination; stress tolerance; stress physiology; proline content



Citation: Giovenali, G.; Kuzmanović, L.; Capoccioni, A.; Ceoloni, C. The Response of Chromosomally Engineered Durum Wheat-*Thinopyrum ponticum* Recombinant Lines to the Application of Heat and Water-Deficit Stresses: Effects on Physiological, Biochemical and Yield-Related Traits. *Plants* **2023**, *12*, 704. <https://doi.org/10.3390/plants12040704>

Academic Editor: Daniela Trono

Received: 19 December 2022

Revised: 27 January 2023

Accepted: 30 January 2023

Published: 5 February 2023



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1. Introduction

Global population growth, coupled with reduced availability of arable land and water, are all critical challenges to urgently deal with in order to cope with food security. Projected objectives indicate the need to reach a 60% production increase for staple crops by 2050, and doubled yields for wheat in particular [1]. However, climate changes pose increasing threats to plant vitality and fertility. Under the different climate scenarios of the second half of this century, global cereal production is projected to decrease by 1–11% [2], and, as for wheat, each degree-Celsius of temperature increase is estimated to imply a 6% decline of global yields [3]. Particularly affected by exacerbating weather conditions is the Mediterranean region, where heat waves accompanied by drought phenomena are foreseen to be more and more severe and frequent [4], resulting in the conspicuous loss of crop production and increased yield volatility [5,6]. In the Mediterranean and bordering Middle East countries, a large proportion of the total wheat surface is covered by durum wheat (*Triticum durum* L., $2n = 4x = 28$), a species that traditionally provides important social and commercial benefits to the whole area [7–9]. In fact, durum wheat (DW) produced in this area accounts for more than half of the global production [2,10,11]. To assure profitable and stable economic returns to farmers and the industry of traditional as well as emerging markets (e.g., [7]), major efforts are needed to counter the projected DW yield losses due to

a warmer and drier climate. Such losses are expected to range from -8% to -50% in the Mediterranean area [2]. Releasing varieties with high and stable yields under unfavourable environmental conditions is therefore a primary breeding objective in the Mediterranean regions. However, the required genetic diversity for meaningful interventions is very limited in the extant crop gene pool, thus resulting as insufficient to adequately respond to current and future challenges. On the other hand, wild wheat relatives are still endowed with ample and largely untapped diversity, allowing them to withstand a wide range of harsh environmental conditions [12,13]. A strong diversity reduction emerges from comparison of DW with its closest wild ancestor, *T. dicoccoides* [14,15]. This member of DW primary gene pool, possessing the same AB genomes as the crop species, and so diploid wild relatives sharing similarity with either genome, allow easy access to their adaptive attributes. Hence, they have been rather extensively and successfully harnessed in DW breeding programs addressing yield stability under abiotic stresses [16–18].

A further and major novelty potentially able to generate climate-resilient wheat cultivars consists of the utilization of the little-explored genetic diversity present in wild grasses belonging to the wheat tertiary gene pool. To access the defined chromosomal regions and target genes of such more distantly related species, such as those belonging to the *Thinopyrum* genus, pre-breeding approaches referred to as “chromosome engineering” are required [19,20]. Through this strategy, we have recently transferred into bread and durum wheat defined segments of *Thinopyrum* chromosomes, specifically of decaploid *Thinopyrum ponticum* (Popd.) Barkworth and D.R. Dewey ($2n = 10x = 70$) and, in a “nested” fashion within the above ones, of diploid *Thinopyrum elongatum* (Host) D.R. Dewey ($2n = 2x = 14$). The target traits for introgression were primarily resistances to major wheat diseases, namely leaf rust, stem rust and Fusarium head blight [19–22]. However, when field trialled across contrasting environments (including abiotic stress-prone) and water-regimes, such DW-*Thinopyrum* spp. recombinant lines revealed improved yield-contributing traits and higher yield stabilities than the control lines lacking the alien segment(s) [23,24]. This evidence was suggestive of a potential involvement of these *Thinopyrum* spp. segments in abiotic stress tolerance. However, while better known for their ability to withstand salinity stress [25], this group of species has been less explored and characterized for heat and drought tolerance [26,27]. Certainly, little knowledge is available of specific stress tolerance/adaptation mechanisms and of the possible association of the tolerant phenotype(s) with the presence of particular alien segments [28,29].

Plants, as sessile organisms, cannot escape from unfavourable environments; therefore, the ability to activate effective adaptive mechanisms against abiotic stress holds great importance in view of yield preservation. Both high temperature peaks and heat waves occurring during the reproductive phase, particularly at flowering, are detrimental to wheat yield formation, with excess temperatures above the optimal $21\text{ }^{\circ}\text{C}$ leading to floret abortion, pollen sterility and finally decreased grain number and yield [30,31]. Similarly, water-deficit occurrence around anthesis affects many key traits related to spike and plant fertility, such as fertile florets per spikelet and grain number per spike and per plant [32,33]. Likewise, drought at the grain filling stage leads to a reduction of grain weight, grain yield and harvest index [34]. On the other hand, under field conditions, heat and drought phenomena often occur simultaneously and usually aggravate the negative impacts of single stresses on crop yield; furthermore, the plant reaction to multiple stress combinations is hardly predictable, and cannot be directly extrapolated from the response to individual stresses [35–37].

The drop in spike fertility and other yield traits under hot and dry conditions is strongly related to physiological and biochemical perturbations occurring in stressed plants. As in practically all green plants, photosynthetic efficiency is highly reduced in wheat subjected to high temperatures and water limitations, due to accelerated leaf senescence, which restricts green-photosynthetic areas, as well as to disruption of chloroplasts’ structure and function, implying reduction of pigments such as chlorophyll and carotenoids, inactivation of key enzymes and membrane injury (e.g., [38] and references therein). Hence, chlorophyll

fluorescence analysis, which enables the assessment of several parameters related to photosynthetic efficiency, is a commonly used tool to monitor the physiological status of plants under abiotic stress conditions [39–41].

Stomata, determining the rate of CO₂ uptake in leaves and, consequently, the amount available at the Rubisco site through their opening/closing mechanism, are key players in photosynthetic efficiency and plant transpiration [42]. Genotypic variation and a positive relation with grain yield have been reported for stomatal conductance in wheat under well-watered conditions [43]. Under prolonged heat occurrences, stomatal conductance is usually enhanced to promote evaporative cooling, thus reducing the inner temperature [44,45]. However, there is also evidence of stomata closure under heat exposure, suggesting a fine and sensitive regulation of this physiological mechanism, subject to intra- and inter-specific variations [46]. In water-limited conditions, stomata closure helps reduce water loss and maintain cell turgor, but leads to decreased CO₂ influx and carbon fixation hindrance in the Calvin cycle [47,48]. As a result, chloroplasts become exposed to excessive excited energy and enhance the production of toxic reactive oxygen species (ROS), which, in turn, cause oxidative damage [49].

In addition to oxidative damage, both drought and heat phenomena trigger a secondary osmotic stress in plants, resulting in the loss of cell turgor. To overcome this, a wide range of molecules known as compatible solutes, including sucrose, trehalose, glycine, betaine and proline, can accumulate at high concentrations in tissues, thus protecting cells against osmotic imbalance. Among them, proline (Pro) is a very versatile compound, whose beneficial effects against different abiotic stresses, not only as an osmolyte, but also as a stabilizer of sub-cellular structures, buffer of cellular redox potential, ROS scavenger, signal molecule and protein folding chaperone, are widely demonstrated [50,51]. An increment of Pro content has been consistently reported in the leaves of heat- or drought-stressed wheat plants, and the activation of this defence mechanism was strongly correlated with a higher stress tolerance (e.g., [52,53]). However, less is known about Pro accumulation in sink organs, such as spikes, directly related to yield, and about its role in plants subjected to heat stress and water-deficit simultaneously.

In view of exploiting novel and exotic germplasms for improving the abiotic stress resilience of wheat crops, it is particularly relevant to study how wild gene introgressions impact the critical defence parameters and ultimately the yield of the recipient crop species. To this aim, and to validate the preliminary evidence from field trials [23,24], in the present study we have evaluated, under controlled conditions, the performance of DW-*Th. ponticum* near-isogenic recombinant lines (NIRLs), containing differently sized fractions of the alien 7e1L chromosome arm, when subjected to heat stress alone and in combination with water-deficit stress. Physiological and biochemical parameters of leaf and spike tissues, as well as several plant and spike yield-contributing traits, were assessed. As a result, thanks to the type of the experimental materials employed, the research allowed us to associate the observed phenotypic changes following the single and combined stress application to specific 7e1L regions, and hence to highlight the best performing recombinant lines as candidates for stress-tolerant DW breeding.

2. Materials and Methods

2.1. Plant Materials

Three DW-*Th. ponticum* NIRLs were employed in the present study, named R5-2-10, R112-4 and R23-1 [54], and are here referred to as R5+, R112+ and R23+, respectively. They were developed in the background of the Italian DW cv. Simeto by repeated backcrossing (BC₅ for R5+ and R112+ and BC₄ for R23+) followed by several self-generations. The NIRLs have different portions of the *Th. ponticum* 7e1L chromosome arm on the distal end of the recipient DW 7AL arm (Figure 1). The symbol “+” indicates homozygous carriers of the respective 7e1L segment, while homozygous sib lines, non-carriers of the given 7e1L segment, are accompanied by the “−” symbol. An additional DW genotype used was the ICARDA cv. Margherita, introduced in the analysis because it expressed a remarkable heat

tolerance ability in a range of stressful environments, including the Senegal River Basin, characterized by severe temperature extremes during the flowering stage [55].

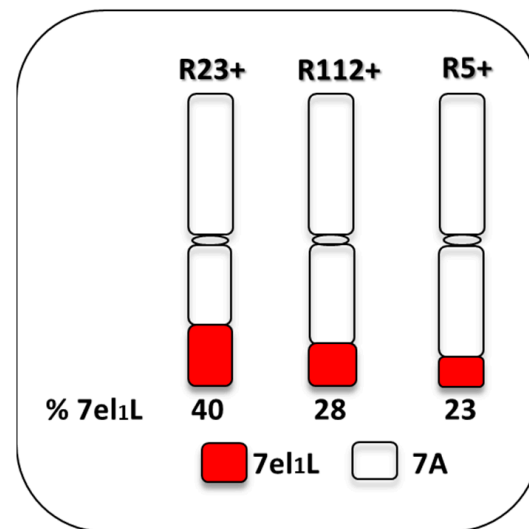


Figure 1. Durum wheat-*Thinopyrum ponticum* recombinant lines tested under heat and water-deficit conditions. 7el1L: chromosome segment derived from *Th. ponticum* and transferred via a chromosome engineering strategy; 7A: durum wheat recipient chromosome.

2.2. Plant Growing Conditions and Stress Treatments

Two separate experiments were carried out to investigate plant behaviour towards heat stress (HS) alone or combined with water-deficit and heat stress (WH). For both experiments, sterilized seeds were placed to germinate on moistened filter paper (22–24 °C), and then transferred in pots containing soil, sand and perlite in a 6:1:1 ratio for vernalization in a cold cabinet at a 8°/6 °C (day/night) temperature and 12 h photoperiod for 15 days. Afterwards, the plants were moved into a walk-in controlled environment chamber, where they were given varying temperature and light regimes, depending on the phenological state and the treatment conditions. The plants were uniformly fertilized and watered, except for those subjected to the water retention treatment (see below).

In the HS experiment, to simulate field conditions, plants whose main culm had reached anthesis (Zadoks scale 61–65, [56]; Figure 2A) were subjected to a gradual increase in temperature in a dedicated chamber. The treatment consisted of a progressive increase in temperature over a 2 h interval, from 22 °C up to 38 °C (+2 °C every 15 min), followed by 2 h at 38 °C and by 2 h of a gradual decrement from 38 °C to 22 °C (−2 °C every 15 min), totalling 6 h/day of HS applied over 3 consecutive days (Figure 2B). After the HS application, the plants were brought back to the original chamber and kept there until harvest. In the same chamber, the control (non-stressed) plants were maintained at 22 °C/18 °C during anthesis, and the whole set of materials were subsequently grown as illustrated in Figure 3.

In the combined stress (WH) experiment, water withholding started at the booting stage (about 10 weeks from germination) and ended at maturity, mimicking a prolonged stress condition. At anthesis, HS was applied as described above on two subsets of plants for either 3 (WH3) or 7 (WH7) consecutive days (3d, 7d; Figure 3). For the water-deficit application, the pots were initially watered to maximum capacity, left to drain overnight and then the soil surface was covered with an aluminium foil to avoid soil evaporation. The pot weight upon drainage completion was taken as 100% Soil Water Content (SWC). The pots were weighed every 2 days to maintain the stressed plants at 30% SWC and the control plants at 70% SWC. Fifteen–twenty plants per genotype and experimental condition (control and stress) were grown and used for data recording, totalling about 300 plants in each of the two experiments.

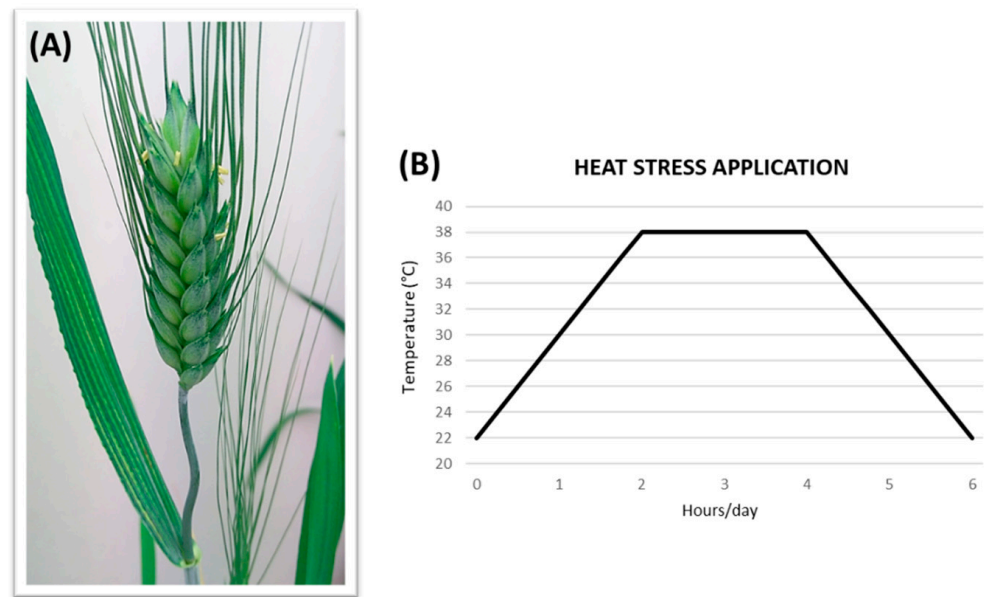


Figure 2. Heat stress application: (A) Phenological phase of the main tiller at the time of stress application, corresponding to anthesis (Zadoks scale 61–65). (B) Procedure followed for heat treatment, performed for three consecutive days in the heat stress experiment, and for three or seven consecutive days in the combined heat + water-deficit stress experiments.

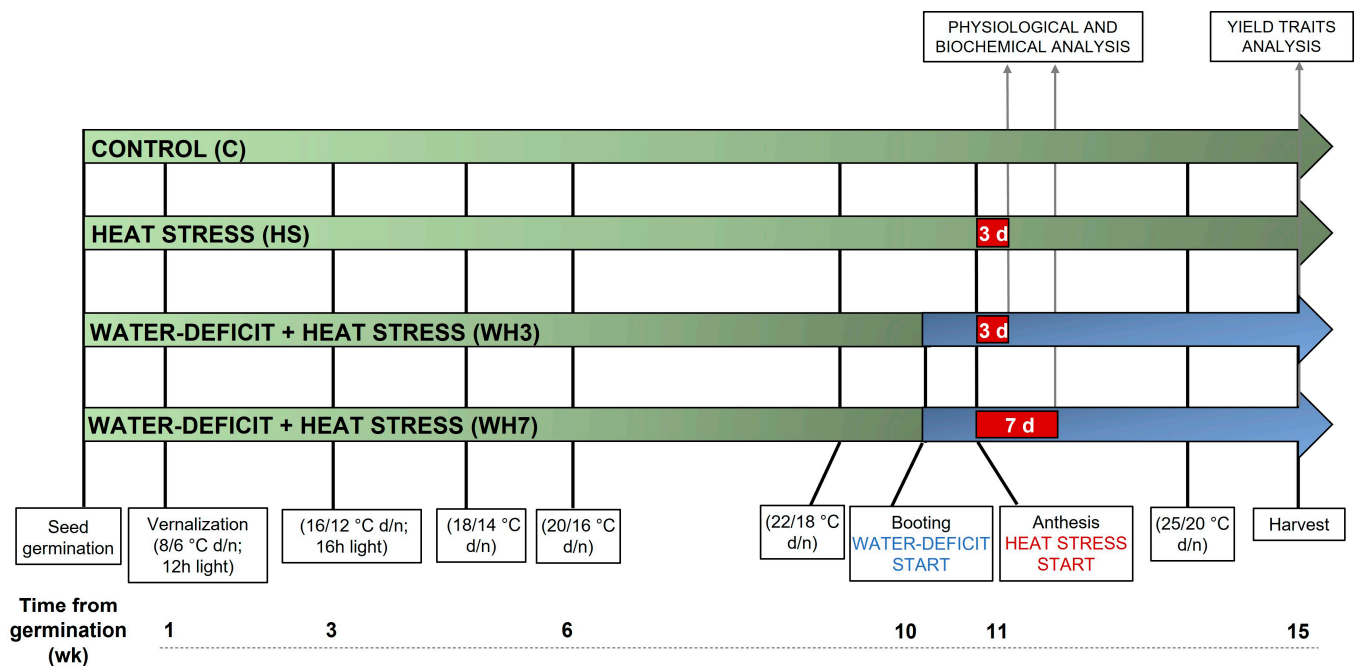


Figure 3. Timeline and features of experimental plant growth conditions, stress treatments application and data recording. Approximate duration (in weeks, wk) of main growth intervals and of the total cycle since seed germination is indicated. d = days.

2.3. Physiological and Biochemical Measurements

2.3.1. Physiological Parameters

Measurements of the physiological parameters were taken on 10 stressed and control plants after the 3rd day of stress application in the HS, and after the 3rd and 7th day in the combined WH stress experiments. Chlorophyll content (SPAD) was measured on 3 positions along the adaxial surface of flag leaf (FL) using a hand-held chlorophyll meter

(SPAD- 502 Plus; Konica-Minolta, Japan). Stomatal conductance (SC, $\text{mmol}/[\text{m}^2\text{s}]$) and leaf temperature (TL, $^{\circ}\text{C}$) were measured in the middle of the FL adaxial surface using a SC-1 leaf porometer (METER Group, Inc. USA). To evaluate the photochemical response of plants, the OJIP curve (Fluorescence Transient) was recorded using a portable fluorometer (PAR-FluorPen FP110; PSI- Photon Systems Instruments, Drásov, Czech Republic) on the FL adaxial surface after a dark adaptation for 30 min with detachable clips. The OJIP curve provided parameters of the status and function of photo-system II (PSII) reaction centres, such as F_v/F_m (Max photochemical efficiency), F_v/F_0 (Potential photochemical efficiency) and Performance Index (PI). The latter expresses the energetic bifurcations of PSII [39–41], being a combined measure of the quantity of the photosynthetic reaction centres (RCs), maximum energy flow reaching the RCs and electron transport at the beginning of illumination.

Relative water content (RWC) was assessed on 5 fully expanded penultimate leaves (FL-1) of the main culm per genotype and treatment and calculated as $\text{RWC} = (\text{FW} - \text{DW})/(\text{TW} - \text{DW})$, where FW, DW and TW stand for fresh, dry and turgid leaf weight, respectively. Namely, a 5 cm-long segment from the middle portion of each FL-1 was excised, its FW recorded and then put at 4°C overnight in tubes with cut ends immersed in distilled water for the next day's TW measurement. Finally, turgid leaf segments were oven-dried for two days at 65°C to assess DW.

In the WH experiments, water use (WU) and water use efficiency (WUE) were also evaluated on 5 plants per genotype and treatment. First, the WU of the control and stressed plants was obtained by quantifying the total amount of water (g) given to each pot from the beginning of drought stress application (booting stage) until plant maturity, and was normalized per plant dry biomass unit. The amount of water added to each pot was calculated on the basis of differences in pot weight, so as to maintain the 30% or 70% SWC for stressed or control plants, respectively, as described above. Second, the water use efficiency (WUE) was calculated at harvest as the ratio between plant grain yield and WU [57,58].

2.3.2. Proline Quantification in Leaves and Spikes

Flag leaf (FL) and spike (awns excluded) sampling was performed immediately after stress application; tissues were collected in tubes, suspended in liquid nitrogen and stored at -80°C until use. Proline (Pro) content was measured in FL (Pro-FL) and spikes (Pro-SP) of stressed and control plants following a standardized ninhydrin-based colorimetric assay [59]. The absorbance of 200 μL of the obtained Pro extract was read on a 96-well plate by a microplate reader at 520 nm, using toluene as a reference. Pro concentration of samples was determined in technical duplicates using a standard concentration curve. Pro content corresponded to $\mu\text{mol}/\text{g}$ FW. At least 3 plants/genotypes/treatment conditions were used for Pro quantification.

2.4. Evaluation of Yield-Related Parameters

Plant height (H, cm) and above-ground plant dry biomass (BM, g) were measured at harvest. For the main culm, i.e., the one that was subjected to HS exactly at anthesis in both experiments, grain number per spike (GNS1), grain number per spikelet (GNSL1), spike fertility index (SFI1), thousand grain weight (TGW1, g) and grain yield per spike (GYS1, g) were measured. The same parameters were also assessed at the whole plant level (GNS, GNSL, SFI and GYS). The number of tillers per plant (TNP) and of productive tillers per plant (PTP), grain number per plant (GNP), thousand grain weight (TGW), grain yield per plant (GYP) and harvest index (HI) were also recorded on harvested plants. SFI, a spike fertility parameter positively correlated with fruiting efficiency, was calculated as the ratio between GNS and chaff weight [23], and HI as the ratio between GYP and BM. The measurements of all the yield-related parameters were derived from at least 10 plants for each genotype/treatment combination.

2.5. Stress Indices

To better evaluate and identify stress-tolerant/susceptible genotypes, the following stress indices were calculated by applying the formulas reported in Table 1 (as from [60]): tolerance index (TOL), mean productivity (MP), geometric mean productivity (GMP), stress tolerance index (STI), harmonic mean (HM), stress susceptibility index (SSI), yield index (YI), yield stability index (YSI) and relative stress index (RSI). All the indices are yield-based and commonly used to estimate the tolerance or susceptibility to abiotic stresses (e.g., [60–62]). The Plant Abiotic Stress Index Calculator (iPASTIC) software [60] was used to calculate all indices, by setting the input model with mean data derived from GYP, obtained in both control ($\bar{Y}_p$) and stress ($\bar{Y}_s$) conditions. Through the estimation of the average of ranks (AR), based on all indices' values, iPASTIC also allowed for a comprehensive and reliable ranking of genotypes (the lower the AR value, the more stress tolerant the genotype).

Table 1. Stress indices calculated with the iPASTIC software with corresponding formulas [60].

Index	Formula
Tolerance index (TOL)	$TOL = \bar{Y}_p - \bar{Y}_s$
Mean productivity (MP)	$MP = (\bar{Y}_p + \bar{Y}_s)/2$
Geometric mean productivity (GMP)	$GMP = \sqrt{(\bar{Y}_s \times \bar{Y}_p)}$
Harmonic mean (HM)	$HM = 2(\bar{Y}_s \times \bar{Y}_p)/(\bar{Y}_s + \bar{Y}_p)$
Stress susceptibility index (SSI)	$SSI = 1 - (\bar{Y}_s/\bar{Y}_p)/1 - (\bar{Y}_s/\bar{Y}_p)$
Stress tolerance index (STI)	$STI = (\bar{Y}_s \times \bar{Y}_p)/(\bar{Y}_p)^2$
Yield index (YI)	$YI = \bar{Y}_s/\bar{Y}_s$
Yield stability index (YSI)	$YSI = \bar{Y}_s/\bar{Y}_p$
Relative stress index (RSI)	$RSI = (\bar{Y}_s/\bar{Y}_p)/(\bar{Y}_s/\bar{Y}_p)$

2.6. Statistical Analysis

One-way analysis of variance (ANOVA) was performed to estimate the differences ascribable to the genotype (G) effect, while two-factor ANOVA was applied to analyse the effect of the G $\times$ treatment (T) interaction. Whenever a significant F value was obtained for single factors or their interaction, a Tukey HSD test was performed at $p < 0.05$ level. All statistical analyses were performed by JASP software (JASP Team, 2022; JASP Version 0.16.1). For all the measured parameters, two statistical comparisons (ANOVA) were performed: in the first one, the datasets of the individual NIRLs (+) and of the corresponding non-carrier sib lines (−) were compared, to associate possible differences in stress behaviour with the presence of a specific alien segment; in the second one, the three NIRLs' datasets were compared to that of cv. Margherita, to evaluate their performance with respect to a known heat tolerant and productive cultivar.

Pearson's correlation analysis was performed for a subset of yield-related and physiological traits. Each pair of variables was correlated by calculating Pearson's correlation coefficients (r value), and four different conditions were separately considered: control, HS, WH3 and WH7. Four principal component analyses (PCA), one for each condition, were also made for NIRLs + and Margherita genotypes in the R Environment (R Project for Statistical Computing 4.2.1), using the missMDA package.

3. Results

3.1. Heat Stress (HS) Application

3.1.1. Physiological and Biochemical Response to HS

Heat treatment (T) and genotype differences (G) had a significant individual impact on most of the physiological traits and on proline (Pro) content (Table 2). HS decreased most of the parameters, notably chlorophyll content (SPAD) and stomatal conductance (SC), as shown by both of the statistical comparisons performed (Table 2). The presence/absence of alien segments (G) was significant for SPAD, SC and Pro-SP (Table S1, Tukey test). In particular, a positive difference was detected for SC in R5+ vs. R5- (+33%) and for Pro-SP in R23+ vs. R23- (+55%). In comparison with cv. Margherita, Pro-SP of the R23+ and R112+

also proved to be noticeably superior (+82% and +44%, respectively; Table S2), while the SC of all three recombinants showed lower values (−15% to −36%).

Table 2. ANOVA mean squares for physiological, biochemical and yield-related traits measured under control and HS treatment: (A) comparison between alien segment carrier (NIRLs+) and non-carrier (NIRLs-) lines, and (B) comparison between NIRLs+ and cv. Margherita. *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively (df, degrees of freedom; G, genotype; T, treatment). For trait acronyms, see the Materials and Methods section.

Factors	A				B			
	G	T	G × T	Residuals	G	T	G × T	Residuals
	df	5	1	5	3	1	3	
SPAD	718.3 ***	149.9 ***	52.1 ***	11.2	750.6 ***	119.3 **	7.3	13.4
SC	24341.2 ***	280889.3 ***	14015.7 **	3489.9	105054.2 ***	491996.4 ***	38365.4 ***	3525.7
TL	1.12	2.15	2.14	1.02	2.40 *	9.71 ***	9.58 ***	0.76
Fv/Fm	0.00008	0.00028 *	0.00005	0.00006	0.00004	0.00004	0.00010	0.00006
Fv/F0	0.15	0.29 *	0.07	0.07	0.07	0.06	0.09	0.06
PI	15.2 ***	1.41	7.50 *	2.38	9.47 **	1.03	8.11 **	1.87
RWC	0.001 ***	0.0001	0.0005 ***	0.00005	0.001 ***	0.0003 *	0.0005 ***	0.0001
Pro-FL	9.47	145.1 ***	5.65	4.61	34.9 **	196.4 ***	6.80	5.62
Pro-SP	2737.6 ***	1468.1 *	803.9 *	293.4	6369.7 ***	149.3	631.0	380.4
GNS1	60.7	25.4	158.2 **	36.5	162.9 **	252.8 **	169.50**	31.2
GNSL1	0.73 ***	0.10	0.50 *	0.17	0.39	0.73 *	0.58 *	0.16
SFI1	1884.6 ***	291.6	555.5 *	181.9	3554.9 ***	1368.4 **	1165.25***	181.7
TGW1	385.9 ***	85.6	91.9	47.5	843.3 ***	166.6	257.1 **	46.1
GYS1	0.34 *	0.04	0.27 *	0.12	0.45 *	0.12	0.16	0.11
GNS	171.4 *	383.6 *	123.1	65.1	183.4 *	794.5 ***	195.4 *	60.9
GNSL	1.58 ***	2.07 **	0.49	0.25	1.03 **	4.68 ***	0.77 *	0.24
SFI	5718.6 ***	3357.2 **	1506.1 ***	316.2	20441.3 ***	6067.4 ***	2997.9 ***	365.5
GYS	0.49 *	0.001	0.34	0.18	1.57 ***	0.20	0.38	0.17
TNP	1.87 *	6.23 **	1.60 *	0.64	0.81	3.38 *	1.30	0.49
PTP	2.94 ***	1.35	0.33	0.49	3.23 ***	0.85	0.32	0.50
GNP	2809.1 ***	8015.9 ***	1224.1 **	375.7	868.9	8335.6 ***	2391.6 ***	359.3
TGW	381.6 ***	282.1 *	103.9	46.6	854.2 ***	301.1 *	257.8 **	48.7
GYP	1.74 ***	4.23 ***	1.59 ***	0.36	9.07 ***	7.03 ***	2.08 ***	0.32
H	2191.3 ***	401.9 ***	49.8	26.3	1110.1 ***	171.2 **	39.1	17.7

The results of the Tukey test for significant G×T interactions (Figure 4 and Tables S3 and S4) showed, as a whole, R5+ and R112+ to have the most tolerant response to HS among NIRLs. This was particularly sustained by their better turgor maintenance vs. the control (“−”) NIRLs (RWC, Figure 4A), comparable to that of cv. Margherita (Figure 4B). Higher leaf hydration under HS of the two recombinants, ascribable to their 7e1L segments’ presence, was accompanied by good performance of their photosynthesis-related traits. For example, although the chlorophyll content (SPAD) in R5+ was significantly lower than in R5- under HS (−7%), this did not seem to affect its photosynthetic efficiency, as the chlorophyll fluorescence traits Fv/Fm and PI did not appear to be altered by HS (Table S3). For all physiological traits, R5+ and R112+ showed similar values to those of cv. Margherita, which confirms their positive response to the heat stress (Table S4).

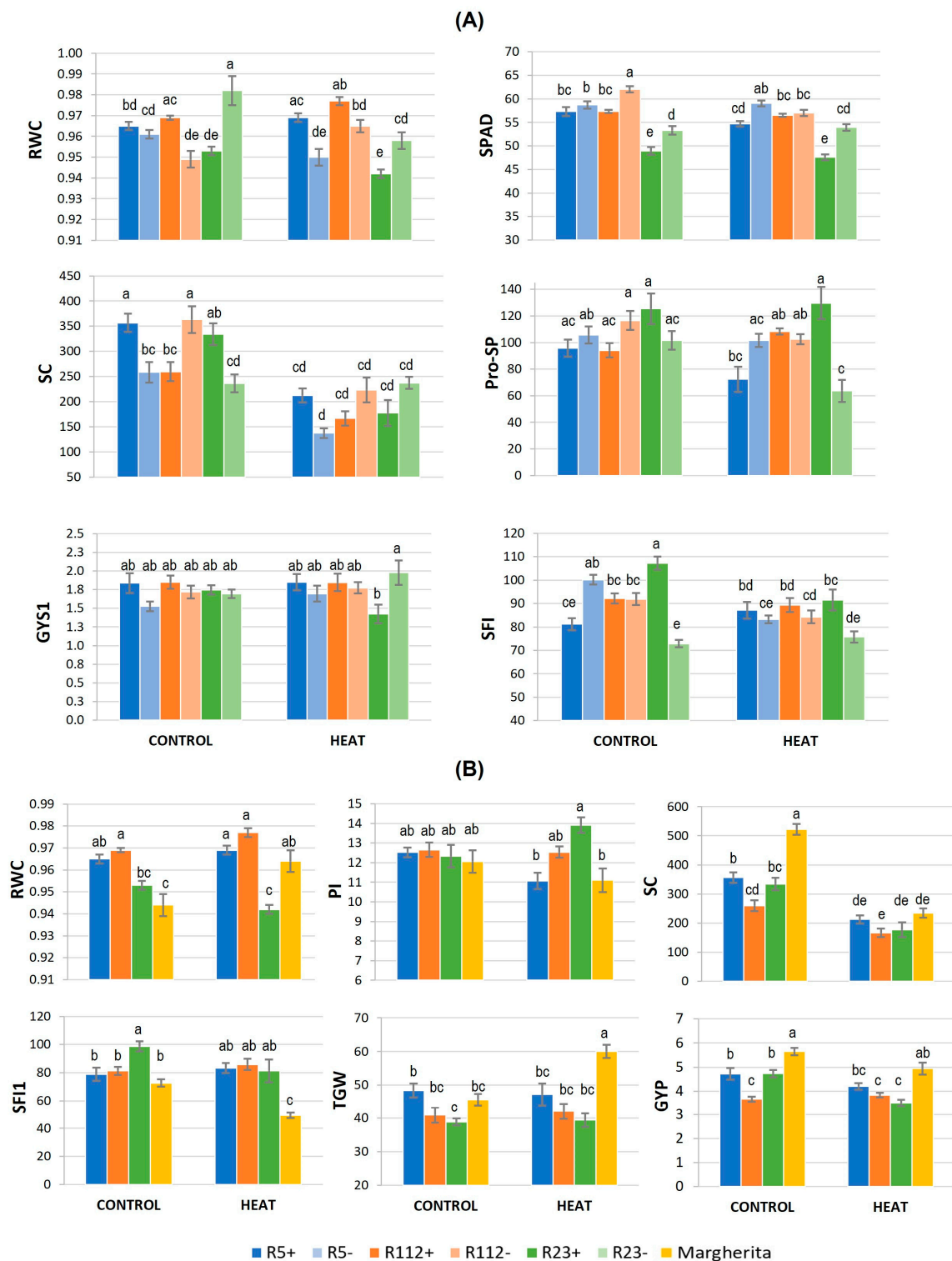


Figure 4. Significant genotype $\times$ treatment effects on physiological and yield-related parameters under 3-day heat stress: (A) comparison between alien segment carrier (+) and non-carrier (−) NIRLs, and (B) comparison between NIRLs+ and cv. Margherita. The error bars represent standard errors of the means, while the letters above histograms correspond to the ranking of Tukey HSD test at $p < 0.05$ significance level. For traits acronyms, see the Materials and Methods section.

In contrast, the presence of the longest 7el₁L segment, as in R23+, had a negative impact on both RWC and SPAD, as shown by the observed significant decrease under HS (−2% and −13%, respectively, Table S3). However, a comparison of R23+ values under the

HS and control (unstressed) conditions highlighted the ability of this recombinant line to maintain the leaf hydration level of the control samples under HS (RWC, Figure 4A). The same was observed for Pro-SP of R23+, not altered under HS vs. control. Spike Pro content of R23+ resulted as 103% higher under the stress vs. R23- (Figure 4A), which, instead, had a significant 40% decrease in HS vs. control conditions. Other alien segments did not confer noticeable advantages for Pro accumulation in spike or flag leaf, and, in comparison with cv. Margherita, the three recombinants showed similar values for both Pro-FL and Pro-SP (Table S4).

In the absence of HS, stomatal conductance (SC) was significantly increased by the presence of alien segments in R5+ and R23+ recombinants compared to their negative controls (+38% and +42%, respectively), while the opposite trend was observed in the case of R112+ (−28%) (Table S3 and Figure 4A). In comparison with cv. Margherita, all three recombinants showed 32–50% lower SC in the control condition (Table S4 and Figure 4B), suggesting their constitutively lower leaf gas exchange. However, HS caused a significant reduction in all genotypes (about −40% in NIRLs+ and −50% in Margherita), leading to comparable SC values between them, indicating similar reactions to the stress through this mechanism (Tables S3 and S4 and Figure 4).

3.1.2. Main Fertility and Yield-Related Traits Involved in HS Response

Most fertility traits of the main culm, the one principally targeted by HS at anthesis, were affected by the alien segment presence (G, Table 2A). Variations due to the treatment (T) were only observed in the comparison between NIRLs+ and cv. Margherita (Table 2B). However, G × T interaction was significant for several traits of the main culm, highlighting differences among genotypes in both of the statistical comparisons performed (Table 2A,B). Grain yield of the main culm (GYS1) appeared to be similar across all genotypes in both stress and control conditions (Tables S3 and S4), except for R23+, which had 28% lower GYS1 vs. R23- under stress, the negative effect being thus ascribable to the presence of the 40%-long 7e1L segment (Figure 4A). R23+ exhibited significantly higher values for grain number-related traits (GNS1, GNSL1 and SFI1) vs. R23- under control conditions (Table S3). In comparison with cv. Margherita, all the recombinants displayed a higher fertility of the main culm under HS, as proven by their average 70% higher SFI1 (Table S4 and Figure 4B) and, as for R112+, 36% and 26% higher GNSL1 and GNS1, respectively (Table S4). Spikelet fertility (GNSL1) of all recombinants under HS remained similar to that of the control condition (Tables S3 and S4), suggesting a stabilizing role of alien segment presence under stress for this trait.

Similarly to what observed for the main culm, also at the whole plant level R23+ had, under control conditions, a better performance than R23- for SFI (+47%), GNP (+53%) and GYP (+23%), evidently due to its alien segment (Table S3). Under HS, R23+ maintained a 20% higher SFI than R23- (Figure 4A), though not the GYP advantage (Table S3). The presence of the other alien segments did not show significant effects on whole plant yield traits. With respect to control conditions, the heat stressed Margherita plants significantly reduced many traits related to grain number (GNS, GNSL, GNP) and spike fertility (SFI), and were shown to be, on average, 86% inferior to all three recombinants for the latter trait (Table S4). Conversely, under HS, Margherita displayed a significant 31% higher grain weight vs. control conditions (TGW, Figure 4B), which likely enabled its high GYP, despite a conspicuous decrease of GNP (−35%, Table S4; Figure 4B). In the same NIRLs+ vs. Margherita comparison, apart from absolute values (depending on background genotypes), both R5+ and, even more, R112+ revealed a remarkable stability of plant yield traits under HS vs. control conditions, mainly due to the maintenance/increase of grain number and fertility parameters both at the spike and plant level (Table S4).

3.2. Combined Water-Deficit and Heat Stress Application

3.2.1. Physiological and Biochemical Response to Combined Stress

The combination of water-deficit (W) and heat (HS) stress (= WH) treatments (T) had, as expected, a significant impact on more traits (Tables 3 and 4) than those affected by HS alone (Table 2). For both experiments, in which HS was imposed for 3 (WH3) or 7 (WH7) days at anthesis, ANOVA analyses showed the effect of genotype alone (G) to be significant for about half of the physiological traits measured (Tables 3 and 4). Among the genotype-dependent outcomes, the presence of 7e1L chromosome segments negatively affected SPAD of NIRLs R112+ (−11% and −13% in WH3 and WH7, respectively) and R5+ (−9% in WH7) compared to their R112- and R5- control lines (Tables S5 and S9). With respect to cv. Margherita, all three NIRLs+ showed significantly lower water use (WU) (Tables S6 and S10), suggesting a higher water uptake and transpiration of the control cultivar. This was supported by the significantly higher SC of Margherita vs. all NIRLs+ in WH3 (average 23%) and vs. R112+ and R23+ in WH7 (+15%) (Tables S6 and S10). As for traits related to photosynthetic efficiency, i.e., Fv/Fm, Fv/F₀ and PI, the R112+ recombinant outperformed Margherita in WH3 (Table S6), whereas in WH7, R112+ had 36% lower Pro-FL (Table S10).

Table 3. ANOVA mean squares for physiological, biochemical and yield-related traits measured under control and combined water-deficit and 3-day HS (WH3): (A) comparison between alien segment carrier (+) and non-carrier (−) NIRLs, and (B) comparison between NIRLs+ and cv. Margherita. *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively. For trait acronyms, see the Materials and Methods section.

Factors	A				B			
	G	T	G × T	Residuals	G	T	G × T	Residuals
df	5	1	5		3	1	3	
SPAD	1017.7 ***	168.9	126.9 *	55.6	671.1 ***	408.5 **	139.1	58.2
SC	10722.8	154764.8 ***	16595.3 *	5440.0	53776.6 ***	359666.3 ***	32983.9 ***	4722.6
TL	4.52	70.80 ***	1.64	2.17	6.09 *	71.12 ***	0.64	1.66
Fv/Fm	0.00017 ***	0.00001	0.00005	0.00004	0.0002 ***	0.0000001	0.0001 *	0.00004
Fv/F ₀	0.25 ***	0.003	0.15 *	0.05	0.34 ***	0.08	0.09	0.05
PI	11.89**	3.24	2.58	3.51	23.8 ***	23.8 **	10.7 *	3.1
RWC	0.00002	0.00015	0.0001	0.00009	0.00003	0.00006	0.0001	0.0001
WU	137.4	8958.2 ***	439.8 ***	58.6	1049.5 ***	11465.6 ***	670.9 ***	61.4
WUE	0.0005 **	0.0003	0.001 ***	0.0002	0.0001	0.0001	0.0007 *	0.0002
Pro-FL	1.15	2.12	2.13 *	0.62	2.25 *	6.08 **	2.16 *	0.59
Pro-SP	701.5 *	33878.8 ***	475.6	243.0	1132.9 *	15349.5 ***	996.3 *	271.1
GNS1	375.9 ***	4700.6 ***	193.6 ***	39.0	69.5	2072.7 ***	268.6 ***	32.6
GNSL1	2.22 ***	19.1 ***	1.00 **	0.28	0.52	6.83 ***	1.23 **	0.27
SFI1	2616.9 ***	12260.6 ***	1778.0 ***	268.3	159.9	5843.2 ***	1596.3 ***	209.5
TGW1	482.7 ***	803.3 ***	80.6	53.1	455.7 ***	32.1	8.73	54.8
GYS1	0.90 ***	6.17 ***	0.40 **	0.12	0.82 ***	3.21 ***	0.21	0.11
GNS	364.5 ***	6432.5 ***	216.9 **	57.9	47.9	2614.0 ***	252.2 **	57.8
GNSL	1.94 ***	25.1 ***	0.84 *	0.31	0.24	9.80 ***	0.78	0.32
SFI	4931.4 ***	14469.5 ***	1991.1 ***	281.6	1746.5 ***	5137.5 ***	1217.9 **	293.6
GYS	0.61 **	9.00 ***	0.31	0.16	0.71 **	3.74 ***	0.13	0.15
TNP	3.40 ***	0.31	4.22 ***	0.50	6.25***	5.11 **	3.93 **	0.67
PTP	3.45 ***	2.22 *	2.84 ***	0.48	6.13 ***	6.08 **	2.52 **	0.60
GNP	4203.4 ***	22969.3 ***	5385.4 ***	204.2	5182.7 ***	24323.4 ***	6268.4 ***	268.2
TGW	357.3 ***	734.1 ***	22.6	30.9	298.4 ***	294.2 **	17.1	32.9
BM	45.8 ***	114.5 ***	16.3 **	3.97	61.7 ***	173.5 ***	13.3 **	3.69
HI	0.06 ***	0.05 ***	0.03 ***	0.004	0.04 ***	0.07 ***	0.02 **	0.003
GYP	4.54 ***	34.2 ***	8.44 ***	0.71	7.03 ***	41.3 ***	7.9 ***	1.02
H	1002.7 ***	52.4	22.9	16.4	765.9 ***	33.5	55.1 *	16.1

Table 4. ANOVA mean squares for physiological, biochemical and yield-related traits measured under control and combined water-deficit and 7-day HS stress (WH7): (A) comparison between alien segment carrier (+) and non-carrier (−) NIRLs, and (B) comparison between NIRLs+ and cv. Margherita. *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively. For trait acronyms, see the Materials and Methods section.

Factors	A				B			
	G	T	G × T	Residuals	G	T	G × T	Residuals
	df	5	1	5	3	1	3	
SPAD	515.6 ***	583.5 ***	84.9 *	28.1	265.2 ***	602.4 ***	48.7	36.5
SC	15069.4 **	760720.9 ***	9298.6 *	3700.3	34393.1 ***	614880.3 ***	10975.7	4858.0
TL	2.02	73.7 ***	7.08 *	2.94	10.0 **	52.4 ***	3.12	2.46
Fv/Fm	0.00004	0.0005 **	0.00005	0.00006	0.00007	0.0003 *	0.00005	0.00006
Fv/F0	0.09	0.68 **	0.06	0.07	0.13	0.47 *	0.05	0.07
PI	4.64	46.50 ***	3.05	3.37	3.94	18.5 *	17.9 **	3.80
RWC	0.0004 *	0.00004	0.0002	0.0001	0.0002	0.001 **	0.0002	0.0001
WU	205.2 **	5666.1 ***	419.7 ***	54.0	1033.9 ***	6972.3 ***	678.1 ***	33.4
WUE	0.0004 **	0.002 ***	0.001 ***	0.0001	0.0001	0.003 ***	0.0007 ***	0.0001
Pro-FL	3.28 *	23.8 ***	2.10	1.01	5.17 *	37.3 ***	1.18	1.40
GNS1	335.8 ***	6199.9 ***	209.9 ***	39.4	12.8	4117.6 ***	225.2 **	43.6
GNSL1	2.27 ***	25.6 ***	1.00 ***	0.20	0.30	17.7 ***	0.80 **	0.25
SFI1	2620.6 ***	14652.8 ***	1660.3 ***	220.6	313.2	6230.6 ***	2093.9 ***	235.9
TGW1	489.9 ***	28.4	16.4	37.9	590.0 ***	0.34	2.60	42.7
GYS1	0.82 ***	12.7 ***	0.40 **	0.09	0.87 ***	8.61 ***	0.23	0.09
GNS	221.7 **	9992.5 ***	190.3 *	64.1	42.3	9909.4 ***	209.3 *	65.3
GNSL	1.64 ***	40.3 ***	0.72 *	0.29	0.13	41.3 ***	0.73	0.33
SFI	3232.2 ***	28549.0 ***	2528.4 ***	276.6	1141.1 *	19765.9 ***	3232.8 ***	331.7
GYS	0.78 ***	18.6 ***	0.25	0.14	0.76 **	17.7 ***	0.17	0.14
TNP	5.17 ***	1.39	3.35 ***	0.46	5.78 ***	3.08 *	5.08 ***	0.60
PTP	4.72 ***	3.43 **	2.55 ***	0.42	4.83 ***	4.23 **	3.82 ***	0.53
GNP	5414.3 ***	35354.0 ***	4694.8 ***	200.9	6196.3 ***	36513.4 ***	6394.2 ***	282.2
TGW	412.3 ***	194.3 **	5.6	18.8	515.4 ***	155.3 **	3.83	17.5
BM	20.7 ***	177.2 ***	32.6 ***	3.5	19.2 **	279.3 ***	37.3 ***	3.76
HI	0.03 ***	0.07 ***	0.01 ***	0.002	0.01 **	0.05 ***	0.01	0.003
GYP	3.57 ***	69.5 ***	9.6 ***	0.58	4.13 **	78.0 ***	12.6 ***	0.85
H	860.7 ***	251.0 ***	59.3 *	19.6	741.4 ***	495.3 ***	49.1 *	16.6

When the $G \times T$ interactions were considered, significant differences were observed concerning photosynthesis- and water management-related traits (Tables 3 and 4). However, only a limited number of significant effects turned out to be conferred by the alien segments (Tables S7 and S11). Under WH3 stress conditions, the 7eL1 chromosome segment conferred a slight, yet significantly higher PSII potential photochemical efficiency to R112+ compared with R112- (+5% Fv/F0, Figure 5A). Moreover, R112+, as well as R5+, were also better performing than Margherita for PI (+30% and +17%, respectively; Figure 5B), which indicates their greater ability to maintain photosynthetic activity under stress. Comparing WH3 vs. the control conditions, R5+, R23+ and Margherita decreased their WU and SC values more than R112+ (Figure 5B), suggesting the maintenance of water status and water economy to be key mechanisms in their adaptation to the combined stress.

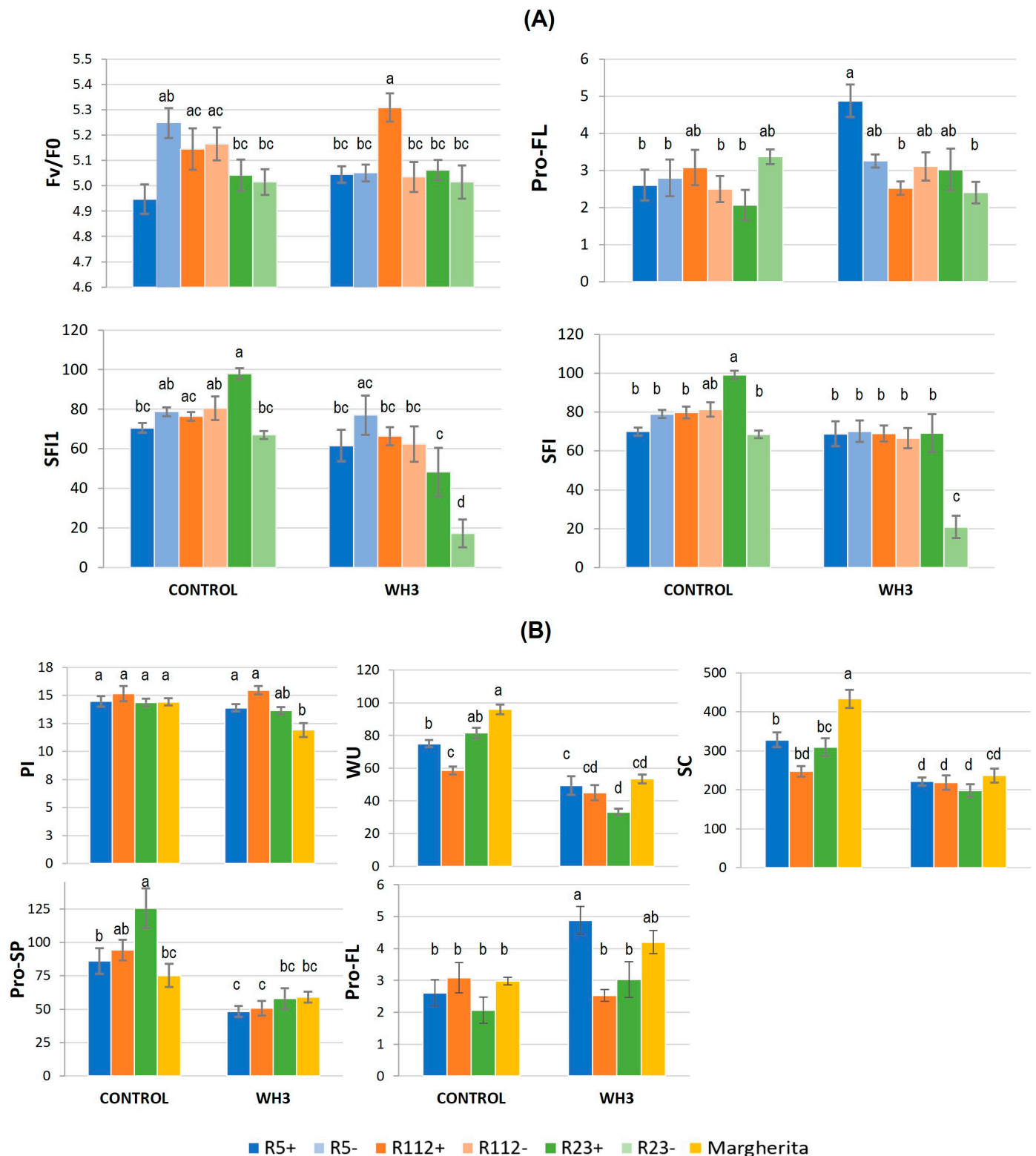


Figure 5. Significant genotype $\times$ treatment effects on physiological and yield-related parameters under combined water-deficit and 3-day heat stress (WH3): (A) comparison between alien segment carrier (+) and non-carrier (−) NIRLs, and (B) comparison between NIRLs+ and cv. Margherita. The error bars represent standard errors of the means, while the letters above the histograms correspond to the ranking of Tukey HSD test at $p < 0.05$ significance level. For trait acronyms, see the Materials and Methods section.

Proline accumulation under combined stress was not as much of a differential between the genotypes as under HS application alone. None of the three alien segments significantly altered the Pro concentration in the WH3 or WH7 experiments (Pro-FL and Pro-SP, Tables S7 and S11). In the WH3 experiment only, R23+ was shown to have a constitutively higher Pro amount in the spike (Pro-SP) than cv. Margherita (+48%) and R5+ (+58%) (Table S6), but the potential advantage was apparently lost under the stress (Table S8 and Figure 5B). As for Pro in the flag leaf (Pro-FL), R5+ nearly doubled its amount under stress vs. control conditions (Table S8 and Figure 5B). A similar behaviour was exhibited by cv. Margherita, whereas R23+ only had a mild increase and R112+ a non-significant reduction.

Under more intense heat stress, as in the WH7 experiment, the traits associated with plant water management were shown to be even more important than in WH3 for stress tolerance. Lower values under stress vs. control conditions were in general observed in all NIRLs+ for SC (Table S11). As in the WH3 experiment, R23+ was confirmed to be the best water-saving genotype under stronger stress conditions due to the presence of its 7e1L segment (−50% WU in R23+ and −15% in R23- vs. their respective control conditions, Table S11 and Figure 6A). A significantly decreased WU under stress vs. control conditions was also exhibited by R5+ and cv. Margherita, indicating the good adaptive ability of the three genotypes to a longer exposure to stress, as opposed to R112+, which maintained an unchanged water request in WH7 (Figure 6B and Table S12). As a whole, the comparison with cv. Margherita showed the three NIRLs+ to be similar to the heat tolerant control genotype for most physiological traits under WH7 stress conditions (Table S12). Interestingly, however, R5+, besides the better WU, also showed an increased PI value under stress with respect to the control conditions (Figure 6B and Table S12), which highlights its putative capacity to increase its photosynthetic efficiency under stress.

3.2.2. Influence of Combined Stress on Spike Fertility and Plant Production

Essentially all spike fertility and plant production traits were strongly affected by the stress combination in the WH3 and WH7 experiments, as shown from all statistical analyses performed (T, Tables 3 and 4). However, seven days of heat treatment (WH7) were more impactful on all genotypes and reduced the differences among them. The significance of the genotype effect alone (G) was also observed for all traits in NIRLs+ vs. NIRLs-analysis (Tables 3A and 4A), and, for most traits, in NIRLs+ vs. Margherita comparisons (Tables 3B and 4B). Specifically, in both WH3 and WH7, the effect of the presence of the alien segment was almost exclusively observed for the R23+ recombinant vs. its control line R23-. The effect was remarkably positive for fertility traits of the main culm (average +36% for GNS1 and GNSL1, +71% for SFI1) and of the whole plant (+107% GNP), but also for the tiller number (average +61% TNP and PTP) and grain yield (average +68% GYP) (Tables S5 and S9). In WH3 only, the 40%-long segment of R23+ also increased GNS, GNSL (33–37%) and SFI (76%) at the whole plant level (Table S5), while in WH7, this was the case for HI (+47%) (Table S9). These results indicated that the alien segment present in R23+ constitutively enhances the grain and tiller number, likely directing the allocation of nutrients into grains rather than to biomass. By contrast, the grain weight (TGW) significantly decreased in R23+ vs. R23- in both experiments (−27%; Table S5), while under prolonged HS stress, the TGW of R5+ was 9% higher vs. R5- (Table S9).

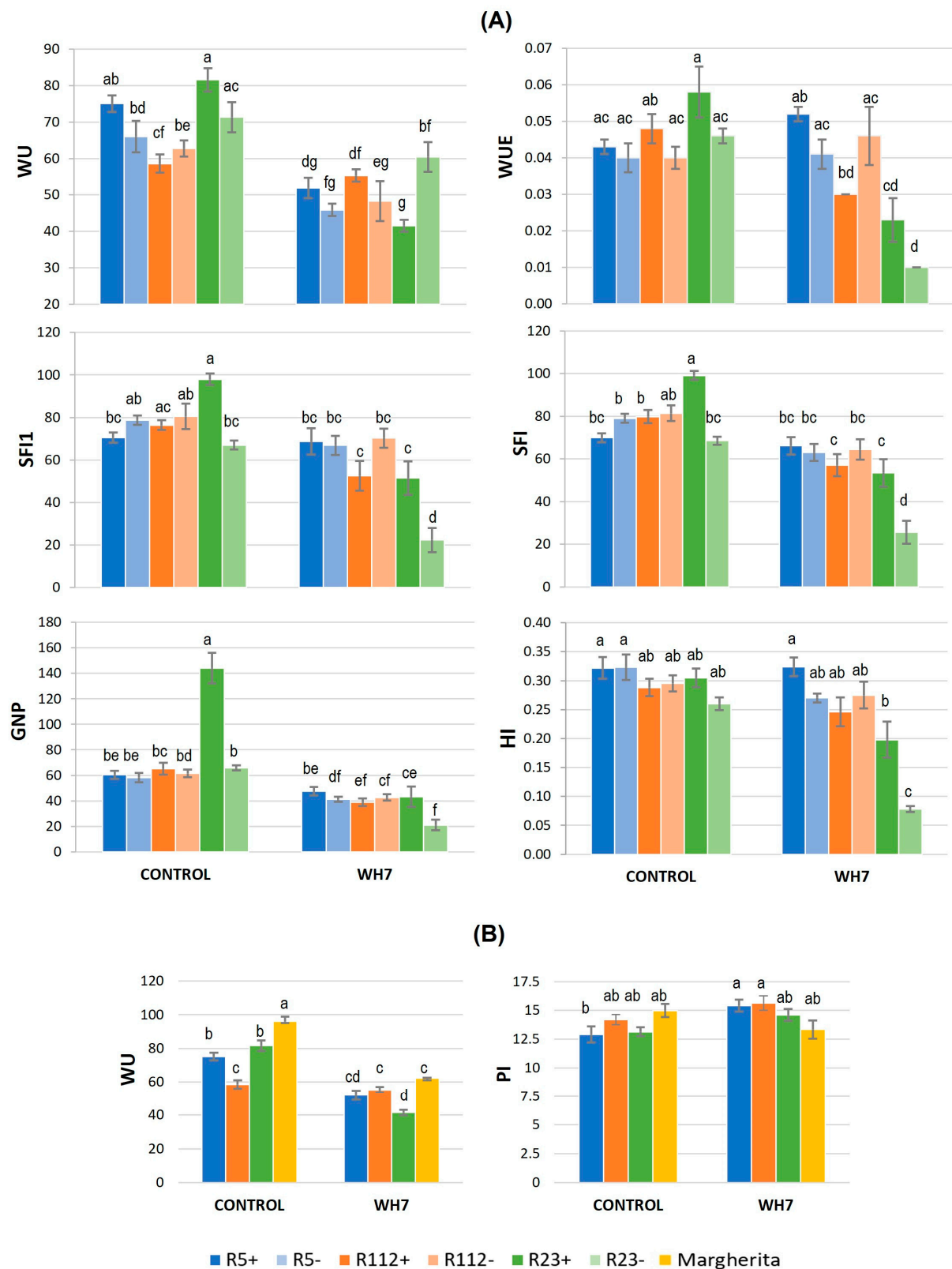


Figure 6. Significant genotype $\times$ treatment effects on physiological and yield-related parameters under combined water-deficit and 7-day heat stress (WH7): (A) comparison between alien segment carrier (+) and non-carrier (−) NIRLs, and (B) comparison between NIRLs+ and cv. Margherita. The error bars represent standard errors of the means, while the letters above the histograms correspond to the ranking of Tukey HSD test at $p < 0.05$ significance level. For trait acronyms, see the Materials and Methods section.

In comparison with Margherita, the Tukey test for the G factor revealed N1R1L+ to be not significantly different for the spike grain number and fertility, while differences were more evident for some plant traits (Tables S6 and S10). The results indicated that, with the increase of heat stress intensity, as in WH7, R5+ resulted as more tolerant than R112+, possibly due to the positive impact of its 23%-long 7e1 segment on the grain weight under stress (TGW) and to the lower water economy of R112+, as described above (§ 3.2.1). On the other hand, although R23+ had significantly lower TGW, TGW1 and GYS1 than Margherita under both WH3 and WH7, this recombinant appeared to be similar to the heat tolerant cultivar for the plant yield components TNP, PTP, BM and the final yield (GYP) (Tables S6 and S10). This was likely due to R23+ having significantly higher SFI (+23 in WH7, +29% in WH3) and also GNP (+20%) in WH7, confirming that the higher spike fertility of R23+ determines its productivity under combined stress conditions.

For the majority of traits, ANOVA indicated a significant $G \times T$ interaction, especially in the comparison between the N1R1Ls (Tables 3A and 4A). Overall, while in the WH3 condition R23+ was shown to be the most affected by the stress (comparisons between control and stressed values) for a number of traits (−30% to −70% for GNS1, GNSL1, SFI1, GYS1, GNS, GNSL, SFI, TNP, PTP, GNP, BM, GYP; Table S7), in the WH7 experiment, this was also true for R112+. On the other hand, R5+ plants proved to be rather stable between the control and stress conditions, only displaying the negative stress effect on the spike grain number in WH7 (GNS, GNSL, Table S11). The only alien segment associated with a significant increase under the combined stress was that of the R23+ recombinant. In both stress experiments, extraordinarily 1–2.3-fold higher values of SFI1 and SFI were displayed by R23+ in comparison with R23- (Tables S7 and S11, Figures 5A and 6A). In WH7 only, the same was observed for the total grain number/plant (GNP), 1.2-fold higher in R23+ than in R23- (Table S11 and Figure 6A). SFI1, SFI and GNP were the only traits that under both stress and control conditions confirmed the positive association between spike fertility (and consequently total grain number/plant) and the presence of the 40%-long Th. ponticum chromosome segment. Considering the three N1R1Ls+ vs. Margherita, their response to stress was highly similar to that of the cultivar (Tables S8 and S12), with no depressing effect on the yield traits vs. control values (particularly in WH3). In WH3, only R23+ showed a remarkably lower grain number of the main culm (−37% GNS1 and −40% GNSL1) and harvest index (−62%) vs. Margherita, yet still reaching an overall comparable plant grain yield (Table S8). In WH7, the four genotypes were even more similar for all traits (Tables S8 and S12).

3.3. Correlations between Yield and Physiological Traits under Stress

Pearson's correlation heatmaps for control, HS, WH3 and WH7 treatments are reported in Figure 7. Under all the conditions considered, yield per spike and per plant (GY1, GYS, GYP) were highly positively correlated with grain number (GNS1, GNS, GNP; $r = 0.37^{**}$ – 0.90^{***}), the latter being in all cases negatively correlated to grain weight (TGW1, TGW). Interestingly, only under the HS treatment (Figure 7B) were GY1 and GYP also positively correlated to TGW1 and TGW ($r = 0.39^{**}$ and 0.46^{***} , respectively). Among the physiological traits positively correlated to yield under stress conditions, this was the case for RWC, showing a significant correlation with GY1, SFI and GNS1 in the HS treatment ($r = 0.45^{**}$, $r = 0.38^{*}$ and $r = 0.34^{*}$, respectively, Figure 7B), confirming the importance of leaf hydration for good productivity. In control conditions only (Figure 7A), stomatal conductance (SC) was correlated with yield ($r = 0.35^{***}$). With the addition of water-deficit to heat stress in WH3 and WH7 treatments (Figure 7C,D), physiological parameters such as SPAD and WUE turned out to be positively correlated to yield at both the main culm and whole plant levels ($r = 0.34^{**}$ – 0.55^{***}), revealing their contribution to overcoming heat and drought combination by the tested genotypes. Interestingly, in the most extreme WH7 condition (Figure 7D), SC was shown to be negatively correlated with grain number-related traits GNP ($r = -0.28^{*}$) and SFI ($r = -0.28^{*}$), which, in turn, were highly positively related to the final yield ($r = 0.83^{***}$ and 0.47^{***} , respectively). A positive correlation was

also observed between RWC and GNS1 ($r = 0.34^*$) in WH7 conditions, confirming the importance of RWC in grain number production in harsh environments. Regarding the proline content in flag leaf (Pro-FL), this turned out to be positively correlated to GYP under HS ($r = 0.34^*$; Figure 7B), as well as WH3 ($r = 0.54^{**}$; Figure 7C) and WH7 ($r = 0.47^*$; Figure 7D) conditions.

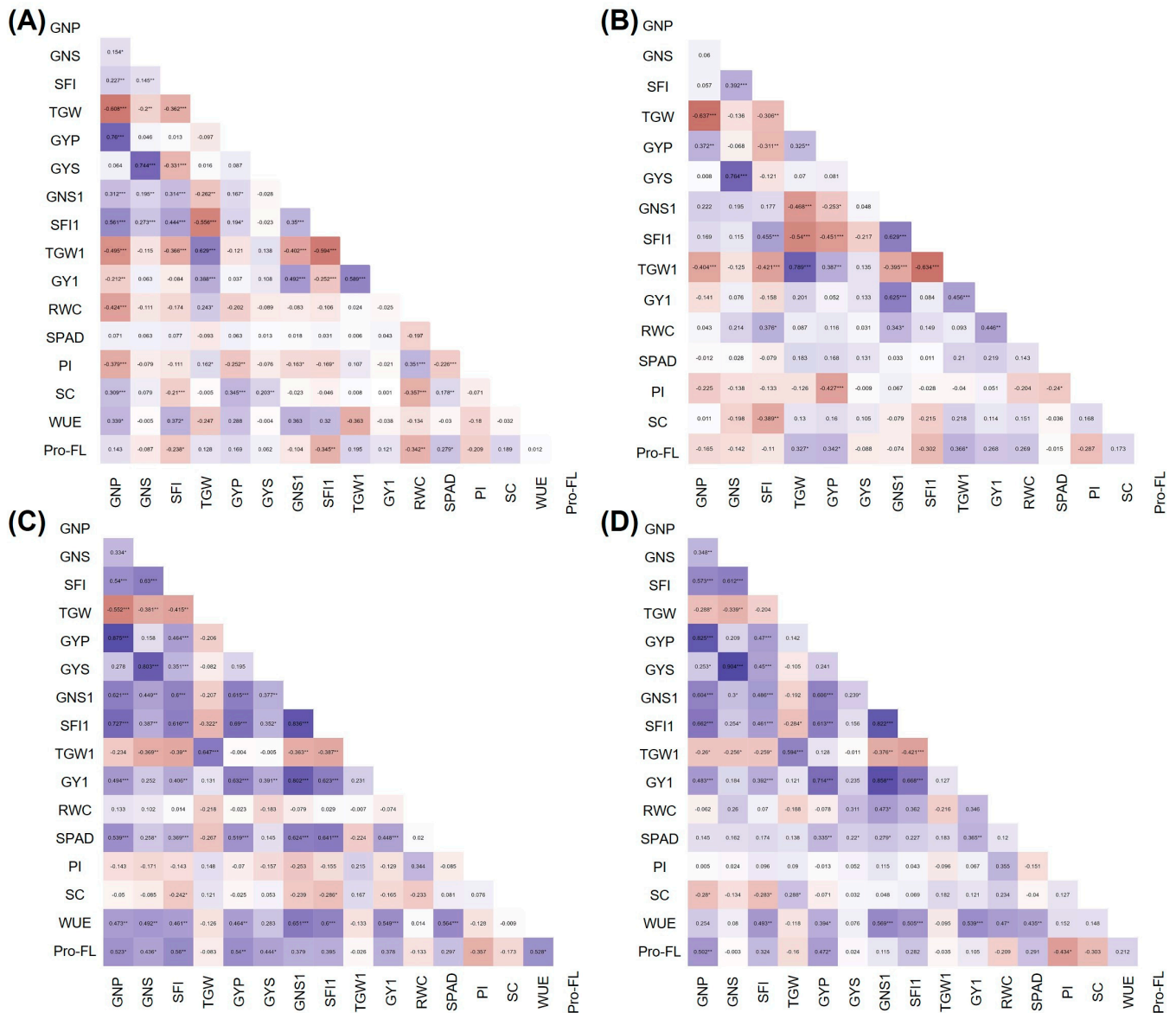


Figure 7. Heatmaps of Pearson's correlation coefficients (r) between pairs of yield-related traits and physiological parameters under (A) control condition, (B) 3-day heat stress, (C) combined water and 3-day heat stress (WH3); and (D) combined water and 7-day heat stress (WH7). Violet and brown colours correspond to positive and negative correlation, respectively. The saturation of colours reflects the absolute value of r . *, ** and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$ significance levels, respectively. For trait acronyms, see the Materials and Methods section.

3.4. Principal Components Analysis (PCA) of Yield-Related Traits under Stress

In order to identify the recombinants with the best productive potential under different stress conditions and traits that mostly affect yield, PCA analyses were performed using a subset of traits of the main culm, the one primarily targeted by stress application at anthesis (Table S13 and Figure 8). The selected traits were those enhanced by the presence of one or more *Th. ponticum* segments as from the ANOVA analyses performed. The first two components of the PCAs explained 59.8%, 74.2%, 66.2% and 59.2% of the total variance for the control, HS, WH3 and WH7 conditions, respectively (Figure 8). Under non-stressed conditions, PC1 (principal component 1) was largely and positively related to GY1, TGW1 and SPAD, and to a lesser extent to Pro-FL and SC. Instead, PC1 was negatively related to GNS1, SFI1, RWC and less to PI. A strong overlap between the genotype clusters could be observed under control conditions, indicating similarities between the alien segment-carriers and the heat tolerant cv. Margherita in the absence of stress. Overall, PCA showed that differences between genotypes in all four conditions were mainly determined by their grain yield (GY1) and 1000-grain weight (TGW1), as these two traits were commonly found on the positive side of PC1 (x axis). Only under water-limited conditions (WH3 and WH7) did PC1 (43% and 32.4% of total variance, respectively) also indicate a positive relation of yield with grain number and spike fertility (GNS1, SFI1), and the positive contribution of the same parameters was confirmed by PC2 (23.2% of total variance) in the WH3 case. In all conditions, physiological traits such as Pro-FL and SC were positively related to GY1, and for SC this relation was particularly clear-cut under the most severe WH7 stress (see the similar angle between the two traits in Figure 8). The importance of leaf hydration (RWC) was highlighted mainly under stress conditions and was closely related to photosynthetic efficiency (PI), as shown by their consistent and close clustering (see PC2 in the control, WH3 and WH7 conditions). Each stress condition was characterized by the specific modulation of relations between parameters. Under HS, PC1 explained 48.8% of the total variance, underlining a strong interrelation between TWG1 and SC, and between GY1 and RWC. In the most extreme WH7 conditions, all physiological and yield-related traits showed a high and positive interrelatedness on PC1, suggesting that the plant productivity under these conditions depends on all the resources available. Based on the observed results under stress, the biplots revealed that, due to TGW1 values, R23+ (light blue cluster, Figure 8) was the most different and low-yielding genotype in all cases, while Margherita (red cluster) was confirmed to be better performing vs. the others, especially under HS only. Under HS alone, due to a higher grain number, R112+ (green cluster) revealed a slightly better yielding ability than the other two recombinants. In contrast, genotype grouping under combined stress showed R5+ (violet cluster) to have an overall comparable (WH7) or better (WH3) performance than Margherita for the main culm traits analysed.

3.5. Stress Indices and Tolerance Ranking

The nine stress indices were calculated using the values of GYP in control (Y_p) and stress (Y_s) conditions (Table 5). The variety Margherita, known for its good performance in hot environments [55], was the best genotype under the present HS conditions for many stress indices, such as MP, GMP, HM, STI and YI (Table 1). After the ranking analysis, Margherita also had the lowest value of the overall average of ranks (AR), which qualifies it as the most tolerant genotype in the HS condition (AR = 2.82), with R5+ (AR = 3.18) and R112+ (AR = 3.73) ranking second and third, respectively. However, according to the TOL, SSI, YSI and RSI indices, reported as most suitable to discriminate well-performing wheat genotypes in heat stressed environments [62], R112+ resulted as the best performing of all genotypes under HS. In the combined WH3 treatment, Margherita was confirmed to be the most tolerant genotype, as indicated by the lowest AR (2.36) and the highest values, as in the HS conditions, of the GMP, HM, STI and YI indices. The second- and the third-ranking genotypes were R5- and R5+ (AR = 2.45 and 3.45, respectively). However, under the most intense WH7 conditions, it was R5+ that resulted as the top ranking line, with high values

for the TOL, SSI, YI, YSI and RSI indices and an AR value (1.73) corresponding to less than 60% of the second-ranking Margherita (Table 5).

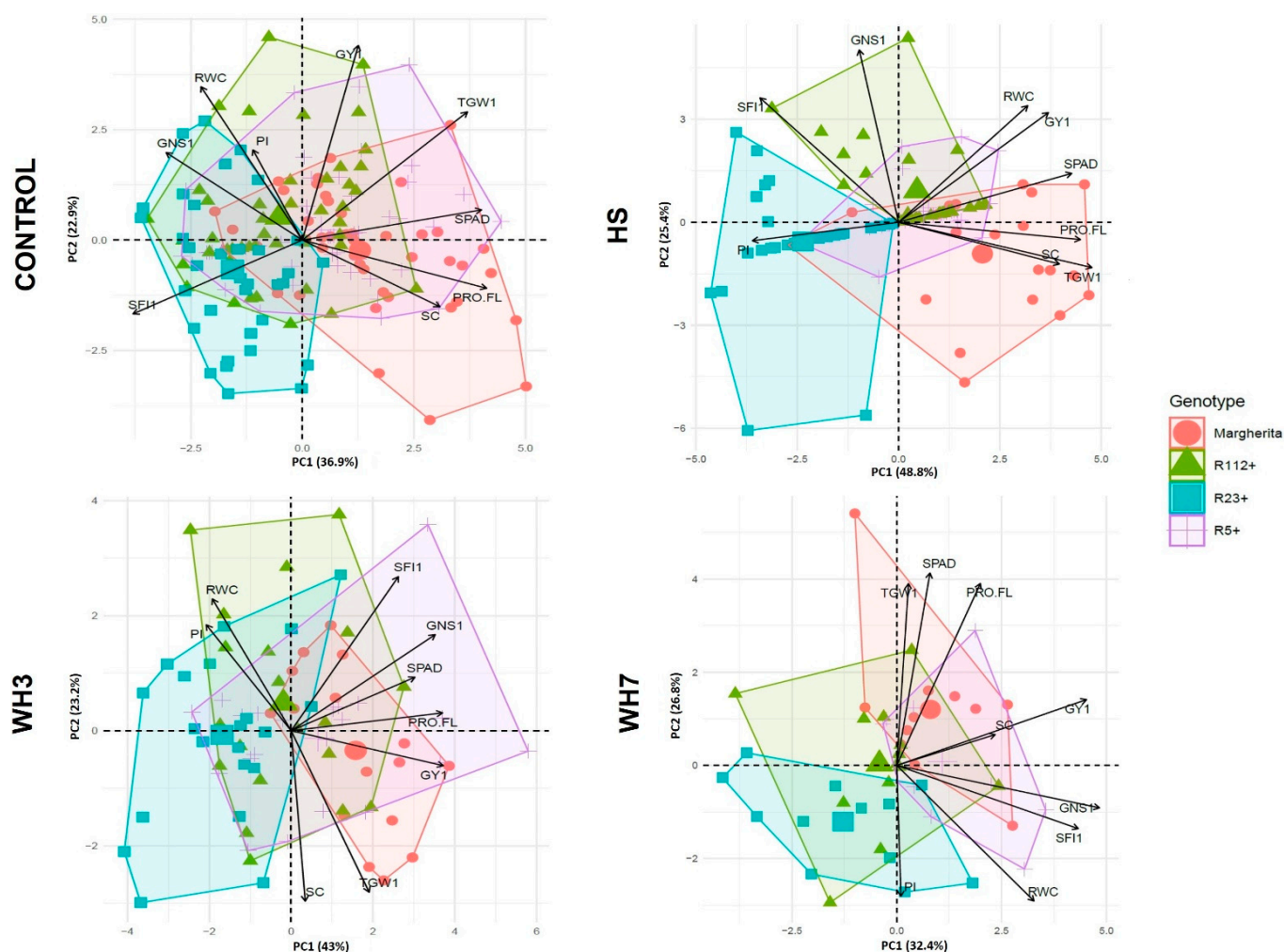


Figure 8. Principal component analysis (PCA) for physiological and yield parameters of the main culms of the three NIRLs+ and cv. Margherita, in control and three stress conditions, i.e., heat stress (HS), water-deficit + 3-day heat stress (WH3) and water-deficit + 7-day heat stress (WH7). The acronyms of the vectors correspond to traits described in the Materials and Methods section.

Table 5. Stress indices' values and ranking of the genotypes tested in the three stress experiments performed: HS, heat stress; WH3, water-deficit + 3-day heat stress; and WH7, water-deficit + 7-day heat stress. The best value for each index appears in bold. For acronyms of the stress indices see the Materials and Methods section; SR, sum of ranks; SD, standard deviation; AR, average of ranks.

Treatment	Genotype	Stress Tolerance Indices																								
		Yp		Ys		TOL		MP		GMP		HM		SSI		STI		YI		YSI		RSI		SR	SD	AR
		Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank			
HS	Margherita	5.65	1	4.94	1	0.71	6	5.30	1	5.29	1	5.27	1	1.38	6	1.48	1	1.25	1	0.87	6	0.96	6	31	2.52	2.82
	R5+	4.72	3	4.20	2	0.52	5	4.46	2	4.45	2	4.45	2	1.22	5	1.05	2	1.06	2	0.89	5	0.98	5	35	1.47	3.18
	R5-	3.75	6	3.76	4	−0.01	2	3.76	5	3.75	5	3.75	5	−0.04	2	0.75	5	0.95	4	1.00	2	1.10	2	42	1.54	3.82
	R112+	3.67	7	3.84	3	−0.17	1	3.75	6	3.75	6	3.75	6	−0.52	1	0.74	6	0.97	3	1.05	1	1.15	1	41	2.49	3.73
	R112-	4.06	4	3.75	5	0.31	4	3.90	4	3.90	4	3.90	4	0.84	4	0.80	4	0.95	5	0.92	4	1.02	4	46	0.40	4.18
	R23+	4.73	2	3.51	7	1.22	7	4.12	3	4.08	3	4.03	3	2.84	7	0.88	3	0.89	7	0.74	7	0.82	7	56	2.21	5.09
	R23-	3.84	5	3.66	6	0.19	3	3.75	7	3.75	7	3.75	7	0.53	3	0.74	7	0.93	6	0.95	3	1.05	3	57	1.83	5.18
WH3	Margherita	4.39	2	3.06	1	1.33	5	3.73	2	3.67	1	3.61	1	0.85	4	1.10	1	1.36	1	0.70	4	1.08	4	26	1.57	2.36
	R5+	2.95	4	2.49	3	0.47	3	2.72	4	2.71	4	2.70	4	0.44	3	0.60	4	1.10	3	0.84	3	1.31	3	38	0.52	3.45
	R5-	2.60	7	2.98	2	−0.38	1	2.79	3	2.78	3	2.78	3	−0.42	1	0.63	3	1.33	2	1.15	1	1.78	1	27	1.75	2.45
	R112+	2.94	5	1.87	5	1.07	4	2.40	6	2.34	6	2.28	6	1.03	5	0.45	6	0.83	5	0.63	5	0.99	5	58	0.65	5.27
	R112-	2.80	6	2.46	4	0.34	2	2.63	5	2.63	5	2.62	5	0.34	2	0.56	5	1.09	4	0.88	2	1.37	2	42	1.54	3.82
	R23+	5.62	1	1.86	6	3.76	7	3.74	1	3.23	2	2.79	2	1.88	6	0.85	2	0.82	6	0.33	6	0.51	6	45	2.43	4.09
	R23-	3.17	3	1.04	7	2.13	6	2.10	7	1.81	7	1.56	7	1.89	7	0.27	7	0.46	7	0.33	7	0.51	7	72	1.21	6.55
WH7	Margherita	4.39	2	2.12	2	2.27	6	3.25	2	3.05	1	2.86	1	1.05	5	0.76	1	1.19	2	0.48	5	0.95	5	32	1.92	2.91
	R5+	2.95	4	2.37	1	0.59	1	2.66	3	2.64	2	2.63	2	0.40	1	0.57	2	1.33	1	0.80	1	1.58	1	19	1.01	1.73
	R5-	2.60	7	1.92	4	0.68	2	2.26	6	2.23	6	2.21	4	0.53	2	0.41	6	1.08	4	0.74	2	1.46	2	45	1.92	4.09
	R112+	2.94	5	1.75	5	1.19	4	2.34	5	2.27	5	2.19	5	0.82	4	0.42	5	0.99	5	0.59	4	1.17	4	51	0.50	4.64
	R112-	2.80	6	2.04	3	0.76	3	2.42	4	2.39	4	2.36	3	0.55	3	0.47	4	1.15	3	0.73	3	1.43	3	39	0.93	3.55
	R23+	5.62	1	1.16	6	4.45	7	3.39	1	2.56	3	1.93	6	1.61	7	0.54	3	0.66	6	0.21	7	0.41	7	54	2.43	4.91
	R23-	3.17	3	1.06	7	2.11	5	2.12	7	1.83	7	1.59	7	1.35	6	0.28	7	0.60	7	0.34	6	0.66	6	68	1.25	6.18

4. Discussion

In the present work, the yield performance and the physiological response of three DW-*Th. ponticum* NIRLs, having 23%, 28% and 40% of alien 7e₁L chromatin on their 7AL telomeric region, were evaluated following the application of heat stress (HS) alone and in combination with water-deficit stress (WH) under controlled conditions. Overall, the presence of each of the three alien segments was revealed to be a stabilizing or even enhancing factor for various productivity components, which makes the three NIRLs useful sources for improving wheat abiotic stress tolerance. The results not only largely confirmed previously identified effects on yield-related traits of the same *Th. ponticum* segments when NIRLs were field trialled [23,24,63,64], but also provided novel insights into the stress adaptive mechanisms of the tested recombinants. The present research, based on controlled and targeted stress application at the critical anthesis phase, has for the first time enabled us to minimize interactions with non-controlled factors and, hence, allowed a more precise dissection and appraisal of the stress effects on yield and related physiological parameters. As a result, different response mechanisms could be identified that appeared to be genotype-dependent, and thus ascribable to specific *Th. ponticum* 7e₁L chromosome segments individually present on the NIRLs' 7AL arms. To achieve this, the use of near-isogenic lines, carrier (NIRLs+) and non-carrier (NIRLs-) of a given alien segment in the common genetic background of cv. Simeto, was an essential tool. In addition to this, the set of tested physiological parameters allowed the evaluation of key plant features for yield formation, i.e., water status (RWC), photosynthetic potential (SPAD) and efficiency (Fv/Fm, PI), stomatal conductance (SC) and stress sensing and signalling (Pro content). All these mechanisms, greatly affecting plant vitality and fertility, play a fundamental role in tolerance acquisition, being strongly modulated by heat and drought stress [65].

As a whole, under HS, the most important traits affecting yield in association with *Th. ponticum* introgressions were grain weight (GW) and grain number (GN), which exhibited a positive correlation with GY at both the main culm and whole plant levels (Figure 7). In particular, GW of the main culm (TGW1), the one targeted for stress application, turned out to be a major determinant of the observed differences between the genotypes (Figure 8). Even if in wheat it is the seed set that is mostly affected by heat stress at anthesis [66], GW was greatly impactful on yield in our HS experiment, possibly due to the procedure followed in stress application. In fact, a 3-day heat application at anthesis could have reduced GN, with the consequent nutrients allocation being concentrated on the set seeds. On the other hand, GN as a sink trait is the key component determining yield in cereals [67], and in wheat, especially under hot conditions, it was shown to represent the limiting factor in raising yield, with most cultivated genotypes resulting as sink-limited (e.g., [55]). Although GN is generally determined in pre-anthesis phases, it can be negatively affected by abiotic stresses at anthesis, in particular during anther dehiscence [67]. Failure to release pollen from anthers, but also reduced pollen viability, limit the successful fertilization and final seed formation [66–68]. *Th. ponticum* 7e₁L introgressions in both bread and durum wheat were shown to contribute to the increase in GN in heat-affected natural environments [23,28,64,69], and those tested here have been validated as a source of useful grain traits for yield stability under such stress.

Under the combined WH stress, most decisive and discriminating yield traits turned out to be GN and the positively correlated spike fertility index (SFI, Figure 7). Moreover, as highlighted by PCA (Figure 8), all physiological parameters displayed an essential role in yield preservation, emphasizing how strongly the maintenance of flag leaf physiology impacts on wheat production under adverse conditions (see § 3.2). Normally, the combination of heat and drought stress has a negative and cumulative impact on plant phenology and physiology [37,70]. However, the impact on yield components depends on both stress duration and severity and on the plant developmental stage. In wheat, episodes of drought and heat stress around anthesis, as in our experimental design, generally reduce the final seed set in a more severe way than the heat stress alone does [37]. Moreover, if water limitation is prolonged during grain filling, it reduces photosynthesis, induces

early senescence, shortens the grain-filling period and can concomitantly increase the remobilization of assimilates from leaves and stems to the grains, raising the grain filling rate [71]. In the WH experiments performed here, a strong impact on GNP was detected (from -20 to -70% in WH7 vs. control conditions, Figure 6A and Tables S11 and S12), which was remarkably superior to that detected under the single HS (Tables S3 and S4). In contrast, an overall increase in TGW was observed in all three experiments, but its slight increment detected in WH3 and WH7 was not able to compensate for the decrease of all other yield components. Regarding the physiological parameters, those associated with plant water management (WU, WUE, SC) were especially critical to achieve tolerance and preserve yield (see also [37,42]).

Considering the stress effects more specifically concerning each recombinant line, the 23%-long segment present in R5+ was found to be associated with the highest yield stability and overall physiological activity across all stress conditions examined here. The alien segment favoured R5+ particularly under the combined stress experiments (Figure 8), when considerable Pro accumulation in the flag leaf (Pro-FL) and an efficient stress perception leading to stomatal closure were observed (Figure 5, Tables S7, S8, S11 and S12). The Pro increase was higher than that of the heat tolerant cv. Margherita and was strongly and positively correlated with GYP in all stress experiments performed here (Figure 7) and in other studies (e.g., [72]). R5+ proved to be the top performing genotype, especially in the most extreme WH7 condition, when it showed the highest values for WUE, PI and HI (Figure 6), besides that of GW and final GY (Tables S9–S12). The enhanced TGW of R5+ was previously observed under rainfed field conditions [24,73], where drought and heat naturally occurred at the time of grain filling. The R5+ advantage for HI confirms previous observations in a low-yielding environment of South Australia, characterized by a short season, low rainfall, high temperatures and recurrent heat waves [23]. This suggests a putatively higher ability of R5+ to allocate dry matter more towards grains than biomass under unfavourable conditions. It is well established that, in water-limited environments, plants put in place water-saving mechanisms, including regulation of stomatal aperture and consequent reduction in the rate of water vapour dispersion from leaves [46–48]. The same tolerance mechanism was also observed under a combination of heat and water-deficit conditions, when plants generally close stomata to limit water loss [74]. In our observations, the combination of prompt stomata closure together with an efficient use of available water for grain production (WUE) have likely given R5+ a remarkable yield advantage (Figures 5B and 6A, Tables S11 and S12). The general high yield stability exhibited by R5+ in the present HS and WH experiments is in agreement with a previous observation of the highest yield gains (up to 30%) that R5+ displayed vs. R112+ and R23+ across a range of nine contrasting and mostly stressful environments [23].

Regarding the R112+ recombinant line, a positive effect of its 28%-long *Th. ponticum* segment was evident under HS, with all yield-related traits revealing their high stability (Table S3). Compared with Margherita and the other two NIRLs, R112+ largely compensated for its lower TGW with superior values for all parameters describing grain number (GNS, GNSL, SFI, GNP). The superiority of R112+ for GN was previously observed in multi-environment rainfed trials, which indicated R112+ to be best suited to environments characterised by heat stress not accompanied by major water scarcity [23,63,64]. In the HS experiment, R112+, as R5+, maintained all yield parameters unchanged in the stressed vs. control conditions (Table S3). Both recombinant NIRLs, like the heat tolerant cv. Margherita (Figure 4B), displayed higher RWC under HS as compared to their NIRL-controls (Figure 4A). In addition, the *Th. ponticum* chromatin in common between R112+ and R23+ (Figure 1) was associated with a high photosynthetic efficiency under HS, as shown by their PI index (Figure 4B). PI remained unchanged (R112+) or even increased (R23+) in HS vs. control conditions, suggesting the remarkable efficiency of the photosynthetic apparatus when coping with the HS condition. A high photosynthetic potential and rate of R112+ were previously observed in field trials across several environments, in which enhanced values for traits such as flag leaf area, chlorophyll content [63,64] and

photosynthetic efficiency [24] were detected. On the other hand, while R112+ was one of the best performing genotypes under the HS alone, the same was not observed under the combined WH stress. This could already be perceived in the WH3 treatment (Tables S5–S8) and became more evident with prolonged stress duration (WH7), particularly in comparison with the behaviour of R5+ (Tables S9–S12). The unchanged amount of water use (WU) and decreased WUE in WH7 vs. the control condition (Figure 6A, Tables S9–S12) are suggestive of the higher amounts of water required by R112+ vs. the other genotypes in both optimal and stress conditions, and of lower efficiency in activating water-saving mechanisms that are fundamental to cope with water-limited conditions [75].

The NIRL carrier of the longest *Th. ponticum* chromosome segment, R23+, resulted as the most sensitive among the three recombinants under both HS and combined WH stress, with many of the yield traits significantly reduced with respect to non-stress conditions (Tables S3, S7 and S11). This is at least in part ascribable to linkage drag associated with the most proximal portion of its alien segment, not shared with the other NIRLs+ [23,63,64], as well as to its genetic background, in common with R23–, but somewhat less isogenic with respect to the remaining NIRLs [23]. Nonetheless, apart from the low absolute values, when compared to its R23– sibling, R23+ confirmed some exceptionally positive effects of its 7el₁L segment on spike fertility (SFI) and plant GN. SFI, in particular, remained significantly higher in R23+ vs. R23– under both control ($\approx +45\%$) and, notably, stress conditions ($+105\%$ to $+230\%$, depending on the stress treatment, Tables S3, S7 and S11). This result is in line with the high increase of GN that R23+ displayed in dry environments (South Australia, Morocco), characterized by both heat and drought stress at critical developmental phases [23]. Higher GN, GNSL and SFI, consistently displayed by field grown R23+ plants, were tentatively associated with the recombinant's constitutively higher floret survival rate [23,63,64]. This hypothesis is strongly sustained by the present report, and further studies are underway to determine the genetic and physiological bases of R23+ reproductive resilience. SFI has been proposed as a promising trait to select for the best genotypes in wheat breeding programs [76], as increased SFI implies improved partitioning of assimilates, fundamental to support wheat yield potential in hot environments [77]. Besides SFI, HI and GNP also turned out to be statistically superior in R23+ vs. R23– in the WH7 experiment, suggesting that the presence of the 40%-long 7el₁L chromosome segment might cause preferential nutrients allocation in seeds rather than in spike chaff under unfavourable conditions. Moreover, lower water use by R23+ vs. R23–, a behaviour that may have contributed to the maintenance of high spike fertility in the former, was for the first time revealed under WH7 stress (Figure 6A). A further new insight concerned Pro accumulation in R23+ spike tissues (Pro-SP). This NIRL+ was the genotype with the highest Pro-SP in absolute terms under both control and HS conditions and, unlike its NIRL– sib, maintained Pro-SP unchanged after the HS treatment (Figure 4A). Whether Pro accumulation in spikes is a specific R23+ mechanism to cope with stress and might be correlated with its increased fertility under HS remains to be further elucidated.

Our analysis of the Pro content in both leaves and spikes of the durum wheat-*Th. ponticum* recombinant lines, under control and stress conditions, showed the dynamics of Pro accumulation in spikes to be more complex. In fact, in line with many other studies on leaves of heat- and/or drought-stressed wheat plants (e.g., [52,72]), Pro-FL was very similar across the genotypes under control conditions, and exhibited an overall increase under all applied stresses, with major increments detected in R5+ and Margherita (Tables S3, S4, S7, S8, S11 and S12). As for Pro-SP, the present experiments clearly showed that in all conditions much larger Pro amounts were present in spikes than in leaves (10–30-fold greater across genotypes), confirming previous evidence in wheat and barley [78,79]. However, while showing only minor variations under HS alone (Figure 4), when the three days of heat stress were accompanied by a water-deficit condition (WH3 experiment), a considerable decrement of Pro-SP was detected in all genotypes (Figure 5B). The rate and direction of Pro accumulation under combined heat and drought stress are still unclear and are probably connected with the intensity/timing of stress application,

as well as with the plant tissue analysed. For example, *Arabidopsis* plants subjected to a combination of heat and drought accumulate sucrose instead of proline, suggesting that sucrose replaces proline as the major osmoprotectant, probably to safeguard the overactive and sensitive mitochondria from the potentially toxic pyrroline-5-carboxylate, an intermediate in Pro biosynthesis and degradation [80]. Other studies on various species provide controversial results for Pro accumulation under individual or combined heat and drought stresses (e.g., [81,82]). Proline's role in stressed spike tissues is poorly understood, not least because most analyses have been focused on leaves. A recent work on the response to drought stress by different wheat organs [78] underlined how, compared with flag leaf, smaller amount of ROS and higher amounts of antioxidant enzymes and proline were accumulated in spike organs under stress, associating these response mechanisms with a better water status, higher photosynthesis and lower membrane damage. As above recalled, also in our experiments both control and stressed spikes exhibited a superior Pro content than in flag leaves in absolute terms, even if the combination of heat and drought caused a general Pro decrease in spikes compared to unstressed conditions. Given the relevance of spike traits for yield performance under stress, and the special stress-responsive behaviour apparently associated with this organ (e.g., [83]), the mechanisms exerted at the spike level by our recombinant lines will be further investigated in a future study.

5. Conclusions

As a whole, the results of the present study showed that, among the three chromosomally engineered durum wheat-*Th. ponticum* recombinant lines, the one possessing the smallest amount of alien 7eL1L introgression, namely R5+, already known to possess very effective disease resistance genes within its alien segment [19,54], exhibited excellent yield stability across all the applied abiotic stress conditions. This was primarily due to highly efficient plant water use, but also to an effective flag leaf physiology (chlorophyll activity, Pro accumulation) and high grain weight. R5+ also proved to be comparable for productivity and some physiological adaptive mechanisms to the elite heat tolerant line Margherita, which validates its readiness to be introduced in breeding programs for abiotic-stress prone environments, such as those of the Mediterranean basin. Further confirmation of the very promising behaviour of R5+, besides that of R5-derived secondary recombinants, and, to some extent, of R112+, comes from the recent results of field trials carried out in the natural stressful conditions of Algeria, Italy and Turkey [84,85]. No doubt, improving abiotic stress tolerance in wheat is essential to achieve yield stability and to decrease the yield gap under changing climate [1,6,31,86]. The evidence reported here substantiates how, for this and other key targets, the use of chromosome engineering to harness wild relatives' useful attributes [19–22] is an effective strategy to develop stable and functional genotypes for large-scale applications.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants12040704/s1>. Table S1: Heat stress experiment: statistical comparison for G effect performed between the NIRLs ("+" vs. "−"). Table S2: Heat stress experiment: statistical comparison for G effect performed between the NIRLs+ and cv. Margherita. Table S3: Heat stress experiment: statistical comparison for genotype × treatment (G × T) effect performed between the NIRLs ("+" vs. "−"). Table S4: Heat stress experiment: statistical comparison for G × T effect performed between the NIRLs+ and cv. Margherita. Table S5: Combined water-deficit + heat stress experiment, with 3 days of heat stress (WH3): statistical comparison for G effect performed between the NIRLs ("+" vs. "−"). Table S6: Combined water-deficit + heat stress experiment, with 3 days of heat stress (WH3): statistical comparison for G effect performed between the NIRLs+ and cv. Margherita. Table S7: Combined water-deficit + heat stress experiment, with 3 days of heat stress (WH3): statistical comparison for genotype × treatment (G × T) effect performed between the NIRLs ("+" vs. "−"). Table S8: Combined water-deficit + heat stress experiment, with 3 days of heat stress (WH3): statistical comparison for G × T effect performed between the NIRLs+ and cv. Margherita. Table S9: Combined water-deficit + heat stress experiment, with 7 days of heat stress (WH7): statistical comparison for G effect performed between the NIRLs ("+" vs. "−"). Table S10: Combined water-

deficit + heat stress experiment, with 7 days of heat stress (WH7): statistical comparison for G effect performed between the NIRLs+ and cv. Margherita. Table S11: Combined water-deficit + heat stress experiment, with 7 days of heat stress (WH7): statistical comparison for genotype $\times$ treatment ($G \times T$) effect performed between the NIRLs (“+” vs. “−”). Table S12: Combined water-deficit + heat stress experiment, with 7 days of heat stress (WH7): statistical comparison for $G \times T$ effect performed between the NIRLs+ and cv. Margherita. Table S13: Correlations between five principal components’ variants and main culm physiological/yield-related traits. (A) Control condition; (B) Heat stress treatment (HS); (C) Water-deficit + heat stress for 3 days (WH3); (D) Water-deficit + heat stress for 7 days (WH7).

Author Contributions: Conceptualization, C.C. and L.K.; formal analysis, G.G. and L.K.; investigation, G.G. and A.C.; resources, C.C.; data curation, G.G., L.K., A.C., and C.C.; writing—original draft preparation, G.G. and L.K.; writing—review and editing, L.K. and C.C.; visualization, G.G. and A.C.; supervision, C.C.; project administration, C.C.; funding acquisition, C.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was carried out under the frame of the PRIMA —Partnership for Research and Innovation in the Mediterranean Area—programme supported by the European Union, Call 2018—Project “IMPRESA” (IMProving RESilience to Abiotic stresses in durum wheat: enhancing knowledge by genetic, physiological and “omics” approaches and increasing Mediterranean germplasm biodiversity by crop wild relatives-based introgressions).

Data Availability Statement: The data presented in this study are available in supplementary material here.

Acknowledgments: Bioinformatic help for the analysis in R Environment from Bruno Bellisario is gratefully acknowledged.

Conflicts of Interest: The authors declare no conflict of interest.

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**Chapter 4: Targeted chromosomal introgressions from
Thinopyrum spp. increase durum wheat tolerance to intense heat
stress applied at anthesis**

Chapter 4: Targeted chromosomal introgressions from *Thinopyrum* spp. increase durum wheat tolerance to intense heat stress applied at anthesis

Abstract

Durum wheat (DW) is one of the major crops grown in the Mediterranean area, a climate-vulnerable region where extreme day/night temperature peaks are increasingly and severely threatening DW yield stability. In order to improve DW heat tolerance, the introgression of chromosomal segments derived from the wild gene pool is a promising strategy. Here, four DW-*Thinopyrum* spp. near-isogenic recombinant lines (NIRLs) were assessed for their physiological response and yield performance after an intense heat stress (IH) applied for 3 days at anthesis. The NIRLs included two primary types (R5, R112) carriers (+) and non-carriers (–) of a differently sized *Th. ponticum* 7el₁L segment on DW 7AL arm, and two corresponding secondary NIRLs (R69-9/R5, R69-9/R112), possessing a *Th. elongatum* 7EL segment distally inserted into the 7el₁L ones. Results showed the best stress-responding genotypes to be R112+, R69-9/R112+ and R69-9/R5+, which stood out for maintenance of leaf relative water content, but also for proline accumulation in flag leaf (especially R69-9/R5+) and for photosynthetic efficiency preservation (R112 types). Furthermore, all the above three NIRLs essentially maintained a stable yield under IH compared with non-stressed plants and turned out to be the best-performing genotypes from stress indices analysis. This work largely confirmed the value of exploiting *Thinopyrum* species as useful source for the improvement of DW heat tolerance.

4.1. Introduction

Durum wheat (DW), *T. durum* L. ($2n = 4x = 28$), with a global production of 32.9 million tonnes in 2022 (International Grains Council, <https://www.igc.int/en/default.aspx>), is one of the most important crops worldwide, and definitely in the Mediterranean area (Lidon et al., 2014; Zampieri et al., 2020). Since DW is predominantly grown in rain-fed conditions, environmental factors have a great impact on its proper development and on final yield. Current climate projections predict that drought and heat waves/high temperature peaks will become more common and intense in the next future, especially in the Mediterranean basin. Here, future annual warming rates are projected to be 20% higher than the global average (Hilmi et al.,

2022), and this will contribute to heavily reduce DW arable land and productivity. In fact, for each degree-Celsius of temperature increase, a 6% decline on wheat yields is expected (Zhao et al., 2017). Therefore, considering the pressing need to reach a 60% production increase for staple crops by 2050 to feed the increasing human population (Ray et al., 2013), improving heat tolerance in wheat, and DW in particular, is one of the most urgent goals to accomplish.

In order to confer efficient stress tolerance mechanisms to modern DW varieties, and hence adequately respond to current and future challenges, the enlargement of wheat genetic basis via exploitation of little explored genetic resources of wild relatives offers good promise (Zhang et al., 2017; Dempewolf et al., 2017). In particular, the *Thinopyrum* genus, which is part of the wheat tertiary gene pool, includes several perennial species native and naturally adapted to harsh environments of southern and eastern Europe, western Asia and northern Africa (Monsen et al., 2004; Wang, 2011). In the recent years, a number of DW-*Thinopyrum* spp. recombinant lines, have been developed through the “chromosome engineering” strategy (Ceoloni et al., 2005; Ceoloni et al., 2014). This cytogenetic approach, consisting of the transfer of segments of alien chromosomes carrying particular desired genes to wheat chromosomes (Sears, 1972), allows to incorporate into the crop genome only small amounts of the wild chromatin, thus substantially reducing the occurrence of unwanted linkage drag. The DW-*Thinopyrum* spp. recombinant lines obtained, already proven to possess disease resistance attributes, besides yield and quality advantages (see general introduction §1.2.5.; Ceoloni et al., 2014, 2015; Kuzmanovic et al., 2018; Kuzmanovic et al., 2019), have been recently field tested under abiotic stress conditions (Kuzmanovic et al., 2021) and to heat and combined heat+drought stresses applied under controlled conditions at the critical stage of anthesis (Giovenali et al., 2023; see Chapter 3).

Generally, the temperature at which thermal stress occurs is dependent on wheat developmental stage. At anthesis the optimum temperatures is 21°C (Jacott and Boden, 2020) and exposure to temperatures above 24°C already results in a substantial floret damage (Janni et al., 2020). The reproductive stage is one of the most stress susceptible phases in wheat and it is strictly connected to yield formation and final yield: abnormalities occurring in pollen grains and/or ovules and ovaries due to heat phenomena heavily hamper proper fertilization and hence negatively affect potential harvest (Dolferus et al., 2011). Thus, a severe and prolonged heat imposition during the extremely heat-sensitive flowering period, is expected to be detrimental for overall plant vitality, altering the normal plant physiology by causing photosynthetic decline and accelerated senescence, but also affecting spike tissues and functions, ultimately reducing seed set and development (see Chapter 1, § 1.3.2.). In order to counteract the stress factors, a

dense response network, going from stress perception to cellular genome-wide reprogramming, is generally activated (see Chapter 1, § 1.3.3.). Among the stress response mechanisms, the accumulation of specific molecules called “osmolytes” (amino acids, sugars and others) could be extremely beneficial, since they contribute to maintain a proper cellular water status in key source and sink tissues, such as flag leaves and spikes. Although flag leaves, as the major photosynthetic organs in wheat, are the most widely studied, recent works highlighted that the spikes have an enhanced tolerance to abiotic stress, such as heat and drought. In fact, the spike proved to present many advantages in response to drought in comparison to the flag leaf, including a lower ROS accumulation, a better osmotic adjustment, a higher antioxidant system and a delayed senescence (Li et al., 2020). Furthermore, unlike leaves, the spike metabolome is strongly responsive to water stress, through a strong coordination between carbon and nitrogen metabolisms, culminating in active biosynthesis of organic acids and amino acids (Vergara-Diaz et al., 2020). In particular, a higher Pro accumulation in wheat spikes than in flag leaves was reported under heat and water-limited conditions (Kumar et al., 2017; Frimpong et al., 2021). As for sugar content in wheat spikes, El-Habti et al. (2020) reported that water soluble carbohydrates (WSC) were predominantly allocated into the spikes of modern varieties, even if heat and drought stress combination did not further increase their amount, unlike observed in older genotypes. Despite that, the superior sugar content in the spike could be indicative of improved floret fertility even under high temperatures (Dreccer et al., 2014), thus further confirming the predominant role of osmolytes accumulation in stress tolerance acquisition.

In the present work the overall performance of some *DW-Thinopyrum* spp. near-isogenic recombinant lines (NIRLs) was evaluated under the application of a severe heat stress applied at anthesis stage. In order to elucidate stress response mechanisms activated by the recombinant lines and tentatively associate them with presence of specific wild chromatin introgressions, the physiological activity of flag leaves was monitored during the three days of heat stress imposition and the osmolytes content (proline, sugars) of flag leaf and spike tissues was quantified at the end of the third day of stress. Several yield parameters were also measured on control and stressed plants with the aim to select the best-performing genotypes under a high temperature regime throughout the day and night. In fact, climate models predict that night-time temperatures will increase more than day-time temperatures in the near future, making it necessary to select thermally tolerant genotypes that can withstand heat stress not only during the day but also at night (Jacott and Boden, 2020).

4.2. Materials and methods

4.2.1. Plant materials

In the present experiment, different DW-*Thinopyrum* spp. recombinant lines were used as main target genotypes. Among them, two primary DW-*Th. ponticum* NIRLs were employed, named R5-2-10 and R112-4, here referred to as R5+ and R112+, respectively. The NIRLs were previously obtained through a chromosome engineering strategy (Ceoloni et al., 2005) and developed in the background of the Italian DW cv. Simeto by repeated back-crossing (BC₅ for R5+ and R112+ and BC₄ for R23+) followed by several self-generations. The NIRLs possess different portions of the tall wheatgrass species *Th. ponticum* 7e₁L chromosome arm replacing 23% and 28% of the distal end of the recipient DW 7AL arm, respectively (see Chapter 2, **Figure 2.1**). The symbol “+” indicates homozygous carriers of the respective 7e₁L segment, while the symbol “–” indicates homozygous sib lines non-carrier of the given 7e₁L segment. Besides them, two secondary NIRLs were also employed, named R69-9/R5+ and R69-9/R112+ and derived from the primary types R5+ and R112+, respectively (Kuzmanovic et al., 2019). Such NIRLs present a *Th. elongatum* 7EL chromosome segment distally inserted (“nested”) into the 7e₁L ones (see Chapter 2, **Figure 2.1**). The choice to include R69-9 types in the present IH experiment, that is the one in which adult plants were subjected to the highest day/night temperature regime, was based on two circumstances. The first one consisted of the recent reaching of a sufficient rounds of backcrossing (BC₄) of the “nested” NIRLs to the recurrent cv. Simeto for reliable comparisons between corresponding + and – NIRLs (i.e. presence/absence of the given alien segment); the second one regarded the promising results observed for the R69-9 types in the framework of a PRIMA project (acronym IMPRESA), during which they showed a remarkable performance under extremely hot conditions in north African field environments. Finally, the DW cv. Margherita, bred by ICARDA, has been introduced as a positive tolerant control, since it expressed a remarkable heat tolerance ability in a range of stressful environments, including the Senegal River Basin, characterized by temperature extremes during most of the growth cycle (Sall et al., 2018a, b).

4.2.2.Methods

4.2.2.1. Plant growing conditions

All plant materials assayed were grown under controlled conditions into walk-in growth chambers. This allowed for standardization of growth conditions and minimization of environmental variability. Seeds sterilization was carried out for 5 min in a sodium hypochlorite solution (80% distilled water, 20% bleach), followed by two rinses and two hr of soaking in distilled water. Sterilized seeds were germinated for few days in Petri dishes on a double layer of soaked filter paper and then transplanted in pots containing soil, sand and perlite in 6:1:1 ratio. The potted plantlets were then put for about 2 weeks into a cold climatic chamber at 8/6°C day/night (d/n) and 12 hr photoperiod to simulate vernalisation. After that, plants were grown at 16/12°C d/n and 16 hr photoperiod until tillering, at 20/16°C d/n until booting, at 22/18°C d/n until the early grain filling period and finally at 25/20°C until full ripening. During plant growth cycle, several fertilizer, with different N:P:K ratios depending on plant phenological stage, were dispensed.

4.2.2.2. Application of IH stress treatment

The intense heat (IH) stress treatment started when plant main culms reached the anthesis stage (Zadoks scale 61-65; Zadoks et al., 1974). Plants at that stage were moved into an adjacent chamber set at 37/27°C d/n and remained there for 3 consecutive days. The same temperature regime was used in many other similar studies (e.g. Lu et al., 2020; El Habti et al., 2020; Liu et al., 2019). Stressed plants were regularly watered to avoid any stress due to water limitation. A set of control plants (C) remained into the first growth chamber under the optimal temperature regime of 22/18°C d/n. Features of the experimental plant growth conditions, stress application and data recording are illustrated in **Figure 4.1**.

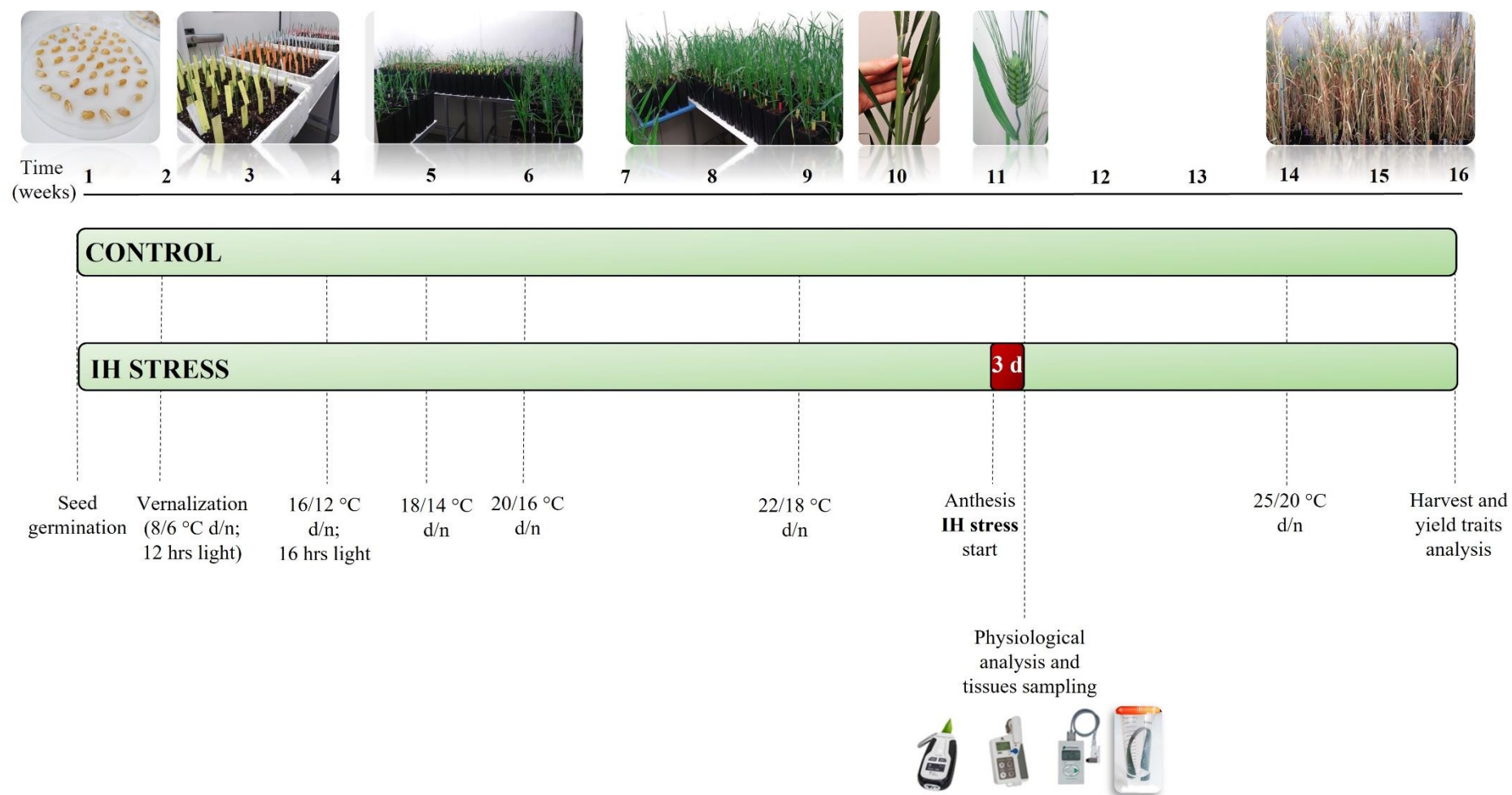


Figure 4.1. Timeline and features of experimental plant growth conditions, stress application and data recording.

4.2.2.3. Physiological analyses performed during and at the end of IH application

4.2.2.3.1. *Chlorophyll content measurements*

Chlorophyll content (SPAD) was measured during the 1st, 2nd and 3rd day after anthesis on stressed and control plants. Measurements were taken on 3 different positions along the adaxial surface of flag leaf using a hand-held chlorophyll meter (SPAD- 502 Plus; Konica-Minolta, Japan). The 3 measurements performed on each flag leaf were then averaged, thus obtaining a mean value from each plant. At least 10 plants/genotype/treatment condition were used for chlorophyll content measurements.

4.2.2.3.2. *Chlorophyll fluorescence parameters analysed*

Chlorophyll fluorescence parameters were assessed during the 1st, 2nd and 3rd day after anthesis on stressed and control plants. In order to evaluate the photochemical response, OJIP curve (Fluorescence Transient) was recorded using a portable fluorometer (PAR-FluorPen FP110; PSI- Photon Systems Instruments, Czech Republic) on flag leaf adaxial surface after a dark adaptation of 30 min with detachable clips. OJIP curve is obtained by exposing the sample to saturating light (= high irradiance) after a dark adaptation period and provides detailed information about the status and functionality of photo-system II (PSII) reaction centres, allowing to extrapolate key parameters, such as Fv/Fm (max photochemical efficiency of PSII), and Performance Index (PI). The latter is a combined measure of three factors: quantity of the photosynthetic reaction centres (RCs) of PSII, maximum energy flow reaching the RCs and electron transport at the beginning of illumination. Thus, PI substantially expresses the energetic bifurcations of PSII (Kalaji et al., 2016; Sharma et al., 2014; Zivcak et al., 2008). At least 10 plants/genotype/treatment condition were used for chlorophyll fluorescence measurements.

4.2.2.3.3. *Stomatal conductance measurements*

Stomatal conductance was measured during the 1st, 2nd and 3rd day after anthesis on stressed and control plants. Stomatal conductance was taken in the middle of FL adaxial surface using a SC-1 leaf porometer (METER Group, Inc. USA), previously calibrated into the climatic

chamber according to the company's instructions. At least 10 plants/genotype/treatment condition were used for stomatal conductance measurements.

4.2.2.3.4. Relative Water Content (RWC) determination

Leaf sampling for RWC determination was performed 3 days after anthesis on stressed and control plants. RWC was assessed on a 5 cm-long section cut from the middle portion of the fully expanded penultimate leaf (FL-1) of the main culm and calculated with the same formula and modalities reported in Chapter 2 (see § 2.2.2.2.).

4.2.2.4. Biochemical analysis performed after the IH stress application on target tissues

4.2.2.4.1. Proline quantification

Proline (Pro) was quantified on flag leaves (Pro-FL) and spikes (Pro-SP) of stressed and control plants. Tissues sampling was performed immediately after the stress application: flag leaves were collected in tubes, whereas spikes were wrapped in aluminium foils. Each plant tissue was immediately suspended in liquid nitrogen and stored at -80°C until further use. Pro content was measured following the ninhydrin-based colorimetric assay (see Chapter 2; § 2.2.2.3.1.)

4.2.2.4.2. Water soluble carbohydrates (WSC) quantification in spikes

In order to quantify the WSC content in plants after the IH experiment, both control and IH-stressed spike samples were evaluated. Spike samples were collected at the end of the third day of IH application, frozen in liquid nitrogen and stored at -80°C . One hundred mg of frozen spike powder were freeze-dried for two consecutive days using a FreeZone Benchtop Freeze Dryer (Labconco corporation; temperature of the collector set at -50°C , vacuum set point at 0.200 mbar). Twenty mg of the obtained freeze-dried materials were used for WSC extraction and quantification, following the modified anthrone method reported by El Habti et al. (2020). One ml of 80% ethanol was added to each sample, which was then put at 80°C for 1 hr. After 10 min of centrifugation at 3400 rpm, the first supernatant was collected. One ml of MQ water was added in the tube containing the pellet and the samples were incubated at 60°C for 1 hr. After 10 min of centrifugation at 3400 rpm, the resulting supernatant was collected. In total, 4

consecutive water extractions were performed to reach an extract amount of 90 to 99% of the total WSC present in the samples. Finally, 1 ml of cold anthrone was added to 200 µl of the sum of the supernatants and the samples were put immediately at 100°C for 10 min. The samples were put in ice to stop the anthrone reaction and 200 µl of the resulting solutions were transferred into a 96-well microplate to read their absorbance at 630 nm. WSC concentration of samples was determined in technical duplicates using a calibration curve of increasing concentration of glucose and was expressed as g/g DW.

4.2.2.4.3. Lipid peroxidation (*Thiobarbituric acid -TBARS*) assay

The lipid peroxidation assay was performed on flag leaves sampled 3 days after anthesis on stressed and control plants, in order to evaluate the rate of membrane damage caused by the severe heat imposition. Flag leaves were collected in tubes, immediately suspended in liquid nitrogen and stored at -80°C until further use. The TBARS assay, previously reported in Chapter 2 (see § 2.2.2.3.2.), was performed. At least 3 plants/genotype/treatment condition were used for malondialdehyde (MDA) quantification.

4.2.2.5. Yield-related traits measurements

All yield-related traits were measured when plants reached the complete ripening and in post-harvest. They consisted of: 1. traits describing whole plant features and 2. traits assessed on main culms, i.e. those subjected to IH stress exactly at anthesis. Traits and their respective acronyms are reported in **Table 4.1**. Spike fertility index (SFI) was calculated as the ratio between grain number per spike (GNS) and chaff weight, and harvest index (HI) as the ratio between grain yield per plant (GYP) and biomass (BM). Measurements of all yield-related parameters derived from at least 10 plants/genotype/treatment combination.

	Yield-related traits measured	Acronyms
Main culm traits	Grain Number per Spike	GNS1
	Spike Fertility Index	SFI1
	Thousand Grain Weight	TGW1
	Grain Yield per Spike	GY1
Whole plant traits	Grain Number per Plant	GNP
	Spike Fertility Index	SFI
	Thousand Grain Weight	TGW
	Harvest Index	HI
	Grain Yield per Plant	GYP

Table 4.1. Yield traits measured in the experimental activities performed on adult plants.

4.2.2.6. Yield-based stress indices evaluation

With the aim to further evaluate and identify tolerant/susceptible genotypes under stressful conditions, calculation and analysis of stress indices is a useful and practical tool, used mostly in field experiments but also under controlled conditions. The stress indices analysed here, calculated through the Plant Abiotic Stress Index Calculator (iPASTIC) software (<https://manzik.com/ipastic/>), together with corresponding formulas, are reported in **Table 4.2**. Following the instructions of software developers, input model was set using mean data derived from GYP, detected in both control (Y_p) and stress (Y_s) conditions. Besides calculating the value of each index for each genotype, iPASTIC also allows genotypes' ranking. Furthermore, it provides the average of ranks (AR), thus indicating potentially superior genotypes on a broader basis (the lower the AR value, the more stress tolerant the genotype).

INDEX	FORMULA
Tolerance index (TOL)	$TOL = Y_p - Y_s$
Mean productivity (MP)	$MP = (Y_p + Y_s)/2$
Geometric mean productivity (GMP)	$GMP = \sqrt{Y_s * Y_p}$
Harmonic mean (HM)	$HM = 2 * (Y_s * Y_p) / (Y_s + Y_p)$
Stress susceptibility index (SSI)	$SSI = 1 - (Y_s / Y_p) / 1 - (\bar{Y}_s / \bar{Y}_p)$
Stress tolerance index (STI)	$STI = (Y_s * Y_p) / (\bar{Y}_p)^2$
Yield index (YI)	$YI = Y_s / \bar{Y}_s$
Yield stability index (YSI)	$YSI = Y_s / Y_p$
Relative stress index (RSI)	$RSI = (Y_s / Y_p) / (\bar{Y}_s / \bar{Y}_p)$

Table 4.2. Stress indices calculated through iPASTIC software with related formulas (from: Pour-Aboughadareh et al., 2019).

4.2.2.7. Statistical analysis of data

For all datasets, one-way analysis of variance (ANOVA) was performed to estimate differences ascribable to the genotype (G) effect, while two-factor ANOVA was applied to analyse the effect of $G \times$ treatment (T) interaction. For those physiological parameters for which measurements were repeated over time (SPAD, SC, Fv/Fm, PI, see **Table 4.3**), a repeated measures ANOVA was also performed. In fact, the repeated measures ANOVA permits to compare means across one or more variables that are based on repeated observations. Whenever a significant F value was obtained for single factors or their interaction, the Tukey HSD test was performed at $P < 0.05$ level. Before performing ANOVAs, obvious outliers were eliminated. All statistical analyses were performed by JASP software (JASP Team, 2022; JASP Version 0.16.1). For all parameters, two statistical comparisons (ANOVAs) were performed: in the first one, the datasets of each individual NIRL (+) and of the corresponding non-carrier (sib) line (–) was compared, to associate possible differences in stress behaviour with presence/absence of a specific alien segment; in the second one, the three NIRLs' datasets were compared to that of cv. Margherita, to evaluate their performance with respect to a known heat tolerant and productive cultivar.

4.3. Results

4.3.1. Genotype (G), treatment (T) and $G \times T$ impact

Almost all the physiological, biochemical and yield-related traits (see **Table 4.3**) resulted to be highly significant for the G factor in both the statistical comparisons (**Table 4.4A** and **4.4B**), revealing strong differences among the genotypes tested. Moreover, the heat treatment applied at anthesis (T factor) resulted to be highly impactful on all parameters, with only the Pro-SP trait apparently not affected by the IH stress imposition (**Table 4.4A** and **4.4B**).

Acronyms	Measurement/Analysis performed
SPAD	Chlorophyll content (<i>SPAD unit</i>)
Fv/Fm	Max photochemical efficiency of PSII
PI	Performance Index
SC	Stomatal Conductance (<i>mmol/[m²s]</i>)
RWC	Relative water content
GNS1	Grain Number/Spike (main culm)
SFI1	Spike Fertility Index (main culm)
TGW1	Thousand Grain Weight (main culm, <i>g</i>)
GY1	Grain Yield (main culm, <i>g</i>)
GNP	Grain Number/Plant
SFI	Spike Fertility Index
TGW	Thousand Grain Weight (<i>g</i>)
HI	Harvest Index
GYP	Grain Yield/Plant (<i>g</i>)
Pro-FL	Flag Leaf Proline Content (<i>μmol/g FW</i>)
Pro-SP	Spike Proline Content (<i>μmol/g FW</i>)
MDA	Flag Leaf Malondialdehyde Content (<i>μmol/g FW</i>)
WSC	Spike Water Soluble Carbohydrates Content (<i>g/g DW</i>)

Table 4.3. Acronyms used in text and figures, with relative significance.

When the $G \times T$ interaction was considered, a different modulation of RWC, proline and sugar content (Pro-FL, Pro-SP, WSC) was revealed among the NIRLs+ and – after the stress, this in turn probably having different impacts on grain yield (GY1, GYP) and GNP variation (**Table 4.4A**). However, a higher number of statistically significant differences for the $G \times T$ interaction were observed in the comparison performed between NIRLs+ and Margherita. In fact, besides those mentioned above, also other parameters (Fv/Fm, SC, GNS1, SFI and HI) turned out to be differently altered in the genotypes by the IH imposition (**Table 4.4.B**).

A

Factors	G	T	G × T	Residuals
df	7	1	7	
SPAD	649.44 ***	445.73 **	69.43	42.24
Fv/Fm	0.0003 ***	0.03 ***	0.0001	0.0001
PI	31.9 ***	3668.1 ***	15.79	8.21
SC	13640.76	2790000 ***	7750.73	8682.56
RWC	0.002 ***	0.02 ***	0.002 **	0.0005
Pro-FL	63.40 ***	488.84 ***	61.58 ***	5.24
Pro-SP	355.1 *	148.63	1261.11 ***	153.06
MDA	3.08 **	13.11 ***	1.23	0.795
WSC	0.004 *	0.02 ***	0.003 *	0.001
GNSI	93.23 ***	4200.84 ***	34.71	22.83
SFI1	212.64	6749.03 ***	116.06	129.18
TGW1	148.4 ***	1245.34 ***	22.58	24.13
GY1	0.31 ***	6.06 ***	0.17 *	0.08
GNP	1295.29 ***	32307.56 ***	873.28 ***	223.31
TGW	122.50 ***	2825.59 ***	49.36	31.22
SFI	1152.06 ***	33708.75 ***	414.42	241.48
HI	0.01	0.14 ***	0.01	0.01
GYP	3.15 ***	30.41 ***	1.51 **	0.44

B

Factors	G	T	G × T	Residuals
df	4	1	4	
SPAD	873.4 ***	155.42 *	23.47	26.84
Fv/Fm	0.0003 *	0.02 ***	0.0003 *	0.00009
PI	25.74 **	2342.83 ***	9.97	6.72
SC	43702.65 ***	2505000 ***	59210.66 ***	5415.28
RWC	0.004 ***	0.02 ***	0.002 *	0.0005
Pro-FL	77.78 ***	332.30 ***	94.41 ***	4.952
Pro-SP	497.46 *	222.46	604.38 *	159.29
MDA	3.24 *	7.58 **	0.76	0.85
WSC	0.003 *	0.01 ***	0.003 *	0.0008
GNSI	238.56 ***	3374.38 ***	59 *	23.44
SFI1	135.35	4798.37 ***	77.67	109.69
TGW1	163.24 ***	728.05 ***	29.04	24.31
GY1	0.83 ***	5.44 ***	0.27 **	0.07
GNP	1167.38 ***	23907.18 ***	1033.35 ***	190.23
TGW	131.49 **	1768.4 ***	52.54	26.37
SFI	2455.73 ***	31770.72 ***	1129.67 ***	220.19
HI	0.004	0.07 ***	0.009 *	0.004
GYP	2.76 ***	28.26 ***	2.79 ***	0.44

Table 4.4. ANOVA mean squares for the traits measured under control and IH treatment. **A:** comparison between alien segment carrier (NIRLs+) and non-carrier (NIRLs-) lines; **B:** comparison between NIRLs+ and cv. Margherita. *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively (df, degrees of freedom; G, genotype; T, treatment).

4.3.2. Physiological variations detected in control and stressed flag leaves

Whereas the majority of the physiological traits measured on flag leaves showed significant differences for the G factor (**Table 4.4**), the presence of the *Thinopyrum* spp. chromosome segments affected only the chlorophyll content (SPAD) and Performance Index (PI) values (**Table 4.5A**). In fact, R69-9/R5+ and R69-9/R112+ exhibited inferior values than the corresponding sibs- for the PI and SPAD traits, respectively. When the statistical comparison between NIRLs+ and Margherita was considered, the latter exhibited significantly higher values than all R69-9 types for SPAD and a greater stomatal conductance (SC) than R5+ and R69-9/R5+ (**Table 4.5B**). By contrast, R69-9/R112+ turned out to possess a higher PI than Margherita, suggesting an improved PSII efficiency in the recombinant line.

A

Trait		SPAD			FvFm			PI			SC		
Time		day 1	day 2	day 3	day 1	day 2	day 3	day 1	day 2	day 3	day 1	day 2	day 3
R5+	Mean	54.532	55.126	54.952	0.828	0.829	0.815	11.766	10.83	9.63	113.414	162.178	185.607
	SE	0.791	0.739	1.01	0.003	0.004	0.005	0.906	1.162	1.164	27.995	33.175	37.016
	Tukey	a			b			ab			ns		
R5-	Mean	54.092	54.833	54.691	0.834	0.825	0.821	12.963	11.135	10.116	174.865	201.128	252.912
	SE	1.124	1.047	0.802	0.002	0.0040	0.003	0.67	0.906	1.09	35.185	32.035	43.522
	Tukey	a			b			b			ns		
R112+	Mean	52.264	53.039	52.207	0.838	0.837	0.827	14.775	13.684	11.775	188.088	181.571	184.146
	SE	0.504	0.516	0.516	0.002	0.0020	0.005	1.116	0.662	1.456	34.184	26.69	39.342
	Tukey	abc			ab			ab			ns		
R112-	Mean	53.898	53.993	54.491	0.832	0.829	0.823	12.08	11.3	10.774	181.807	196.058	227.422
	SE	0.488	0.635	0.618	0.003	0.003	0.003	0.739	0.823	0.904	30.014	26.812	42.066
	Tukey	ab			ab			ab			ns		
R69-9/R5+	Mean	47.316	46.102	46.387	0.835	0.83	0.83	13.518	11.254	11.434	179.976	218.8	200.076
	SE	0.969	1.097	0.881	0.003	0.003	0.002	0.857	0.852	0.727	31.332	31.23	28.304
	Tukey	de			ab			b			ns		
R69-9/R5-	Mean	48.792	47.456	45.394	0.84	0.831	0.827	15.032	12.534	11.379	175.481	194.88	214.61
	SE	1.575	2.023	2.067	0.002	0.0030	0.003	0.994	1.317	1.143	27.01	24.809	28.797
	Tukey	cde			a			a			ns		
R69-9/R112+	Mean	45.194	44.579	43.844	0.838	0.839	0.828	14.271	13.754	11.626	194.911	257.388	193.55
	SE	0.915	1.175	0.834	0.002	0.0020	0.003	1.024	0.854	0.731	32.738	31.836	27.049
	Tukey	e			a			ab			ns		
R69-9/R112-	Mean	49.798	48.394	49.577	0.836	0.831	0.827	13.919	12.176	10.883	213.517	233.279	211.032
	SE	1.227	1.131	1.2	0.003	0.002	0.002	1.041	0.799	0.605	41.74	38.3	26.851
	Tukey	bcd			ab			b			ns		

B

Trait		SPAD			FvFm			PI			SC		
Time		day 1	day 2	day 3	day 1	day 2	day 3	day 1	day 2	day 3	day 1	day 2	day 3
R5+	Mean	54.532	55.126	54.952	0.828	0.829	0.815	11.766	10.83	9.63	113.414	162.178	185.607
	SE	0.791	0.739	1.01	0.003	0.004	0.005	0.906	1.162	1.164	27.995	33.175	37.016
	Tukey	a			b			ab			b		
R112+	Mean	52.264	53.039	52.207	0.838	0.837	0.827	14.775	13.684	11.775	188.088	181.571	184.146
	SE	0.504	0.516	0.516	0.002	0.0020	0.005	1.116	0.662	1.456	34.184	26.69	39.342
	Tukey	a			ab			ab			ab		
R69-9/R5+	Mean	47.316	46.102	46.387	0.835	0.83	0.83	13.518	11.254	11.434	179.976	218.8	200.076
	SE	0.969	1.097	0.881	0.003	0.003	0.002	0.857	0.852	0.727	31.332	31.23	28.304
	Tukey	b			ab			b			b		
R69-9/R112+	Mean	45.194	44.579	43.844	0.838	0.839	0.828	14.271	13.754	11.626	194.911	257.388	193.55
	SE	0.915	1.175	0.834	0.002	0.0020	0.003	1.024	0.854	0.731	32.738	31.836	27.049
	Tukey	b			a			a			ab		
Margherita	Mean	53.06	53.955	53.727	0.828	0.825	0.827	10.209	10.107	10.315	184.131	172.908	203.554
	SE	0.689	0.622	0.775	0.004	0.0040	0.003	0.98	0.909	0.904	44.554	44.759	50.183
	Tukey	a			ab			b			a		

Table 4.5. Means, standard error (SE) and ranking of Tukey test at $P < 0.05$ level for flag leaf physiological traits. **A:** Statistical comparison for G factor performed between the NIRLs + vs. -; **B:** Statistical comparison for G factor performed between the NIRLs + and cv. Margherita.

The results of the repeated measures ANOVA and the subsequent Tukey test for significant $\text{Time} \times \text{G} \times \text{T}$ interactions (**Table 4.6**) showed that, among the NIRLs' pairs (each NIRL+ vs. its NIRL-), the two parameters associated with chlorophyll fluorescence (Fv/Fm and PI) were strongly influenced by both the timing of the measurements and the IH stress (**Table 4.6A**). Whereas almost all genotypes significantly decreased Fv/Fm and PI under IH vs. unstressed control conditions (C) during the 1st or 2nd day after the anthesis date, R112+ and R69-9/R112+ maintained them unaltered for a longer time, showing a decrease on the 3rd after anthesis (**Figure 4.2**). This observation is indicative of a more efficient PSII preservation in the course of stress application ascribable to the presence of the *Th. ponticum* segment in common between R112+ and R69-9/R112+ (see Chapter 2, **Figure 2.1**). Interestingly, the heat tolerant

Margherita had a significantly decreased Fv/Fm value already from the 1st day of IH imposition (Table 4.6B), highlighting the distinctively positive photosynthetic behaviour of R112+ and R69-9/R112+.

A

Factors	Time	Time × G	Time × T	Time × G × T	Residuals
SPAD	8.02	1.85	8.56	1.86	5.77
Fv/Fm	0.002 ***	0.0001 *	0.0001	0.0001 *	0.00006
PI	121 ***	3.50	2.02	6.46 *	3.12
SC	26402.46 **	2962.08	4854.23	4051.06	4049.43

Trait		SPAD			FvFm			PI			SC		
Time		day 1	day 2	day 3	day 1	day 2	day 3	day 1	day 2	day 3	day 1	day 2	day 3
R5+	Mean	57.35	57.39	57.07	0.844	0.844	0.837	17.79	17.19	15.96	294.25	340.38	361.12
	C SE	0.72	0.83	0.51	0.002	0.003	0.003	0.41	0.67	0.57	20.21	30.54	29.93
	Tukey	ns	ns	ns	ab	abc	abcde	abc	abc	abcde	ns	ns	ns
	Mean	53.59	54.37	54.12	0.822	0.818	0.803	9.76	8.18	6.75	43.86	79.93	89.87
	III SE	0.71	0.73	0.88	0.003	0.003	0.004	0.57	0.74	0.50	5.80	21.17	23.39
R5-	Tukey	ns	ns	ns	efg	fg	g	h	h	h	ns	ns	ns
	Mean	56.17	55.30	56.37	0.842	0.840	0.829	16.16	14.57	13.80	372.96	328.43	402.80
	C SE	1.29	1.36	0.93	0.003	0.002	0.003	0.55	0.51	0.85	37.62	32.39	21.73
	Tukey	ns	ns	ns	abcd	abcd	bcdef	abcde	abcdef	bdefg	ns	ns	ns
	Mean	53.22	54.62	54.24	0.831	0.818	0.815	11.63	9.42	6.96	92.33	137.48	145.85
R112+	III SE	0.95	0.76	0.66	0.003	0.005	0.004	0.57	0.94	0.54	15.19	28.48	33.81
	Tukey	ns	ns	ns	bcdef	fg	fg	de	h	h	ns	ns	ns
	Mean	52.57	53.18	52.95	0.843	0.841	0.841	16.66	15.25	15.29	324.34	300.74	345.72
	C SE	1.23	0.78	0.97	0.002	0.002	0.002	0.49	0.57	0.94	28.61	18.55	31.72
	Tukey	ns	ns	ns	abc	abcd	abcd	abcde	abcde	abcde	ns	ns	ns
R112-	Mean	52.10	52.96	51.96	0.835	0.835	0.811	12.20	12.71	7.12	82.11	115.37	83.16
	III SE	0.57	0.45	0.46	0.002	0.002	0.002	0.84	0.87	0.43	14.45	13.07	12.84
	Tukey	ns	ns	ns	abcde	bcdef	fg	de	h	h	ns	ns	ns
	Mean	54.32	53.12	54.87	0.847	0.844	0.837	16.36	16.19	14.97	303.82	301.28	369.15
	C SE	0.67	1.07	0.78	0.002	0.003	0.002	0.76	0.57	0.73	22.19	28.41	22.98
R112-	Tukey	ns	ns	ns	ab	abc	abcde	abcde	abcde	abcde	ns	ns	ns
	Mean	53.75	54.30	54.36	0.827	0.823	0.816	10.55	9.55	8.68	115.25	143.45	126.19
	III SE	0.57	0.53	0.63	0.002	0.003	0.003	0.53	0.59	0.57	29.34	23.99	34.79
	Tukey	ns	ns	ns	cdef	def	fg	gh	h	h	ns	ns	ns
	R69-9/R5+	Mean	48.89	47.98	47.42	0.842	0.839	0.837	16.07	14.72	14.32	264.46	329.20
C SE		0.95	0.93	0.81	0.002	0.002	0.002	0.56	0.51	0.49	32.72	14.68	25.18
Tukey		ns	ns	ns	abcd	abcd	abcde	abcde	abcde	bcdef	ns	ns	ns
Mean		45.29	42.64	42.30	0.825	0.820	0.820	9.87	7.47	7.83	71.36	108.40	95.20
III SE		1.07	1.50	1.78	0.003	0.003	0.003	0.57	0.53	0.55	16.69	29.71	25.11
R69-9/R5-	Tukey	ns	ns	ns	cdef	efg	efg	gh	h	h	ns	ns	ns
	Mean	52.96	52.50	53.48	0.848	0.846	0.843	18.95	19.44	18.22	297.27	270.87	331.32
	C SE	1.15	1.12	1.17	0.001	0.001	0.003	0.55	0.39	0.77	19.16	22.20	28.20
	Tukey	ns	ns	ns	a	ab	abc	ab	a	ab	ns	ns	ns
	Mean	45.87	44.42	41.82	0.834	0.822	0.819	11.98	8.51	7.96	102.41	141.69	150.95
R69-9/R112+	III SE	1.43	1.64	1.57	0.002	0.003	0.002	0.68	0.67	0.43	15.52	29.44	26.18
	Tukey	ns	ns	ns	abcde	efg	efg	de	h	h	ns	ns	ns
	Mean	46.57	45.00	45.37	0.844	0.842	0.840	17.56	16.25	15.00	331.82	380.01	334.49
	C SE	0.95	1.14	0.94	0.003	0.002	0.002	0.82	0.96	0.64	13.10	13.42	16.56
	Tukey	ns	ns	ns	abc	abcd	abcd	abc	abcd	abcde	ns	ns	ns
R69-9/R112-	Mean	43.81	44.01	42.62	0.832	0.836	0.817	11.35	11.26	8.25	99.08	148.39	92.88
	III SE	0.82	1.02	0.71	0.003	0.003	0.003	1.08	0.88	0.42	26.09	23.08	14.58
	Tukey	ns	ns	ns	bcdef	abcde	fg	de	h	h	ns	ns	ns
	Mean	49.93	48.62	49.23	0.843	0.838	0.836	16.32	14.52	13.23	320.81	381.56	330.14
	C SE	0.91	1.10	1.00	0.002	0.002	0.002	0.90	0.81	0.75	43.04	49.13	31.88
R69-9/R112-	Tukey	ns	ns	ns	abc	abcd	abcde	abcd	bcdef	cdefg	ns	ns	ns
	Mean	49.56	48.13	49.90	0.822	0.822	0.818	8.72	8.79	8.54	44.91	107.81	131.63
	III SE	1.80	1.43	1.25	0.003	0.004	0.002	0.43	0.50	0.35	9.40	25.02	22.33
	Tukey	ns	ns	ns	defg	efg	fg	h	h	h	ns	ns	ns

B

Factors	Time	Time × G	Time × T	Time × G × T	Residuals
SPAD	4.45	0.63	4.56	1.55	4.37
Fv/Fm	0.0008 ***	0.0001 *	0.0002	0.0001 *	0.00007
PI	46.19 ***	8.40 *	1.59	4.58	3.14
SC	5557.30	3152.99	900.72	2043.24	3156.66

Trait		SPAD			FvFm			PI			SC			
Time		day 1	day 2	day 3	day 1	day 2	day 3	day 1	day 2	day 3	day 1	day 2	day 3	
R5+	C	Mean	57.35	57.39	57.07	0.844	0.844	0.837	17.79	17.19	15.96	294.25	340.38	361.12
		SE	0.72	0.83	0.51	0.002	0.003	0.003	0.41	0.67	0.57	20.21	30.54	29.93
		Tukey	ns	ns	ns	ab	ab	ab	ns	ns	ns	ns	ns	ns
	IH	Mean	53.59	54.37	54.12	0.822	0.818	0.803	9.76	8.18	6.75	43.86	79.93	89.87
		SE	0.71	0.73	0.88	0.003	0.003	0.004	0.57	0.74	0.50	5.80	21.17	23.39
		Tukey	ns	ns	ns	cd	de	e	ns	ns	ns	ns	ns	ns
R112+	C	Mean	52.57	53.18	52.95	0.843	0.841	0.841	16.66	15.25	15.29	324.34	300.74	345.72
		SE	1.23	0.78	0.97	0.002	0.002	0.002	0.49	0.57	0.94	28.61	18.55	31.72
		Tukey	ns	ns	ns	ab	ab	ab	ns	ns	ns	ns	ns	ns
	IH	Mean	52.10	52.96	51.96	0.835	0.835	0.811	12.20	12.71	7.12	82.11	115.37	83.16
		SE	0.57	0.45	0.46	0.002	0.002	0.002	0.84	0.87	0.43	14.45	13.07	12.84
		Tukey	ns	ns	ns	abcd	abcd	de	ns	ns	ns	ns	ns	ns
R69-9/R5+	C	Mean	48.89	47.98	47.42	0.842	0.839	0.837	16.07	14.72	14.32	264.46	329.20	295.42
		SE	0.95	0.93	0.81	0.002	0.002	0.002	0.56	0.51	0.49	32.72	14.68	25.18
		Tukey	ns	ns	ns	ab	ab	ab	ns	ns	ns	ns	ns	ns
	IH	Mean	45.29	42.64	42.30	0.825	0.820	0.820	9.87	7.47	7.83	71.36	108.40	95.20
		SE	1.07	1.50	1.78	0.003	0.003	0.003	0.57	0.53	0.55	16.69	29.71	25.11
		Tukey	ns	ns	ns	bcd	cde	cd	ns	ns	ns	ns	ns	ns
R69-9/R112+	C	Mean	46.57	45.00	45.37	0.844	0.842	0.840	17.56	16.25	15.00	331.82	380.01	334.49
		SE	0.95	1.14	0.94	0.003	0.002	0.002	0.82	0.96	0.64	13.10	13.42	16.56
		Tukey	ns	ns	ns	ab	ab	ab	ns	ns	ns	ns	ns	ns
	IH	Mean	43.81	44.01	42.62	0.832	0.836	0.817	11.35	11.26	8.25	99.08	148.39	92.88
		SE	0.82	1.02	0.71	0.003	0.003	0.003	1.08	0.88	0.42	26.09	23.08	14.58
		Tukey	ns	ns	ns	abcd	abc	de	ns	ns	ns	ns	ns	ns
Margherita	C	Mean	53.81	53.32	53.51	0.848	0.846	0.845	15.16	15.34	15.56	454.55	444.15	498.83
		SE	1.50	1.84	1.23	0.001	0.001	0.001	0.29	0.80	0.28	17.04	10.07	18.31
		Tukey	ns	ns	ns	a	ab	ab	ns	ns	ns	ns	ns	ns
	IH	Mean	52.81	54.17	53.81	0.818	0.817	0.820	7.73	8.09	8.13	80.12	68.58	80.53
		SE	0.75	0.85	0.89	0.002	0.003	0.003	0.42	0.57	0.44	24.72	24.72	21.45
		Tukey	ns	ns	ns	de	de	cde	ns	ns	ns	ns	ns	ns

Table 4.6. Means, standard error (SE) and ranking of Tukey test at P < 0.05 level for flag leaf physiological traits. **A:** Statistical comparison (repeated measures ANOVA) for Time × G × T factor performed between the NIRLs + vs. −; **B:** Statistical comparison (repeated measures ANOVA) for Time × G × T factor performed between the NIRLs + and cv. Margherita.

As for SPAD and SC, although the observed differences emerged from the repeated measures ANOVA were not statistically significant (Table 4.6), some trends could be observed. Among the NIRLs+, R112+ had the lowest SPAD reduction at the 3rd of IH treatment (−1.86% in R112+ vs. a mean of −7.34% in the other NIRLs+). Moreover, almost all the NIRLs+, with the only exception of R69-9/R112+, exhibited a stronger reduction of SC than the corresponding sibs−,

starting from the 1st of IH stress. Also Margherita heavily reduced (about –85%) its SC in IH vs. C conditions, suggesting that also a well-known tolerant genotype performed a prompt stomata closure under a severe heat stress occurring at anthesis.

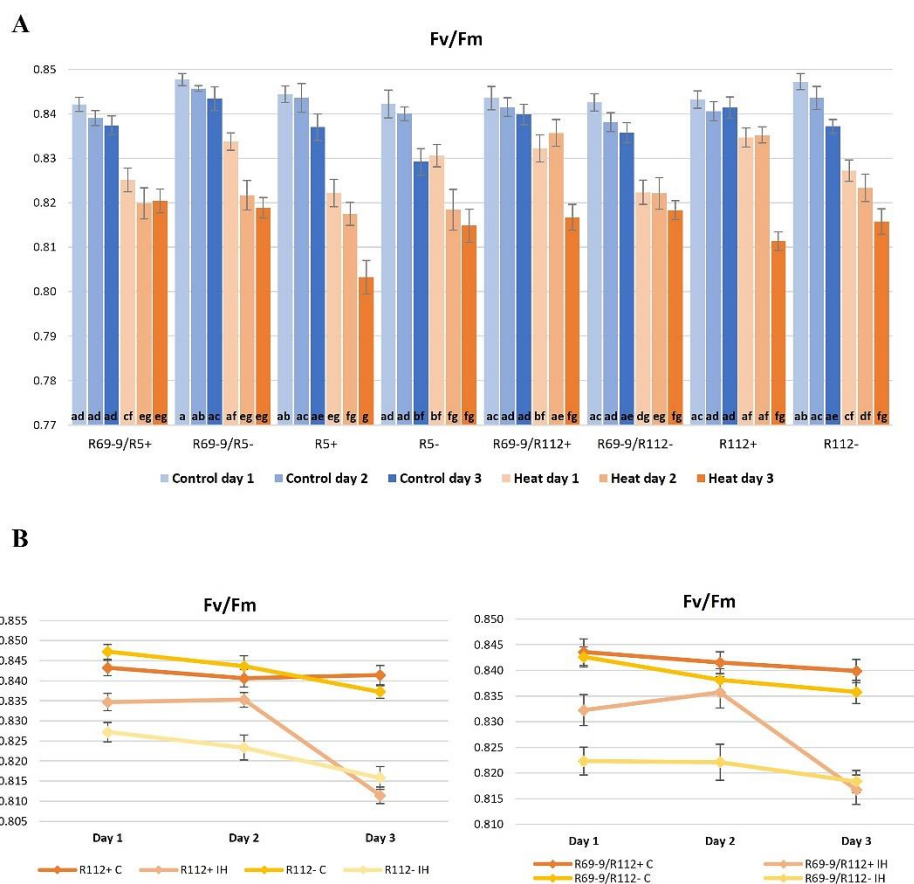


Figure 4.2. Variations detected for Fv/Fm in control and IH stressed flag leaves during the three days after anthesis. **A:** Fv/Fm modulation over time in all the NIRLs+ and -; **B:** Fv/Fm modulation over time in R112+ and R69-9/R112+ (and the corresponding sibs-).

4.3.3. Osmolytes contribution to the heat response of leaves and spikes

The results of G effect on the biochemical traits revealed some differences only for R5+ and R69-9/R5+ genotypes. In fact, presence of the wild segments seemed to determine a reduction of RWC in R5+ and higher Pro accumulation in R69-9/R5+ flag leaves (**Table 4.7A**). From the comparison between NIRLs+ and Margherita, the latter exhibited a lower RWC than R112+, R69-9/R5+ and R69-9/R112+ (**Table 4.7B**) and also an inferior Pro-FL than R69-9/R5+,

suggesting the involvement of an overall more efficient stress response in these NIRLs+. On the other hand, R5+ showed similar values to those of Margherita and a higher lipid peroxidation (MDA) than the latter (**Table 4.7B**), which is suggestive of a less efficient tolerance in R5+ (and Margherita, to some extent) in comparison with the above mentioned NIRLs+ under a severe heat stress application.

A							B						
Trait		RWC	Pro-FL	Pro-SP	MDA	WSC	Trait		RWC	Pro-FL	Pro-SP	MDA	WSC
R5+	Mean	0.907	4.88	53.793	6.844	0.193	R5+	Mean	0.907	4.88	53.793	6.844	0.193
	SE	0.012	0.765	6.177	0.428	0.01		SE	0.012	0.765	6.177	0.428	0.01
	Tukey	b	bc	ns	a	b		Tukey	b	b	ns	a	b
R5-	Mean	0.947	3.39	57.123	6.62	0.205	R112+	Mean	0.948	5.35	57.275	5.288	0.233
	SE	0.005	0.457	8.93	0.4910	0.009		SE	0.006	1.133	6.814	0.3590	0.022
	Tukey	a	c	ns	ab	ab		Tukey	a	b	ns	b	ab
R112+	Mean	0.948	5.35	57.275	5.288	0.233	R69-9/R5+	Mean	0.954	13.844	36.33	5.678	0.245
	SE	0.006	1.133	6.814	0.3590	0.022		SE	0.005	3.843	4.001	0.289	0.009
	Tukey	a	bc	ns	bc	ab		Tukey	a	a	ns	ab	a
R112-	Mean	0.951	2.606	45.891	5.051	0.204	R69-9/R112+	Mean	0.943	6.178	41.644	6.009	0.209
	SE	0.008	0.638	6.295	0.303	0.015		SE	0.006	1.499	4.941	0.2670	0.012
	Tukey	a	c	ns	c	b		Tukey	a	b	ns	ab	ab
R69-9/R5+	Mean	0.954	13.844	36.33	5.678	0.245	Margherita	Mean	0.89	4.702	53.933	5.565	0.205
	SE	0.005	3.843	4.001	0.289	0.009		SE	0.016	0.688	2.896	0.4800	0.017
	Tukey	a	a	ns	abc	ab		Tukey	b	b	ns	b	ab
R69-9/R5-	Mean	0.925	5.404	39.593	5.833	0.266							
	SE	0.013	1.356	6.509	0.6010	0.02							
	Tukey	ab	bc	ns	abc	a							
R69-9/R112+	Mean	0.943	6.178	41.644	6.009	0.209							
	SE	0.006	1.499	4.941	0.2670	0.012							
	Tukey	ab	bc	ns	abc	ab							
R69-9/R112-	Mean	0.921	7.2	43.947	6.379	0.226							
	SE	0.012	1.807	8.022	0.405	0.021							
	Tukey	ab	b	ns	abc	ab							

Table 4.7. Means, standard error (SE) and ranking of Tukey test at $P < 0.05$ level for flag leaf and spike biochemical traits. **A:** Statistical comparison for G factor performed between the NIRLs + vs. –; **B:** Statistical comparison for G factor performed between the NIRLs + and cv. Margherita.

Considering the effects of the $G \times T$ interaction, R5+ confirmed to significantly reduce (–6%) RWC under IH vs. C conditions, compared with R5–, which maintained an almost unchanged RWC value (**Table 4.8A**). On the contrary, both R69-9/R5+ and R69-9/R112+ efficiently maintained their RWC values under the IH application, in contrast with their corresponding sibs– (around – 7% reduction, **Table 4.8A**). As for WSC, a significant 53% increase of WSC content was detected in stressed spikes of R112+, indicating a higher sugar accumulation under hot conditions in the reproductive organs of R112+ compared with all other genotypes (**Table 4.8A; Figure 4.3A**). Interestingly, opposite trends regarding Pro accumulation in flag leaves (FL) and spikes (SP) was observed between the primary recombinants (R5, R112) and the secondary recombinants (R69-9/R5, R69-9/R112). On one hand, all the R69-9 types showed a significantly increment in Pro-FL under IH vs. C conditions, with R69-9/R5+ being the best

Pro accumulator in absolute terms (**Table 4.8A**; **Figure 4.3A**). On the other, whereas all the R69-9 types possessed a lower amount of Pro-SP under C conditions, and increased it after the IH application (**Figure 4.3A**), the primary recombinants showed a higher Pro content in C (unstressed) condition and a strong osmolyte's reduction in IH stressed spikes. In the comparison of NIRLs+ vs. Margherita, the observations previously performed for the G effect alone were confirmed, the latter showing similar values to those of R5+, whereas R112+, R69-9/R5+ and R69-9/R112+ showed higher RWC values and a significant increase in Pro-FL under IH conditions (**Figure 4.3B**).

A								B							
Trait			RWC	Pro-FL	Pro-SP	MDA	WSC	Trait			RWC	Pro-FL	Pro-SP	MDA	WSC
R5+	C	Mean	0.94	3.30	64.70	6.28	0.17	R5+	C	Mean	0.94	3.30	64.70	6.28	0.17
		SE	0.00	0.63	6.33	0.48	0.00			SE	0.00	0.63	6.33	0.48	0.00
		Tukey	ab	cde	abc	ns	c			Tukey	a	bc	ab	ns	c
	IH	Mean	0.89	6.46	39.25	7.59	0.21		IH	Mean	0.89	6.46	39.25	7.59	0.21
		SE	0.01	0.19	1.38	0.57	0.01			SE	0.01	0.19	1.38	0.57	0.01
		Tukey	c	bcde	bcd	ns	abc			Tukey	b	bc	abc	ns	abc
R5-	C	Mean	0.95	3.61	73.49	5.72	0.19	R112+	C	Mean	0.96	2.45	64.81	5.09	0.18
		SE	0.01	0.62	8.06	0.47	0.02			SE	0.01	0.72	8.67	0.51	0.02
		Tukey	a	cde	a	ns	bc			Tukey	a	c	a	ns	c
	IH	Mean	0.94	3.09	35.31	7.81	0.21		IH	Mean	0.94	7.67	42.21	5.43	0.28
		SE	0.01	0.78	3.43	0.00	0.00			SE	0.01	1.13	3.27	0.55	0.02
		Tukey	a	cde	bcd	ns	abc			Tukey	a	b	abc	ns	a
R112+	C	Mean	0.96	2.45	64.81	5.09	0.18	R69-9/R5+	C	Mean	0.97	3.26	31.02	5.42	0.23
		SE	0.01	0.72	8.67	0.51	0.02			SE	0.00	0.46	0.78	0.24	0.01
		Tukey	a	de	ab	ns	c			Tukey	a	bc	c	ns	abc
	IH	Mean	0.94	7.67	42.21	5.43	0.28		IH	Mean	0.94	21.78	40.31	5.83	0.26
		SE	0.01	1.13	3.27	0.55	0.02			SE	0.01	1.61	6.59	0.45	0.01
		Tukey	a	bcd	abcd	ns	ab			Tukey	a	a	abc	ns	ab
R112-	C	Mean	0.96	2.90	58.33	5.22	0.20	R69-9/R112+	C	Mean	0.95	2.31	32.81	5.63	0.22
		SE	0.00	0.89	5.57	0.42	0.02			SE	0.00	0.33	0.90	0.14	0.01
		Tukey	a	cde	abcd	ns	bc			Tukey	a	c	bc	ns	abc
	IH	Mean	0.95	2.31	33.45	4.89	0.21		IH	Mean	0.93	8.50	48.27	6.39	0.20
		SE	0.01	1.08	3.50	0.51	0.02			SE	0.01	1.63	7.13	0.44	0.02
		Tukey	a	de	bcd	ns	bc			Tukey	a	b	abc	ns	bc
R69-9/R5+	C	Mean	0.97	3.26	31.02	5.42	0.23	Margherita	C	Mean	0.95	5.72	54.71	4.40	0.18
		SE	0.00	0.46	0.78	0.24	0.01			SE	0.01	0.62	3.37	0.41	0.03
		Tukey	a	cde	cd	ns	abc			Tukey	a	bc	abc	ns	c
	IH	Mean	0.94	21.78	40.31	5.83	0.26		IH	Mean	0.87	3.68	52.89	6.27	0.23
		SE	0.01	1.61	6.59	0.45	0.01			SE	0.02	0.97	5.92	0.52	0.02
		Tukey	ab	a	bcd	ns	abc			Tukey	b	bc	abc	ns	abc
R69-9/R5-	C	Mean	0.96	1.90	25.76	4.72	0.22	R69-9/R112-	C	Mean	0.96	3.06	25.60	5.82	0.22
		SE	0.00	0.60	2.18	0.20	0.01			SE	0.00	0.88	1.13	0.56	0.01
		Tukey	a	e	d	ns	abc			Tukey	a	cde	d	ns	abc
	IH	Mean	0.89	8.20	53.43	6.95	0.31		IH	Mean	0.89	11.34	57.71	6.94	0.23
		SE	0.01	1.41	3.96	0.73	0.01			SE	0.01	1.74	8.79	0.50	0.03
		Tukey	bc	bc	abcd	ns	a			Tukey	c	b	abcd	ns	abc
R69-9/R112+	C	Mean	0.95	2.31	32.81	5.63	0.22	R69-9/R112-	C	Mean	0.96	3.06	25.60	5.82	0.22
		SE	0.00	0.33	0.90	0.14	0.01			SE	0.00	0.88	1.13	0.56	0.01
		Tukey	a	de	cd	ns	abc			Tukey	a	cde	d	ns	abc
	IH	Mean	0.93	8.50	48.27	6.39	0.20		IH	Mean	0.89	11.34	57.71	6.94	0.23
		SE	0.01	1.63	7.13	0.44	0.02			SE	0.01	1.74	8.79	0.50	0.03
		Tukey	ab	bc	abcd	ns	bc			Tukey	c	b	abcd	ns	abc

Table 4.8. Means, standard error (SE) and ranking of Tukey test at $P < 0.05$ level for flag leaf and spike biochemical traits. **A:** Statistical comparison for $G \times T$ factor performed between the NIRLs + vs. -; **B:** Statistical comparison for $G \times T$ factor performed between the NIRLs + and cv. Margherita.

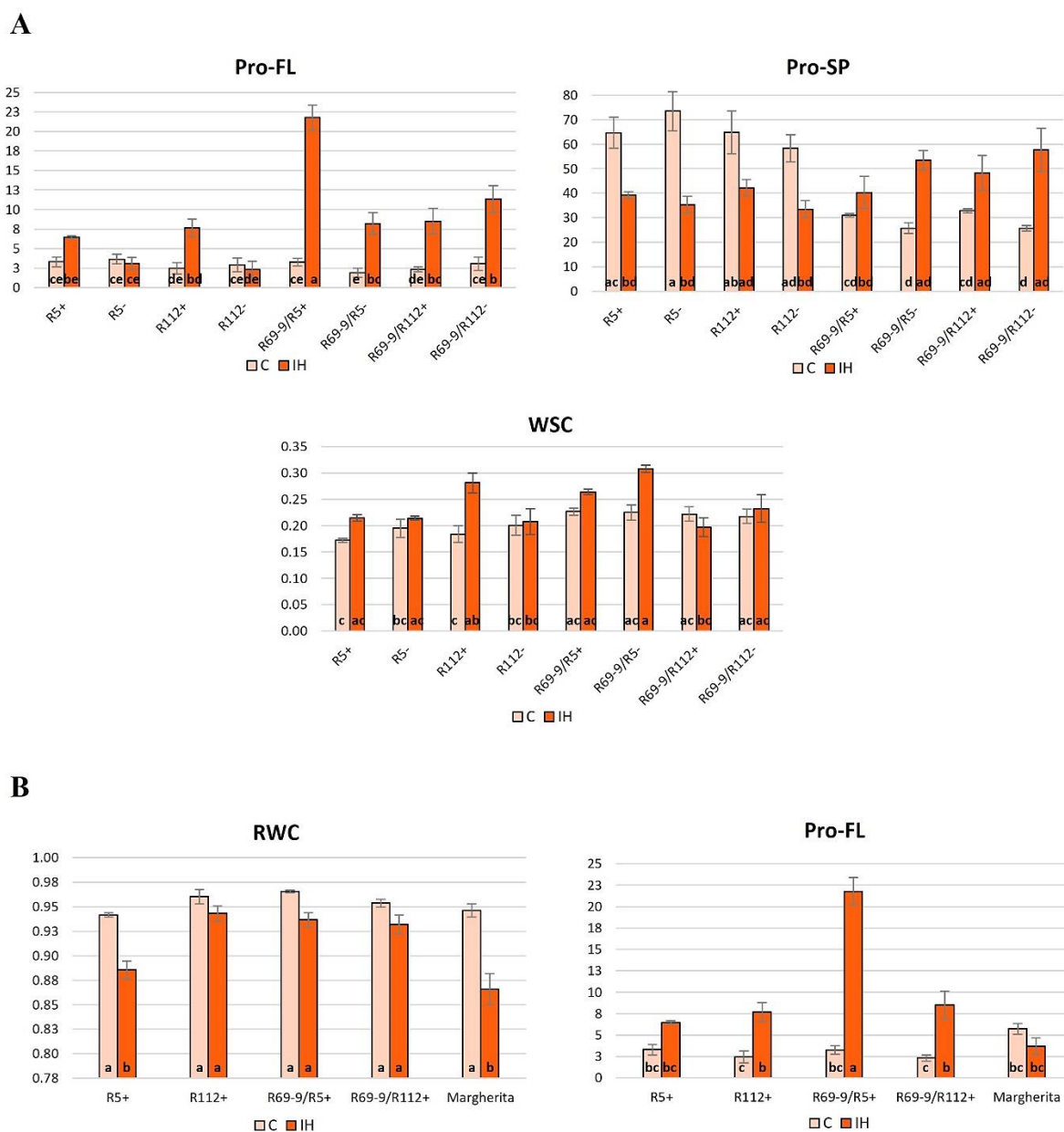


Figure 4.3. Significant $G \times T$ effects on biochemical parameters. **A:** Statistical comparison performed between the NIRLs + vs. -; **B:** Statistical comparison performed between the NIRLs + and cv. Margherita. The error bars represent standard errors of the means, while the letters into the histograms correspond to the ranking of Tukey HSD test at $p < 0.05$ significance level.

4.3.4. Influence of IH application on plant fertility and production

The results of analysis of yield traits revealed the presence/absence of the wild chromosome segments (G effect) not to have a strong impact on NIRLs' fertility and production (**Table 4.9A**), whereas a number of significant differences could be detected in the comparison between

NIRLs+ and Margherita (**Table 4.9B**). In fact, R112+, R69-9/R5+ and R69-9/R112+ exhibited higher values than Margherita for some yield traits of the main culm (GNS1, GY1) and of the whole plant (SFI), confirming the highly positive behaviour of these three genotypes already highlighted in the previously subsections (see § 4.3.2. and 4.3.3.).

A

Trait		GNS1	SFI1	TGW1	GY1	GNP	SFI	TGW	HI	GYP
R5+	Mean	31.214	67.509	55.164	1.686	56.231	59.493	54.027	0.396	3.311
	SE	2.416	3.186	1.592	0.104	5.275	3.39	1.825	0.009	0.236
	Tukey	b	ns	ab	c	c	c	a	ns	cd
R5-	Mean	35.786	73.989	52.503	1.831	62	73.176	50.983	0.326	3.126
	SE	1.566	4.019	1.627	0.085	4.245	3.8	1.702	0.02	0.202
	Tukey	ab	ns	bc	abc	bc	ab	ab	ns	d
R112+	Mean	39.917	74.145	49.238	1.881	70.5	72.737	47.028	0.354	3.421
	SE	1.672	2.505	1.551	0.074	3.253	2.759	1.596	0.016	0.138
	Tukey	a	ns	c	abc	abc	a	b	ns	bed
R112-	Mean	34.385	77.427	52.372	1.784	66.667	74.23	50.143	0.311	3.188
	SE	1.567	2.483	1.537	0.065	3.087	2.227	1.575	0.014	0.135
	Tukey	ab	ns	bc	bc	abc	a	ab	ns	cd
R69-9/R5+	Mean	35.238	68.69	56.34	1.953	69.091	64.492	53.491	0.364	3.699
	SE	1.513	3.357	1.455	0.069	5.4650	2.4980	2.0560	0.0170	0.156
	Tukey	ab	ns	ab	abc	abc	abc	a	ns	abcd
R69-9/R5-	Mean	36.103	72.09	53.882	1.926	75.724	64.418	51.521	0.351	3.709
	SE	1.385	2.705	1.059	0.066	4.7030	2.3670	1.5780	0.0160	0.137
	Tukey	a	ns	bc	ab	a	abc	ab	ns	abc
R69-9/R112+	Mean	35.852	70.128	57.273	2.032	77.037	67.058	54.793	0.369	4.127
	SE	1.633	2.3	0.996	0.082	4.3910	2.3090	1.2510	0.0170	0.185
	Tukey	a	ns	ab	a	a	ab	a	ns	a
R69-9/R112-	Mean	33.833	65.187	58.44	1.96	72.792	61.458	55.513	0.349	3.861
	SE	1.706	3.218	0.979	0.091	6.6950	2.9100	1.4750	0.0190	0.276
	Tukey	ab	ns	a	ab	ab	bc	a	ns	ab

B

Trait		GNS1	SFI1	TGW1	GY1	GNP	SFI	TGW	HI	GYP
R5+	Mean	31.214	67.509	55.164	1.686	56.231	59.493	54.027	0.396	3.311
	SE	2.416	3.186	1.592	0.104	5.275	3.39	1.825	0.009	0.236
	Tukey	bc	ns	a	bc	b	bc	a	ns	b
R112+	Mean	39.917	74.145	49.238	1.881	70.5	72.737	47.028	0.354	3.421
	SE	1.672	2.505	1.551	0.074	3.253	2.759	1.596	0.016	0.138
	Tukey	a	ns	b	ab	ab	a	b	ns	b
R69-9/R5+	Mean	35.238	68.69	56.34	1.953	69.091	64.492	53.491	0.364	3.699
	SE	1.513	3.357	1.455	0.069	5.4650	2.4980	2.0560	0.0170	0.156
	Tukey	ab	ns	a	a	ab	ab	a	ns	b
R69-9/R112+	Mean	35.852	70.128	57.273	2.032	77.037	67.058	54.793	0.369	4.127
	SE	1.633	2.3	0.996	0.082	4.3910	2.3090	1.2510	0.0170	0.185
	Tukey	a	ns	a	a	a	a	a	ns	a
Margherita	Mean	30.071	67.386	52.578	1.556	72.143	54.357	51.525	0.362	3.627
	SE	2.77	4.159	1.25	0.131	8.846	4.187	1.289	0.017	0.406
	Tukey	c	ns	ab	c	ab	c	ab	ns	b

Table 4.9. Means, standard error (SE) and ranking of Tukey test at $P < 0.05$ level for yield-related traits. **A:** Statistical comparison for G factor performed between the NIRLs + vs. -; **B:** Statistical comparison for G factor performed between the NIRLs + and cv Margherita.

When the $G \times T$ interactions were considered, a significant drop in grain yield of the main culm (GY1), the one specifically subjected to IH at the anthesis date, was detected in R5+ (-30%) and in almost all the R69-9 types (from -20 to -27%) in IH vs. control conditions, with the

only exception of R69-9/R5+ (**Table 4.10A; Figure 4.4A**). Similarly, significant reductions in grain yield of the whole plant (GYP) were revealed in all the R69-9 types in IH vs. C conditions, with, again, the only exception of R69-9/R5+, which emerged as the most stable genotype from the productive point of view among such recombinant types. When all NIRLs+ were compared with Margherita, R112+, R69-9/R5+ and R69-9/R112+ confirmed to possess superior values under IH also for yield traits such as GNS1, GY1 and TGW (**Table 4.10B; Figure 4.4B**). Moreover, stressed plants of R112+ showed a higher GNP than Margherita, and stressed plants of R69-9/R5+ and R69-9/R112+ a higher GYP than Margherita (**Table 4.10B**). As a whole, conspicuous reductions in all yield traits were caused by the IH imposition, as expected considering the timing and the severity of the stress applied. Among all genotypes, R112+ emerged as the most stable, showing a significant reduction under IH vs. C condition (−20%) for grain number of the main culm (GNS1) only (**Table 4.10B**). For the same trait, cv. Margherita showed the most severe drop (−44%). In spite of the general GNS1 decrease, a significant reduction in GYP was detected in R69-9/R112+ (best yielding under C conditions) and Margherita, whereas R5+, R112+ and R69-9/R5+ maintained a statistically unchanged GYP in IH vs. C conditions.

A

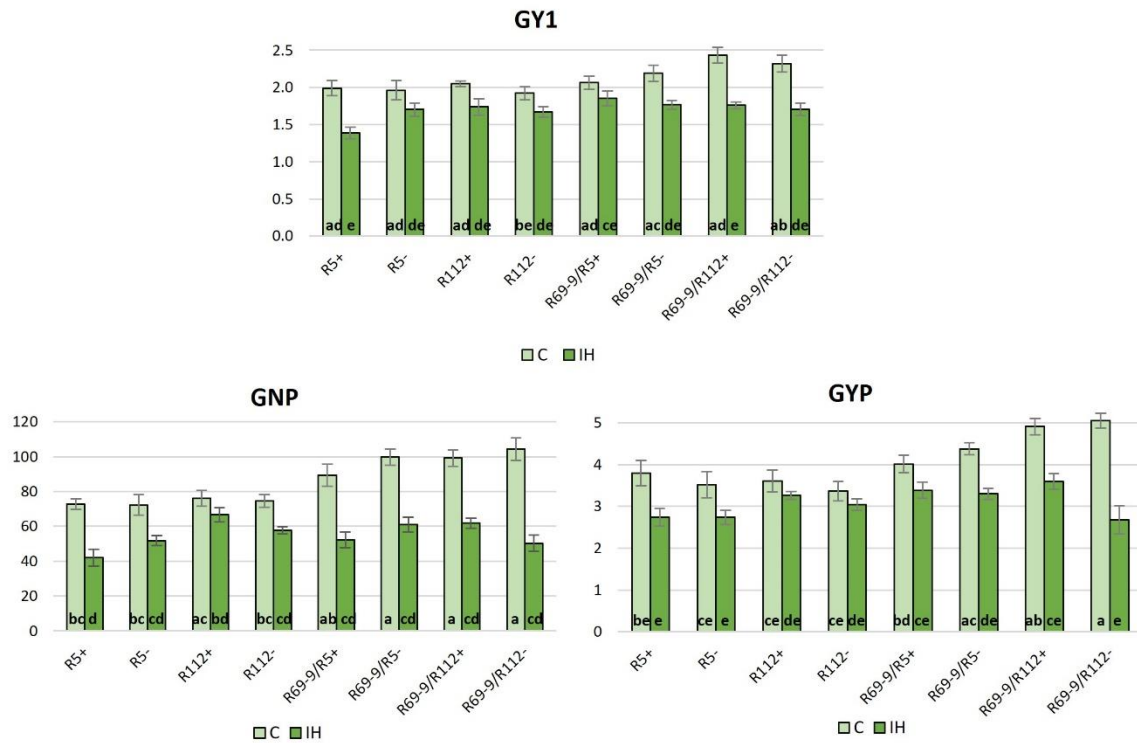
Trait		GNS1	SFI	TGW1	GY1	GNP	SFI	TGW	HI	GYP
R5+	Mean	39.29	76.40	50.60	1.99	72.83	71.44	47.99	0.42	3.80
	C SE	1.57	3.18	1.59	0.10	2.95	3.69	1.27	0.01	0.30
	Tukey	ns	ns	ns	abcd	bc	ns	ns	ns	bcd
	Mean	23.14	58.62	59.73	1.38	42.00	46.79	60.06	0.39	2.74
	IH SE	1.06	2.75	1.23	0.08	4.90	3.78	0.83	0.01	0.21
R5-	Tukey	ns	ns	ns	e	d	ns	ns	ns	e
	Mean	40.29	82.10	48.45	1.96	72.29	82.32	48.88	0.37	3.52
	C SE	1.46	4.25	1.64	0.13	5.91	4.18	2.10	0.02	0.31
	Tukey	ns	ns	ns	abcd	bc	ns	ns	ns	cde
	Mean	31.29	65.88	57.23	1.70	51.71	64.03	53.08	0.29	2.74
R112+	IH SE	1.32	5.48	1.30	0.09	2.81	5.45	2.58	0.02	0.17
	Tukey	ns	ns	ns	de	cd	ns	ns	ns	e
	Mean	44.50	78.08	46.17	2.05	76.00	80.08	43.85	0.35	3.61
	C SE	1.06	3.70	1.44	0.04	4.45	3.14	1.53	0.03	0.27
	Tukey	ns	ns	ns	abcd	abc	ns	ns	ns	cde
R112-	Mean	35.33	70.77	51.86	1.74	66.57	67.12	49.75	0.36	3.26
	IH SE	1.67	3.07	2.23	0.11	4.20	3.76	2.26	0.02	0.10
	Tukey	ns	ns	ns	de	bcd	ns	ns	ns	de
	Mean	40.00	82.53	49.94	1.92	74.63	82.71	46.89	0.35	3.37
	C SE	2.25	2.84	1.76	0.09	3.59	2.09	1.40	0.02	0.24
R69-9/R5+	Tukey	ns	ns	ns	bcd	bc	ns	ns	ns	cde
	Mean	30.71	73.18	54.46	1.67	57.57	68.01	52.93	0.29	3.04
	IH SE	0.99	3.04	2.23	0.07	2.15	2.55	2.24	0.01	0.14
	Tukey	ns	ns	ns	de	cd	ns	ns	ns	de
	Mean	39.80	77.56	52.08	2.06	89.40	74.96	45.96	0.41	4.01
R69-9/R112+	C SE	1.77	4.31	1.42	0.09	6.29	2.70	2.21	0.02	0.21
	Tukey	ns	ns	ns	abcd	ab	ns	ns	ns	bcd
	Mean	31.09	61.30	59.89	1.85	52.17	54.03	59.77	0.32	3.38
	IH SE	1.60	3.98	1.88	0.10	4.50	3.07	1.89	0.02	0.19
	Tukey	ns	ns	ns	cde	cd	ns	ns	ns	cde
R69-9/R5-	Mean	43.00	77.64	50.85	2.19	99.73	75.51	44.43	0.39	4.38
	C SE	1.77	3.74	1.12	0.11	4.57	2.01	1.63	0.02	0.15
	Tukey	ns	ns	ns	abc	a	ns	ns	ns	abc
	Mean	31.89	68.50	55.73	1.77	61.06	56.86	55.85	0.32	3.30
	IH SE	1.10	3.55	1.41	0.06	4.22	3.29	1.66	0.02	0.13
R69-9/R112-	Tukey	ns	ns	ns	de	cd	ns	ns	ns	de
	Mean	44.09	79.20	55.36	2.43	99.18	82.00	49.92	0.43	4.91
	C SE	1.86	2.63	1.54	0.11	4.65	2.00	1.74	0.03	0.20
	Tukey	ns	ns	ns	a	a	ns	ns	ns	ab
	Mean	30.19	63.47	58.59	1.76	61.81	57.72	58.14	0.33	3.59
R69-9/R112+	IH SE	0.98	2.30	1.24	0.05	2.92	2.62	1.16	0.01	0.19
	Tukey	ns	ns	ns	de	cd	ns	ns	ns	cde
	Mean	41.30	77.88	56.00	2.32	104.30	78.90	49.36	0.41	5.05
	C SE	1.61	3.01	0.90	0.11	6.56	2.29	1.92	0.02	0.17
	Tukey	ns	ns	ns	ab	a	ns	ns	ns	a
R69-9/R112-	Mean	28.50	56.12	60.19	1.70	50.29	46.74	59.91	0.30	2.68
	IH SE	1.51	3.43	1.39	0.08	4.60	3.19	1.10	0.02	0.34
	Tukey	ns	ns	ns	de	cd	ns	ns	ns	e

B

Trait		GNS1	SFI	TGW1	GY1	GNP	SFI	TGW	HI	GYP
R5+	Mean	39.29	76.40	50.60	1.99	72.83	71.44	47.99	0.42	3.80
	C SE	1.57	3.18	1.59	0.10	2.95	3.69	1.27	0.01	0.30
	Tukey	ab	ns	ns	b	bc	ab	ns	ab	abc
	Mean	23.14	58.62	59.73	1.38	42.00	46.79	60.06	0.39	2.74
	IH SE	1.06	2.75	1.23	0.08	4.90	3.78	0.83	0.01	0.21
R112+	Tukey	ef	ns	ns	cd	d	de	ns	ab	cd
	Mean	44.50	78.08	46.17	2.05	76.00	80.08	43.85	0.35	3.61
	C SE	1.06	3.70	1.44	0.04	4.45	3.14	1.53	0.03	0.27
	Tukey	a	ns	ns	ab	abc	a	ns	ab	bc
	Mean	35.33	70.77	51.86	1.74	66.57	67.12	49.75	0.36	3.26
R69-9/R5+	IH SE	1.67	3.07	2.23	0.11	4.20	3.76	2.26	0.02	0.10
	Tukey	bcd	ns	ns	bc	c	abc	ns	ab	bcd
	Mean	39.80	77.56	52.08	2.06	89.40	74.96	45.96	0.41	4.01
	C SE	1.77	4.31	1.42	0.09	6.29	2.70	2.21	0.02	0.21
	Tukey	ab	ns	ns	ab	ab	a	ns	ab	ab
R69-9/R112+	Mean	31.09	61.30	59.89	1.85	52.17	54.03	59.77	0.32	3.38
	IH SE	1.60	3.98	1.88	0.10	4.50	3.07	1.89	0.02	0.19
	Tukey	cd	ns	ns	b	cd	cd	ns	b	bc
	Mean	44.09	79.20	55.36	2.43	99.18	82.00	49.92	0.43	4.91
	C SE	1.86	2.63	1.54	0.11	4.65	2.00	1.74	0.03	0.20
Margherita	Tukey	a	ns	ns	a	a	a	ns	a	a
	Mean	30.19	63.47	58.59	1.76	61.81	57.72	58.14	0.33	3.59
	IH SE	0.98	2.30	1.24	0.05	2.92	2.62	1.16	0.01	0.19
	Tukey	de	ns	ns	bc	cd	bcd	ns	b	bc
	Mean	38.57	76.73	50.62	1.95	102.00	75.23	48.32	0.41	4.94
Margherita	C SE	1.43	2.67	1.51	0.07	4.84	2.57	1.29	0.01	0.29
	Tukey	abc	ns	ns	b	a	a	ns	ab	a
	Mean	21.57	56.49	54.53	1.16	42.29	32.44	54.73	0.31	2.32
	IH SE	2.67	5.98	1.80	0.14	4.31	4.36	1.46	0.02	0.25
	Tukey	f	ns	ns	d	d	e	ns	b	d

Table 4.10. Means, standard error (SE) and ranking of Tukey test at $P < 0.05$ level for yield-related traits. **A:** Statistical comparison for $G \times T$ factor performed between the NIRLs + vs. -; **B:** Statistical comparison for $G \times T$ factor performed between the NIRLs + and cv Margherita.

A



B

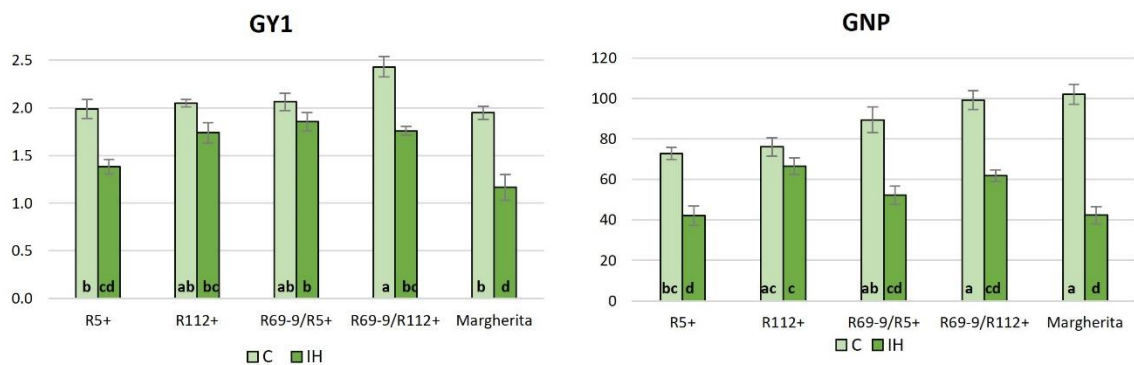


Figure 4.4. Significant $G \times T$ effects on yield-related parameters. **A:** Statistical comparison performed between the NIRLs + vs. -; **B:** Statistical comparison performed between the NIRLs + and cv. Margherita. The error bars represent standard errors of the means, while the letters into the histograms correspond to the ranking of Tukey HSD test at $p < 0.05$ significance level.

4.3.5. Stress indices assessment and tolerance ranking

The results of the analysis of the nine stress indices is reported in **Table 4.11**. The ranking performed on the basis of the values obtained for each stress index for each genotype, substantially confirmed the above reported considerations about the higher heat tolerance

shown by R69-9/R5+, R69-9/R112+ and R112. In particular, R69-9/R5+ and R69-9/R112+ turned out to be the most tolerant genotypes, exhibiting the lowest values (3.09) of the overall average of ranks (AR). With a 3.73 AR value, also R112+ highlighted to possess a remarkable stress tolerance. Among these best performing NIRLs+, R69-9/R112+ ranked first for many of the stress indices considered, such as MP, GMP, HM, STI and YI. Rather surprisingly, the heat tolerant Margherita turned out to be most susceptible among all the genotypes tested under the specific IH stress conditions, showing a high AR value of 7.36.

Genotype	Yp		Ys		TOL		MP		GMP		HM		SSI		STI		YI		YSI		RSI		AR
	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank	
R5+	3.80	6	2.74	6	1.06	5	3.27	7	3.23	7	3.18	7	0.99	7	0.60	7	0.91	6	0.72	7	1.00	7	6.55
R5-	3.52	8	2.74	6	0.78	4	3.13	9	3.11	9	3.08	9	0.79	4	0.55	9	0.91	6	0.78	4	1.08	4	6.55
R112+	3.61	7	3.26	4	0.35	2	3.44	6	3.43	5	3.43	5	0.35	1	0.67	5	1.08	4	0.90	1	1.25	1	3.73
R112-	3.37	9	3.04	5	0.33	1	3.21	8	3.20	8	3.20	6	0.35	2	0.59	8	1.01	5	0.90	2	1.25	2	5.09
R69-9/R5+	4.01	5	3.38	2	0.63	3	3.70	4	3.68	3	3.67	3	0.56	3	0.78	3	1.12	2	0.84	3	1.17	3	3.09
R69-9/R5-	4.38	4	3.30	3	1.08	6	3.84	3	3.80	2	3.76	2	0.88	5	0.83	2	1.10	3	0.75	5	1.05	5	3.64
R69-9/R112+	4.91	3	3.59	1	1.32	7	4.25	1	4.20	1	4.15	1	0.96	6	1.01	1	1.19	1	0.73	6	1.02	6	3.09
R69-9/R112-	5.05	1	2.68	8	2.37	8	3.87	2	3.68	4	3.50	4	1.67	8	0.78	4	0.89	8	0.53	8	0.74	8	5.73
Margherita	4.94	2	2.32	9	2.62	9	3.63	5	3.39	6	3.16	8	1.89	9	0.66	6	0.77	9	0.47	9	0.65	9	7.36

Table 4.11. Values of stress indices and ranking of the genotypes tested. For acronyms of the stress indices, see the Materials and Methods section (§ 4.2.2.6.).

4.4. Discussion

In the present study the overall performance of a set of primary DW-*Th. ponticum* near isogenic recombinant lines (NIRLs), possessing slightly different amounts of the 7el₁L chromosome arm replacing the distal end of the recipient DW 7AL arm, and of secondary DW-*Thinopyrum* spp. NIRLs, carrying a *Th. elongatum* 7EL chromosome segment distally inserted (“nested”) into the 7el₁L ones, was evaluated under a severe heat regime imposed at anthesis. The results here obtained, based on variation of physiological traits detected in flag leaves, osmolytes accumulation into yield-contributing tissues (flag leaves and spikes), and overall plant productivity, largely confirmed the value of specific *Thinopyrum* spp. introgressions in enhancing DW heat tolerance. In fact, the primary NIRL named R112+ (carrier of a 28%-long 7el₁L chromosome segment), substantiated its remarkable yield stability previously observed under a milder heat imposition at the same anthesis stage (Giovenali et al., 2023, see Chapter 3), whereas the secondary NIRLs R69-9/R5+ and R69-9/R112+ turned out to be additional valuable heat-tolerant genotypes.

The heat treatment imposed here (37/27°C d/n applied for 3 consecutive days starting from anthesis date of the main culms) proved to have a strong impact on almost all the physiological, biochemical and yield-related parameters analysed (**Table 4.4**), suggesting the involvement of a number of response mechanisms in the treated genotypes. These observations are in line with those of Narayanan et al. (2015, 2018), who reported that in wheat high temperatures applied during anthesis throughout day and night worsen the negative effects on physiological, biochemical and yield parameters compared to the heat stress imposed at day or night only.

All the physiological measurements performed on control and stressed plants, confirmed that the heat regime applied caused a strong reduction of flag leaves' chlorophyll content (SPAD, up to 21%), photosynthetic efficiency (Fv/Fm and PI, up to -4% and -57%, respectively) and stomatal conductance (SC, up to -86%). As previously reported in the general Introduction (see Chapter 1, § 1.3.2.), photosynthesis is known to be severely hampered in wheat by heat stress, because of chloroplasts disruption and inactivation of key enzymes and complexes, such as the PSII (Akter and Islam, 2017; Ni et al., 2018; Dhakal et al., 2021). Strictly connected with the maintenance of the photosynthetic process is also the rate of CO₂ uptake in the leaves and hence the regulation of stomatal aperture (Faralli et al., 2019). In fact, even if stomatal opening is generally induced by heat stress to promote evaporative cooling (Bahar et al., 2011; Aparecido et al., 2020), here a common stomatal closure was observed in all genotypes response to the intense heat stress applied, even in the heat tolerant cv. Margherita, this probably exacerbating the negative effects of prolonged high temperature stress on the photosynthetic efficiency of the genotypes. Despite this, the R112+ line exhibited the lowest SPAD decrease among the NIRLs+ after 3 days of IH imposition and, together with the R69-9/R112+ derived line, a high photosynthetic efficiency during the first 2 days of stress (**Table 4.6**, **Figure 4.2**). The preservation of both the maximal photochemical efficiency (Fv/Fm) and of the overall PSII performance (PI) appears to be related to the presence of the proximal portion of the *Th. ponticum* segment shared by R112+ and R69-9/R112+ (**Figure 1.8**), since the same behaviour was not observed in the corresponding sibs-. This interesting evidence of photosynthetic stability under an intense heat stress was detected here for the first time and could be one of the underlying mechanisms of the improved heat tolerance observed for these two genotypes, also in terms of yield stability. In fact, the selection of tolerant plants on the base of high Fv/Fm values under heat stress conditions is a widely used strategy (Sharma et al., 2014; Zhou et al., 2015).

Confirming the negative impact that the intense heat stress had on plant physiology, a general increase of the flag leaf MDA content, and hence of membrane lipid peroxidation, was detected

in almost all genotypes (**Table 4.8**). In detail, R112+, R112– and R69-9/R5+ showed the minimal MDA variation (from –6 to + 7%) in IH vs. C conditions, while the opposite was true for Margherita and R69-9/R5– (about +45% MDA increase). Moreover, a statistically significant drop in RWC was detected in R5+ and both the NIRLs– of R69-9 type (**Table 4.8A**). Since the same severe decrement was not exhibited by R5–, a negative impact of the 23%-long 7el₁L chromosome segment (carried by R5+ but not by R5–) can be inferred for RWC under the influence of the severe heat treatment at anthesis, even if under a milder heat treatment the opposite trend was reported (Giovenali et al., 2023).

On the other hand, the 7EL chromosome segment derived from *Th. elongatum*, common to R69-9/R5+ and R69-9/R112+, seemed be involved in RWC preservation, since these two recombinants did not significantly decrease their RWC under IH vs. C conditions (**Table 4.8A**). From the results of RWC and MDA parameters, it clearly emerged the positive performance under the IH stress of R69-9/R5+, showing a minor MDA increase and RWC reduction compared with the other genotypes. This behaviour might also be associated with the observed Pro increase in flag leaves (Pro-FL). In fact, R69-9/R5+ exhibited the highest osmolyte content in flag leaves under stress, a general response adopted by R69-9 types, though to a minor degree compared with R69-9/R5+ (**Figure 4.3**). Thus, this trait can be ascribed to a genetic control associated with presence of the specific 7EL segment. Generally, Pro accumulates in plants under several stress conditions (heat included) and high accumulation of this osmolyte is linked to a superior tolerance (see Chapter 1, § 1.3.3.). In fact, heat-tolerant wheat plants tend to accumulate high amount of Pro and sugars in leaves (Dwivedi et al., 2019; Qaseem et al., 2019; Ru et al., 2023), this promoting plant growth and ameliorating the stress effects through osmotic adjustment, RWC maintenance and membrane stability. A positive effect on Pro accumulation in flag leaves could be also observed in association with presence of the 7el₁L segments of R5+ and R112+ recombinants, which increased their Pro-FL values under IH (+95% in R5+ and +210% in R112+), while their sibs– decreased them (–15% in R5–, –20% in R112–).

In the present work, also Pro (Pro-SP) and water soluble carbohydrates (WSC) contents into the spikes were analysed. The spikes, besides being vital sources of sugars for the developing grains, prove to be able to withstand abiotic stress, also thanks to a high osmolyte accumulation (Kumar et al., 2017; Li et al., 2020; Frimpong et al., 2021). Here, a higher Pro content was detected in the spikes than in flag leaves (**Figure 4.3A**), confirming previous results (Giovenali et al., 2023; see Chapter 3). Interestingly, while all the R69-9 types had a lower amount of Pro-SP than the primary types (R5, R112) under control conditions (constitutive), the IH stress caused a Pro increase (induced) in the former genotypes while a Pro decrease in the latter

genotypes (**Figure 4.3A**). The highly contrasting behaviour emerged between the tested genotypes for spike Pro content could be associated with the difference in their chromosomal makeup (*Th. ponticum* vs. *Th. elongatum*) at the distal chromosome ends (see **Figure 1.8**). A more uniform situation involved the control of the WSC content. In fact, all genotypes exhibited a similar WSC in unstressed conditions (values ranging from 0.17 to 0.22 g/g DW; **Table 4.8**) and, although almost all of them increased this trait after the IH stress imposition, only for R112+ a statistically significant increase (+53%) was highlighted (**Figure 4.3A; Table 4.8**). Dreccer et al. (2014) reported that an elevated sugar content in the spike could be indicative of an improved floret fertility (higher number of grains per spike) under high temperatures. In line with this hypothesis, from the analysis of the yield-related parameters, R112+ emerged as the only genotype among the NIRLs+ able to maintain a statistically unchanged grain number per plant (GNP) value (**Table 4.10B, Figure 4.4B**). In fact, R112+ stressed plants slightly decreased the GNP parameter (−12%), whereas all the other genotypes remarkably reduced it (from −23 to −59%). The superiority of R112+ for grain-number traits across different environmental conditions was already detected in field trials (Kuzmanovic et al., 2018) and in controlled experimental conditions (Giovenali et al., 2023), indicating yield stability of R112+ even in hot (but well-watered) environments. Yield stability across different temperature regimes was also exhibited by R69-9/R5+, which significantly reduced only the GNP trait under IH vs C conditions, yet maintaining GYP (**Table 4.10A**). A somewhat lower contribution to maintenance of yield performance under stress was due to the *Thinopyrum* spp. segment of line R69-9/R112+, which, however, had a lower GYP decrease compared to its sib− (**Figure 4.4A**). In summary, the results of the present work showed that, among the chromosomally engineered DW-*Thinopyrum* spp. recombinant lines, the one possessing the 28%-long 7el₁L segment, i.e. R112+, exhibited a remarkable heat tolerance during the extremely heat-sensitive stage of flowering. At the basis of its tolerance, maintenance of photosynthetic efficiency and an improved sugar content in the spike appear as major mechanisms. The secondary recombinants R69-9/R5+ and R69-9/R112+, here tested for the first time under a severe heat stress at anthesis, also showed interesting tolerance mechanisms, particularly the former. The 7EL chromosome segment (derived from *Th. elongatum*) present in both R69-9+ types, revealed to be mainly involved in RWC maintenance and a superior Pro accumulation in flag leaves (mainly in R69-9/R5+) under heat conditions.

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**Chapter 5: Physiological evaluation and yield performance under
rainfed and irrigated field conditions of durum wheat-
Thinopyrum ponticum recombinant lines**

Chapter 5: Physiological evaluation and yield performance under rainfed and irrigated field conditions of durum wheat-*Thinopyrum ponticum* recombinant lines

Abstract

Drought stress is a global problem that seriously affects crop productivity. Durum wheat (DW) cultivation is severely hampered by water shortage, especially in semiarid areas of Mediterranean basin, where precipitation scarcity and heat waves are frequent issues in the spring-summer period. Thus, screening and selection of new tolerant genotypes that maintain yield stability under drought is a breeding priority. In order to widen wheat genetic basis and potentially improve its stress tolerance, the introgression of novel genes/QTLs derived from wild relatives represents a suitable and sustainable strategy. In this work, some DW varieties and three DW-*Thinopyrum ponticum* near-isogenic recombinant lines (NIRLs), carriers (+) and non-carriers (–) of differently sized 7el₁L chromosome segments transferred from the wild species on DW 7AL arms, were tested for two consecutive growing seasons under irrigated and rainfed field conditions. Physiological measurements (chlorophyll content and photosynthetic efficiency) performed on flag leaves and the analysis of yield-contributing traits (i.e. grain number, grain weight and yield) highlighted differences in the genotypes responses to water limitation. In particular, the NIRL carrier of the 23%-long 7el₁L chromosome segment, named R5+, turned out to be one of the most promising among the genotypes tested, mainly thanks to the maintenance of chlorophyll content and grain weight across different water regimes. In fact, R5+ ranked within the first positions for thousand grain weight in both the field trials and also for grain yield in the second one, thus confirming results obtained from experiments under controlled conditions, altogether indicating a remarkable tolerance to water deficit associated with this introgression.

5.1 Introduction

The increment in frequency and severity of drought phenomena is a serious threat for the agricultural system. Several areas worldwide, including semiarid Mediterranean regions, are characterized by strong seasonal variability in precipitation and severe peaks of drought and heat in the spring-summer period, and such phenomena are expected to further increase in the next future (Hasanuzzaman et al., 2018; Naumann et al., 2018). Water shortage, often

accompanied by high temperature, low humidity and high irradiance, is one of the most adverse, yield-limiting factors for crop species such as wheat, considering that about 9-10% of cereal losses are due to drought and extreme weather conditions (Hasanuzzaman et al., 2018). The effects of drought phenomena on wheat productivity may differ depending on the growth stage in which the stress occurs, but also on stress duration and intensity (Khadka et al., 2020). Generally, drought stress prevails in wheat during the grain filling period, when, due to low winter rainfall, high evaporation and/or shortage of irrigation (Saeidi and Abdoli, 2015), the plant photosynthetic system is strongly impaired and early leaf senescence induced. In fact, water shortage promotes stomata closure, reducing carbon assimilation (see Chapter 1, §1.3.2.), hence reducing photosynthetic rate (Sikder et al., 2015; Saeidi and Abdoli, 2015). In order to achieve the food security goals for an increasing human population (see Chapter 1, § 1.1.3.), breeding for drought-resistant wheat genotypes is among the priority goals (Hammer et al., 2021; Varshney et al., 2021; Xiong et al., 2022). In breeding activities, grain yield is at the basis of selection, despite yield is a complex trait, affected by several factors (Khadka et al., 2020). The evaluation of the physiological response to drought stress, as to other abiotic stresses, may be more efficient, especially if targeted on flag leaf. In fact, flag leaves' photosynthesis in wheat contributes 30% to 50% of the assimilates for grain filling, thus being a main contributor to grain yield (Biswal et al., 2013; Saeidi and Abdoli, 2015; Murchie et al., 2023). For this reasons, measurements based on flag leaf chlorophyll content, that is positively correlated with yield even under water-limited conditions, and on chlorophyll fluorescence, can be employed as useful, non-destructive methods to assess the wheat physiological response to drought (Sikder et al., 2015; Khadka et al., 2020).

Here, the physiological and yield response of DW-*Thinopyrum ponticum* near-isogenic recombinant lines (NIRLs), together with some DW varieties cultivated in Italy and in harsh environments of Algeria and Tunisia, was evaluated in field trials carried out in one environment of central Italy across two seasons and under two water regimes, rainfed and irrigated.

5.2 Materials and methods

5.2.1. Plant materials

Three DW-*Thinopyrum ponticum* NIRLs were evaluated. The NIRLs, originally named R5-2-10, R112-4 and R23-1 and here referred to as R5+, R112+ and R23+, respectively, were

previously obtained via chromosome engineering strategy (Ceoloni et al., 2005). They were developed by repeated back-crossing (BC) in the background of the Italian DW cv. Simeto (BC₅ for R5+ and R112+ and BC₄ for R23+) followed by several self-generations. The NIRLs+ possess different portions of the tall wheatgrass species *Th. ponticum* 7el₁L chromosome arm replacing 23%, 28% and 40% of the distal end of the recipient DW 7AL arm, respectively (see Chapter 2, **Figure 2.1**). Besides the 3 NIRLs “+”, homozygous carriers of the respective 7el₁L segment, corresponding NIRLs “–”, i.e. homozygous sib lines, non-carrier of the given 7el₁L segment, were field tested. In addition to NIRLs, whose comparison (“+” vs. “–”) was aimed at attributing possible trait variation to presence/absence of the respective alien segment, some representative DW varieties originating from Italy (Simeto, Ettore, Monastir) and from north-African countries (cv. Waha from Algeria and cvs. Om Rabia and Khiair from Tunisia) were also included in the comparative analysis.

5.2.2. Methods

5.2.2.1. Field trials and trait measurements

Seasonal and trial characteristics

Plant physiological and main yield traits were analysed in two growing seasons at the experimental farm of the University of Tuscia (VT, Italy). The growing area is characterized by a Mediterranean climate, with mean annual air temperature of about 14.5 °C and precipitation of 790 mm. On average, total rainfall and mean air temperature during the growing season of durum wheat (from mid-late November to early July) in this area are 468 mm and 11°C, respectively. However, climatic conditions greatly vary from year to year. In the 2019-20 season, precipitations from December 1st to end of June totaled 377 mm, more than 20% less than the site average for the same period, whereas in 2020-21 season, the 688.6 mm amount was well above the average for the site, and 80% more abundant than that of the previous season (**Table 5.1**). Average minimum and maximum temperatures, instead, were not remarkably different between the two experimental seasons, although their values varied across months (**Table 5.1**).

Year	Month	Decade	Rain (mm)	Mean Min. Temp. (°C)	Mean Max. Temp. (°C)
2019	December	1	28.6	5.5	14
2019	December	2	28.2	4.4	13.8
2019	December	3	39.8	3.8	12.6
2020	January	1	0.4	-0.2	12.8
2020	January	2	13.6	2.2	12.3
2020	January	3	10.4	3.5	13.8
2020	February	1	1.4	3.3	14.5
2020	February	2	3.6	2.3	16.5
2020	February	3	8	1.5	16
2020	March	1	56.6	4.6	14.5
2020	March	2	0	4.7	17.5
2020	March	3	15	2.7	14.1
2020	April	1	0.4	4.3	18.9
2020	April	2	19.3	7.1	20.3
2020	April	3	19	8.2	19.3
2020	May	1	0	9	23.6
2020	May	2	24.2	14.3	24.4
2020	May	3	22.2	11.4	24.2
2020	June	1	54.8	12.2	24.3
2020	June	2	28.5	12.5	25.1
2020	June	3	3	14.8	28.5
			TOT. 377	6.3	18.1

Year	Month	Decade	Rain (mm)	Mean Min. Temp. (°C)	Mean Max. Temp. (°C)
2020	December	1	176	4.1	12.3
2020	December	2	0.6	1.4	12.8
2020	December	3	58.6	1.6	11.9
2021	January	1	76.8	1.7	7.45
2021	January	2	0.4	-1.5	9.5
2021	January	3	90.8	0.6	12.21
2021	February	1	39.8	3.3	15.2
2021	February	2	32.8	-0.1	10
2021	February	3	1.4	3.3	18.6
2021	March	1	39.8	3.5	14.4
2021	March	2	3.2	1.4	13.7
2021	March	3	0.8	3.4	17.3
2021	April	1	2.8	3	16.8
2021	April	2	44	4.9	15
2021	April	3	32.6	7.8	19.7
2021	May	1	1.8	7.8	21.1
2021	May	2	16.2	7.7	20.4
2021	May	3	3.4	10.5	23.8
2021	June	1	49.4	12.2	26.2
2021	June	2	16.4	15.3	29.3
2021	June	3	1	16	31.1
			TOT. 688.6	5.1	17.1

Table 5.1. Precipitations and temperatures in the 2019-20 and 2020-21 wheat growing seasons registered at the experimental farm of the University of Tuscia (VT, Italy).

During the first growing season (2019-20), spaced plants were arranged in triplicated, 1.2 m-long rows per genotype and per water regime. Distance between rows was 25 cm, and each row contained 13 plants at 10 cm distance along the row. Two water regimes were compared: rain-fed (RF) and irrigated (IRR) (**Figure 5.1**). During the second (2020-21) growing season, plants (350 seeds sowing density) were arranged in triplicated plots of 3.75 m² (1.5 m × 2.5 m) per genotype and per water regime. Irrigation treatments consisted of 4 (2019-20) and 5 (2020-21) applications, starting from the onset of anthesis, and the amount of water added corresponded to the water necessary to replace the total evapotranspiration (ET) net of precipitations. Total ET was calculated by adding up daily ET occurred between two subsequent irrigations (Kuzmanović et al., 2021 and unpublished). Irrigation was applied through pipes placed between the spaced rows or plots' rows (**Figure 5.2**). All through both the growing seasons, common agronomic practices (e.g. fertilization, weed control) for DW cultivation were adopted. Natural disease pressure was negligible in both experimental years; therefore, no fungicide treatment was applied.

Traits analysed

Physiological parameters measured during the 2019-20 season included chlorophyll content recorded at anthesis (SPAD-0), at 10 (SPAD-10) and 20 (SPAD-20) days post-anthesis (dpa), and quantum yield (QY) of PSII of light-adapted flag leaves recorded at 10 dpa (QY-10) and 20 dpa (QY-20). During the 2020-21 season, physiological measurements included chlorophyll content at anthesis (SPAD-0), 7 (SPAD-7), 14 (SPAD-14), 21 (SPAD-21) dpa and QY at anthesis (QY-0), 7 (QY-7), 14 (QY-14) and 21 (QY-21) dpa. A hand-held chlorophyll meter (SPAD- 502 Plus; Konica-Minolta, Japan) and a portable fluorometer PAR-Fluorpen 110-LM/D (Photon Systems Instruments, Drasov, Czech Republic) were used for SPAD and QY measurements on flag leaves, respectively.

Main production traits, namely grain number/plant (GNP), thousand grain weight (TGW) and grain yield/plant (GYP), were measured during the first growing season on five plants/row; grain number/m² (GNM²), TGW and grain yield/m² (GYM²) were measured during the second growing season on each of the triplicate plots per water regime (RF and IRR).

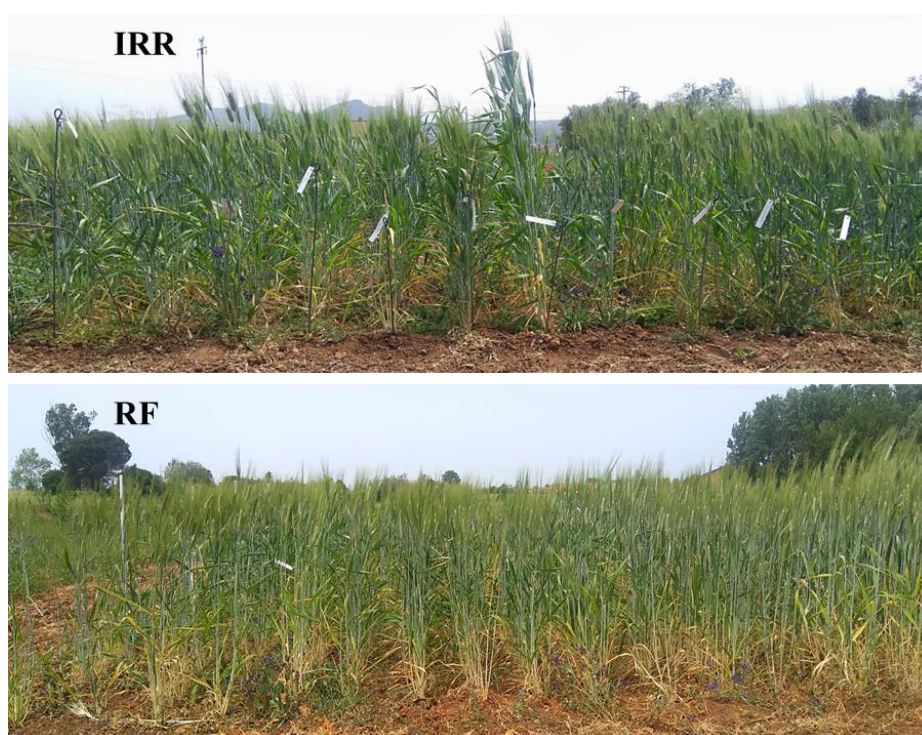


Figure 5.1. Comparative view of irrigated (IRR) and rainfed (RF) rows in the 2019-20 season.



Figure 5.2. Irrigation system applied to plots in the 2020-21 season.

5.2.2.2. Statistical analysis

For all the datasets, one-way analysis of variance (ANOVA) was performed in order to estimate differences ascribable to the genotype (G) effect, while two-factor ANOVA was applied to analyse the effect of the $G \times$ treatment (T) interaction. Before performing ANOVAs, obvious outliers were eliminated. All statistical analyses were performed by JASP software (JASP Team, 2022; JASP Version 0.16.1).

5.3 Results

5.3.1. Traits showing statistically significant differences for genotype (G), treatment (T) and $G \times T$ interaction

The results of the ANOVA for the parameters measured during the 2019-20 (**Table 5.2A**) and the 2020-21 (**Table 5.2B**) field trials, highlighted a number of statistically significant traits, especially for the 2019-20 growing season. Indeed, almost all the traits (with the only exception of GYP) showed to be significant for the G factor, suggesting that they were strongly affected by the genotype considered (**Table 5.2A**). Moreover, the treatment (T = IRR vs. RF) applied during the 2019-20 trial resulted to be effective in the modulation of both the physiological and yield parameters. Considering the $G \times T$ interaction, significant differences were observed for some physiological traits/timing of measurement (SPAD-20, QY-10), as well as for GNP and GYP. Thus, tested genotypes tended to respond in differential manner under the two water

regimes. A lower number of statistically significant parameters emerged from the 2020-21 plot trial (**Table 5.2B**). In fact, among the physiological measurements, only the chlorophyll content at 14 dpa (SPAD-14) resulted to be statistically significant for the G factor. On the other side, all the three yield-related traits (GNM², TGW and GYM²) were statistically significant for the single G and T factors, though the same was not true for their interaction.

A

Factors	G	T	G × T	Residuals
df	11	1	11	
SPAD-0	329.44 ***	0	0	57.68
SPAD-10	151.93 ***	208.29 **	29.43	26.20
SPAD-20	288.57 ***	1557.94 ***	87.36 **	41.14
QY-10	0.01 ***	0.00001	0.01 *	0.004
QY-20	0.05 *	0.43 ***	0.03	0.02
GNP	20502.75 ***	165918.92 ***	17677.65 **	6884.84
TGW	1442.02 ***	4945.06 ***	39.17	52.18
GYP	28.75	1322.36 ***	49.87 **	20.39

B

Factors	G	T	G × T	Residuals
df	11	1	11	
SPAD-0	28.09	68.64	13.45	22.83
SPAD-7	24.08	0.52	22.55	15.27
SPAD-14	49.17 *	19.01	7.07	21.07
SPAD-21	27.37	202.68 **	40.56	21.40
QY-0	0.01	0.00	0.02	0.01
QY-7	0.02	0.03	0.01	0.01
QY-14	0.02	0.01	0.02	0.01
QY-21	0.01	0.08 *	0.03	0.02
GNM ²	21660000 *	129200000 ***	10400000	9859000
TGW	89.94 ***	57.96 *	12.12	8.63
GYM ²	28786.62 **	250719.33 ***	5302.34	10200.76

Table 5.2. ANOVA mean squares for the traits measured under IRR and RF conditions. **A:** Results from the 2019-20 field trial; **B:** results from the 2020-21 field trial. *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively (df, degrees of freedom; G, genotype; T, treatment).

5.3.2. Effects of 7el₁L segments on the traits measured on single plants under irrigated and rainfed conditions

The evaluation of physiological parameters performed on single plants grown along IRR and RF rows (2019-20 season) confirmed some well-known characteristics of the DW-*Th. ponticum* recombinant lines, but at the same time also highlighted new interesting physiological mechanisms involved in their response to water availability. An expected overall decrease of both chlorophyll content (SPAD) and photosynthetic efficiency (QY) can be observed in RF vs. IRR plants, especially in this relatively dry season (see **Table 5.1**). As to the former parameter, slight differences were observed among the genotypes at 10 dpa (SPAD-10), but more significant differences emerged at 20 dpa (SPAD-20; **Figure 5.3**). While the NIRL carrying the

40%-long 7e₁L chromosome segment, i.e. R23+, exhibited the lowest SPAD value across different conditions and time points, those carrying smaller 7e₁L segments, namely R5+ and R112+, showed a high chlorophyll content in RF conditions (SPAD-10 and SPAD-20; **Figure 5.3**). In fact, SPAD values exhibited by R5+ and R112+ (the latter, top ranking) plants under RF conditions resulted even higher than those of the Algerian and Tunisian DW varieties (SPAD-20; **Figure 5.3**). However, this promising aspect of R5+ and R112+ seemed not to be associated with their wild segments' presence, but rather with the interaction between wild segments and the genetic background, as the sibs- of the two recombinants were also among the top performing genotypes having similar values for SPAD (SPAD-20; **Figure 5.3**). In RF conditions, R5+ and R112+ ranked among the best genotypes for photosynthetic efficiency at 10 dpa (QY-10), together with the Italian variety Monastir. On the other hand, at 20 dpa, while R112+ and, more markedly, R5+ had severe drops in QY-20 (-18% and -37%, respectively), R23+ maintained a relatively high photosynthetic efficiency (in spite of low chlorophyll content, see above), together with the Algerian cv. Waha and the Tunisian cv. Khiair (**Table 5.3**; **Figure 5.3**).

	SPAD-0				SPAD-10				SPAD-20			
	IRR		RF		IRR		RF		IRR		RF	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
R5+	51.99	2.04	51.99	2.04	51.79	2.72	51.42	1.83	50.06	2.11	46.23	2.56
R5-	50.65	1.82	50.65	1.82	55.26	2.26	57.46	0.68	52.19	0.65	46.51	2.92
R112+	51.51	1.65	51.51	1.65	52.96	0.97	52.49	0.92	53.83	1.90	50.96	2.12
R112-	53.97	2.80	53.97	2.80	55.06	0.99	52.58	0.99	51.53	1.87	47.27	1.89
R23+	43.47	1.93	43.47	1.93	48.14	1.22	41.48	0.89	39.17	1.15	38.24	2.74
R23-	49.30	1.78	49.30	1.78	51.54	1.19	47.46	1.92	48.67	1.30	44.53	1.84
Simeto	60.60	5.75	60.60	5.75	56.42	1.21	52.26	2.14	56.76	2.45	41.03	2.78
Ettore	45.70	1.06	45.70	1.06	50.86	1.45	50.16	1.11	47.91	1.86	41.03	2.99
Monastir	49.64	1.64	49.64	1.64	51.03	0.85	49.62	1.01	49.19	0.76	43.09	0.93
Waha	53.01	0.93	53.01	0.93	54.41	3.69	50.09	1.33	48.64	1.48	42.46	1.97
Om-Rabia	53.42	3.08	53.42	3.08	48.77	2.62	50.17	2.36	39.30	2.81	41.18	2.78
Khlar	51.06	2.17	51.06	2.17	52.67	1.84	50.11	0.92	47.64	2.07	37.90	2.70
Mean	51.19				52.41		50.44		48.74		43.37	

	QY-10				QY-20			
	IRR		RF		IRR		RF	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
R5+	0.66	0.01	0.70	0.02	0.56	0.06	0.35	0.04
R5-	0.61	0.03	0.69	0.02	0.44	0.04	0.47	0.06
R112+	0.69	0.02	0.71	0.01	0.50	0.04	0.41	0.06
R112-	0.64	0.03	0.63	0.02	0.43	0.05	0.40	0.06
R23+	0.71	0.02	0.68	0.02	0.63	0.01	0.48	0.05
R23-	0.70	0.02	0.72	0.01	0.56	0.05	0.43	0.06
Simeto	0.66	0.03	0.62	0.03	0.42	0.05	0.39	0.06
Ettore	0.70	0.01	0.62	0.02	0.53	0.07	0.40	0.05
Monastir	0.72	0.01	0.71	0.01	0.61	0.03	0.41	0.07
Waha	0.69	0.02	0.67	0.02	0.51	0.06	0.51	0.04
Om-Rabia	0.66	0.02	0.66	0.02	0.49	0.05	0.46	0.05
Khlar	0.65	0.02	0.69	0.01	0.63	0.04	0.54	0.06
Mean	0.67		0.67		0.53		0.44	

	GNP				TGW				GYP			
	IRR		RF		IRR		RF		IRR		RF	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
R5+	198.75	18.23	150.36	15.01	67.32	2.00	59.56	2.43	13.54	1.30	8.77	0.94
R5-	170.17	18.38	159.64	16.16	65.54	2.39	57.03	2.70	10.97	1.19	8.93	0.87
R112+	203.13	25.76	156.39	18.52	62.72	1.57	54.66	2.98	12.49	1.53	8.26	0.99
R112-	225.13	22.76	144.69	18.17	65.73	0.82	58.04	1.95	14.78	1.49	8.29	0.92
R23+	237.18	27.38	224.86	15.36	43.29	1.21	39.37	1.21	9.67	1.24	8.62	0.64
R23-	207.53	13.84	181.71	18.82	60.85	1.36	51.09	2.75	12.64	0.84	9.50	1.19
Simeto	191.88	28.83	94.07	9.49	72.10	0.54	63.72	1.65	13.78	2.04	5.68	0.69
Ettore	229.27	25.35	161.33	20.50	54.02	1.45	46.88	2.71	12.30	1.39	8.10	1.17
Monastir	284.07	26.89	143.87	19.03	55.35	1.18	51.63	1.98	15.63	1.46	7.46	1.05
Waha	289.17	39.34	203.92	23.09	53.60	0.55	46.04	2.00	15.42	2.06	9.13	0.94
Om-Rabia	148.25	18.14	194.00	31.47	59.59	0.76	47.36	3.56	8.72	0.98	8.78	1.38
Khlar	200.93	25.97	217.62	35.29	52.64	2.05	41.94	1.93	10.21	1.14	9.44	1.71
Mean	215.45		169.37		59.39		51.44		12.51		8.41	

Table 5.3. Means and standard errors (SE) for physiological and yield traits measured during the 2019-20 season. IRR, irrigated; RF, rain-fed. For the significance of traits acronyms see Materials and Methods, § 5.2.2.1.

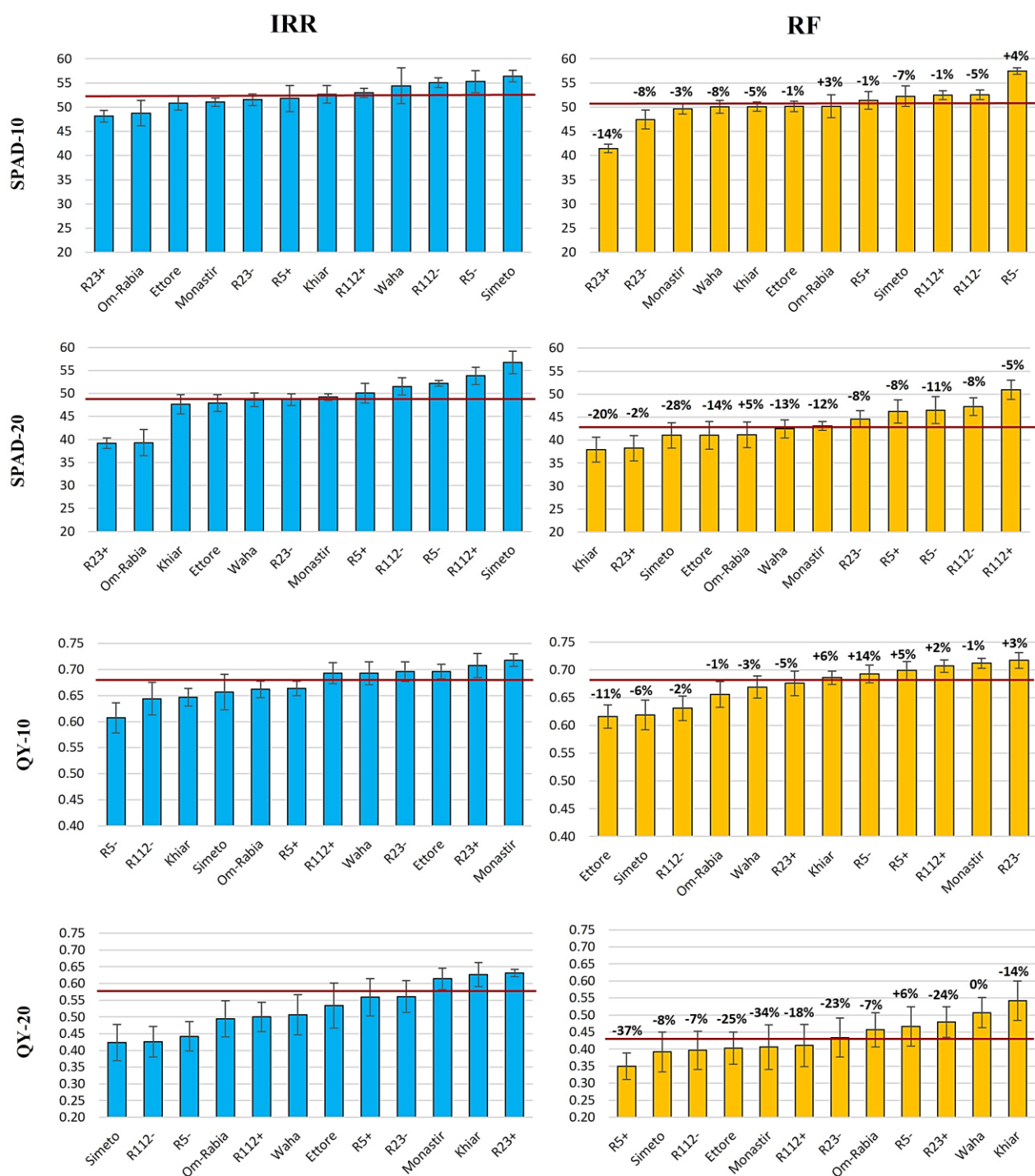


Figure 5.3. Physiological evaluation of DW genotypes under irrigated (IRR) and rainfed (RF) conditions in the 2019-20 field trial. For the significance of traits acronyms see Materials and Methods, § 5.2.2.1. The red lines on the bars indicate the mean value among the genotypes. The numbers above the bars indicate the % variations of the corresponding genotype in RF vs. IRR conditions.

Considering the yield performance of the tested genotypes, an overall decrease in grain yield (GYP) and in its components (grain number/plant, GNP and thousand grain weight, TGW) was observed in RF vs. IRR conditions (**Table 5.3**). During the 2019-20 field trial, a 21% reduction was detected for GNP (means of 215 in IRR and 169 in RF), 13% for TGW (means of 59 g in IRR and 51 g in RF) and 33% for GYP (means of 13 g in IRR and 8 g in RF). Among the NIRLs+ (and among all the genotypes in RF condition), R23+ showed the highest GNP and only a slight decrease (–5%) of this trait in water-limited conditions (**Figure 5.4**). Moreover, R23–, the genetic control of R23+ (non-carrier of the alien segment), showed a higher 12% decrease in GNP in RF vs. IRR conditions, this suggesting the involvement of the 40%-long 7el₁L segment in grain number maintenance under water shortage. On the other hand, stressed plants of both R5+ and R112+ had about a 23% decrease in GNP, settling on similar absolute values of the corresponding sibs– (**Figure 5.4**; **Table 5.3**). Interestingly, the two Tunisian varieties tested, that is Om Rabia and Khiair, were the only ones to increase (+31% and +8%, respectively) the GNP parameter under RF. Moreover, the Algerian variety Waha, although showing a heavy decrement (–29%) vs. IRR condition (where it ranked first for GNP), had one of the highest GNP value among all the genotypes in RF condition (**Figure 5.4**). On the other hand, to maintain a relatively high TGW in the RF vs. IRR comparison, were the Italian cv. Simeto, followed by R5 and R112 NIRLs, having Simeto as background (**Figure 5.4**). Confirming the trade-off existing between grain number and weight, R23+ exhibited the lowest TGW in both IRR and RF conditions. Finally, considering the GYP trait, all the NIRLs+ showed similar values under RF conditions (**Table 5.3**), even if R23+ had the lowest % variation (–11%) among the NIRLs+ (–35% in R5+ and –34% in R112+). The Tunisian variety Om Rabia emerged as the most stable genotype, though not the top yielding, across different water regimes.



Figure 5.4. Yield performance of DW genotypes under irrigated (IRR) and rainfed (RF) conditions in the 2019-20 field trial. For the significance of traits acronyms see Materials and Methods, § 5.2.2.1. The red lines on the bars indicate the mean value among the genotypes. The numbers above the bars indicate the % variations of the corresponding genotype in RF vs IRR conditions.

5.3.3. Effects of 7el₁L segments on the traits measured on plots under irrigated and rainfed conditions

The evaluation of physiological traits performed on IRR and RF plots during the 2020-21 field trial partially confirmed the observations of the previous season. Whereas R23+ plants confirmed to possess low SPAD values, a lower decrement in chlorophyll content than in R23– plants was detected in RF vs. IRR conditions (SPAD-7, SPAD-21; **Table 5.4**; **Figure 5.5**). Moreover, also R5+ and R112+ confirmed the trend of chlorophyll content (i.e. high SPAD

values in RF plants and better percent variations than the sibs–). On the other hand, new evidence emerged about the photosynthetic efficiency of the genotypes. In fact, although R23+ was not ranked among the best-performing genotypes for the QY trait (QY-7 and QY-21; **Figure 5.5**), it only had a slight variation in RF vs. IRR conditions. Moreover, RF plants of R5+ and, to a minor extent, R112+ displayed a strong QY increase vs. IRR conditions (**Figure 5.5**), while in the previous field trial a severe drop was observed for these genotypes. The observed differences in genotypes' response between seasons have to take into account the influence of conspicuous weather differences, particularly precipitation amount (see **Table 5.1**).

	SPAD-0				SPAD-7				SPAD-14				SPAD-21			
	IRR		RF		IRR		RF		IRR		RF		IRR		RF	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
R5+	52.93	2.22	49.27	6.01	53.00	2.02	55.27	1.67	50.73	3.13	56.67	1.78	46.07	1.60	50.40	2.76
R5-	54.63	1.86	54.23	2.50	53.17	1.19	49.10	5.19	50.23	1.64	52.27	2.48	50.33	2.29	48.47	3.94
R112+	51.23	0.81	51.53	0.52	50.80	1.06	51.93	0.22	53.73	1.15	52.90	1.39	51.03	3.21	47.63	2.32
R112-	52.40	3.50	52.73	1.19	51.20	2.48	49.00	2.60	52.50	1.54	51.97	2.80	52.70	0.84	45.47	3.09
R23+	46.87	0.94	46.93	0.43	49.40	1.56	47.60	0.67	43.57	1.75	44.20	0.58	44.53	3.38	42.20	2.06
R23-	53.63	1.41	49.40	1.96	54.80	0.12	48.67	2.64	50.60	1.53	49.10	1.07	54.53	0.75	45.37	3.54
Simeto	52.83	1.92	48.60	4.76	52.67	1.39	54.27	1.50	49.03	3.06	49.43	5.53	54.13	4.73	46.03	0.76
Ettore	54.30	0.61	49.27	2.48	54.63	0.88	50.17	1.92	48.60	2.38	51.00	0.49	46.97	1.38	47.93	0.62
Monastir	50.13	1.87	49.03	2.83	44.07	3.53	50.07	3.11	49.70	0.61	49.20	1.47	47.40	1.08	43.13	4.57
Waha	45.63	4.13	48.67	2.11	49.30	0.91	51.40	0.92	48.87	3.73	51.30	3.53	46.40	0.85	46.60	4.09
Om-Rabia	52.73	2.90	45.10	5.18	50.53	0.37	48.57	3.37	48.77	0.83	47.73	1.86	46.70	2.55	50.00	1.00
Khlar	51.63	1.71	50.77	2.53	49.00	4.02	54.50	1.71	44.27	5.92	47.17	3.54	51.80	2.69	39.10	2.48
Mean	51.58		49.63		51.05		50.88		49.22		50.24		49.38		46.03	

	QY-0				QY-7				QY-14				QY-21			
	IRR		RF		IRR		RF		IRR		RF		IRR		RF	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
R5+	0.56	0.05	0.64	0.02	0.48	0.03	0.59	0.04	0.60	0.05	0.61	0.05	0.49	0.04	0.62	0.02
R5-	0.58	0.03	0.58	0.04	0.54	0.05	0.58	0.03	0.62	0.04	0.62	0.03	0.51	0.03	0.53	0.04
R112+	0.60	0.04	0.51	0.03	0.55	0.05	0.60	0.04	0.64	0.03	0.65	0.03	0.56	0.05	0.58	0.03
R112-	0.63	0.02	0.58	0.03	0.59	0.03	0.57	0.04	0.56	0.04	0.59	0.03	0.51	0.06	0.52	0.04
R23+	0.56	0.04	0.51	0.04	0.56	0.04	0.53	0.04	0.62	0.03	0.61	0.03	0.55	0.04	0.57	0.04
R23-	0.55	0.05	0.58	0.03	0.61	0.04	0.54	0.04	0.63	0.05	0.54	0.04	0.55	0.04	0.57	0.04
Simeto	0.60	0.03	0.64	0.03	0.50	0.05	0.56	0.05	0.50	0.04	0.55	0.04	0.53	0.05	0.59	0.05
Ettore	0.56	0.03	0.60	0.04	0.61	0.03	0.59	0.04	0.56	0.05	0.65	0.03	0.48	0.04	0.67	0.02
Monastir	0.61	0.04	0.55	0.04	0.50	0.04	0.49	0.04	0.60	0.03	0.62	0.04	0.48	0.04	0.58	0.03
Waha	0.63	0.04	0.63	0.04	0.61	0.04	0.62	0.02	0.56	0.04	0.67	0.02	0.57	0.04	0.49	0.05
Om-Rabia	0.62	0.03	0.57	0.04	0.52	0.04	0.61	0.03	0.70	0.01	0.60	0.03	0.61	0.04	0.57	0.04
Khlar	0.51	0.05	0.60	0.03	0.49	0.05	0.53	0.04	0.62	0.05	0.63	0.03	0.60	0.04	0.60	0.04
Mean	0.58		0.58		0.55		0.57		0.6		0.61		0.54		0.57	

	GNM2				TGW				GYM2			
	IRR		RF		IRR		RF		IRR		RF	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
R5+	17131.16	683.20	13806.90	644.38	50.25	1.37	48.23	1.33	658.00	34.86	549.48	46.67
R5-	17099.66	576.48	16377.07	1474.88	47.81	2.77	45.65	0.36	485.05	60.74	421.57	53.28
R112+	17728.49	718.62	14718.84	2676.64	48.35	0.29	43.86	2.05	656.75	8.99	523.03	49.18
R112-	15361.01	1475.45	11182.62	1962.61	45.70	0.83	44.47	0.97	557.02	26.43	477.20	25.16
R23+	18487.76	527.98	15383.61	708.23	37.10	0.29	36.34	1.12	524.88	46.88	391.69	3.96
R23-	20312.37	2268.47	13491.93	2442.80	45.26	3.24	46.04	1.57	684.60	79.99	470.45	28.13
Simeto	16198.84	1177.48	13837.29	639.49	49.58	1.17	44.41	3.20	601.82	92.28	457.38	3.06
Ettore	19989.25	2128.32	14653.99	1397.36	46.28	2.72	41.46	1.74	695.56	52.99	588.97	10.15
Monastir	18668.59	1918.00	22189.61	901.43	43.63	3.24	42.82	0.58	696.04	53.10	529.21	31.79
Waha	18125.35	672.95	17864.49	3647.33	40.22	0.78	37.81	0.60	434.05	117.70	454.64	48.82
Om-Rabia	16541.23	1344.95	13594.20	1061.86	38.55	0.58	43.71	1.57	549.82	121.42	384.80	55.06
Khlar	20659.21	3348.62	17056.67	3034.29	40.45	0.19	36.85	0.83	597.06	88.96	475.97	35.49
Mean	18025.24		15346.44		44.43		42.64		595.05		477.03	

Table 5.4. Means and standard errors (SE) for physiological and yield traits measured during the 2020-21 field trial. IRR, irrigated; RF, rain-fed. For the significance of traits acronyms see Materials and Methods, § 5.2.2.1.

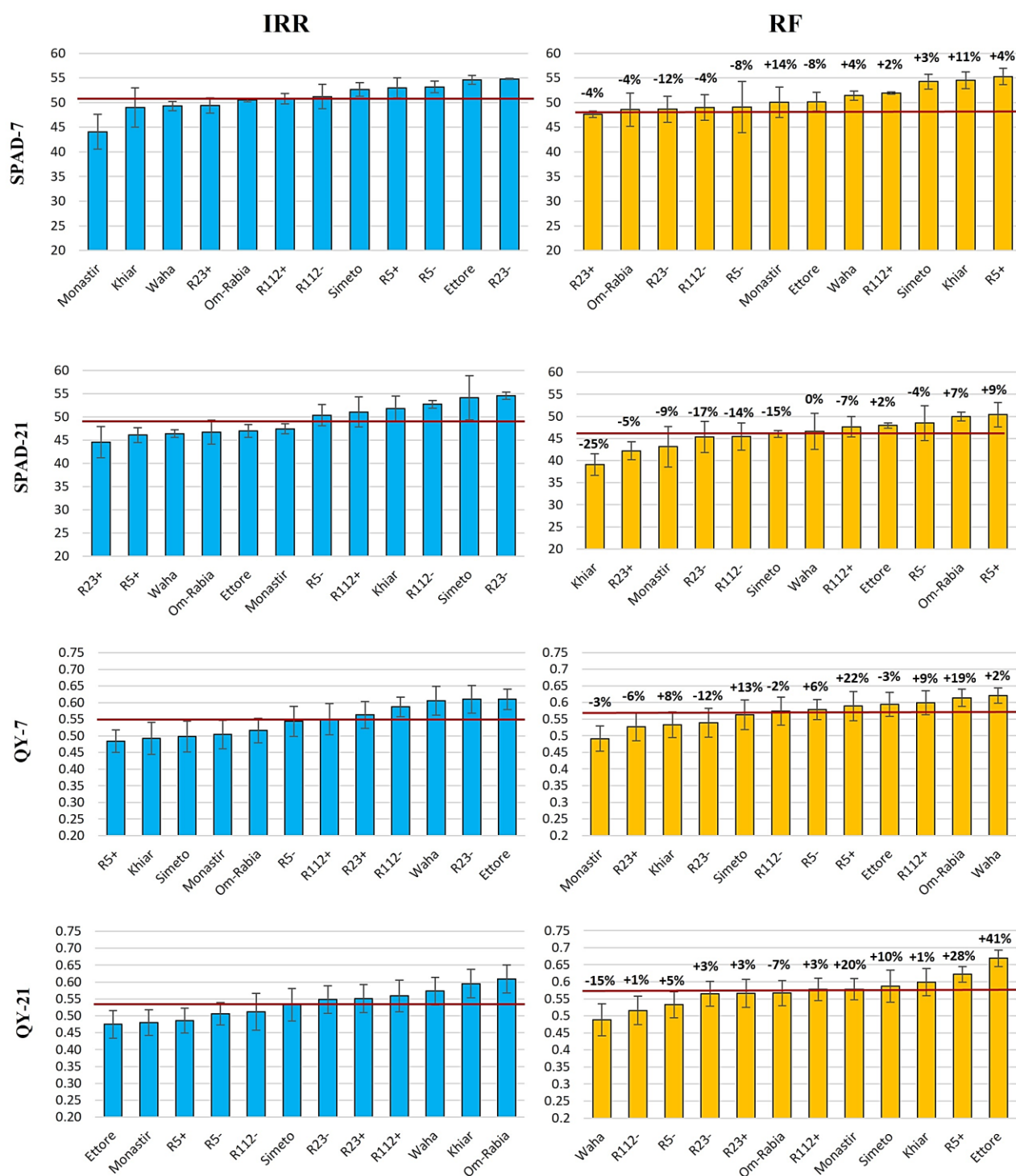


Figure 5.5. Physiological evaluation of DW genotypes under irrigated (IRR) and rainfed (RF) conditions in the 2020-21 field trial. For the significance of traits acronyms see Materials and Methods, § 5.2.2.1. The red lines on the bars indicate the mean value among the genotypes. The numbers above the bars indicate the % variations of the corresponding genotype in RF vs IRR conditions.

As observed in the 2019-20 growing season, an overall decrease in the yield-related parameters was evident in all the water-stressed genotypes. However, lower reductions were detected during the 2020-21 field trial, likely due to the more abundant precipitations, with a 15% decrement for GNM² (means of 18025 in IRR and 15346 in RF), 4% for TGW (means of 44 g in IRR and 43 g in RF) and 20% for GYM² (595 g in IRR and 477 g in RF). RF plants of R23+ confirmed to have an absolute higher grain number (GNM²) value and a lower decrement compared with its sib- (**Figure 5.6**), together with a lower TGW than R23- in both IRR and RF conditions. A strong GNM² increment (+19%) was detected in RF plants of the Italian variety Monastir (opposite behaviour in the previous season, **Figure 5.4**), whereas both Khiair and Waha confirmed a good grain number value in water-limited environments (**Table 5.3**). Looking at the TGW trait, Simeto and all the NIRLs- (highly isogenic to Simeto), confirmed their high grain weight under RF conditions. Among the NIRLs+ and all the other genotypes, R5+ turned out to have the highest seed weight, conferring it the second-best position in GYM² ranking under RF conditions, the first one being the Italian cv. Ettore (**Table 5.4; Figure 5.6**). Unlike the grain yield results of the 2019-20 field trial, which identified Om Rabia as the most stable genotype across the two water regimes, in 2020-21 it was Waha to benefit from RF rather than IRR conditions. As above outlined, the different precipitation amounts in the two experimental years may have considerably influenced the observed differences in genotypes' yield behaviour.

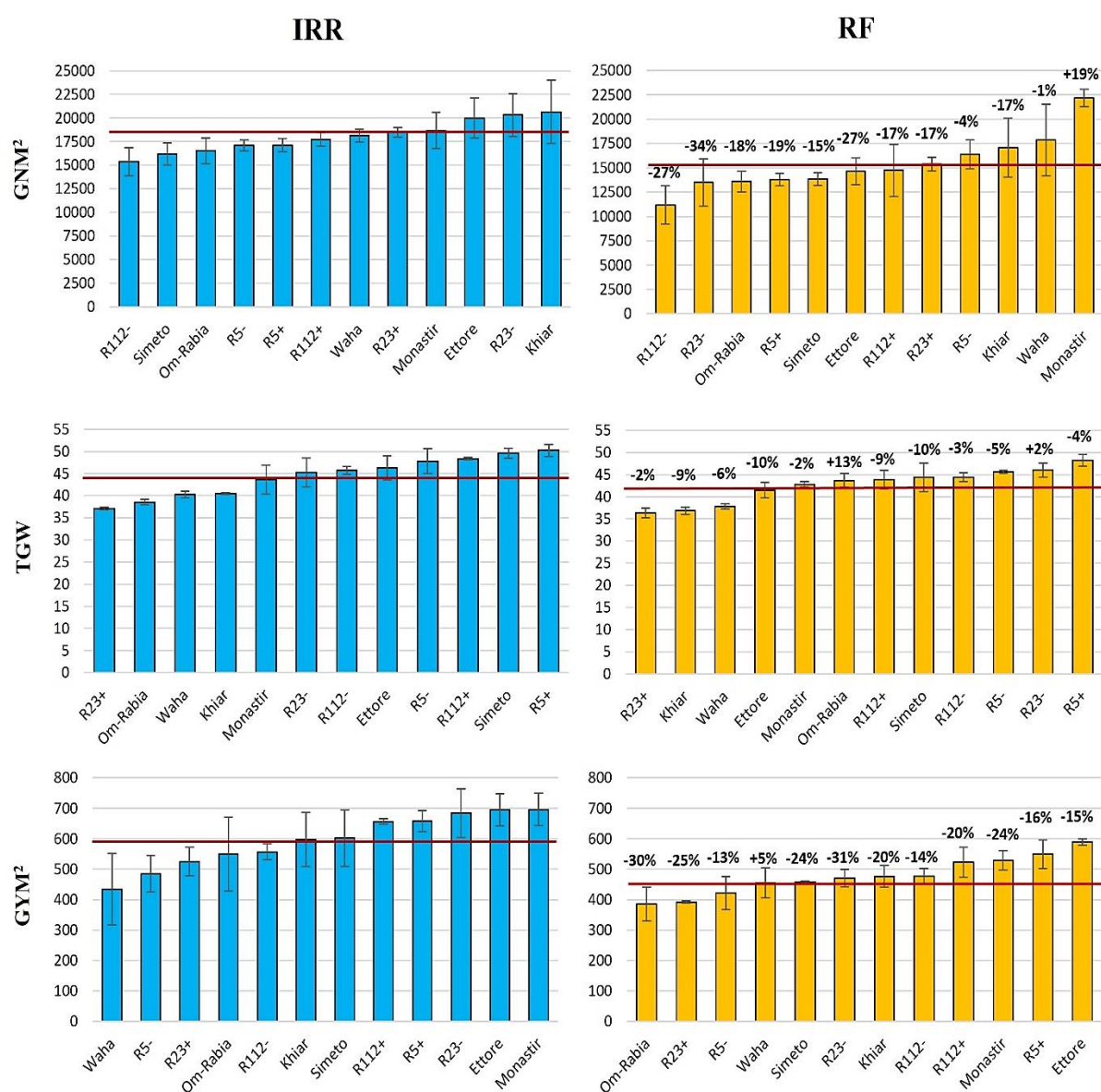


Figure 5.6. Yield performance of DW genotypes under irrigated (IRR) and rainfed (RF) conditions in the second field trial. For the significance of traits acronyms see Materials and Methods, § 5.2.2.1. The red lines on the bars indicate the mean value among the genotypes. The numbers above the bars indicate the % variations of the corresponding genotype in RF vs IRR conditions.

5.4 Discussion

In the present work, three DW-*Th. ponticum* NIRLs+, carriers of a differently sized 7e1L chromosome segment derived from the wild species on DW 7AL arm, were evaluated from the physiological and productive point of views during two growing seasons (2019-20 and 2020-21) in a central-Italy environments where DW is a typical crop. In both seasons, two water

regimes were applied, i.e. rainfed (RF) the one normally adopted by farmers, and supplementary irrigation (IRR, control). In the test environment, as in many Italian DW crop areas, terminal drought phenomena frequently occur, which of course can have variable impacts on final yield depending on the seasonal amount of precipitations. To this respect, the two experimental seasons analysed here were characterized by very contrasting amounts, the first year (2019-20) being much drier than the second one (2020-21). However, in considering the differences observed between the two seasons in genotypes' behaviour (even under the same water regime), not only weather differences must be taken into account, but also the different planting management (spaced plants and plots). While further and larger-scale plot trials are being carried out, some considerations can here be made, with particular focus on NIRLs' behaviour. These are enabled by the parallel analysis of recombinant lines (+) and corresponding sib lines lacking the alien segments (-), which permits to associate possible differences to presence/absence of the wild 7el₁L segments. Moreover, inclusion of some DW varieties allowed for comparison of the overall NIRLs+ behaviour to that of DW varieties well adapted to even stressful Mediterranean areas.

In general, an overall decline in the photosynthetic activity (chlorophyll content, SPAD; chlorophyll efficiency, QY) and a penalty of yield parameters (grain number per plant, GNP, and per unit area, GNM²; thousand grain weight, TGW; grain yield per plant, GYP, and per unit area, GYM²) was observed in RF vs. IRR conditions, this confirming the negative impact of water shortage on wheat productivity. In the 2019-20 trial, i.e. the one with the lowest rainfall (**Table 5.1**), the greatest mean reduction in grain yield was recorded (-33% in RF vs. IRR conditions). However, also in the 2020-21 trial, despite the higher amount of rain, a yield reduction of -20% was detected. Thus, water availability was undoubtedly confirmed as one of the main determining factors for DW yield. In this frame, the behaviour of DW-*Th. ponticum* recombinants somewhat confirmed previous observations on their yield performance, and also highlighted new interesting features.

First of all, presence of the 23%-long 7el₁L segment in the R5+ NIRL, confirmed to be associated with an elevated TGW under RF conditions (**Tables 5.3 and 5.4**; Kuzmanovic et al., 2020; Kuzmanovic et al., 2021). Secondly, the most proximal portion of the 40%-long 7el₁L segment, specific to the R23+ NIRL, confirmed to include genetic factor(s) enhancing the grain number (GN) trait. In fact, a high increase in GN was detected in R23+ plants grown in the dry environments of South Australia and Morocco (Kuzmanovic et al., 2018) and this characteristic has been tentatively associated with the recombinant's constitutively higher floret survival rate (Kuzmanovic et al., 2014; Kuzmanovic et al., 2016; Kuzmanovic et al., 2018). Here, R23+

emerged as the best genotype for GNP (**Figure 5.4**) and among the best genotypes for GNM² (**Figure 5.6**) in RF conditions, showing in both seasons a lower trait decrement in the IRR vs. RF comparison than its corresponding sib⁻. However, due to the usually occurring trade-off between grain number and weight, R23+ ranked last for the TGW trait in both years (**Tables 5.3** and **5.4**). It is well-known that wheat yield is mainly the result of grain number and grain weight, but several studies report that GN as a sink trait is the key component determining yield in cereals (e.g. Dolferus et al., 2011; Vahamidis et al, 2019 and references therein). Moreover, grain number per spike and number of fertile spikelet per spike have been identified as the most affected parameters under drought conditions (Vahamidis et al, 2019). Thus, a genotype that exhibits the ability to maintain high grain number across different and stressful conditions, such as R23+ does, represents an extremely valuable genetic material.

Among the new evidence emerged from the present work is that R5+ and R112+ were among the best performing genotypes for chlorophyll content in RF conditions during the 2019-20 (SPAD-10 and SPAD-20; **Figure 5.3**) and the 2020-21 (SPAD-7 and SPAD-21; **Figure 5.5**) growing seasons. Indeed, SPAD values of R5+ and R112+ were not significantly different from those of the corresponding "-" sibs (**Tables 5.3** and **5.4**); however, R112+ in the spaced-plants trial, and R5+ in the plot trial ranked first for SPAD in RF condition (**Figures 5.1** and **5.3**), reaching higher values than all DW varieties. One of the negative effects of drought stress observed on wheat physiology is the reduction in chlorophyll content (Huseynova, 2012; Khakwani et al., 2013), mainly due to a decreased expression of genes encoding enzymes involved in chlorophyll biosynthesis (Khadka et al., 2020 and references therein). Sensitive varieties showed remarkable chlorophyll loss under drought conditions, contrary to tolerant genotypes, whose high chlorophyll content positively correlates with grain yield (Khadka et al., 2020). This appears to be the case for R5+ and R112+ as from the results of their GYM² observed in RF plots (**Table 5.4**). In the same trial, also QY values of R5+ and R112+ RF plants were above average and, as for R5+, largely higher than those of IRR plants (**Figure 5.5**). Considering the pivotal role that photosynthesis plays in wheat drought tolerance (Huseynova, 2012; Saeidi and Abdoli, 2015; Allahverdiyev et al., 2017), the present observations are in line with additional evidence of tolerance some DW-*Th. ponticum* recombinant lines, notably R5+, towards heat and water-deficit stresses (Giovenali et al., 2023; see also Chapters 2 and 3). Among the NIRLs+, R5+, thanks to the grain weight contribution, was the best yielding line across the two field trials (GYP, **Figure 5.4**; GYM², **Figure 5.6**) under RF conditions, and showed an above average yield also in the IRR environment. Especially in the plot trial, the yield of R5+ was higher than those of all the Tunisian and Algerian DW varieties tested, which

further validates the highly positive contribution of the 7e1L R5+ segment to withstand water-limited conditions, also emerged in the experiments described in Chapters 2 and 3 and in previous trials (Kuzmanovic et al., 2018). As a whole, the results obtained from the field trials highlighted the different behaviour of the three DW-*Th. ponticum* recombinant lines grown in contrasting water regimes. Certainly, the ability to maintain grain number (as shown by R23+) or grain weight (as shown by R5+) across different irrigation conditions is an extremely interesting skill to exploit in DW breeding programs.

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Chapter 6: Concluding Remarks

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As a whole, the results obtained in this thesis substantiate the value of exploiting the wild gene pool to improve durum wheat (DW) tolerance to abiotic stresses, such as high temperatures and water deficit. In fact, a number of physiological and biochemical response mechanisms, which are recognized as essential to achieve yield stability, turned out to be associated with the presence of specific wild chromatin segmental introgressions. The DW-*Thinopyrum* spp. recombinant lines (NIRLs) tested here, previously obtained through the cytogenetic strategy of chromosome engineering, displayed several major contributing traits to stress tolerance, a summary of which is provided in the following figure (**Figure 6.1**).

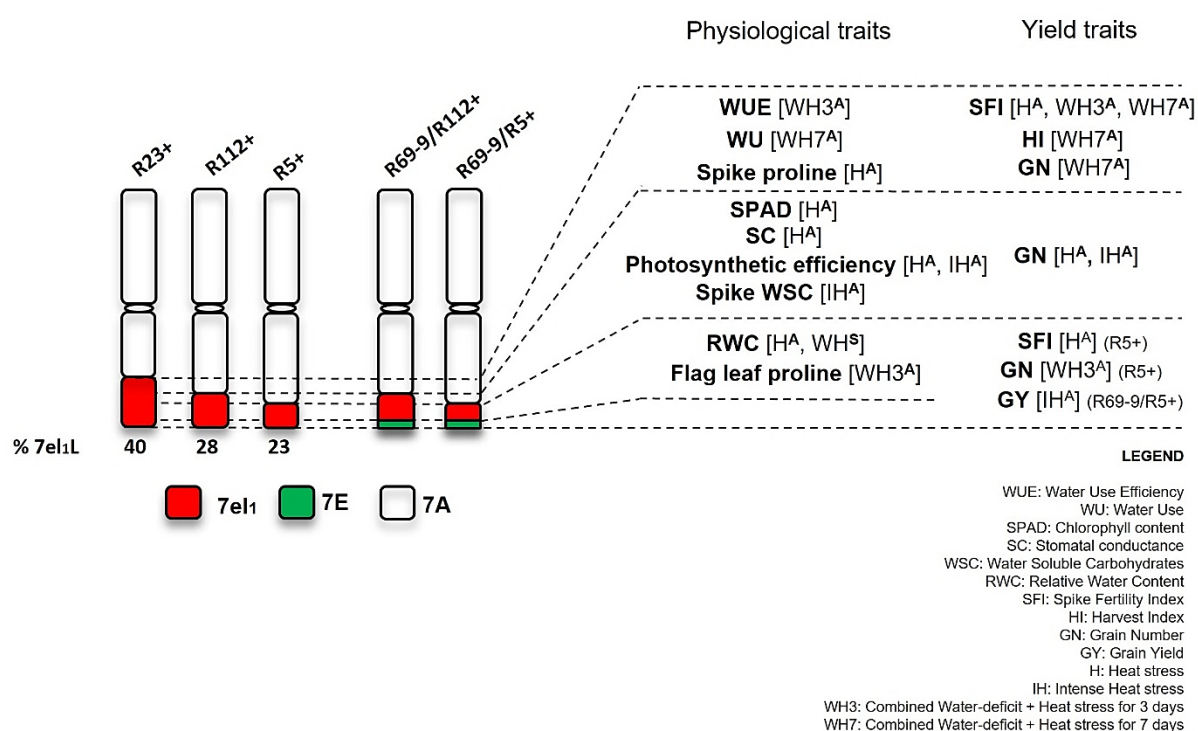


Figure 6.1. Structural-functional dissection of the *Thinopyrum* spp. chromatin transferred. The main traits reported were analysed under controlled conditions. The stress experiments in which the traits were observed are reported into the square brackets. ^A corresponds to the anthesis stage, ^S to seedling stage.

The graphical evidence corresponds to a structural-functional dissection of the *Thinopyrum* spp. chromatin transferred into the 7AL arm of the various recombinant lines when subjected to stress conditions. The availability of various breakpoints between 7AL and 7el1L or 7el1L/7EL

chromatin, differentiating the various recombinant chromosomes (see Chapter 1, §1.2.5.), enables specific attribution of given stress-responsive traits, of both physiological/biochemical nature and yield-related, to defined portions of the alien chromatin. In conclusion, the tested NIRLs, already known to possess traits of resistance to major wheat diseases (rusts, *Fusarium* spp.), revealed so far unexplored characteristics towards abiotic stress. This evidence, besides representing an important achievement in terms of knowledge of location of wild genes/QTL in defined regions, a prerequisite for their identification and molecular characterization, also indicates the readiness of the selected tolerant NIRLs to be introduced in breeding programs for abiotic stress-prone environments, such as those of the Mediterranean basin.

Ringraziamenti

Non potevo concludere lo scritto della mia tesi di Dottorato senza prima ringraziare tutte le persone che mi hanno accompagnato in questo percorso. Il primo grande ringraziamento va al mio tutor, la professoressa Carla Ceoloni, per avermi coinvolta in questo interessantissimo progetto, nonché per avermi costantemente seguita e indirizzata. La sua immensa passione e dedizione per questo lavoro è di grande esempio per tutti. Un grande grazie va al co-tutor della mia tesi, la dottoressa Ljiljana Kuzmanovic, una guida sicura e sempre disponibile. Ringrazio di cuore tutti i colleghi del Laboratorio di Citogenetica Vegetale e tutte le persone che, per un lungo o breve periodo, hanno lavorato al mio fianco. Carissimi Sandra, Alessandra, Miriam, Marilia, Marco, Ambra e Francesco, la vostra complicità ed il vostro supporto sia morale che pratico sono stati la spinta di cui avevo bisogno, GRAZIE! Infine, ringrazio per il loro supporto i pilastri della mia vita quotidiana, le persone per me più care in assoluto: la mia famiglia, Daniel e gli amici tutti (compresi quelli a quattro zampe, compagni inseparabili di lunghe passeggiate meditative e scaccia-pensieri).