M.Sc. Organic Agriculture and Food Systems (EUR-Organic)

Genotype x Environment effects on pigment-related traits for adaptation to stressed condition in bread wheat

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Abstract

Bread wheat (Triticum aestivum L.) is a food source for 36% of the global population. Climate change, including drought events and heat stress, poses significant challenges. Excessive solar radiation, coupled with drought and high temperature, may lead to photoinhibition of PSII through the photooxidation of pigment-protein complexes in the thylakoid membrane. This damage to the photosystems restricts wheat yield, emphasizing the vital role of photoprotection in pigment-protein complexes. In order to prevent photooxidation and enhance yield stability under drought and heat stress, leaf pigments serve a dual function in photosynthesis and protection against excessive radiation. Photoprotection mechanisms involve pigment-protein complexes and, especially through the xanthophyll cycle, play a crucial role. Physiological trait selection related to photoprotection is pivotal, but estimating these traits in numerous genotypes is time and labour consuming. Proximal sensing methods can rapidly estimate physiological traits through reflectance indices. The study aims to explore the use of proximal sensing methods to identify genotypic differences in 13 genotypes, estimating photoprotective and photosynthetic traits at two different leaf levels (flag leaf and third leaf) in response to water deficit (WD) and heat stress (HTS) and compared to well-watered (WW), during grain filling. This can provide insights for selecting wheat lines adapted to stress environments. In response to heat and water stress, the Photochemical Reflectance Index (PRI) exhibited a higher de-epoxidation cycle, while Non-Photochemical Quenching (NPQt) values were higher only in WD, supporting PRI-related findings. The leaf's adaptation to high temperature and intense light may hinder NPQ response to HTS. Carotenoid-related indices, with few exceptions, generally exhibit higher values in response to WW, indicating a potential cessation of carotenoid synthesis, such as Lutein. Similar findings were also observed for the third leaf. Negative correlation coefficients between Anthocyanin Reflectance Index and other Carotenoid related indices across all environments suggest a trade-off between these pigments. Despite environmental trends, no genotypic or GxE differences were reported for photoprotective indices and NPQ. This suggests a consistent and stable genetic pool concerning traits associated with photoprotection. Greenness related indices decreased in response to abiotic stress in flag leaf, while ΦPSII followed this tendency in WD. Increased leaf area index and ΦPSII in HTS, along with higher resource allocation to grains (Harvest Index) indicate greater resilience to elevated temperature and relative humidity, contrasted with those evaluated in the WD trial. In response to water deficiency, reduction in grain yield was supported by consistently low stomatal conductance across all genotypes

List of Abbreviations

ABA	Abscisic acid
AnT	Anthocyanins
BM	Biomass
Cars	Carotenoids
Chls	Chlorophylls
FL	Flag Leaf
GY	Grain Yield
HI	Harvest Index
HTS	Heat Stress
LHC	Light Harvesting Complex
Lut	Lutein
OEC	Oxygen Evolving Complex
PSI	Photosystem I
PSII	Photosystem II
ROS	Reactive Oxygen Species
Vio	Violaxanthin
WD	Water Deficit
WW	Well-watered
Zea	Zeaxanthin
ΦPSII	Quantum Yield of PSII

1.Introduction

Wheat (*Triticum aestivum L.*) is the main staple food for 36% of the world population, (Kakraliya & Singh, 2018) and its demand is expected to increase by 50% in the next 30 years (Wheat Research – CIMMYT, n.d.). However, Asseng et al. (2017) reported that the increase in temperature from 1980 to 2010 (0.46 °C in Obregón, the study area) has caused a 10% decrease in wheat yield, and predictions suggest that yields are expected to decline at a rate of 6% for each °C of temperature increase. Climate change will also bring about unprecedented drought events, which combined with heat stress can have a major impact on wheat production by affecting crop growth and yield. Therefore, it is pivotal to address these challenges through a variety of measures, including enhancing yield, selecting genotypes able to withstand the ongoing climate change (Coast et al., 2019). Plant adaptive responses to both, drought and heat involve several organisation levels, from cell to entire organs, making it difficult to identify single heat- or drought-tolerance genes. Thus, selection based on physiological traits associated with heat and drought stress represents one of the best approaches available to breed for drought and heat resilient wheat (Cossani & Reynolds, 2015). This approach poses the challenge of accurately estimating physiological traits in a large number of genotypes (M. Reynolds et al., 2009). Besides, screening for high-yielding varieties may not take into consideration GxE interaction, which is a major determinant of yield stability (Huggins et al., 2018). Remote and proximal sensing can help in filling these gaps by estimation of physiological traits throughout reflectance indices (Hernandez et al., 2015). Drought and heat stress are often coupled with an excess of solar radiation, which can cause photoinhibition of PSII due to photooxidation of pigment-protein complexes in the thylakoid membrane (Bashan et al., 2006). Such a damage to the photosystems will limit wheat yield, as crop biomass and photosynthesis are closely linked particularly under stress conditions (Makino A., 2011). Thus, photoprotection of pigment-protein complexes is crucial to prevent photooxidation and can contribute to stabilise yield under drought and heat stress. Leaf pigments are not only responsible for photosynthesis but also for protecting the leaf against excessive radiation. One of the most important energy dissipation mechanisms in plants, consists of the de-epoxidation of photo-protective pigments, such as the carotenoids involved in the xanthophyll cycle (Zeaxanthin, Violaxanthin, and Antheraxanthin) (Bashan et al., 2006). In addition, xanthophylls protect leaves against UV-induced stress and reactive oxygen species (Tambussi et al., 2002).

Reflectance indices captured by remote sensing devices, have proved useful in estimating leaf greenness and other pigment related attributes at the canopy level showing good correlations with yield under heat stress (Robles-Zazueta et al., 2022). However, in wheat, photosynthetic assimilates produced after anthesis, especially at the first top three leaves, have a major role in final grain yield (Mu et al., 2010). Only a few studies have deepened the relationship between different levels of the canopy (Mulero et al., 2023). In this study we intend to explore (1) if the selection for photoprotective and photosynthetic traits, estimated at leaf level and through proximal sensing methods, may be applied to identify genotypic differences for the selection of adapted wheat lines to stress environments. (2) Taking into consideration the photoprotective response, at lower levels, such as at third leaf, can bring a complete overview of the photoprotective capacity the whole plant and its true response to stressed conditions (Mulero et al., 2023).

2. Literature Review

2.1 Water Deficit effect

It is pivotal, for the sake of definition, to specify that in this study "water deficit" is intended at the atmospheric and soil level (Chaves et al., 2003). When considering field conditions, water shortage is often combined with high irradiance and increased temperature (Ma et al., 2006). Different avoidance mechanisms are adopted by plants in drought conditions, such as, through deep root system and partitioning use of water. Drought tolerance, as expressed through root system architecture, is characterized by traits such as a substantial root angle, heightened root length, increased root density, and an expanded xylem diameter, particularly with greater soil depth (C. Li et al., 2021). Breeding for the ideal root architecture necessitates a consideration on the spatial distribution of water in the soil, a factor influenced by both rainfall patterns and soil type(C. Li et al., 2021). On the other hand, plants are able to build tolerance with dehydration, through a partial wilt and ability to continue the growth process once water source is available again (Nezhadahmadi et al., 2013) .The reaction of plants to drought stress depends on several factors, such as the resilience of genotype, the phenology of the plant at the moment of the stress, whether it is imposed along the whole plant cycle or only in a specific time frame and abiotic factors (Nezhadahmadi et al., 2013). However, the most impactful factor to the response is given by the specific interaction between Genotype and Environment (Chaves et al., 2003). The response to drought stress in wheat, after a study from Zivack and colleagues (2013), is distinguished into stomatal and non-stomatal effects. Stomatal closure is one of the first response and Abscisic acid (ABA) was identified as one of the sentinel chemicals used for stomal regulation. (Chaves et al., 2003). Stomatal closure leads to a reduction in CO2 absorption and it has a direct negative effect on net photosynthetic rate. Whereas, non-stomatal effects include suppression of Rubisco activity, synthesis of ATP and impaired photochemistry (Ma et al., 2005 and Zivack et al., 2013). The combination of these factors will decrease the oxidation of NADPH with several effects, such as formation of Reactive Oxygen Species (ROS), decrease in ATP synthesis, impaired sugars production (NADPH reduces CO2 into sugars) and imbalances in the electron transport chain. All of these effects will impact the overall efficiency of photosynthesis and therefore the carbohydrate metabolism of leaves and carbohydrate partitioning in sink organs. The diminished sink strength is ultimately affecting crop yield and the probability of kernel abortion, such as in maize (Shokat et al., 2020). In particular, the degree of effect on the photosynthetic system depends on the intensity and the duration of the stress

(Zivack et al., 2013). Drought stress was also linked to increases in the xanthophyll pool (Zivack et al., 2013). In fact, xanthophylls, including violaxanthin, antheraxanthin, and zeaxanthin, are involved in a feedback regulating process, dissipating excess light energy as heat through nonphotochemical quenching. This process helps prevent potential damage to the photosynthetic machinery when the availability of water is limited. The effects of drought on plants also extend to morphological characteristics. Research has indicated that factors such as leaf area, height, canopy architecture and stem diameter tend to decrease under decreased availability of water (Anjum et al., 2011). Furthermore, the agronomic response of the plant depends on the phenological stage at which the stress is applied. In fact, at anthesis, the reduced pollination leads to infertile spikelets and lowered grain numbers, while post-anthesis the size of the kernel is most affected, with consequent less content of starch and protein (Wang et al., 2017).

2.2: Heat Stress effect

The response to heat stress is highly dependent on the genotypes, for example those ones selected for their tolerance to high temperature are characterized by efficient transpiration rate (Abdelhakim et al., 2022). However, the developmental stage of the wheat plant plays a role as well. During anthesis high temperature can put at risk the build-up of carbohydrates, which are addressed for the final grain production (Posch et al., 2019). Under high temperature, development is accelerated, thus reduced growth and not filled grains are common effect under heat (Chakrabarti et al., 2022). In wheat, metabolic and physiological processes that can impact the growth of the plant usually happen after 30 °C (X. Zhang et al., 2023), when this temperature threshold is surpassed, there is a decrease in grain yield (Abdelhakim et al., 2022).Typically, high light conditions are often coupled with increased temperatures, and this combination of factors has physiological implications (Y.-E. Chen et al., 2017).This double factor can lead to damages to the photosynthetic machinery and consequent impairs such as the decrease in chlorophyll content, respiration and therefore carbon fixation. In fact, elevated temperatures can initially boost the maximum rate of Rubisco, nevertheless there is a threshold beyond which excessive heat can have harmful effects on the enzyme function (Correia et al., 2020). Therefore, this affects photosynthesis and plant growth (Zhang et al., 2023). In addition, the effect of heat stress on the increase of stromal reducing power in the thylakoid membrane, due to the high consumption of NADPH in the Calvin-Benson cycle, results in overreduction of Plastoquinone and production of ROS (Marutani et al., 2017). ROS can lead to a series of

oxidative damages to pigments, proteins and enzymes. However heat stress at early stage in wheat may create an adaptation to heat stress through antioxidative activity (Amini et al., 2023). Direct effects of heat are translated in morphological changes in cell differentiation and elongation with reduction of leaf area (Zhang et al., 2023).

2.3 Photosystem Machinery

Photosystem I (PSI) and Photosystem II (PSII) are a matrix of proteins and pigments subunits within the chloroplast internal lipid membrane, the thylakoid. The main two functions of these photosystems are light harvesting and the conversion of photo energy into chemical energy. The main subunit of the photosystems are the pigment-proteins imbedded within the thylakoid membrane, the so-called reaction centre or PSII core (Tatsuya Tomo and Suleyman I. Allakhverdiev, 2021). While, as extrinsic subunits there are other complexes, such as the light harvesting antenna complexes, the cytochrome complex, ATP synthase and other units required in the electron transport chain. The core is formed by membrane-spanning subunits are participating to the electron transfer of the water-splitting reaction. In the core, facing the lumen of the thylakoid membrane, there are few protein subunits responsible for the stabilization of OEC (Oxygen Evolving Complex), essential enzyme for the synthesis of molecular oxygen (Shen et al., 2021). The central part of PSII is surrounded by several light harvesting complexes (LHC) associated with the peripheral antenna complexes, such as carotenoids (Cars) and chlorophylls (Chl), located within the thylakoid membrane, required to harness light energy and transfer to the PSII core (Shen et al., 2021). Cars are subdivided into oxygen-free carotenes and oxygen-containing, xanthophylls. The ratio between Cars and Chl varies between 0.1 and 0.5 with B-carotene being the most important between the carotens and Zeaxanthin (Zea), Violaxanthin (Vio) and Lutein (Lut), the most abundant for the Xanthophyll family (Sytar et al.,, 2021). Cars are entitled of some important roles in the structure of the thylakloyd memberane through the protection of phospholipids from cellular damage, quenching activities for excited Chl and oxygen, light harvesting role and energy transfer (Gitelson et al., 2001). LHCs are able to absorb the sun light, thus the excitation energy released drives the transport Carotens such as B-carotene are also liked to photoprotective mechanisms (Flagella et al., 1996) of electrons to the primary electron donor of Chl-a of the reaction centres of PSI and PSII. Among the LHCs, Light Harvesting complex II (LHCII) is the most abundant in PSII. LHCII is characterised by great plasticity, in order to adapt to limited light and high-light conditions (Bos et al., 2017). In addition, light variations can happen in terms of seconds, therefore is required extreme fast regulation. Several Chl-a molecules in the core of PSI and PSII serve as

receptors for the energy transferred through the electron transport chain originating from LHCII. This energy transfer leads to charge separation, triggering a sequence of events at the foundation of the conversion of light energy in the 400-700 nm range into chemical energy. Along with these processes a transmembrane difference of pH and an electric potential gradient is formed, this favours the synthesis of ATP, through the phosphorylation of ADP by ATP-synthase (Vinzenz Bayro-Kaiser and Nathan Nelson, 2021).

2.4 Photoprotection under stressed conditions

In nature, conditions such as high light along with other stressed conditions such as water shortage and high temperatures is a frequent combination (Flexas & Medrano, 2002).C3 plants, such as wheat, have three different pathways to use the light: photosynthesis, photorespiration and thermal dissipation. Photorespiration usually covers a small share. On the other hand, thermal dissipation is a pivotal mechanism, which includes an array of photoprotective strategies in order to cope with photoinhibition (Davis & Hangarter, 2012). Wheat is considered by several studies, to have an acclimated photosynthetic machinery to stress (Zivack et al., 2013). Furthermore, wheat possesses a range of preventive mechanisms to adapt to excessive light conditions and other abiotic stresses., such as the thickness of the epidermal cuticle, waxes, trichomes and leaf rolling behaviours (Demmig-Adams, 1992).

2.4.1 NPQ

High light can lead to excess excitation and photoinhibition, as decreased efficiency of photosynthesis. (Adams et al., 2013). Plants can go under light variations in terms of seconds or for longer periods (Kromdijk et al., 2023). As a defensive mechanism plants dissipate excess excitation through Non-photochemical quenching (NPQ). NPQ is considered as the key photo-protection mechanism, and is pivotal to maintaining the compromise between damage and efficiency (Y.-E. Chen et al., 2017).When NPQ occurs, the efficiency of PSII to covert absorbed light into chemical energy is estimated to decrease by 60-80% (Petar H. Lambrev et al., 2021). Under high light, reduced stomatal conductance, therefore limited CO2 availability, slow the synthesis of ATP enzyme. The result is decreased efflux of protons in the thylakoid membrane and consequent acidification of the latter. This mechanism is a component of NPQ, named qE (Demmig-Adams, 1998). This phenomenon triggers the enzymatic de-epoxidation of the xanthophyll pigment Vio to Zea. The role of Zea is to improve the dissipative potential of RS of PSII. Under environmental stresses, such as increased or decreased temperature and under

high light, the content of Vio rises and therefore the degree of heat dissipated through de-epoxidation (Yamamoto et al., 1999). In particular, the incidence of water stress coupled with high irradiation can influence plant photosynthetic efficiency and as a result the energy dissipated through the de-epoxidation of xanthophyll cycle (Demmig-Adams et al., 1998).In an experiment on wheat, in which the Xant cycle was inhibited through injection of DDT, it was suggested that with leaf aging (60 days after anthesis), a greater portion of their NPQ mechanism became less dependent on the xanthophyll cycle (Dai et al., 2004) . In fact, Quick and Stitt (1989) defined two additional types of quenching: Photoinhibitory Quenching (qI) and State Transition Quenching **(**qT)**.** The first is the result of photoinhibition affecting PSII, leading to a decrease in the flow of electrons through the PSII electron transport chain. The second estimates the shift of LHC of PSII to PSI in order to decrease the excitation state of PSII (Tietz et al., 2017). The values of NPQ on early development stage in winter wheat increase exponentially with the rate of water stress (Zivcak et al., 2013). Furthermore, wheat genotypes under pre-stressed water holding environment during vegetative stage, showed low levels of NPQ, as demonstration of great adaptability of leaves to stress (Cui et al., 2015).

2.4.2 Photoprotective Indices

Xanthophyll cycle is considered one of the main photoprotective mechanism. The wavelength of 531 nm is significantly correlated with the changes of the xanthophyll cycle (Garrity et al., 2011). Photochemical Reflectance Index (PRI), uses this wavelength. PRI is coupled with radiation use efficiency as well (Huggins et al., 2018). Considering optimal condition of water regime, the correlation between PRI and yield is confirmed by a study of Gutiérrez-Rodríguez and colleagues (2004). While in stressed conditions, PRI is expected to rise in stressed conditions and to decline towards maturity (Huggins et al., 2018). In the study from Huggins and colleagues (2018) PRI was found to be a more indicative parameter of severe heat stress, primarily because of the reduction in chlorophyll content, which is one of the major influencing factors of the calculation formula. However, Yudina and colleagues (2020) argued that PRI can be biased by cofounding factors such as other pigments and leaf optical properties. Therefore, the study of the relationship between carotenoid-related indices and PRI, may lead to a clear division of Xant and Cars. Due to the role of Cars in LHCII, their estimation can provide insights into the photosynthetic efficiency (Zhou et al., 2017). In addition, Cars actively participate in the protection of the thylakoid membrane quenching Chl in excited state and scavenging of singlet oxygen (Gitelson et al., 2007). The main limit in the spectral reflectance analysis of Cars is the overlapping reflectance bandwidth between Cars with Chl, with the latter

more abundant than Cars, making the detection of Cars more challenging (Gitelson et al., 2007; Steddom et al., 2003). For this reason, in the last decades several studies explored different wavelength of absorption. However, a recent study by Zhou and colleagues (2017) developed CARI index, which exhibited higher correlation when compared to other Cars-related indices. In fact, in CARI the bandwidth of 720 nm was selected to estimate Cars content, because it has the highest correlation with the latters and the least influence of Chls. Although CARI was studied on a large range of species and phenological stages (Zhou et al., 2017), is relatively new and more references are present for Ratio Analysis of Reflectance Spectra for Carotenoids, (RARSc), (Babar et al., 2006; M. P. Reynolds et al., 2007). For this index, it is assumed that at 500 nm there is a high correlation with Carotenoids and it is not influenced by other effects (Zhou et al., 2017). CRI is another spectral index, able to estimate Cars, developed by Gitelson (2007).It is able to remove the Chl contribution (Kong et al., 2015). The estimation of Cars is useful for monitoring the degree of stress. CARI, was applied in this sense, however it did not differentiate among the different degrees of water shortage (Dao et al., 2021). In a study by Zhang and Kirkham (1996), on sunflower and sorghum species under drought, they found that Cars content did not vary significantly, due to the physiological presence of high Cars levels. As leaves undergo the aging process, Chl tend to degrade faster compared to Cars, which are preserved as a protective mechanism (Bibi et al., 2021). Therefore, the ratio between the two pigments and the dynamics of Cars serve as a good indicator of the physiological state of the plant under stress and its photosynthetic acclimation to changing environments (Gitelson et al., 2007). The study of the ratio between Car and Chl was related with response to stressed conditions and aging. In a study by Morgun and colleagues (2022), the two most productive varieties of winter wheat under drought show low values of this ratio. It was considered an adaptive response to water stress. It needs to be highlighted that when leaf tissues lose water, there is an increase in light absorption, leading to a reduction in reflected light (Bibi et al., 2021). Within the category of plant pigments, there is the group of flavonoids. Among these flavonoids are anthocyanins (AnT), which are water-soluble and non-photosynthetic pigments (Koes et al., 2005). An array of role is attributed to Ant. The main photoprotective role is that under excess light they are capable of absorbing light in the UV wavelength and so diminishing the harvest of light by chlorophylls (Zheng et al., 2021). It can maintain the carbon-sink in senescent leaves and so lengthening leaf lifespan. Furthermore, Ant has an antioxidant role against ROS, however, this function diminishes as leaves undergo senescence (Kong et al., 2015). Among the reflectance indices, ARI can give an estimation of red pigments, such as AnT (Steddom et al., 2003). In the literature, there is large space for research for AnT and the

physiological response of these pigments in crops of relevance, such as wheat (Falcioni et al., 2023). However, few studies were found about application of ARI in plant research and ecophysiology in wheat, such as the one of Falcioni and colleagues (2023), in which ARI and AnT show limited linkage. The transient nature of Ant may play a role, due to changes in photoperiod and temperature may explain the lack of linkage (Chalker-Scott, 2002).

2.4.2 Greenness related indices and ΦPSII

Quantum yield of PSII photochemistry (ΦPSII) gives an estimation of light converted for chemical reaction and therefore the efficiency of photosynthesis (Kramer et al., 2004). Flagella and colleagues (1996) found that durum wheat under sever water stress showed decrease in ΦPSII. Furthermore, in light of the relation between percentage decrease of ΦPSII and yield, this trait can be used to screen drought-resistant variety (Flagella et al., 1996). In general, under high temperature the efficiency of PSII photochemistry decreases. The reasons according to Mathur and colleagues (Mathur et al., 2011) are modifications for charge separation, detaching of antenna from PSII and the tendency of free radicals to convert radicals in pairs in the reaction centres, which is a waste of energy. Further, as leaves undergo aging, with degradation of green tissue, and consequent structural changes of chloroplasts, there is a decline in the photosynthetic rate (Kong et al.,2015) Among the spectral reflectance indices, NDVI is undoubtfully one of the most found in the literature (De Santis et al., 2021). It is often associated with canopy greenness, biomass (De Santis et al., 2021; Kumar et al., 2023). So, often lower values of NDVI, indicate senescent phenotypes (Adamsen et al., 1999). Higher levels of NDVI during grain filling, indicate good level of assimilation of photosynthates, which are crucial in this phase for grain development. However, under heat stress, the grain filling phase tends to be shorter and hence less accumulation of photosynthates and decreased yield (Kumar et al., 2023). Therefore, the identification of lines with higher NDVI under these conditions can be a proxy for selecting higher yield genotypes. Further there is strong evidence about the association between NDVI and Chl (Kumar et al., 2023). However, the relationships between Chl and other traits are different. Under heat stress, Chl loss was significantly correlated with the photosynthesis capacity and yield in spring wheat (M. Reynolds et al., 1994). Under drought stress, the same degree of correlation was confirmed (Gutiérrez-Rodríguez et al., 2004). On the other hand, relative chlorophyll content estimated under grain filling in wheat under water potential was not correlated with grain yield (M. P. Reynolds et al., 1999) (Kumar et al., 2023). In addition, differences in SPAD loss may give an estimation of stay-green phenotypes (Kumar et al., 2023).

However, Barutçular and colleagues (2016), in a study on wheat highlighted that the varieties analysed were more affected by the phenological stage and by higher temperature than water stress in terms of relative chlorophyll content.

2.5 Relevance of Handheld Methods

Remote sensing technologies play a crucial role in real-time plant phenotyping by capturing rapid physiological changes and abiotic factors (Magney et al., 2016). In particular, remote techniques placed within or near the crop surface offers more detailed temporal and spatial related information compared to satellite-based sensing (Magney et al., 2016). Therefore, these tools are considered pivotal in agricultural decision-making and research (Mulla, 2013). Advancements in electromagnetic wavelengths have led to the development of hyperspectral indices, enabling non-invasive estimation of crop status, including nutrient and water content, chlorophyll levels, and photoprotective responses (Cotrozzi & Couture, 2019). Fluorescence measurements offer a rapid and non-intrusive estimation of photoprotective mechanism, such as NPQ (Adams et al., 2013). While these indices allow to fast screening and are easy to interpret, the mathematical formulas do not account the variation in reflectance depending of other factors like phenological stage and environmental conditions (Hernandez et al., 2015). However, this method requires a time of dark adaptation, when it comes to large panel it is a time-consuming procedure. New visions arise to solve this time limitation. Tietz and colleagues (2017) thanks to the use of PAM fluorimeter supplied with far-red light which oxidize the PSII reaction centres, without any time of leaf dark adaptation required were able to quantify NPQ without any time of dark adaptation. Further, the porometer, in this study a steady-state type, is another proximal sense device used. It is able to increase data collection of magnitude at once, thanks to the ability of measure a broad range of traits related to stomatal conductance, VPD leaf, Temperature of the air and at leaf. However, variations in stomatal conductance under high humidity and temperature differences between the leaf and surroundings may affect data reliability

2.6 Correlation between traits

It is crucial to determine whether an increase in photoprotective and photosynthetic traits is coupled or uncoupled with a variable indicating water and temperature stress to determine the adaptability to the stressed environment. Furthermore, traits such as the agronomic data (in this study Grain Yield, Biomass and Harvest index) are traits of interest and have high heritability, therefore can be used as an indirect selection trait, which is more advised than use only one selection trait (in this case photoprotective and photosynthetic) (Falconer & Mackay, 1996).

2.6.1 Water and Temperature indicators

The physiological responses of the plant to high temperature and water deficit change if combined or taken singularly. In the first case, higher plant use to respond with cooling mechanisms to low leaf temperature, while under water scarcity plants are prone to close their stomata (Correia et al., 2021). In case of water deficit, stomatal closure, triggered by ABA signalling at the root level, increases Vapor Pressure Deficit (VPD) constraining photosynthetic activity (Liu et al., 2023). When both effects coincide, a cause-effect reaction mechanism is established: stomatal closure results in a reduction of intracellular CO2 and canopy temperature(CT) is enhanced (Correia et al., 2021). Cooler leaves and canopy temperature under high temperature tend to be coupled with greater stomatal conductance (gsw) (Rebetzke et al., 2000). VPD is coupled with high temperature and/or low relative humidity, which triggers the stoma to open in order to raise the cooling effect by transpiration (Liu et al., 2023). Stomatal conductance is a reliable indicator of the water-carbon status of the plant (Toro et al., 2019), because it regulates the CO2 assimilation and water evaporation at the leaf level. This was proven by a long-term study of CIMMYT on bread wheat, enhanced leaf conductance was associated with an increase in grain yield (Rebetzke et al., 2000). At the same time, genotypes with high biomass production were associated with cooler CT (Babar et al., 2006). Further, CT at grain filling may show linear association with stay-green canopy during heat in the same site of this study and it was associated with grain yield (Lopes et al., 2012; Babar et al., 2006). Higher presence of carotenoids is linked with improvement of transpiration efficiency (M. P. Reynolds et al., 2007). Heat susceptible cultivars were shown an enhanced stomatal closure and subsequent increased leaf temperature (Abdelhakim et al., 2022). The identification of genotypes with low stomatal closure can be a parameter to consider to select heat tolerant genotypes.

2.7 Third leaf as screening method

The flag leaf (FL), the upper leaf, is usually taken as the representative leaf of the whole plant. It is recognized to be highly correlated with carbohydrate production and so grain yield ((Wang et al., 2022). However, due to the distinct light exposure leaves within the canopy layers might vary in their response (Mulero et al., 2023). Therefore, it is suggested as a screening tool to take into consideration the performance in terms of photoprotection intra canopy. At the third leaf the fraction of diffuse light increases, which is better used compared to direct sunlight, and this can increase photosynthetic efficiency at intra-canopy levels (Li et al., 2010). Different studies highlighted that under low light, such as in shaded leaves, there is an increase of Chl in order to maximize the light use efficiency. In particular, Chl-b shows higher levels compared to Chl-a, due to better efficiency in harvesting of light in the blue region (H. Li et al., 2010). Another study on shaded winter wheat plants showed acclimation of the lower canopy levels, third leaves enhanced their photosynthetic rates after anthesis in response to the reduced photosynthesis of flag leaf (Mu et al., 2010). At the same time, the photoprotective activity of the xanthophyll cycle is less in shaded leaves due to the difference in pool size of these pigments compared to sun adapted leaves (Demmig et al., 1987). Shaded leaves have a lower capacity of deal with photoinhibition compared to more exposed ones (Davis & Hangarter, 2012). Most of the carotenoid are highly dependent to light environment, such as the xanthophyll pool, especially Zea, and B-carotene. Xant pigments are most abundant in light exposed leaves because of the greater need to dissipate excess excitation energy (Demmig et al., 1987).

3. Materials and Methods

3.1 Site Condition, environments and field management

The experiment was conducted at the N. Borlaug CIMMYT Experimental station (27 o 20' N; 109 o 54' W; 38 m above sea level) in Ciudad Obregon, Sonora, Mexico. The area in the north-western of Mexico, is characterised by an arid climate with variable rainfall (see Table 1). The experiments were carried out, from December 2021 to June 2022. The experiment consisted of three replicates or plots, sown directly in the soil under the control (well-watered), water deficit (WD) and heat stress (HTS). The latter was sown on 28/02/2022, while WD and WW were planted on 14/12/2022 and 09/12/2022 respectively. Hence, the heat stress corresponds to the late sowing and consequent raise of temperature (around 10° C of difference for the maximum mean temperature, see Table 1). All the environments were irrigated at sowing and after the emergence. In WW and HTS the irrigation was supplied every 10-14 days, while in WD the irrigation was supplied at the initial phases of tillering and heading. Each plot measured 4.5 m in length, with six rows sown, and 1.5 m wide. The soil goes under the classification of Hyposodic Vertisol (Calcaric, Chromic) (as described in Honsdorf et al., 2022)). In order to lessen the biotic risk, herbicides, fungicides and pesticides were applied when needed. Irrigation was provided by a drip irrigation system. Fertilizers were applied in January in WD and WW and in March in HTS.

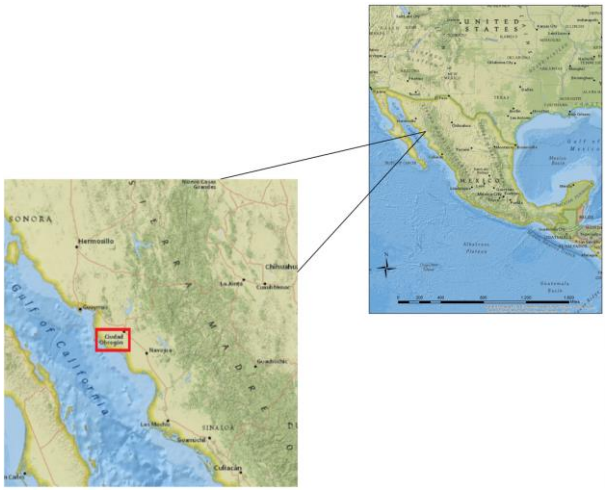


Figure 1: Location of the experiments

Table 1: List of weather and phenological data from day of emergence to day to maturity, retrieved from meteorological station of the CIMMYT´s experimental station.

	WW	WD	HTS
Emergence Date (ED)*	16.12.2021	24.12.2021	11.03.2022
Days to Maturity from ED**	121	110	80
Number of Irrigations	8	4	8
Rainfall (mm)	17,1	16,9	0,3
Temp (Max/Min °C)	34,93/1,69	33,16/1,69	41,96/3,22
Mean Temp (Max/Min °C)	22,61/11,01	19,53/11,01	29,14/13,23
Relative Humidity (Max/Min %)	80,61/50,69	80,61/50,70	75,28/27,49
Solar Radiation (Max/Min MJ/m² d)	12,00/2,74	11,30/2,75	12,90/8,90
Solar Radiation (Mean MJ/m² d) ***	8,5	8,19	11,55

*for more than 50% of the plots

** for the last plot that reached maturity

Table 2:Vapor Pressure Deficit (VPD), measured by Porometer.

Genotype	Environment	VPD (kPa)
CIMMYT5	WW	4.21 ± 0.603
	WD	6.32 ± 1.134
	HTS	4.68 ± 0.524
CIMMYT10	WW	5.02 ± 0.744
	WD	6.41 ± 1.206
	HTS	4.73 ± 0.607
CIMMYT12	WW	4.72 ± 0.656
	WD	6.41 ± 0.644
	HTS	4.33 ± 0.785
CIMMYT13	WW	4.44 ± 0.580
	WD	7.12 ± 0.700
	HTS	4.38 ± 0.686
CIMMYT23	WW	4.44 ± 0.553
	WD	6.78 ± 0.590
	HTS	4.96 ± 0.321
CIMMYT36	WW	5.02 ± 0.134
	WD	6.79 ± 0.583
	HTS	3.99 ± 0.249
CIMMYT41	WW	4.99 ± 0.039
	WD	7.01 ± 0.734
	HTS	4.63 ± 0.692
CIMMYT48	WW	5.00 ± 0.333
	WD	7.15 ± 0.723
	HTS	5.68 ± 0.211
CIMMYT59	WW	5.15 ± 0.293
	WD	6.71 ± 0.817
	HTS	5.17 ± 0.575
CIMMYT61	WW	4.98 ± 0.163
	WD	6.36 ± 0.161
	HTS	4.92 ± 0.694
CIMMYT63	WW	5.34 ± 0.208
	WD	6.94 ± 0.392
	HTS	5.13 ± 0.783
CIANO	WW	5.04 ± 0.368
	WD	7.08 ± 0.199
	HTS	5.31 ± 0.187
BOURLAUG	WW	5.20 ± 0.068
	WD	7.67 ± 0.119
	HTS	5.01 ± 0.436

3.2 Lines Selection and experimental design

The genotypes or lines studied were part of the "Best Physiological Traits + Parents" (BESTPT+PDS) Panel developed by CIMMYT, which comprised a total of 75 lines of *Triticum aestivum*. Lines selected for this study came from CIMMYT´s Wheat Physiology Group, identified for their performance in physiological traits in different trials in irrigated heat (late sowing), drought stress and yield potential (normal conditions) in Mexico and internationally. Seven contrasting genotypes were selected among the panel lines according to the grain yield of the year 20-21 and the evapotranspiration estimation measured as Canopy Temperature (CT) in the WD trial during the vegetative stage (see Figure 2). High Grain Yield- High CT (H-GYCT), Medium Grain Yield-Medium CT(M-GYCT) and Low Grain Yield-Low CT (L-GYCT). Further, two control lines or checks were selected and four genotypes were selected were selected based on the availability of relevant data obtained from a colleague (indicated as AS-Another Subset) (see Table 3. List of genotypes investigated, GID=CIMMYT Genotypic identificationTable 3). "Checks" were designated in the previous years from the physiology department of CIMMYT for their high performance in the breeding experiments in different environments in Mexico or in other trials outside of Mexico (Reynolds et al., 2017). The original design of the BESTPT+PDS Y22 panel followed the replicated block design, the 75 lines were replicated three times (see Figure *3*: Diagram of field experimental design, used in all the environments. Letter "C" stays for CIMMYT (i.e. C5= CIMMYT5). Replications are indicated with different colours: first replication in green, second replication in yellow and third replication in orange. In white, the lines selected.Figure 3). "Checks" were designated in the previous years from the physiology department of CIMMYT for their high performance in the breeding experiments in Mexico or in other trials outside of Mexico (Reynolds et al., 2017).

Figure 2: Photo of the WW plot (right), WD plot (centre) and HTS plot (left)

Table 3. List of genotypes investigated, GID=CIMMYT Genotypic identification

Lines	CIMMYT GID	Criteria of Selection
CIMMYT5	8931712	AS
CIMMYT10	8931724	AS
CIMMYT12	8931727	AS
CIMMYT13	8931717	AS
CIMMYT23	7946966	H-GYCT
CIMMYT36	8823449	H-GYCT
CIMMYT41	8602398	M-GYCT
CIMMYT48	8600341	M-GYCT
CIMMYT59	8287885	L-GYCT
CIMMYT61	8071629	L-GYCT
CIMMYT63	8198432	M-GYCT
CIANO	7627560	Check
BOURLAUG	7806808	Check

1	2	3	4	C5 (5)	6	7	8	9	C10 (10)	11	C12 (12)	C13 (13)	14	15
30	29	28	27	26	25	24	C23 (23)	22	21	20	19	18	17	16
31	32	33	34	35	C36 (36)	37	38	39	40	C41 (41)	42	43	44	45
60	C59 (59)	58	57	56	55	54	53	52	51	50	49	C48 (48)	47	46
C61 (61)	62	C63 (63)	64	65	66	67	68	69	CIANO (70)	71	BOURLAG (72)	73	74	75
C36 (90)	89	88	C61 (87)	C23 (86)	85	84	83	82	81	C5 (80)	79	78	77	76
91	92	93	94	95	96	C13 (97)	98	99	100	101	102	103	104	105
120	119	118	117	C41 (116)	CIANO (115)	114	113	112	111	110	109	C59 (108)	107	106
121	122	123	124	125	126	127	128	129	C48 (130)	131	132	133	134	135
C10 (150)	149	148	147	146	145	144	C63 (143)	142	C12 (141)	140	BOURLAG (139)	138	137	136
151	152	153	154	155	156	157	158	159	160	161	162	163	164	165
180	C59 (179)	C23 (178)	177	176	CIANO (175)	174	173	C48 (172)	C13 (171)	170	169	168	167	166
BOURLAG (181)	C5 (182)	183	C61 (184)	185	186	187	188	189	190	191	192	193	194	195
210	209	208	207	206	205	204	203	202	201	200	199	C36 (198)	197	196
211	C41 (212)	213	C12 (214)	215	216	C63 (217)	218	219	C10 (220)	221	222	223	224	225

Figure 3: Diagram of field experimental design, used in all the environments. Letter "C" stays for CIMMYT (i.e. C5= CIMMYT5). Replications are indicated with different colours: first replication in green, second replication in yellow and third replication in orange. In white, the lines selected.

3.3 Crop measurements

The measurements of this study were taken around early-middle grain filling equivalent to the range Z65-Z75 according to Zadocks scale (Zadoks et al., 1974). For phenology recordings, it was considered the day in which more than half of the plot exhibits the associated characteristics to the phenological stage of early grain filling. Measurements were taken on the central segment of flag leaf of the main stem. For Leaf reflectance indices, included PRI and SPAD, the same procedure was repeated for the third leaf, moving sequentially from the topmost leaf

downward. All the measurements were taken between 9.00 and 14.00, following the CIMMYT guidelines (Pask et al., 2012).

3.3.1 Fluorescence Parameters

Chlorophyll fluorescence parameters, such as theoretical nonphotochemical quenching (NPQt); quantum yield of photosystem II (ΦPSII) and PAR were measured by the handheld device PAM fluorometer MultispeqQ® Beta by PhotosynQ Inc (East Lansing, MI, USa).

3.3.2 Leaf Reflectance Indices

SPAD readings by a portable chlorophyll meter SPAD-502 by Minolta (Tokyo, Japan). It estimates the relative chlorophyll content. In WW and WD environment two reading per leaf were averaged, and this was repeated on three plants in total per each plot. In HTS, the same procedure is valid but only for two plants per plot. The SPAD meter is based on the transmittance at 650 nm and 940 nm. Photochemical Reflectance Index (PRI) was measured by the device PlantPen PRI210 by Photon Systems Instruments (Prague, Czech Republic). The reference for this index is Gamon and colleagues (1990) (see Table3). In WW and WD environment two readings per leaf were averaged, and this was repeated on three plant in total per plot. In HTS, the same procedure is valid but only for two plants per plot. Different hyperspectral reflectance indices were calculated thanks to the hyper-spectral ASD FieldSpec FR radiometer by Analytical Spectral Devices, Inc. (Boulder, CO, USA) attached to a Li-Cor Li-1800-12 integrating sphere (Li-Cor, Inc., Lincoln, NB) The spectroradiometer can read from 350 nm to 2500 nm with raises of 1 nm. In WW and WD, two or three measurements were taken while in HTS only one. The following indices were calculated: ARI, CARI, CRI, RARSc and, NDVI. For formulas see Table 4.

3.3.3 Water Status and Temperature Parameters

Traits such as stomatal conductance (gsw) and Vapor Pressure Deficit (VPD) at leaf level were measured by the Porometer Li-600 by LI-Cor Inc (Lincoln, NE, USA). Two or three measurements were taken on the adaxial surface of flag leaf of different plants per plot. The temperature for the whole canopy (CT) was measured remotely by an infrared thermometer, Sixth Sense LT300' IRT. Four readings were taken from anthesis till the end grain filling, every 5-7 days. In this study the average among the four dates is taken.

3.3.4 Production and Grain Yield-Related Parameters

Final aboveground biomass at physiological maturity (BM) and Grain Yield (GY) were measured per plot, following the methods of Pask and colleagues (2012). Harvest Index (HI) was calculated based on BM and GY.

Leaf Area Index (LAI) was calculated according after readings taken by Decagon AccuPAR LP-80 ceptometer. One reading was taken for every plot.

Table 4: Formulas and references used to calculate some of the traits present in the study

Parameter	Formula	Reference
Anthocyanin ratio index (ARI)	(1/R550) -(1/R700)	Steddom et al., (2003)
Carotenoid reflectance index (CRI)	(1/R510) -(1/R550)	Steddom et al., (2003)
Normalised difference vegetation index (NDVI)	(R800-R680)/(R800+R680)	Gamon et al., (1990)
Ratio analysis of reflectance spectra of carotenoids (RARSc)	R760/R500	Blackburn (1999)
Carotenoid index (CARI)	(R720/R521)–1	Zhou et al., (2017)
Photochemical reflectance index (PRI)	(R531-R570)/(R531+R570)	Gamon et al., (1990)
Harvest Index (HI)	Grain Yield/ Biomass	Pask et al., (2012)

3.4 Statistical analysis

Mean± SE of a maximum three measurements for each plot is used. Data were tested for normality using the Shapiro-Wilk test and QQ-plot. Due to the difference in days after emergence DAE to reach the phenological stage considered in the study, this factor was incorporated into the subsequent statistical analysis as a cofactor. The same is applied for the replication factor. Two-way ANOVA was used to identify the significant differences at $P \leq 0.05$ for interaction between Genotype and Environment (GxE), among environments and among genotypes. In addition, Photosynthetically Active Radiation (PAR) was used as a cofactor for NPQt and ΦPSII. If ANOVA showed significant differences, Tukey test was carried out to identify difference between the genotypes and/or the environments and/or their interaction. In order to explain the associations between the different traits, Spearman correlation was performed. Principal Component Analysis (PCA), was performed to assess the contribution of various parameters to the genotypes across different environments, utilizing all measured traits for each leaf. All the tests and graphs of this study were made in RStudio (R Software version 4.1.0).

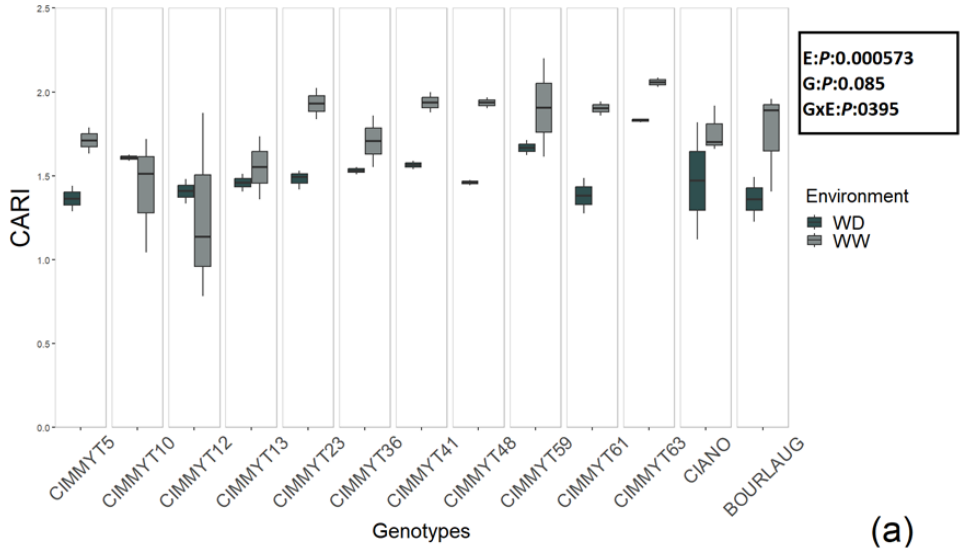
4. Results

4.1 Flag Leaf in response to Water Deficit

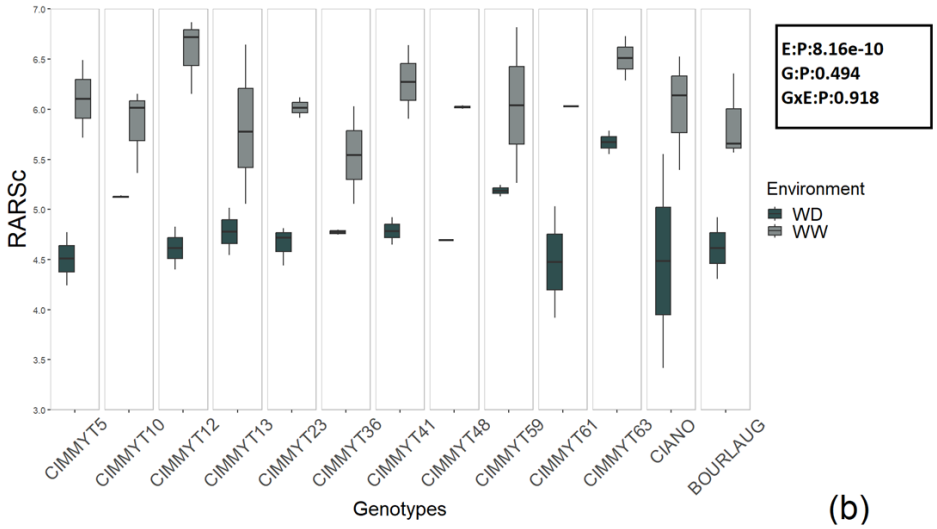
4.1.1 Photoprotection related Indices and NPQt

Four indices related to carotenoids were investigated, CARI, RARSc CRI and PRI. For CRI (see Table 5-Annex), there was no significant effect on genotypes, environment and between them ($p>0.05$). The same is applied to the index related to Ant (ARI) (see Table 5-Annex). Regarding CARI (Fig 4-a) and RARSc (Fig4-b), both showed high significant difference in terms of E (in both cases, $P<0.0001$). On average, RARSc was 26.62% higher under WW, and CARI exhibited a 12.79% increase under WW. However, there are some exceptions in CARI, where CIMMYT10 and CIMMYT12 showed lower mean under WW (respectively,1.42±0.19 and in 1.26 ±0.32) against WD (respectively, 1.60±0.01 and 1.40±0.05). For the same index, CIMMYT61, CIMMYT48 and CIMMYT23 showed the largest percentage difference between the environment means, being WW higher in comparison to WD (respectively, 27.43%, 24.59% and 23. 31%). While CIMMYT13 exhibited the smallest difference (5.84%) between WD (1.45±0.04) and WW (1.54±0.10). RARSc showed the largest differences between genotypes for CIMMYT12 (29.88%, with the largest mean under WW at 6.72±0.21), CIMMYT5 (26.13%) and CIANO with CIMMYT61 around 25% and the smallest average RARSc in response to WD (respectively 4.48±0.87 and 4.47±0.45). The smallest percentage difference was found for CIMMYT10 (12.26%), CIMMYT63 (12.88%, with largest RARSc under WD at 5.67±0.094), for CIMMYT36 (13.89%, with the lowest RARSc under WW at 5.54±0.39). However, these variances were not statistically significant. The WD effect had a significant impact on PRI (Fig4-c), the average PRI among the genotypes under WW was 33.33% higher than the plants under WD. CIMMYT23 was the only exception, showing similar results in terms of PRI in both environments, with an average difference of 0.80%. CIANO, CIMMYT10 and CIMMYT13 exhibited the largest differences among the environments (respectively, 34.38%, 34.37% and 28.44%). Nevertheless, no significant genotype-specific or genotype-environment differences were found. NPQt (Fig4-d) showed higher values under WD, with a mean for all 13 genotypes 4.80±2.19, while under WW of 1.84±0.95. No significant genotype dependent differences were detected. However, CIANO, CIMMYT48 and CIMMYT23 showed the largest differences among environments (respectively 5.47±0.92 under WD vs 1.02±0.27 under WW; 6.66±1.00 vs 1.56±0.04; 5.62±2.98 vs 1.73±0.16). Among the genotypes, CIMMYT5 and CIMMYT12 outstands with the smallest differences of 58.38% and 49.93% between WD and

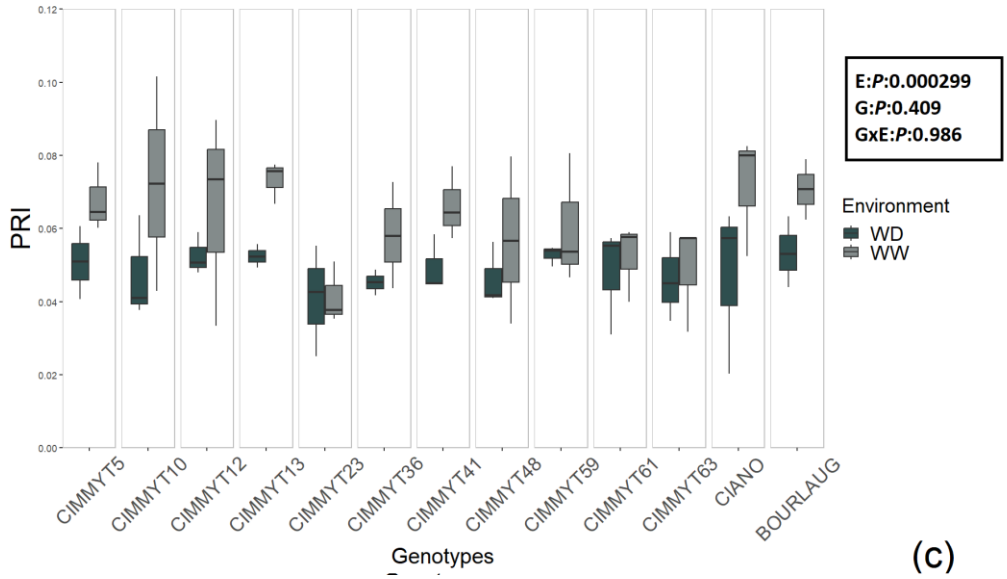
WW. For all the traits described in this paragraph, no significant differences for the same line in the two environments (GxE) were identified.



(a)



(b)



(c)

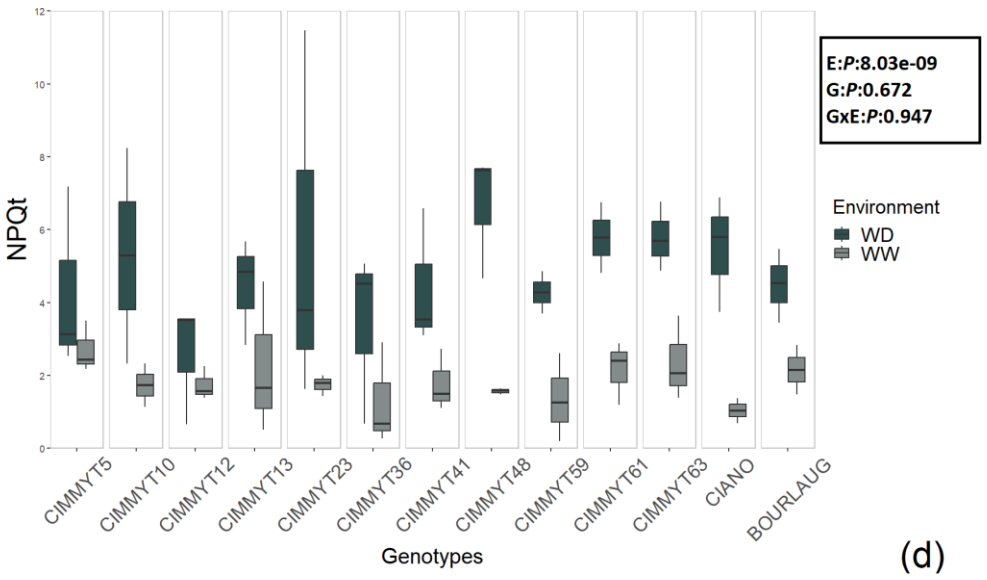


Figure 4: Response of CARI (a), RARSc (b), PRI (c) and NPQt (d) measured on flag leaf in reaction to WD (dark grey) and WW (grey).

4.1.2 Greenness related indices and ΦPSII

NDVI showed highly significant difference only in terms of environments (average mean under WW 0.71±0.02 and WD 0.66±0.03). CIMMYT59 and CIMMYT63 had the smallest difference among environments, 0.33% and 2.19%. In addition, CIMMYT63 had the largest NDVI under WD (0.72 ±0.003). CIMMYT61, CIMMYT12 and CIANO had the largest difference among environments, (respectively, 13.00%, 11.88% and 11.00%). In addition, CIMMYT12 had the largest NDVI under WW (0.73±0.011). The biggest source of variation for SPAD (Fig. 3-b), is represented by E with a p-value < 0.0001. Under WW, relative chlorophyll was, on average, higher 8.52% compared to WD. In addition, the genotypic difference represented part of the variation for SPAD, four groups were identified (see letters in Fig. 3-b). CIMMYT41, CIMMYT10 and CIMMYT36, had the smallest difference between the two environments (expressed as in mean, 3.97%, 5.15% and 6.01%). In addition, CIMMYT10 had the smallest relative chlorophyll content under WW 49.25±1.86, significantly different from CIMMYT48 and CIMMYT59 which have the largest SPAD in response to WW, noted by letter "a". Further, CIMMYT48, CIMMYT59 and CIMMYT61 had the largest difference (respectively, 15.09%, 11.33% and 10.57%). In particular, CIMMYT48 showed the lowest SPAD value under WD 46.2±3.11 and largest under WW 54.46±0.94. Looking at WW, in Fig5-b, genotypes CIMMYT10 and CIMMTYT48 are significantly different, as pointed by the letters (a) and (b). Figure 5 represents ΦPSII, which exhibited significant higher values in response to WW (0.35±0.12) compared to WD (0.26±0.06). The only exception is CIMMYT13, which had slightly higher ΦPSII under WD (0.26±0.04) than under WW (0.25±0.04). However, no significant genotypic or GxE effects were determined. Nevertheless, CIMMYT36, BOURLAG, and CIMMYT59 exhibited the largest differences between environments, respectively (55.57%, 45.90%, and 44.23%), while CIMMYT63 and CIMMYT23, following CIMMYT13, demonstrated the smallest differences (5.68% and 5.25%). For all mean values and ANOVA results, refer to Table 7-Annex.

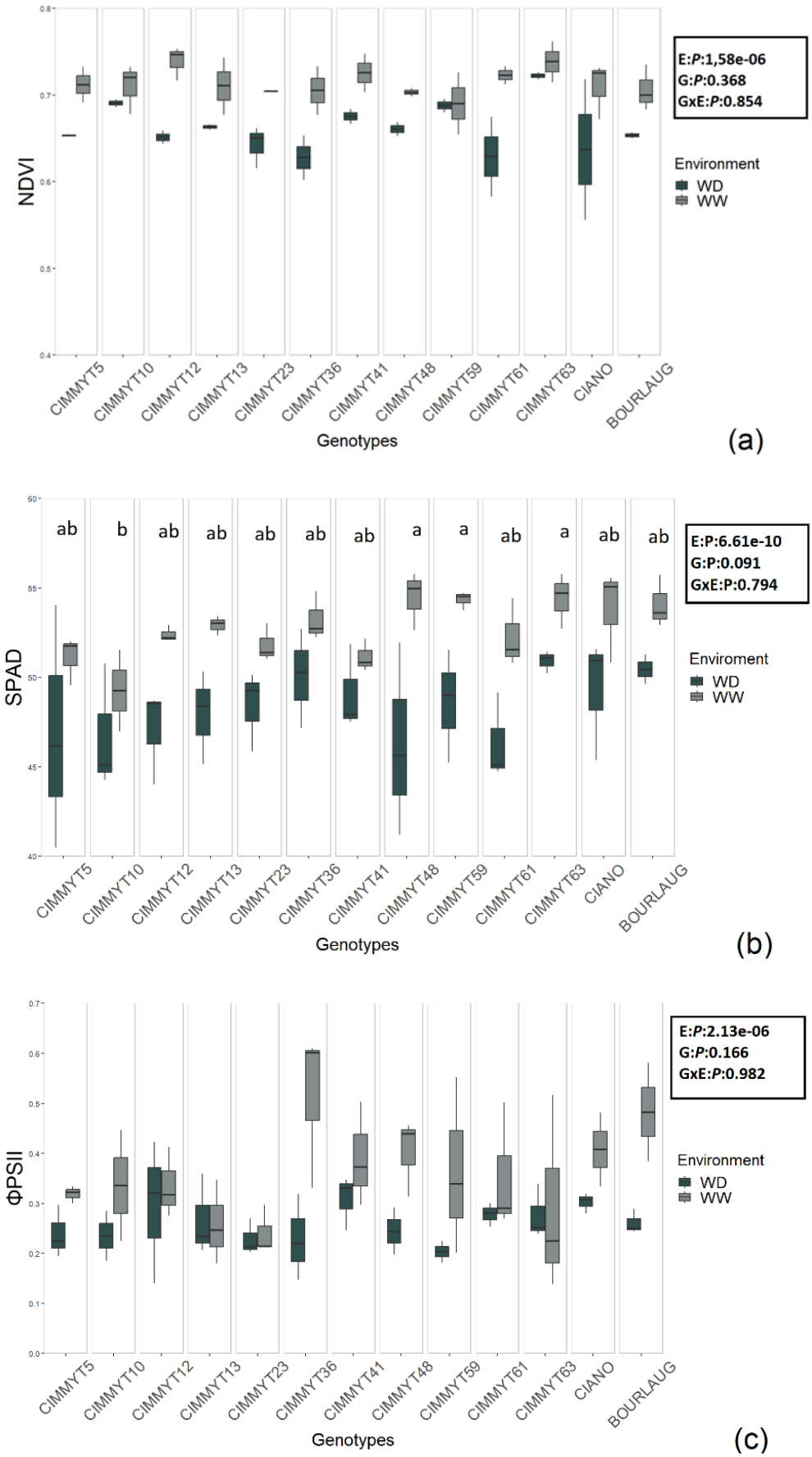
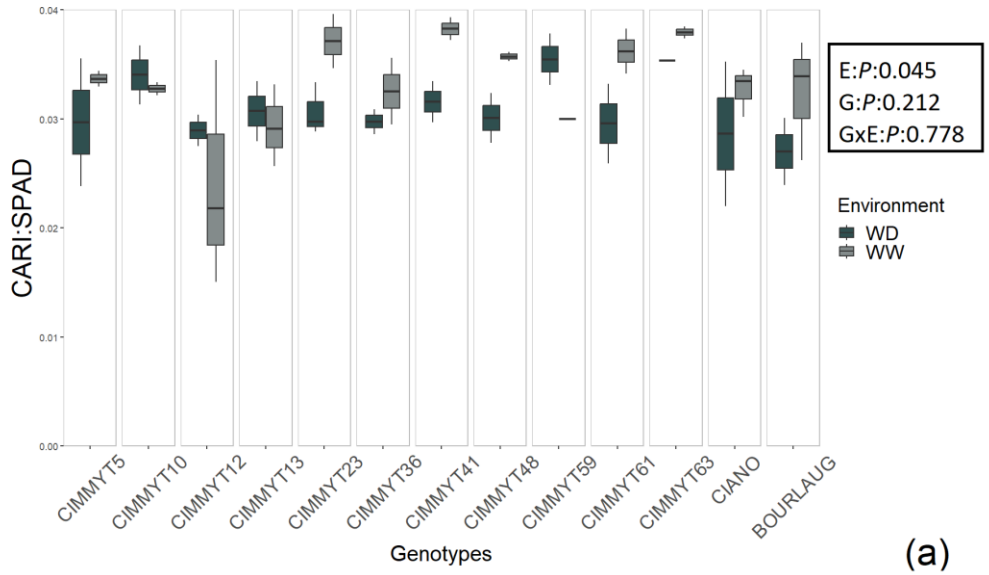


Figure 5: Response of NDVI (a), SPAD (b) and ΦPSII (c) measured on flag leaf in reaction to WD (dark grey) and WW (grey). In Fig5-b, letters refer to significant differences for G within WW

4.1.3 Carotenoid vs Chlorophyll indices relationship

For the ratio CRI:SPAD, there was no significant effect on genotypes, environment and between them (p>0.05) (see Table 6-Annex). CARI:SPAD exhibited a moderate level of significance (P≤0.05 in Figure 6-a) in terms of environments. The significance was due to higher values under WW (on average 0.033±0.005) compared to WD (on average 0.030±0.004). However, CIMMYT12, CIMMYT13, CIMMYT10 and CIMMYT59 showed higher means under WD compared to WW, respectively 20.23%, 4.85%, 3.87% and 1.05%. Although, no significant differences in terms of G and GxE were highlighted, the checks together with CIMMYT12, showed the lowest mean for CARI:SPAD in response to WD. Interestingly, CIMMYT12 showed the lowest mean among the genotypes (0.02±0.005). CIMMYT 41, CIMMYT63 and CIMMYT23 showed the largest CARI:SPAD under WW. In addition, CIMMYT63, showed high CARI:SPAD under WD as well, while CIMMYT41, along with CIMMYT61 showed the largest difference among environments (respectively, 17.52% and 18.36%). The RARSc:SPAD ratio was significantly different only in terms of environment (*P*=5.97e-05; in Figure 6-b) higher under WW (0.11±0.010) compared with plants under WD (0.09±0.012). All the genotypes under WW had higher RARSc:SPAD compared to those under WD. Among those, CIMMYT12 outstands because of the largest difference between the two environments (24.43 %), on the other hand CIMMYT59 exhibited only 0.84%. CIMMYT36 and CIMMYT12, together with the checks showed the lowest values under WD. Interestingly, CIMMYT12, CIMMYT41 and CIMMYT5 had the largest CARI:SPAD under WW. For all the mean values± standard error and results of ANOVA see Table 6-Annex.



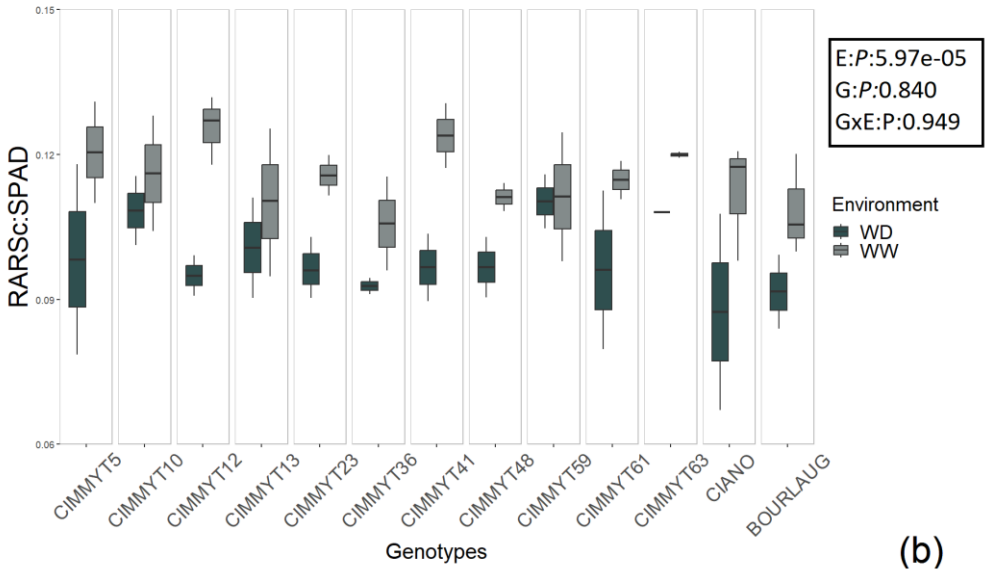
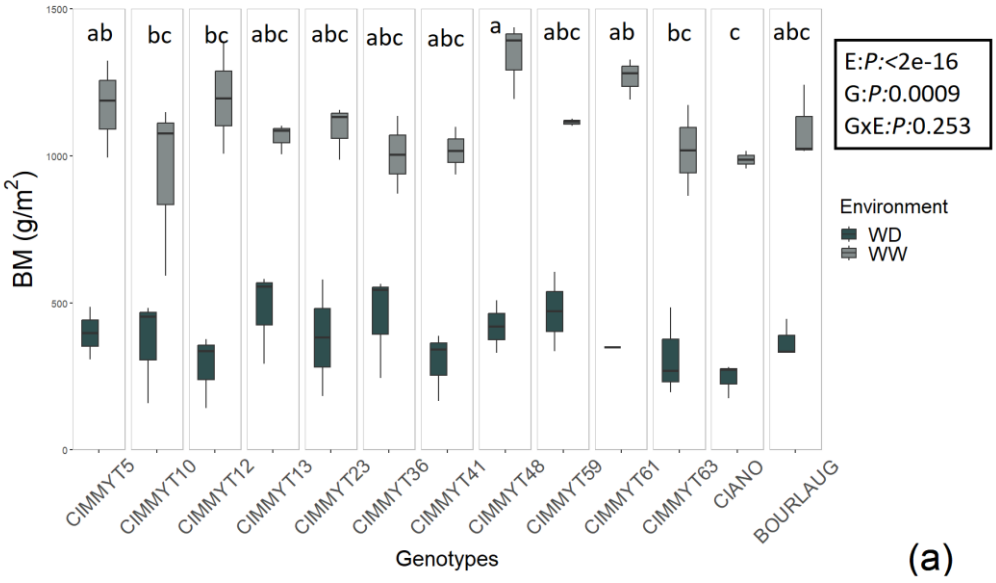


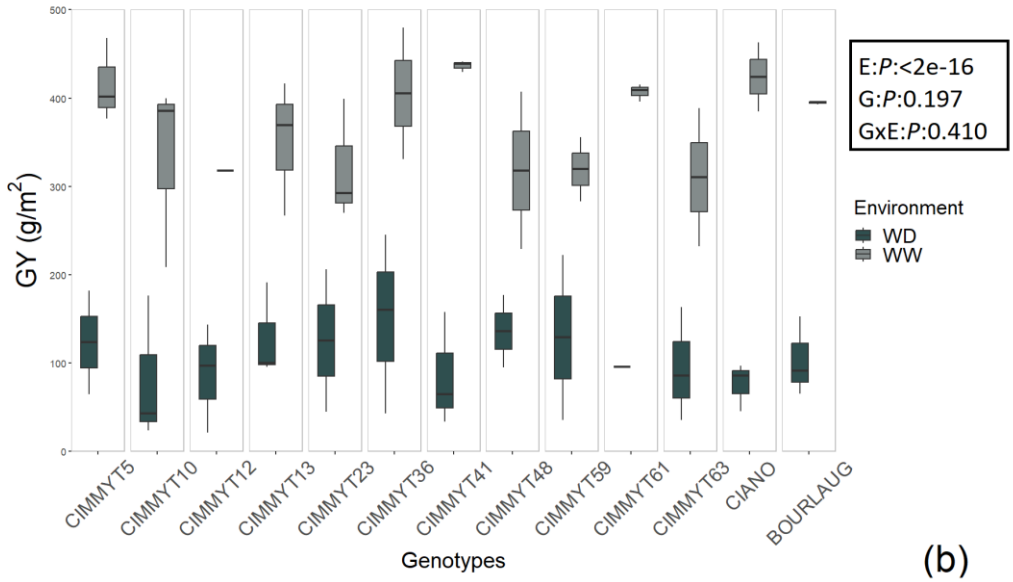
Figure 6: Response of the ratios CARI: SPAD (a) and RARSc: SPAD (b) measured on flag leaf in reaction to WD (dark grey) and WW (grey).

4.1.4 Yield and yield component trend

In all the yield and yield components parameters, plants under WW showed consistently higher values compared to those under WD. Significant differences were determined between environments in biomass (BM), grain yield (GY) and harvest index (HI). The biggest share of variability for BM is determined by the environmental factor (on average, lines in response to WW 1122.41±187.96 vs in response to WD 365.91±137.14). A portion of the variability in BM is attributed to the genotypic effect (*P*= 0.0009).The genotype with the highest BM across the environments was CIMMYT61, which exhibited the highest mean of BM under WW and across environments as noted by the letter "a" in Figure 7-a. Interestingly, CIANO was the genotype most significantly different from CIMMYT61, as noted by the letter "c", due to the result of the check in response to WD (243.00 g/m²). On the other hand, CIMMYT59 (470.50 g/m²) and CIMMYT36 (450.33 g/m²) exhibited the highest BM in response to WD. Grain yield (Fig7-b) differed significantly according to the environment. On average GY was 72.11% higher under WW compared to WD. CIMMYT36 (149.53 g/m²), CIMMYT48 (136.21 g/m²) and CIMMYT13 (128.88 g/m²) showed the largest GY in response WD, opposite to CIANO (75.70 g/m²), CIMMYT10 (80.9 g/m²) and CIMMYT41(85.15 g/m²). Under WW, checks (BOURLAG 445.08 76.82, CIANO 424.00 g/m²) and CIMMYT41(436.56 g/m²) perform better compared to other genotypes. Therefore, CIANO was the genotypes with the largest difference among environments (82.14%). On the other hand, CIMMYT23 (320.49 g/m²), CIMMYT59 (319.4

g/m²) and CIMMYT63 (310.36 g/m²) were the genotypes with lowest yield under WW. A significant environment effect was found for HI as well, on average HI under WW was 22.85% higher compared to WD (WW, 0.35±0.064 vs WD 0.27±0.088). For the majority of the genotypes HI was larger under WW, only CIMMYT48 exhibited 7.27% higher HI under WD compared to WW, opposite to CIMMYT10 which showed the largest differences among the environments (44.37%). However, no significant difference was found in terms of G or GxE. Remarkably, the checks showed largest HI under WW (BOURLAG 0.41±0.0; CIANO 0.44±0.02). CIANO (0.30±0.02), together with CIMMYT48 (0.31±0.02) showed largest HI under WD. CIMMYT10 (0.20±0.08) and CIMMYT59 (0.23±0.10) display the lowest HI under WD. For all the mean values± standard error and results of ANOVA see Table 8-Annex.





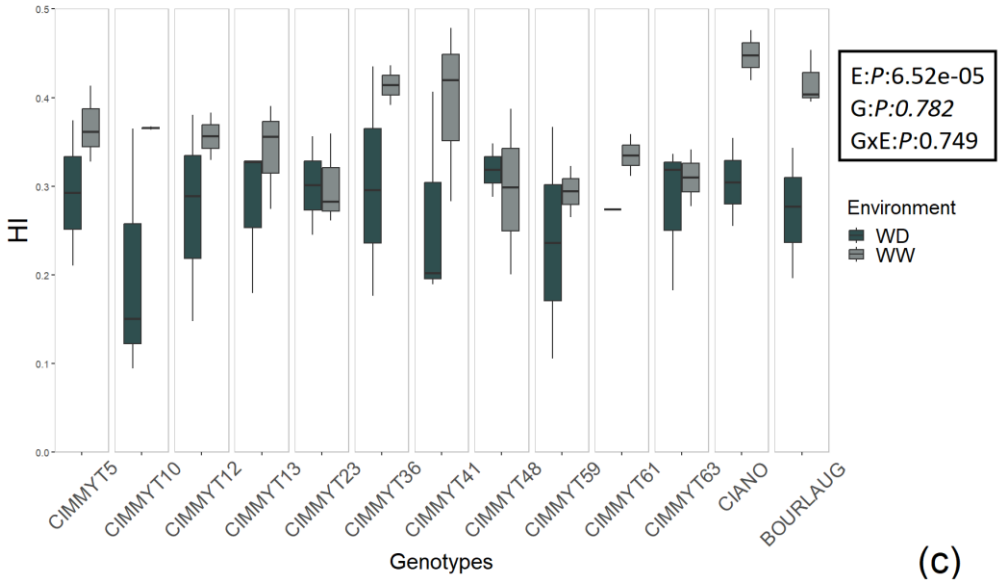


Figure 7: Response of final aboveground biomass (a), grain yield (b) harvest index (c), in reaction to WD (dark grey) and WW (grey). In Fig7-a, letters refer to significant differences for G across environments

LAI showed very significant difference in term of Environments with *P*=0.001. CIANO and CIMMYT61 showed the smallest difference among environments, with slightly higher LAI in response to WD (respectively 1.88% and 0.48%). Instead, CIMMYT48 showed the largest difference (LAI 14.27% higher in response to WW). This line, opposite to CIANO, showed the largest LAI (5.51±0.37), while the check exhibited the lowest LAI in response to WW (4.78±0.23). CIMMYT36 and CIMMYT63 displayed the most distant differences in terms of LAI response to water deficit, with values of 5.06±0.10 and 4.68±0.08, respectively, although these differences were statistically insignificant.

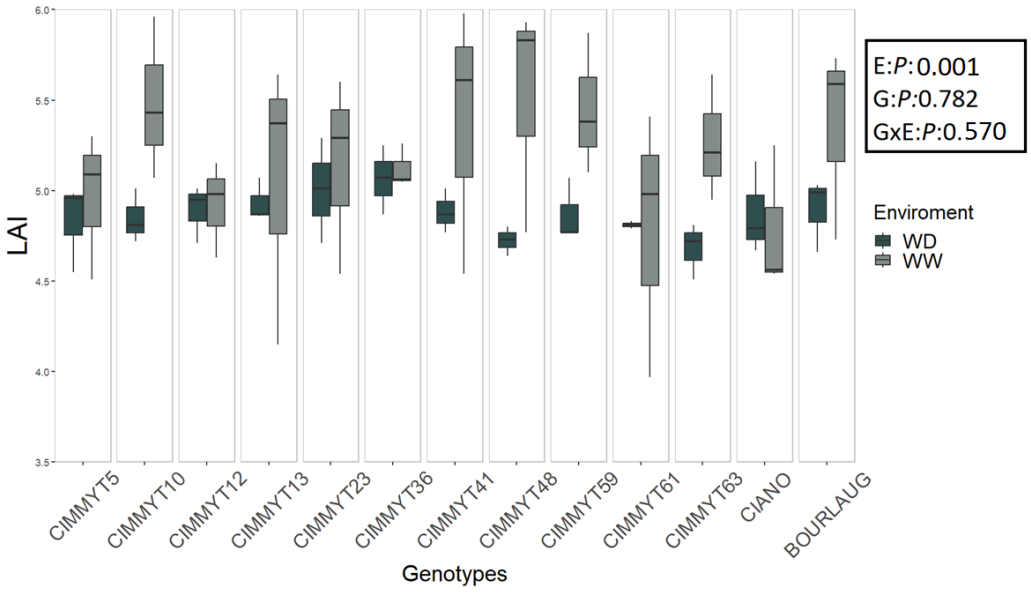


Figure 8: Response of Leaf Area Index (LAI) in reaction to WD (dark grey) and WW (grey).

4.1.5 Correlation between and with agronomic, carotenoids and greenness related traits

Under WD (Figure 10) and WW (Figure 9), NDVI, RARSc and CARI showed highly positive significant correlation dynamics. While under WW, ARI showed a significant negative correlation with CRI and NDVI. Under WD, CRI, exhibited positive significant correlation with NDVI, RARSc and less with CARI (r^2=0.36). A similar pattern was shown under WW as well, however the significance of the correlation is stronger with RARSc and NDVI (*P*=≤0.001) and with CARI (r^2 = 0.39) In addition, CRI is the only exception among the indices related to Cars to show a positive significant correlation with PRI under WD. It was not confirmed under WW. In both environments there were no significant relationships between PRI and NPQt. Spectral reflectance indices associated with carotenoids did not show any significant correlation coefficients with NPQt, however under WD a slight tendency between NPQt and CRI and with CARI was reported (r^2 around - 0.20). Under WW, NPQt and ΦPSII were highly negatively correlated, but under WD it was not confirmed. In both environments, for LAI, no significant correlations were highlighted. Significantly negative correlation dynamics were reported between CRI related indices and agronomic data, such as BM and to GY for *P*=0.01 and *P*=0.05. Under WW, BM exhibited a slight negative correlation with PRI (r^2= - 0.32)

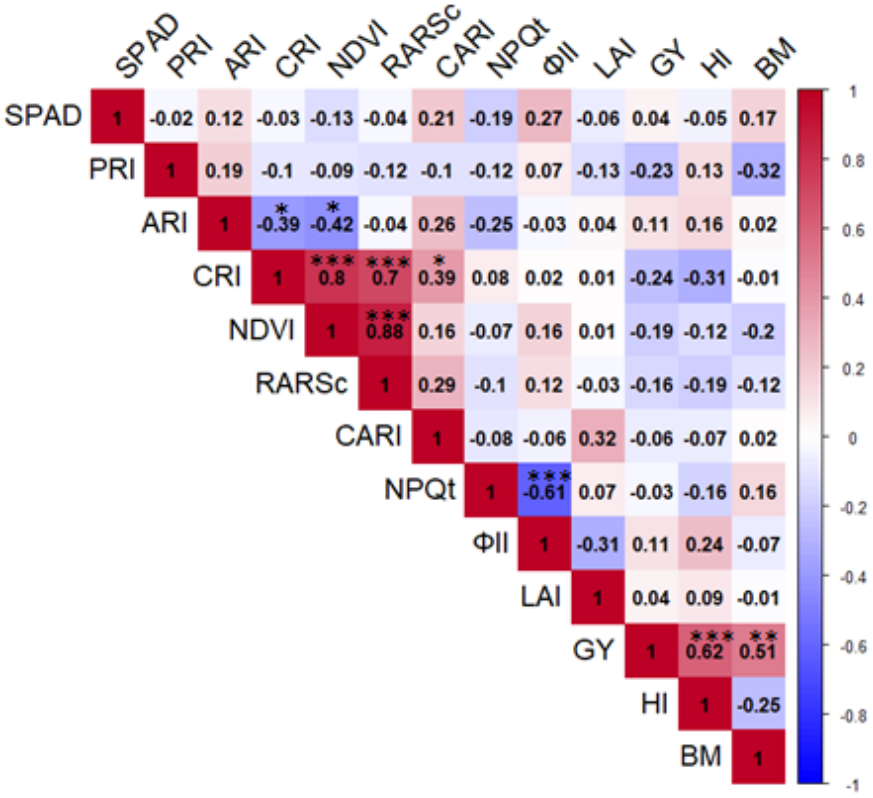


Figure 9 :Heatmap display genotypic correlations for flag leaf among SPAD, PRI, ARI, CRI, CRI, NDVI, RARSc, CARI, NPQt, ΦPSII, LAI, GY (g/m2), HI and BM (g/m2) in reaction to WW. Significance of the codes: 0.0001´***` 0.001 '**' 0.01 '*' 0.05 '.'

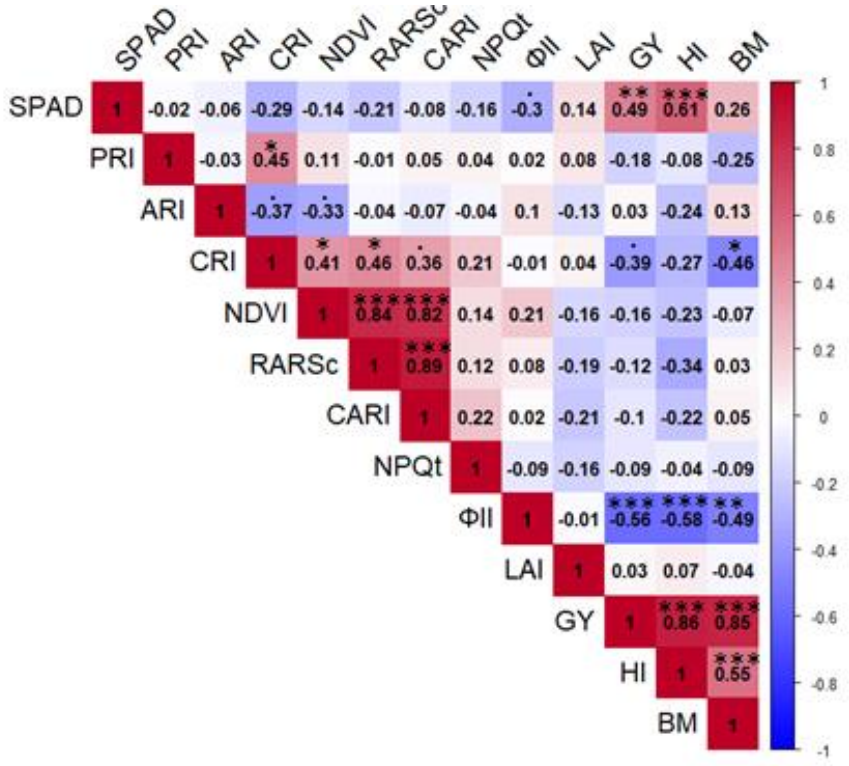
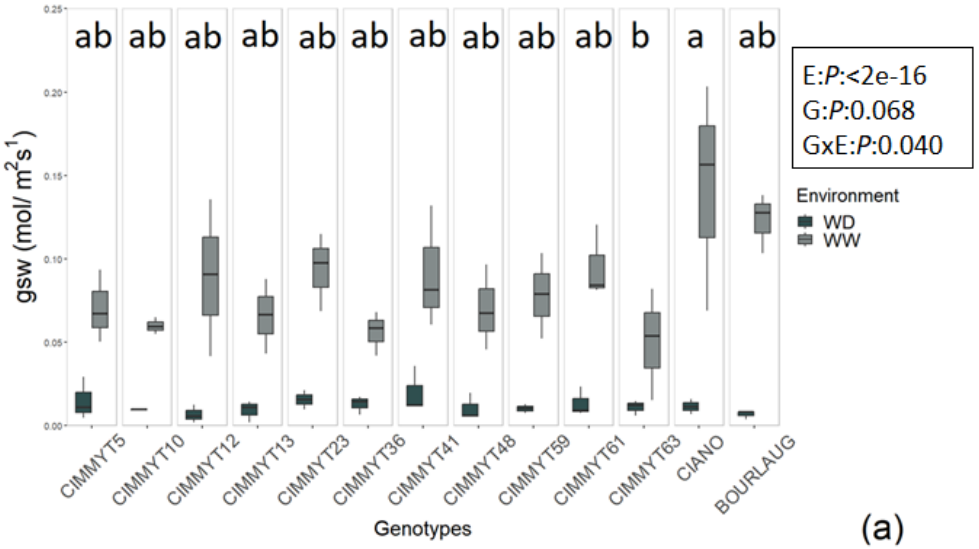


Figure 10: Heatmap display genotypic correlations for flag leaf among SPAD, PRI, ARI, CRI, CRI, NDVI, RARSc, CARI, NPQt, ΦPSII, LAI, GY (g/m2), HI and BM (g/m2) in reaction to WD. Significance of the codes: 0.0001´***` 0.001 '**' 0.01 '*' 0.05 '.'

4.1.6 Stomatal conductance and Canopy Temperature

Stomatal conductance was significantly different among the environments (P<0.0001). On average, gsw under WW was 87.50% higher than in WD. The checks showed the largest difference between environments (P<0.0001). CIMMYT12, CIMMYT23, CIMMYT61 and CIMMYT41 showed as well highly significant difference in terms of GxE. CIMMYT13 (P=0.002), CIMMYT48 (P=0.001), CIMMYT5 (P=0.002) and CIMMYT59 (P=0.001) showed a very significant difference. CIMMYT10, CIMMYT36 and CIMMYT63 showed a significant difference in terms of GxE (respectively, P=0.02, P=0.01 and P=0.03). The variance among genotypes under WW is significantly larger than under WD conditions, with a significance level of P=0.031. CIANO showed the largest stomatal conductance under WW (0.14±0.03), at the second place BOURLAG (0.12±0.01). CIMMYT63 has the lowest gsw under WW (0.05±0.01). Under WD, no significant genotypic effect was found. On average CT showed 18.92% higher values under WD compared to plants under WW, this was a high significant difference in terms of E

environments (*P*<2e-16). CIMMYT63 showed the smallest difference (17.04%), opposite to BOURLAG which exhibited CT 40% higher in WD compared to WW. CIMMYT 12 and BOURLAG showed the highest CT under WD (respectively, 30.31±0.46, and 30.08±0.09), opposite to CIMMYT59 and CIMMYT5 (respectively, 27.85±0.83 and 28.25±0.56). CIMMYT5, together with CIMMYT 41 and BOURLAG, showed the lowest CT under WW as well (22.95±0.77; 22.70±0.39; 21.46±2.38). On the other hand, CIMMYT63 and CIMMYT36 showed the highest CT (respectively, 25.52±0.52 and 24.73±0.91). All the genotypes showed very high significant difference in terms of GxE *(P*<0.0001*).* For all the mean values± standard error and results of ANOVA for gsw, VPD and CT, see Table 9-Annex.



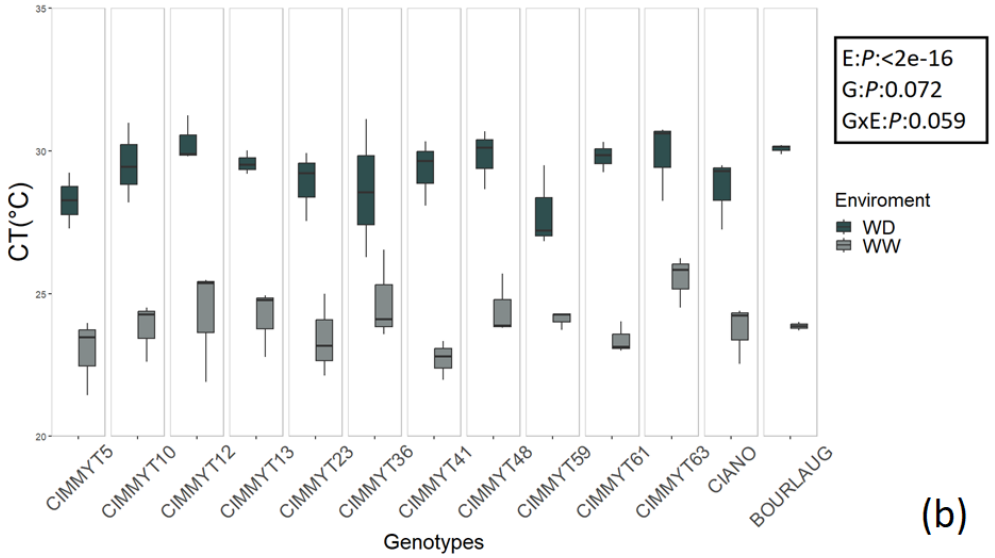


Figure 11:Response of Stomatal Conductance (a) and Canopy Temperature (b) in reaction to WD (dark grey) and WW (grey). In Fig11-a, letters refer to significant differences for G within WW

4.1.7 Water and Temperature Relationships

Under WD, CT displayed a very significant negative association with gsw and highly significant positive with VPD (see Figure 13). The same dynamics are confirmed under WW as well (see Figure 12). As well, within WW, VPD and CT are associated positively with NPQt, but this was not significantly proved. Gsw was significantly positive correlated with ARI within WW. In general, within WW, no other negative or positive association were reported with CT, VPD and gsw and other traits related to photoprotection. Within WD, VPD showed a significant positive association with ARI, whereas with, PRI, CRI and NPQt show a slightly positive non-significant correlation. Similar dynamics were highlighted for CT within WD. Instead, gsw, under WD exhibited a significant negative association with CRI, a slight positive non-significant with CARI and negative non-significant with PRI. Under WD, SPAD and gsw had a significant positive correlation, on the other hand CT and VPD had a significant positive correlation with ΦPSII (respectively for P=0.001 and P=0.05). Considering the grain yield and yield component traits. Under WD and WW, CT showed highly significant negative correlation with GY (r^2=- 0.53 under WD and r^2=- 0.68 under WW) and very significant with HI (r^2=- 0.43 under WD and r^2=- 0.48 under WW). Highly significant negative correlation between CT and BM was confirmed only under WD (r^2=-0.58). Under WD, gsw showed a significant positive correlation with BM and GY (P=0.01) and with HI for (P=0.05). On the other hand, under WW, gsw showed a significant positive correlation with HI (P=0.01) and with GY (P=0.05). whereas VPD, under WD showed a highly significant negative correlation with HI, a very significant negative correlation with GY and significant negative correlation with BM. Under WW, VPD showed only a significant negative correlation with GY for P=0.05. Interestingly, VPD, under WW showed a significant negative correlation with LAI (r^2 =- 0.29) and a positive with NPQt (r^2=0.28). Under WD, a similar correlation coefficient between VPD and NPQt (r^2= 0.31) was reported.

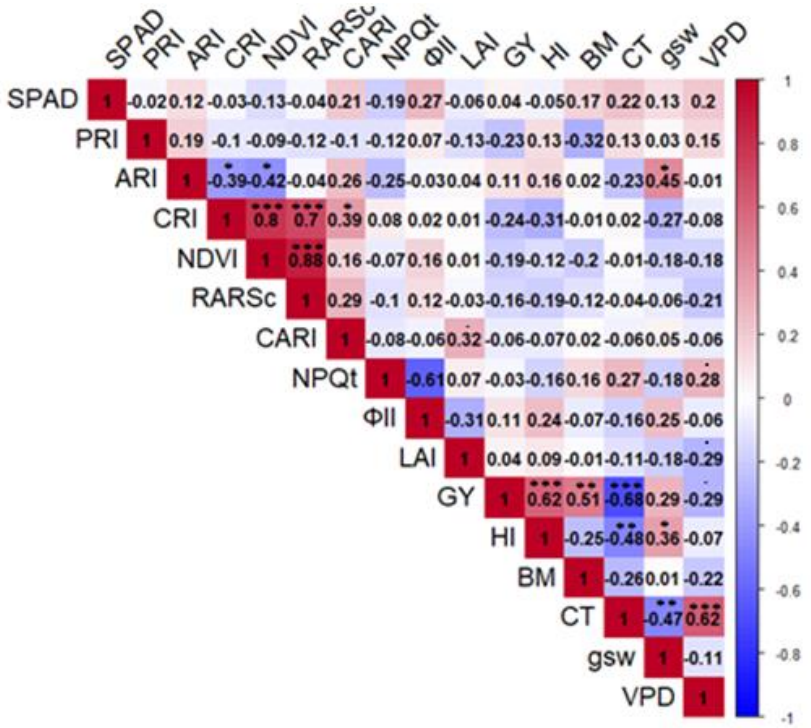


Figure 12:Heatmap display genotypic correlations among SPAD, PRI, ARI, CRI, CRI, NDVI, RARSc, CARI, NPQt, ΦII, LAI, GY (g/m2), HI and BM (g/m2), CT (°C), gsw (mol m-2 s-1) and VPD (kPa) in reaction to in reaction to WW Significance of the codes: 0.0001´***` 0.001 '**' 0.01 '*' 0.05 '.'

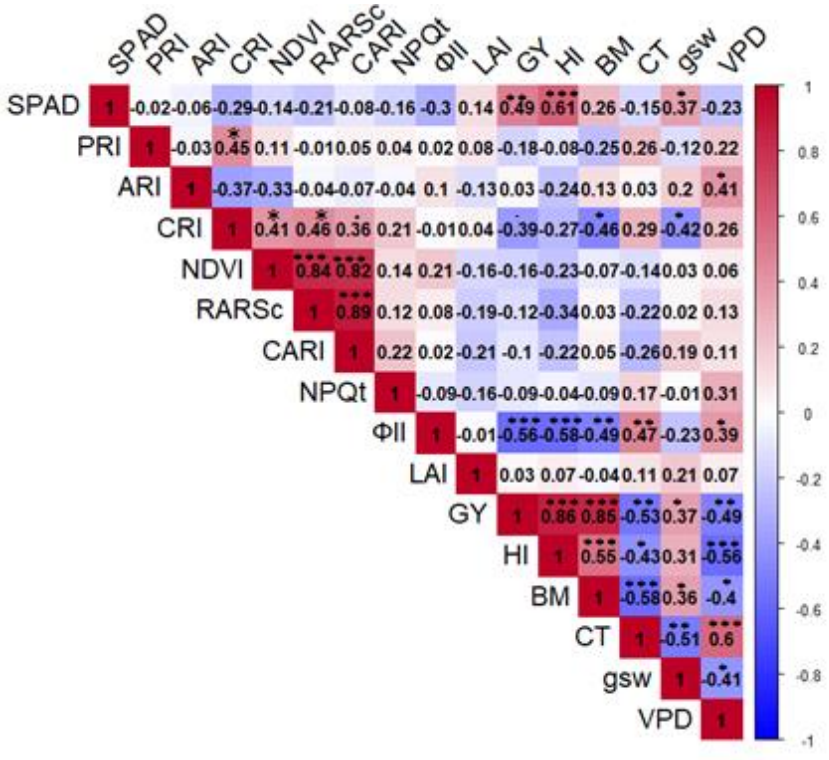


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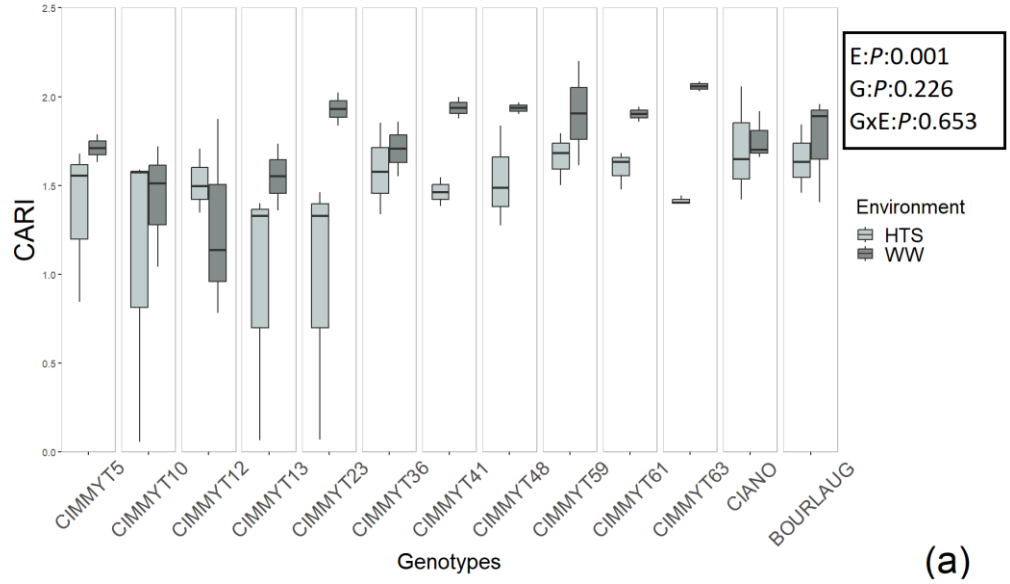
Figure 13:Heatmap display genotypic correlations among SPAD, PRI, ARI, CRI, CRI, NDVI, RARSc, CARI, NPQt, ΦII, LAI, GY (g/m2), HI and BM (g/m2), CT (°C), gsw (mol m-2 s-1) and VPD (kPa) in reaction to WD. Significance of the codes: 0.0001´***` 0.001 '**' 0.01 '*' 0.05 '.'

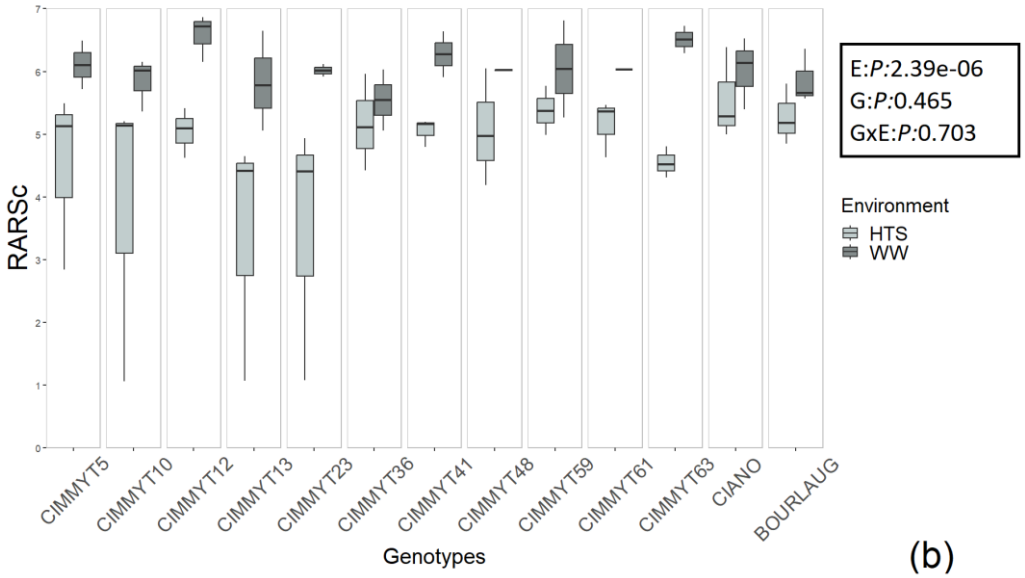
4.2 Flag Leaf in response to Heat Stress

4.2.1 Photoprotection related Indices and NPQt

The environment effect on the spectral indices related to Cars, including PRI, was the only significant effect (see Figure 14). For NPQt, there was no significant effect on G, E and GxE (see Table 10-Annex). The same is valid for ARI (see Table 10-Annex). The average difference between WW and HTS was 18.02% for CARI with plants showing higher CARI in response to WW, however exceptions are present. Although significant difference among G and for GxE were not identified, CIMMYT12 and CIMMYT10 showed 31.52% and 3.97% CARI higher under HTS compared to WW. In fact, both genotypes had the lowest values under WW (1.51±0.19; 1.13±0.32). Instead, CIMMYT63 and CIMMYT41 exhibited the highest CARI in response to WW (2.05±0.02 and 1.93±0.05). Under HTS, CIMMYT59 and CIANO exhibited the highest mean (1.65±0.08 and 1.70±0.18), opposite to CIMMYT13 and CIMMYT23 which were confirmed to had the lowest CARI (0.93±0.43 and 0.95±0.44). On average, plants in response to WW showed RARSc values 27.96% higher than under HTS. CIMMYT13 and CIMMYT23 showed the largest difference among environments (respectively, 42.00% and 42.28%). On the other hand, CIMMYT36 and CIANO showed a certain stability between environments with a difference of, respectively, 6.80% and 7.70%. Further, CIMMYT36 in response to WW showed the lowest RARSc (5.54±0.39) opposite to CIMMYT12 (6.58±0.21). Under HTS, CIANO showed the highest mean values (5.55±0.42) opposite to CIMMYT13, which exhibited the lowest RARSc under HTS (3.37±1.15). However, differences among G were not significant. The significant difference in terms of E for CRI translates to a 27.29% increase in WW compared to HTS. Although significant differences were not found for either G or GxE, CIMMYT13 exhibited 53.83% difference in CRI among the environments, while the checks showed a certain degree of stability between WW and HTS (BOURLAG 2.22% and CIANO 4.66%). Further, checks showed the largest CRI value in response HTS, while CIMMYT13 and CIMYT23 exhibited the lowest values (BOURLAG 3.21±0.18 and CIANO 3.25±0.50 vs 1.69±0.79 and 1.79±0.84). Under WW, CIMMYT36 and CIMMYT61 showed the highest CRI (respectively, 4.19±1.00 and 4.76±0.54), whereas CIANO and BOURLAG display the lowest CRI among all the genotypes (3.10±0.32 and 3.29±0.32). Finally, PRI confirmed a highly significant difference among environments, but not for either G or GxE. On average, plants under WW showed PRI 58.33% higher compared to the lines under HTS. CIMMYT12 showed the largest percentage difference 79.77% and CIMMYT 61 the smallest

(29.91%). CIANO and CIMMYT12 showed the lowest PRI under HTS (0.011±0.009 and 0.013±0.003), opposite to CIMMYT61 and BOURLAG in the same environment, which exhibited the highest PRI (0.037±0.002 and 0.040±0.002). In response to WW, CIMMYT13 showed the highest PRI (0.07±0.003) opposite to CIMMYT23 with the lowest PRI average (0.04±0.004).





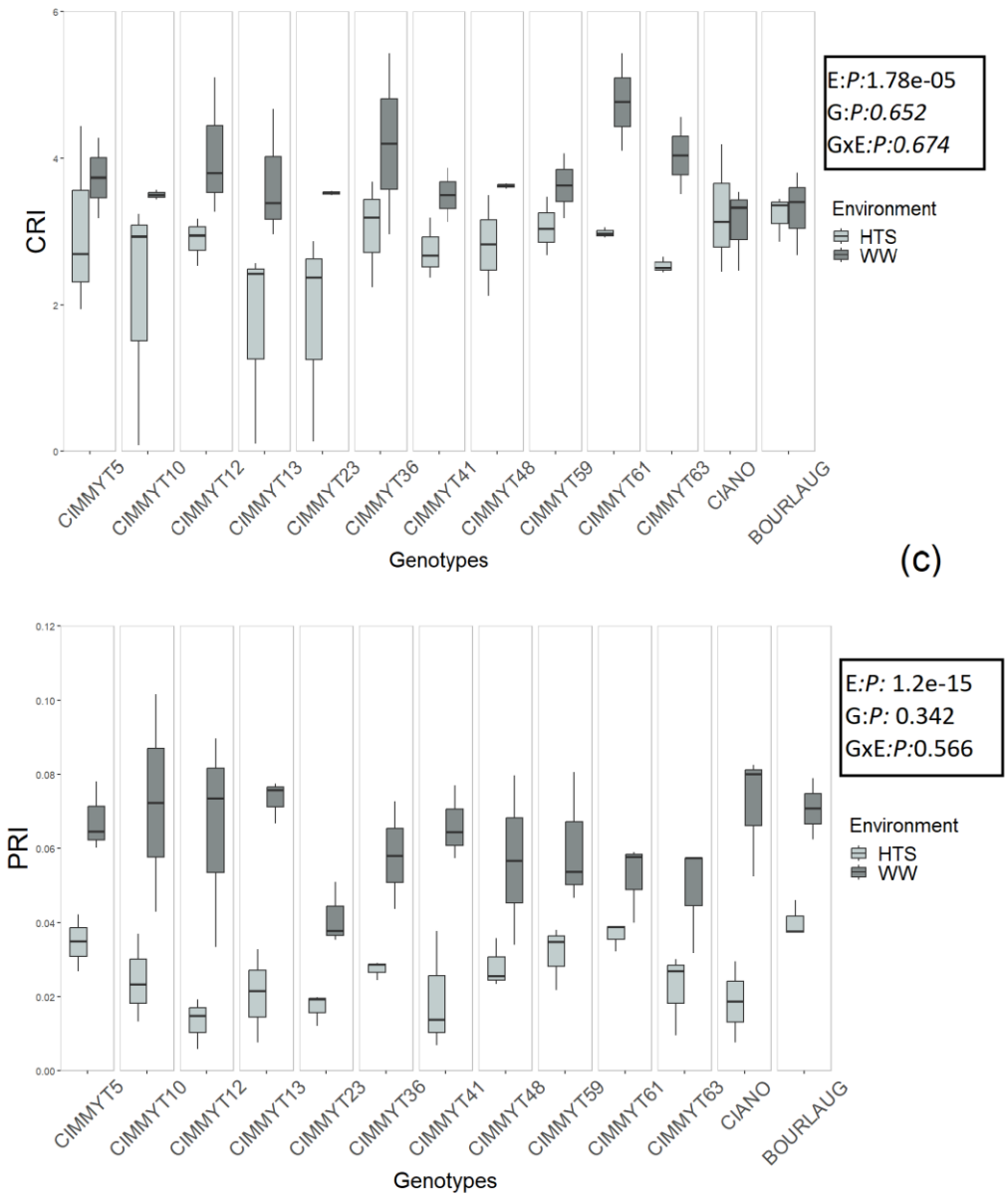


Figure 14:Response of CARI (a), RARSc (b), CRI (c) and PRI (d) measured on flag leaf in reaction to HTS (light grey) and WW (grey).

4.2.2 Greenness related indices and ΦPSII

Variability for NDVI is very significant (*P*=0.006) only for environment. Except for CIMMYT59 where HTS is slightly higher (-0.34%), NDVI under WW is on average higher of 14.08% compared to HTS. The largest difference is for CIMMYT13, with 40.11%. Despite the lack of significant differences in terms of G and GxE, under HTS, CIANO and CIMMYT59 had the highest NDVI (0.69±0.01 and 0.69±0.01), in contrast CIMMYT23 and CIMMYT13 exhibited the lowest values (0.42±0.20 and 0.42±0.20). Under WW, CIMMYT59 and CIMMYT48 showed the lowest NDVI (0.69±0.030 and 0.70±0.003), while CIMMYT12 and CIMMYT63 exhibited the highest NDVI (0.73±0.011 and 0.73±0.020). For SPAD, the first ANOVA resulted in significant differences in term of environments (with WW higher of 12.97 points compared to HTS) and on Genotypes. The second ANOVA, carried specifically on the singular environments, resulted in significant difference in term of Genotypes only in response to WW. The ANOVA test did not identify any significant differences in terms of GxE. On average SPAD under WW was 12.29% higher compared to HTS, except for CIMMYT23, which exhibited 1.84% higher under HTS. Under HTS, CIMMYT10 and CIMMYT13 showed the lowest SPAD (48.8±3.46 and 49.26±1.44), while CIMMYT63 and CIMMYT48 showed the highest values (53.00±1.15 and 54.36±0.72). Instead, in response to WW, CIMMYT10, similar to HTS, and CIMMYT5 showed the lowest SPAD (49.25±1.86 and 51.10±0.77). CIMMYT10, denoted by the letter "b," is the most significantly distant line from CIMMYT63 and CIMMYT48, which exhibited the highest SPAD in response to high temperature, indicated by the letter "a" (54.40±0.90 and 54.46±0.94). For ΦPSII, part of the variability is attributed to the environment factor and partially by the Genotypes effect. Interestingly, the majority of the genotypes exhibited higher values under HTS (on average 14.28%). However, checks, CIMMYT48, CIMMYT59 and CIMMYT61 exhibited higher ΦPSII under WW. Considering the GxE across the environments and the singular environment, no significant effect was reported. CIMMYT 36 (group a) against CIMMYT61 and CIMMYT48 (group b) exhibited the most distinct behaviour in term of ΦPSII. CIMMYT23 with ΦPSII 118.53% higher under HTS compared to WW, which together with CIMMYT48 has also the highest ΦPSII under HTS (0.527±0.12 and 0.528±0.13) is opposite to CIMMYT48 with ΦPSII 38.41% higher under WW, which together with CIMMYT59 has the lowest ΦPSII under HTS (0.24±0.05 and 0.25±0.01). In opposite to the behaviour under HTS, CIMMYT23, under WW, has the second lowest ΦPSII (0.24±0.02) after CIMMYT12 (0.25±0.04). On the other hand, under WW, CIMMYT36 and BOURLAG showed the highest ΦPSII (respectively, 0.51±0.09 and

0.48±0.08). For all the mean values± standard error and results of ANOVA for SPAD, NDVI and ΦPSII , see Table 12-Annex.

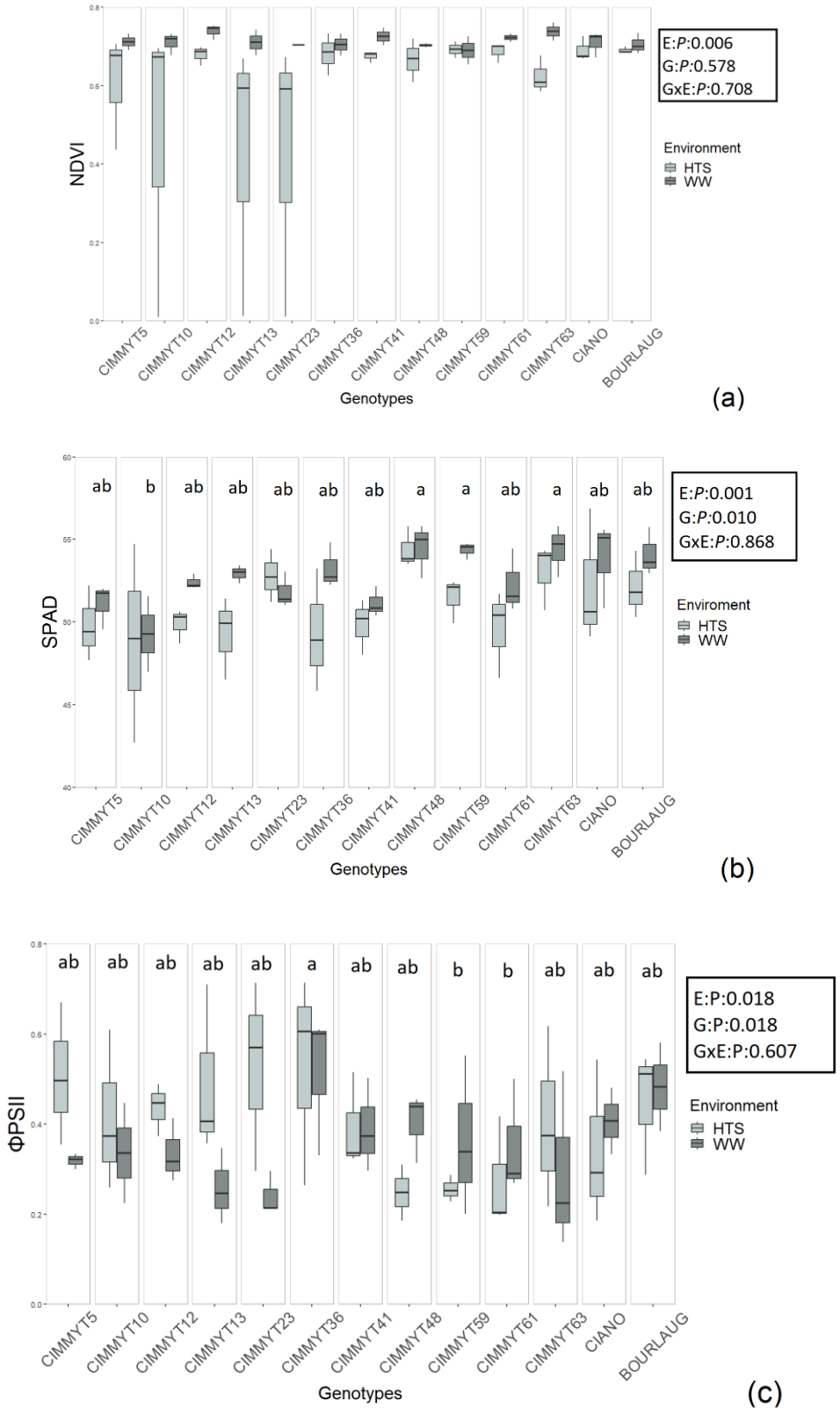


Figure 15:Response of NDVI (a), SPAD (b), and ΦPSII (c) measured on flag leaf in reaction to HTS (light grey) and WW (grey). Letters indicate statistically significant differences, in this case, in Fig.15-b they refer to significant differences for G within WW; in Fig18c, they refer to significant differences for G across the environments.

4.2.3 Carotenoid vs Chlorophyll indices relationships

When examining the various combinations of carotenoid-related indices against SPAD, it becomes evident that the environmental factor plays a significant role in explaining the majority of the variability. However, there is variability in the significance across different ratios. In fact, RARSc:SPAD and CRI:SPAD had a p-value ≤ 0.0001, while CARI:SPAD showed a p-value= 0.005. CARI:SPAD ratios were higher under WW compared to HTS of 39.39%. The only exception is CIMMYT12 with CARI:SPAD on average 26.16% higher under HTS. Checks showed a certain stability, with a small difference between the environments (BOURLAG, 2.29% and CIANO 0.47%). Neither genotype nor genotype-environment interaction (GxE) demonstrated any significant impact on the variability of CARI:SPAD. However, it is important to highlight that under HTS, CIANO and CIMMYT59 were the genotypes with higher CARI:SPAD (respectively 0.0325±0.002 and 0.0322±0.002). Instead, CIMMYT23 and CIMMYT13 were the genotypes the smallest CARI:SPAD (respectively, 0.018±0.008 and 0.019±0.009). CIMMYT13 showed again in response to WW the second lowest CARI:SPAD (0.02±0.002), while CIMMYT41 and CIMMYT63 showed the highest CARI:SPAD (respectively, 0.038±0.0008 and 0.037±0.0004). RARSc:SPAD was significantly higher in response to HTS compared to WW (18.18%). Despite no significant differences were found in terms of G or GxE, some line outstand for their values: such as CIMMYT36 and CIANO, in response to HTS, which showed the highest RARSc:SPAD (respectively, 0.106±0.003 and 0.104±0.007).Further CIMMYT36 shows higher median in response to HTS compared to WW (see Fig 14-b). On the other hand, CIMMYT23 and CIMMYT13 showed the lowest RARSc:SPAD (respectively, 0.065±0.023 and 0.069 0.024). Under WW, BOURLAG and CIMMYT36 showed the lowest average (0.10±0.006 and 0.10±0.007), while CIMMYT12 and CIMMYT41 showed the highest mean (0.12±0.004 and 0.12±0.005). On average CRI:SPAD showed significantly higher values in WW compared to HTS (28.57%), although checks made an exception (respectively, 1.40% and 6.57% higher under HTS). BOURLAG and CIANO exhibited the highest CRI:SPAD under HTS (0.06±0.003 and 0.06±0.006) and lowest in response to WW (0.06±0.006 and 0.05±0.006). CIMMYT13 and CIMMYT23 had the largest difference among environments (49.28% and 48.72%, with CRI:SPAD higher in response to WW). These two genotypes showed the lowest CRI:SPAD in response to high temperatures as well (respectively 0.035±0.01 and 0.034±0.01). For all the mean values± standard error and results of ANOVA for the ratios described in this section, see Table 11-Annex.

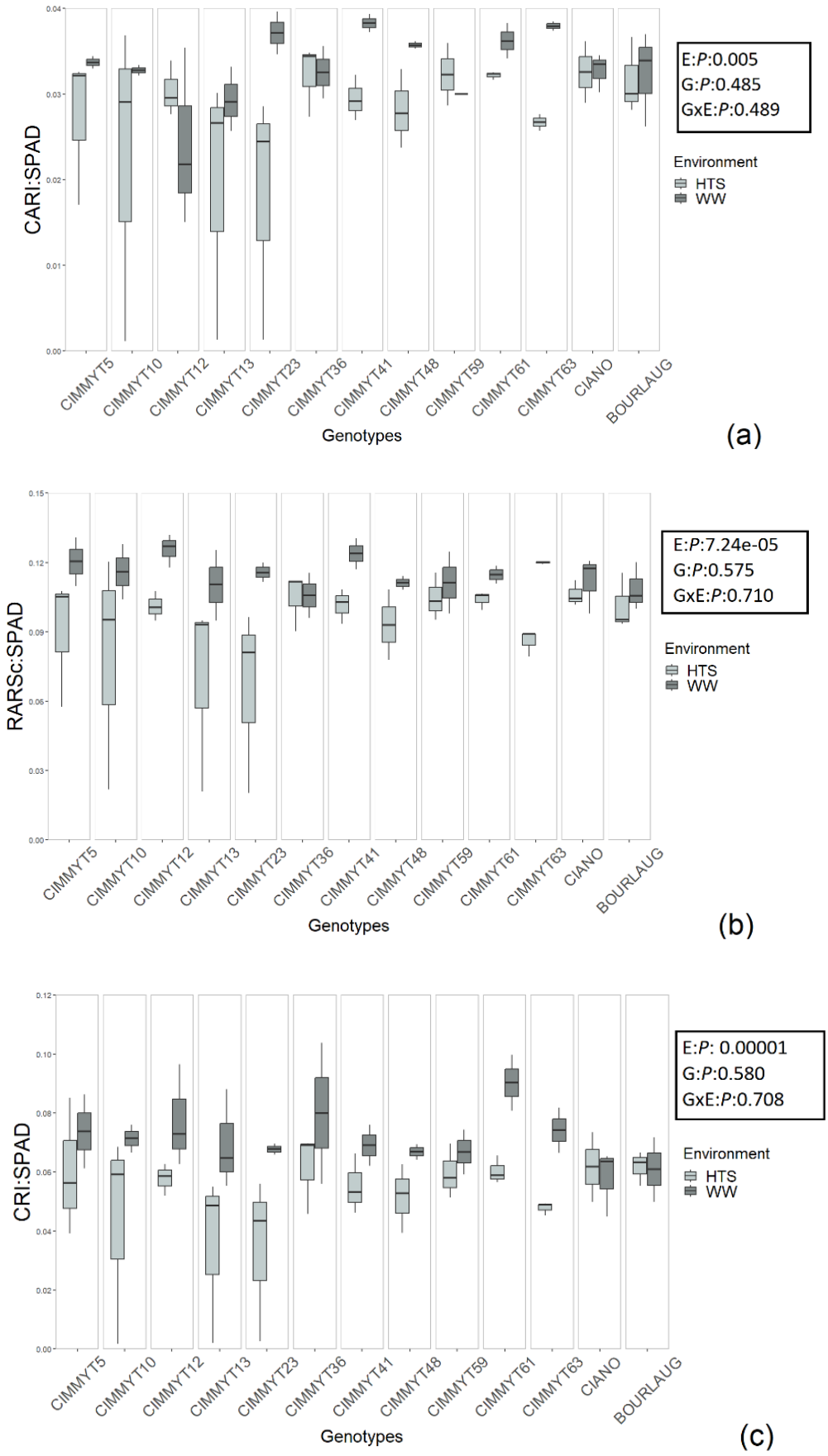


Figure 16:Response of the ratios CARI: SPAD (a), RARSc: SPAD (b) and CRI:SPAD (c) measured on flag leaf in reaction to HTS (light grey) and WW (grey).

4.2.4 Yield and yield component trends

For the biomass, the biggest part of the variation resulted from the environmental effects and the smallest part from the genotypic effects. On average, under WW, there was a 39.18% higher biomass reported compared to HTS. CIMMYT13 showed the smallest difference between the environments (26.29%), opposite to CIMMYT41 (47.25%). CIMMYT13, along with CIMMYT12 exhibited the highest BM in response to HTS (respectively, 784.43 g/m² and 819.79 g/m²) Interestingly, CIANO, under HTS exhibited the lowest BM (570.34 g/m²). Under WW, CIANO showed a similar behaviour, has the second lowest BM after CIMMYT10 (respectively, 987.00 g/m² and 939.34 g/m²), opposite to CIMMYT61 and CIMMYT48 (respectively, 1266.66 g/m² and 1341.00 g/m²). Furthermore, CIMMYT48 exhibited significantly higher biomass compared to the average in response to high temperatures. This was evident through its designation with the letter "a," indicating a high biomass across both environments. In contrast, CIANO, denoted by the letter "b," was statistically different from CIMMYT48 in terms of biomass. Regarding GY, the largest variation was accounted to the environmental impact (P<0.0001). On average, plants under WW showed a 23.23% higher GY compared to those under HTS. Despite no significant differences were reported in term of GxE and G, interestingly CIMMYT13 showed almost no difference in term of GY between the two environments (0.15%), opposite to CIANO which exhibited a difference of 41.90% between the environments. CIMMYT13 and CIANO outstand as well, for, accordingly, the highest (350.44 g/m²) and lowest grain yield (246.33 g/m²) in response to HTS. In contrast, the other check, BOURLAG showed the highest grain yield under WW (445.08 g/m²), opposite to CIMMYT63 (310.36 g/m²). Likewise, for HI, the largest variation is accounted to the environment, while less significance has the genotype effect and the interaction GXE. On average, genotypes under HTS showed a 25.71% higher HI than those under WW. If both of the environments are considered, CIANO (0.46±0.018) and CIMMYT48 with CIMMYT59 (0.37±0.010) exhibited the largest significant difference. CIMMYT5, CIMMYT10, CIMMYT12, CIMMYT13, CIMMYT59, CIMMYT61 and CIMMYT63 showed very significant differences between the environment ($0.05 \leq P \leq 0.0001$), while CIMMYT23, CIMMYT48 showed high significant difference with $P < 0.0001$. For all the mean values± standard error and results of ANOVA for the BM, HI and GY described in this section, see Table 13-Annex.

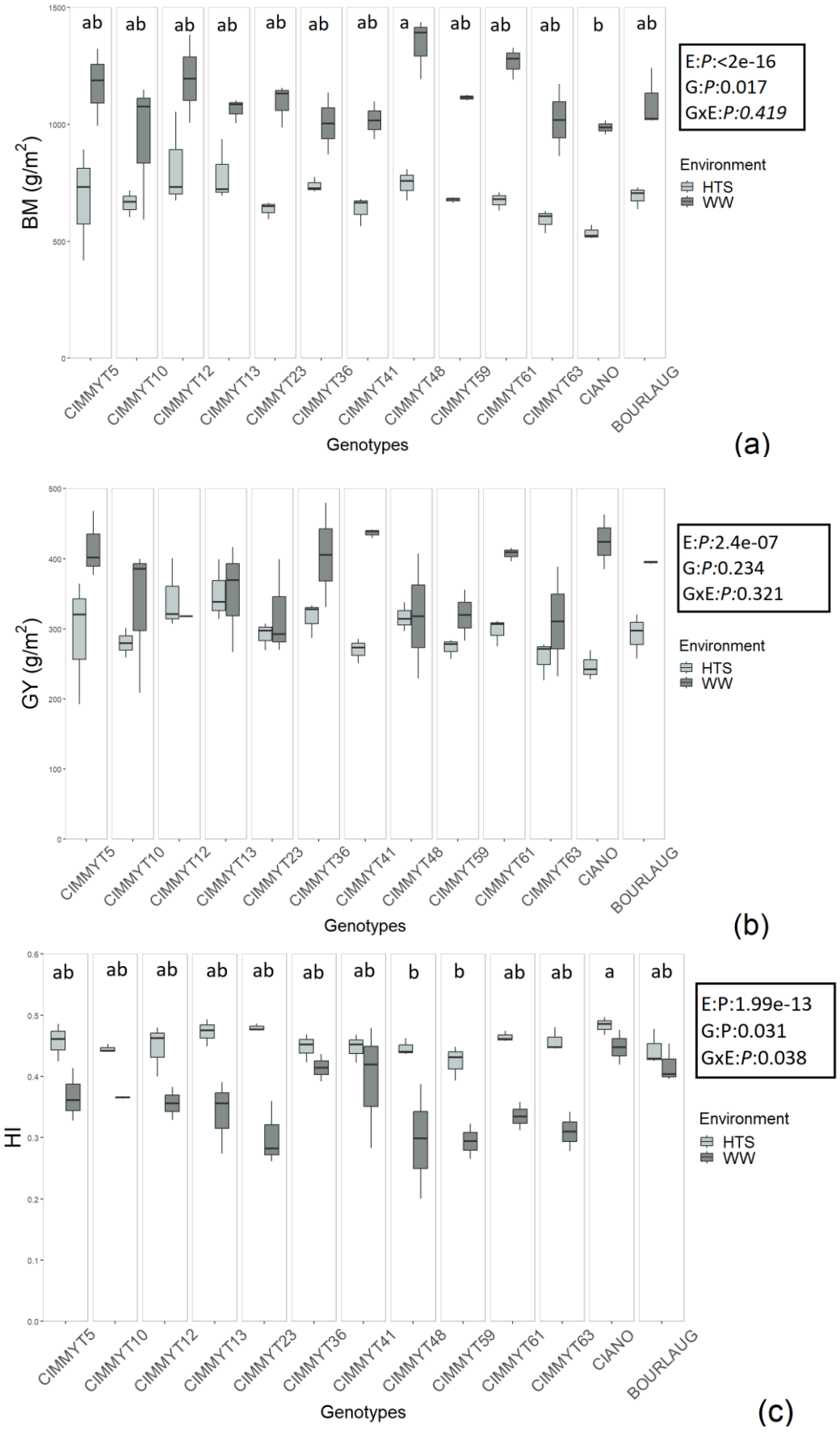


Figure 17:Response of final aboveground biomass (a), grain yield (b) harvest index (c), in reaction to HTS (light grey) and WW (grey). In Fig 17-a and Fig 17-c letters indicate statistically significant differences across environments.

Regarding LAI, the variability is accounted only to the environmental factor (P<0.0001). Within HTS, plants showed on average, LAI 20.69% higher than under WW. The largest difference between the environments was accounted to CIANO (30.52%) and the smallest to CIMMYT59 (13.94%). CIANO (4.78±0.23), showed the lowest LAI under WW, on the contrary CIMMYT48 (5.51±0.37) exhibited the highest value within the same environment Under HTS, CIMMYT5 showed the lowest LAI (6.12±0.06), opposite to CIMMYT12, with the highest average of LAI under the same environment (6.37±0.08). For all the mean values± standard error and results of ANOVA for LAI, see Table 13-Annex.

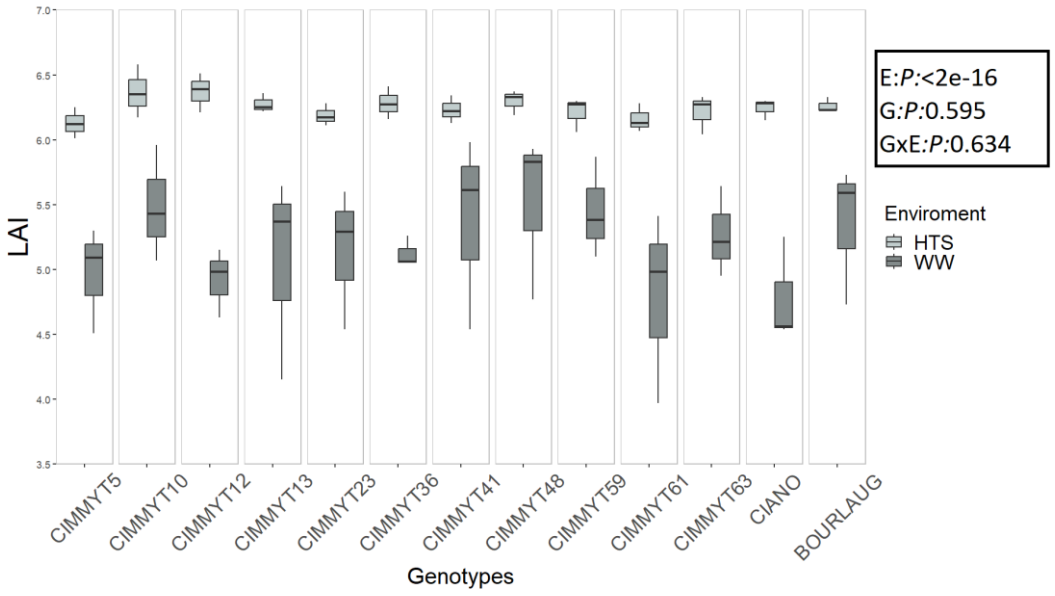


Figure 18: Response of Leaf Area Index (LAI) in reaction to HTS (light grey) and WW (grey).

4.2.5 Correlation between and with agronomic, carotenoids and greenness related traits

Under HTS, highly significant positive correlation was detected among NDVI, RARSc and CRI and CARI. In contrary under WW (Fig.9), CARI showed a significant correlation only with CRI (r^2=0.39). Likewise, under WW, there were no significant relationships between PRI and NPQt, however, opposite to spectral reflectance under WW, which were not associated with PRI, PRI was significant positive associated with NDVI and a significant positive correlation with CARI was found. Such as under WW, LAI did not present any significant correlation with any agronomic traits or Chl and/or Cars related traits. On the other hand, NPQt and ΦPSII were highly negatively correlated. This was exhibited under WW as well. Interestingly, NPQt showed a significant positive correlation with CARI, the other indices show a positive non-significant correlation coefficient. Concerning the agronomic parameters. Interestingly grain yield exhibited a significant negative correlation with indices related to Cars (such as, CRI, RARSc and CARI). Further GY and ΦPSII showed a significant positive correlation. Opposite to the dynamic of GY and HI under WW, under HTS, these two traits did not present any association. Instead, BM, was very significant negatively correlated with HI and highly significant positively correlated with GY.

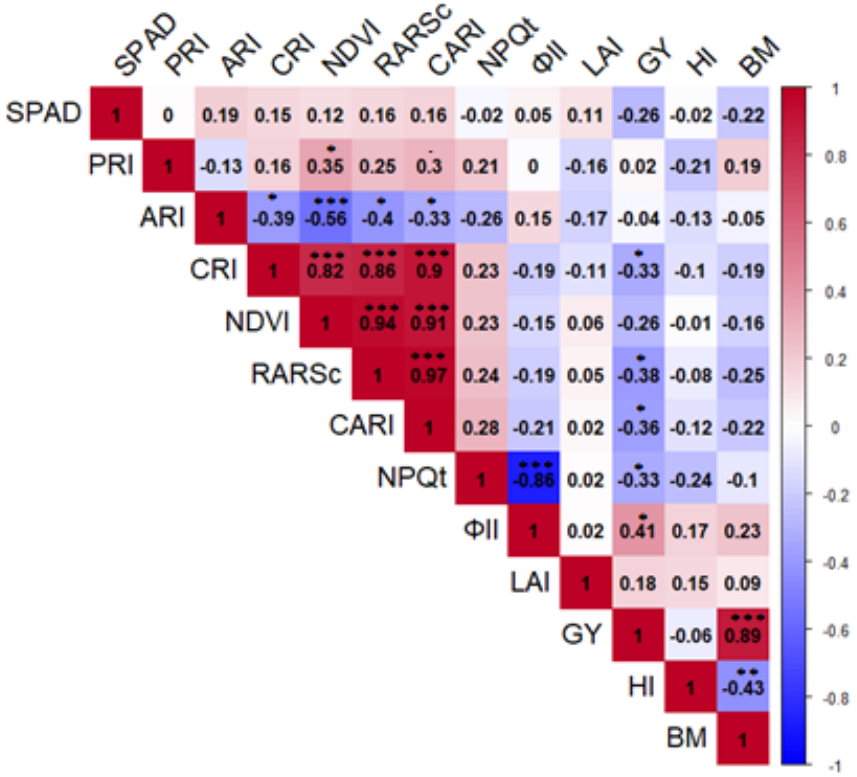


Figure 19:Heatmap display genotypic correlations for flag leaf among SPAD, PRI, ARI, CRI, CRI, NDVI, RARSc, CARI, NPQt, ΦPSII, LAI, GY (g/m2), HI and BM (g/m2) in reaction to HTS. Significance of the codes: 0.0001´***` 0.001 '**' 0.01 '*' 0.05 '.'

4.2.6 Stomatal conductance and Canopy Temperature

The genotypic effect for gsw, across the environments, is a significant source of variation. In this case the environment is not accounted as significant. In addition, considering only WW, a significant variation in term of genotypes was found (*P*=0.03). However, in both analysis the same significance difference in terms of G were found, with CIANO (average 0.10±0.01 across the environment and 0.14±0.01only in WW) and CIMMYT63 (average 0.04±0.01across the environment and 0.05±0.01 only in WW) being the most significant different genotypes. Under HTS, no significant differences were reported, however CIMMYT63 (0.03±0.01) showed the lowest gsw, opposite to CIMMYT36 with the highest gsw (0.10±0.03). CT showed significance in term of genotypes across the environment and very significant difference within the singular environment of HTS (*P*=0.001). Although, the biggest source of variation was the environment (on average plants under HTS showed CT higher of 12.28% compared to those under WW). BOULRAG showed the largest difference between environment (24.48%), while CIMMYT12 exhibited the smallest (6.29%). Looking at the genotypic difference, whether across the environments or under HTS, CIMMYT63 outstands with the highest CT (26.75±0.40, average across the environments and 28.00±0.24 only within HTS). Interestingly, BOULRAG and CIMMYT41 are the most significant different from CIMMYT63, when considering both environments. This scenario is replicated under WW, as well: BOURLAG showed the lowest CT (21.47±2.38), while CIMMYT63 the highest (25.52±0.52). Considering genotypic differences within HTS, the other check, CIANO (26.46±0.30 average within HTS) together with the other genotypes indicated by letter “b” in Fig 18-b are the most significant different compared to CIMMYT63. Contrary to the other traits related to water and temperature. For all the mean values± standard error and results of ANOVA for water and temperature traits, see Table 14-Annex.

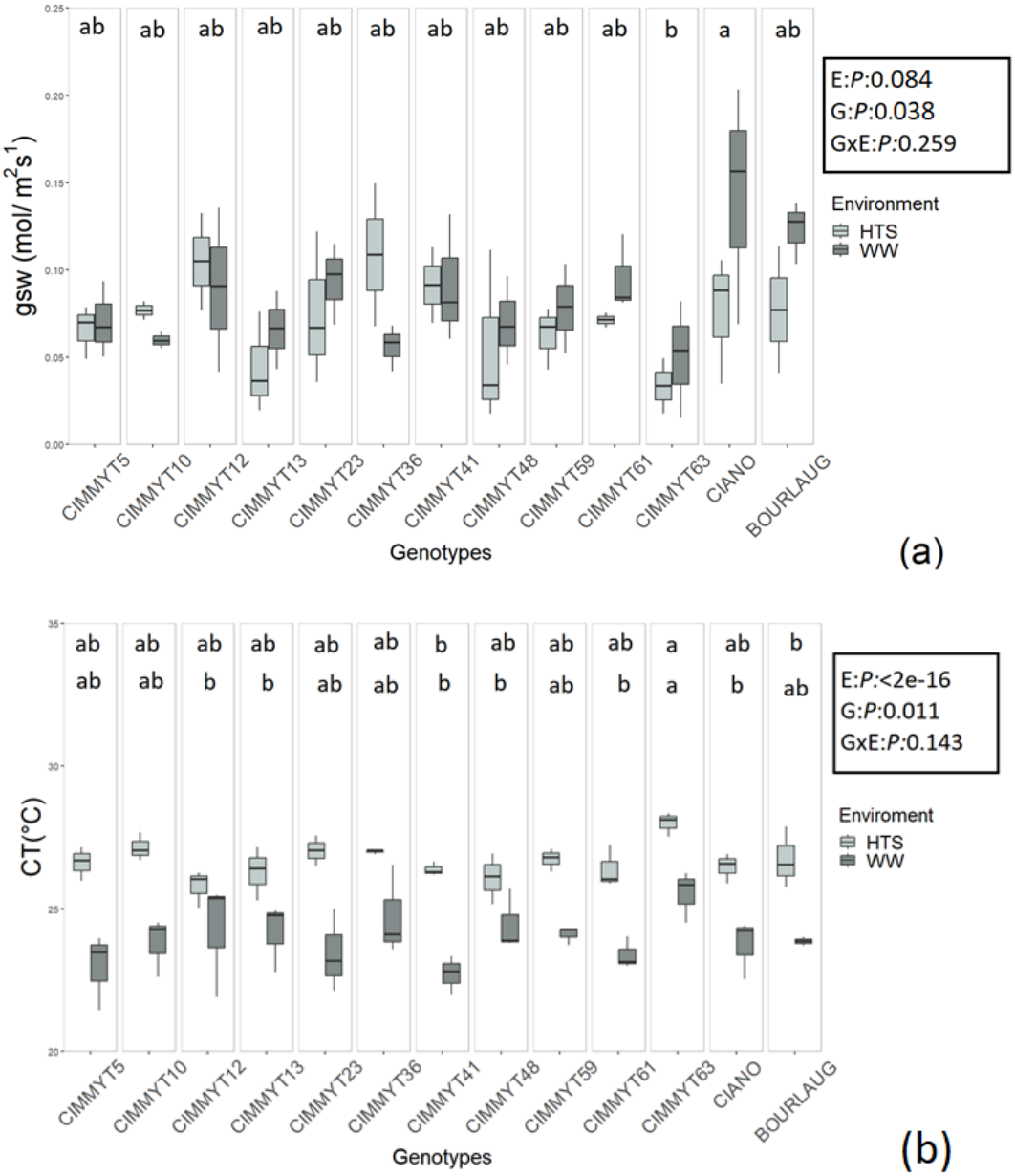


Figure 20:Response of gsw (a) in reaction to HTS (light grey) and WW (grey) measured on flag leaf and of Canopy Temperature (CT) (b). Letters indicate statistically significant differences; in Fig.20-a, the differences across the environments and in WW are consistent, implying that the letters apply to both cases. In Fig.20-b letters on top refer to statistically significant differences for G across the environments, while the letters below indicate statistically significant differences for G within the HTS environment.

4.2.7 Water and Temperature Relationships

In the environment of HTS (Fig.21), CT and gsw do not exhibited any association. Further, no association is observed with Cars related indices, included PRI and NPQt, for any of the traits related to water and/or temperature status. In general, positive relationships are observed between VPD, gsw, and indices related to carotenoids. On the contrary, slightly negative relationships were observed between CT and these indices. Instead, under WW (Figure 12), associations are evident between ARI and gsw and VPD with NPQt. However, SPAD, showed a moderate significant positive correlation with CT and a very significant positive correlation with VPD. VPD, in turn, demonstrates a very significant positive correlation with LAI, instead under WW there is a moderate negative correlation (r^2= -0.29) between these two parameters. VPD and gsw exhibited a very significant negative correlation. Under HTS, likewise under WW, VPD and CT demonstrates a significant positive correlation.

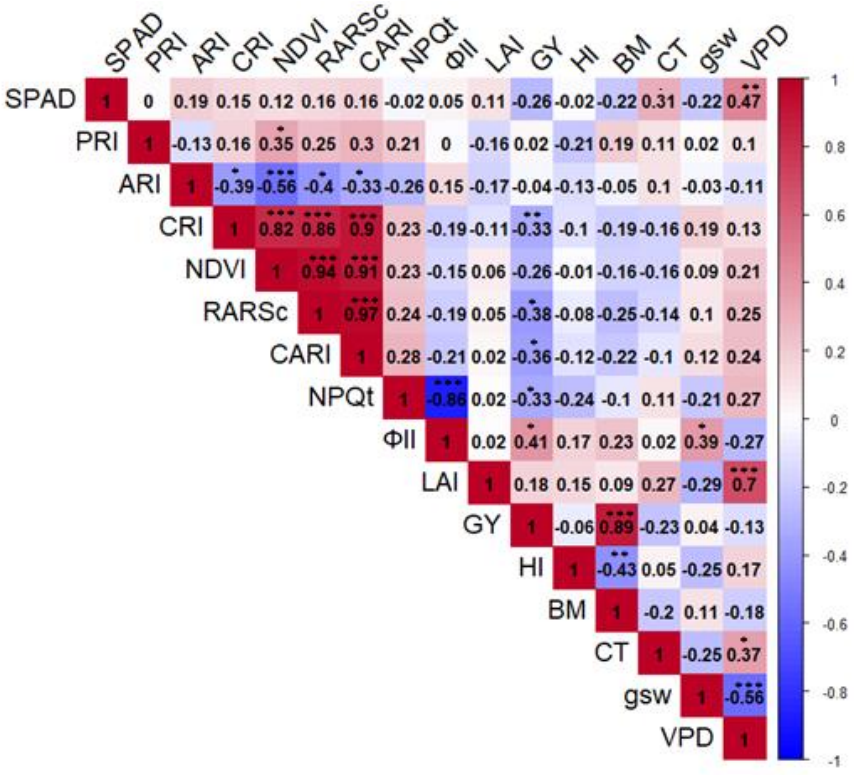


Figure 21:Heatmap display genotypic correlations among SPAD, PRI, ARI, CRI, CRI, NDVI, RARSc, CARI, NPQt, ΦII, LAI, GY (g/m2), HI and BM (g/m2), CT (°C), gsw (mol m-2 s-1) and VPD (kPa) in reaction to HT. Significance of the codes: 0.0001'***` 0.001 '**' 0.01 '*' 0.05 '.'S

4.3 Multivariate analysis over the three environments on flag leaf

Principal Component Analysis (PCA) revealed that Dimension 1 (Dim1) (44.7%) significantly contributed to separating genotypes, measured on the flag leaf, under WW and HTS conditions from those under WD. Additionally, Dimension 2 (Dim2) (32.9%) became relevant for differentiating genotypes between WW and HTS. A clear distinction between WD and WW environments was observed compared to the HTS condition (Figure 23). In fact, lines within the HTS and WW exhibited similar responses in the dimensions represented by the principal components, indicating shared features among genotypes in these specific conditions, as detected by the overlap of the ellipses. When analysing genotypic responses, we found a central tendency in each environment. However, lines within WD and WW were less dispersed compared to HTS. Within HTS, CIMMYT10, CIMMYT13, and CIMMYT23 demonstrated greater distance from the other genotypes. Less distance was noted within WW and WD. However, CIANO and BOULARG exhibited a separated trend in response to WW, while CIMMYT63 and CIMMYT10 showed a distinct trend under WD conditions. Examining the variability of genotypes in response to WD, traits such as NPQt, VPD, and CT emerged as key explanatory factors (Figure 22). In contrast, ARI (trait over all with less contribution), LAI, HI, and ΦPSII played a more significant role in explaining the variability of genotypes under HTS. For genotypic responses to WW, carotenoid-related indices, grain yield, biomass, and SPAD were identified as major contributors.

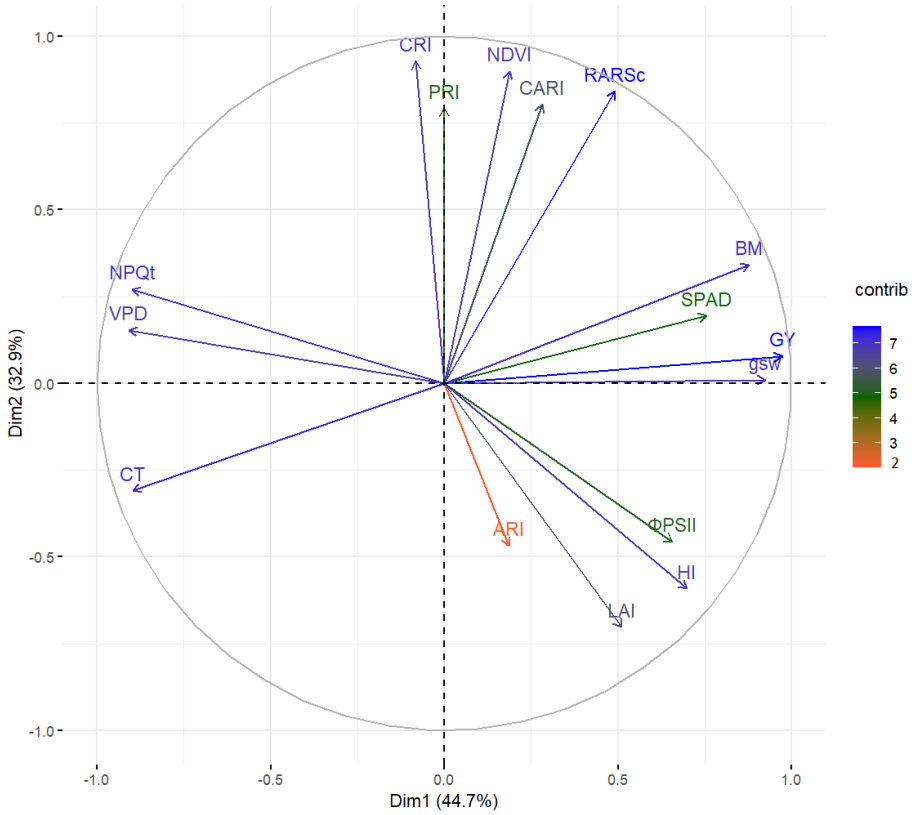


Figure 22:PCA biplot based on flag leaf for variables in all the environments and their contribution according to the scale on the right

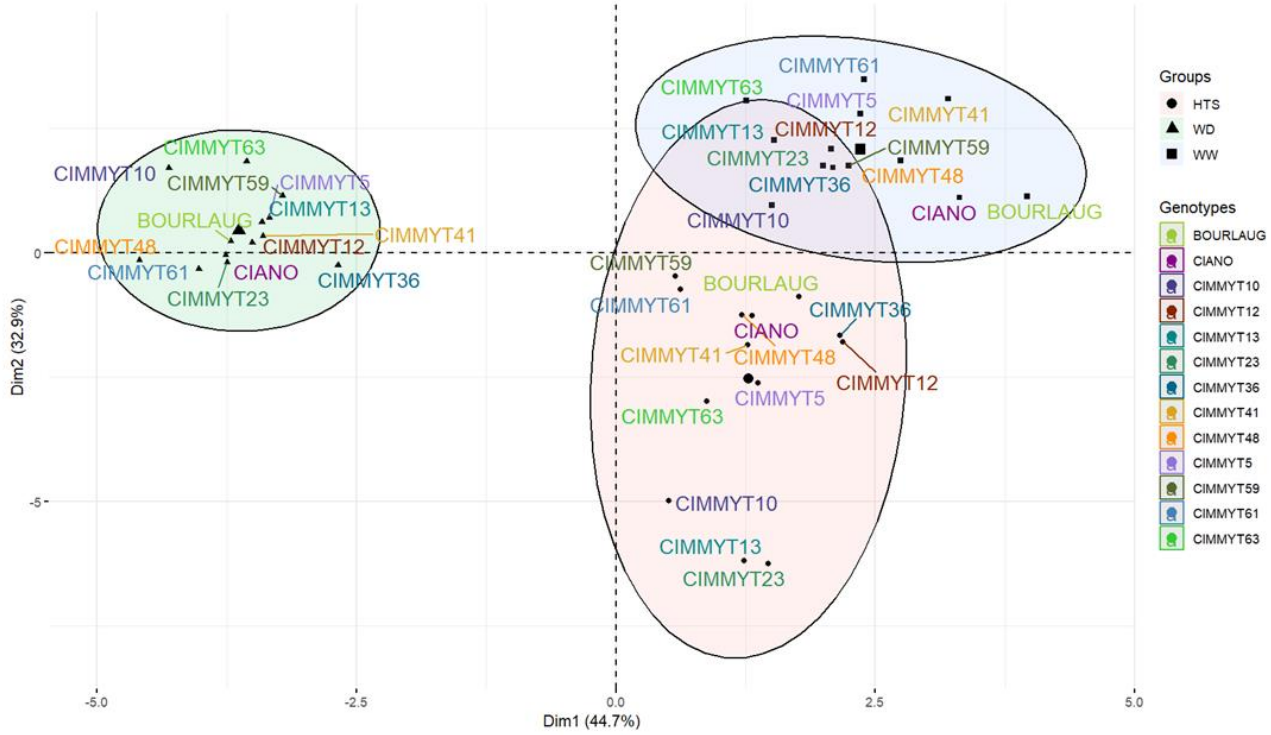


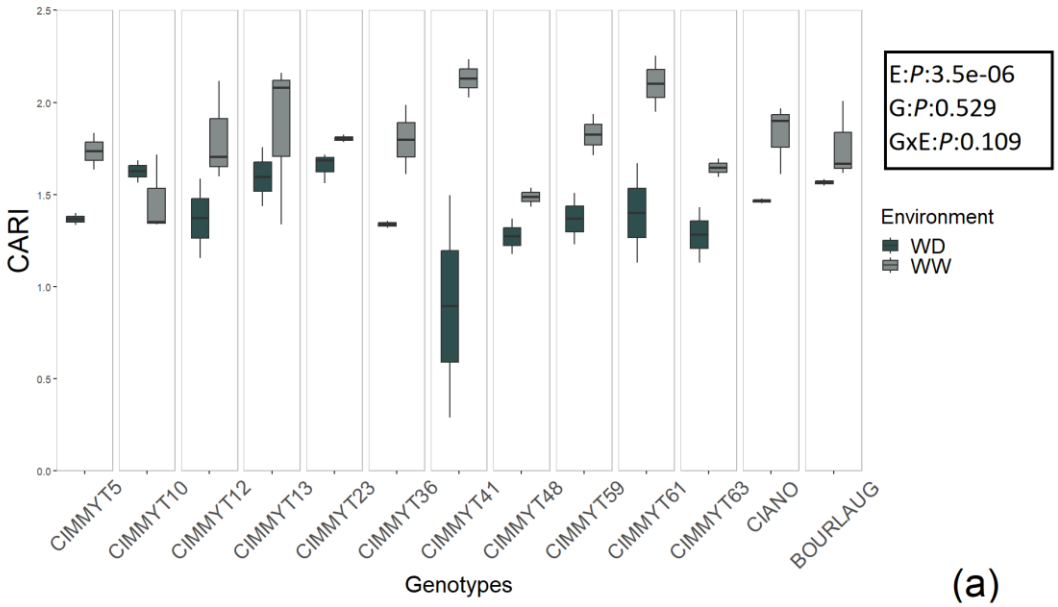
Figure 23:PCA biplot based on flag leaf of spatial position of genotypes in relation with variables of Fig. 22.

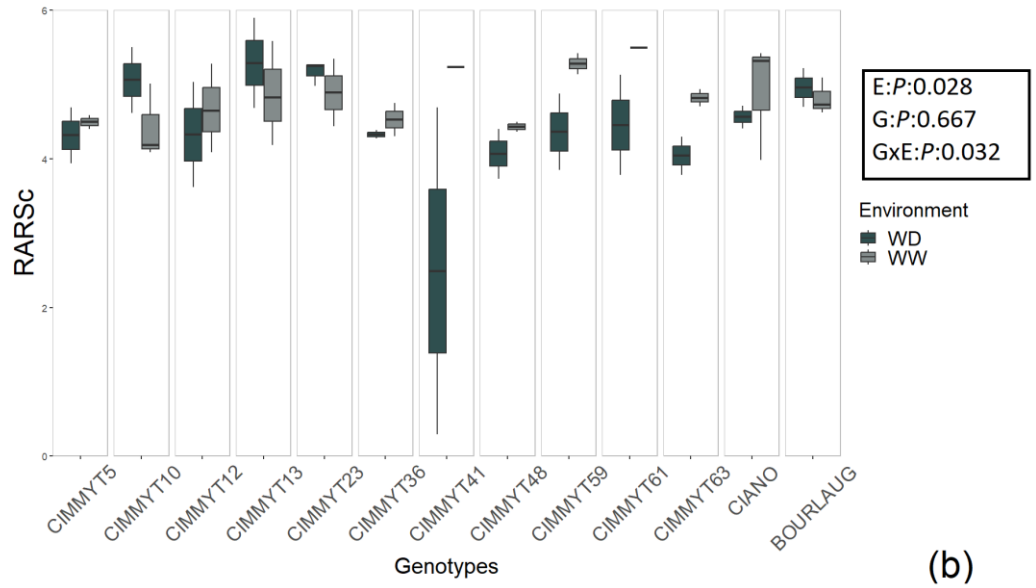
4.4 Third Leaf in response to Water Deficit

4.4.1 Photoprotection related Indices

Measurements of CARI at the third leaf level reveal a significant difference across environments, with plants under well-watered conditions showing a 21.34% increase compared to those under water deficit. However, CIMMYT10 stands out as an exception, exhibiting more than 10% higher CARI under WD compared to WW. On the other hand, CIMMYT41, showed the highest difference between environments (58.10%). However, in this case, no significant GxE difference was found. The difference for CIMMYT41 is explained by the lowest CARI under WD (0.89±0.49) and the highest under WW (2.13±0.08). The variability of RARSc is influenced by both the genetic factor and the GxE effect. In fact, the difference between WD and WW is only of 8.34%, with plants under WW with higher RARSc compared to WD. However, BOURLAG, CIMMYT23, CIMMYT13 and CIMMYT10, showed higher RARSc under WD (respectively, 3.00%; 5.69%; 8.78% and 14.29%). CIMMYT13 exhibited the highest RARSc under WD (5.29±0.49) opposite to CIMMYT41, which outstands with the lowest RARSc (2.48±1.79). While CIMMYT10, exhibited the lowest RARSc mean value in response to WW (4.18±0.29). Interestingly, under WW conditions, CIMMYT41 deviated from the trend observed under WD and exhibited the highest RARS. Due to the large difference, CIMMYT41 displayed a highly significant difference in terms of GxE (P<0.0001). Further, the genotype CIMMYT61, showed a significant difference for GxE (P=0.0161). For CRI, GxE, is the only factor to be significant. The variability is given by CIMMYT41 (P=0.0010) and CIMMYT61 (P=0.0117). Despite the absence of a significant effect to WD, it is crucial to highlight that the studied genotypes did not demonstrate a consistent trend. Specifically, for six genotypes, the CRI was higher under WD compared to WW conditions. On the other hand, the variability of PRI is explained by the E factor, on average plants under WW showed PRI higher of 20% compared to those under WD. Despite the lack of significant differences in terms of G and GxE, some trends are worth highlighting. In fact, BOURLAG showed a certain stability, displaying the smallest difference between environments (5.01%), opposite to CIMMYT5 with the largest difference (39.24%). Further, CIMMYT5 showed the highest PRI under WW (0.07±0.012) opposite to CIMMYT12 (0.04±0.009). While, CIMMYT13 and CIMMYT59 outstand respectively for the lowest (0.034±0.0081) and the highest mean PRI (0.06±0.0007). in response to WD. The analysis on ARI measured on the third leaf under WW and WD display significant difference in terms of E (P<0.0001) with ARI values higher in response to WW. No

differences in terms of GxE interaction were found. However, significant disparities were observed, with CIMMYT12 and CIMMYT10 showing the most contrasting differences between environments (37.59% and 178.96%, respectively). Under WD, the latter line exhibited the lowest ARI for third leaf under WD (-0.15±0.15), opposite to CIANO with the highest (0.15±0.04). Interestingly, CIMMYT10, under WW, displayed the same trend with the lowest ARI among the lines studied (0.19±0.05), while CIMMYT41 showed the highest (0.56±0.09). For the complete table of mean ± standard error of the indices related to photoprotective traits see Table 15-Annex.





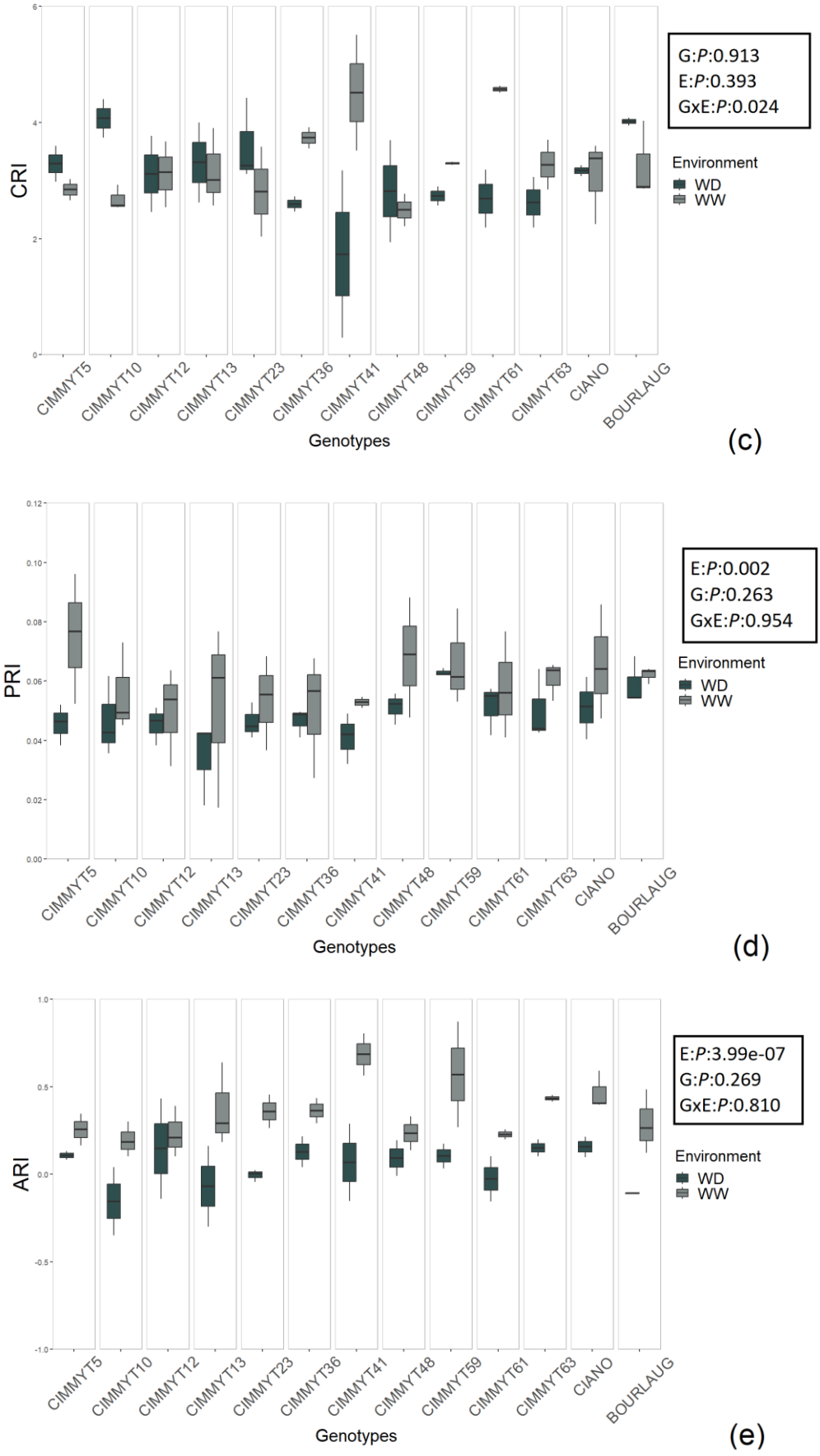


Figure 24:Response of CARI (a), RARSc (b), CRI (c), PRI (d) and ARI (e) for third leaf in reaction to WD (dark grey) and WW (grey).

4.4.2 Carotenoid vs Chlorophyll indices relationships

Interestingly, the analysis on NDVI and SPAD measured on the third leaf under WW and WD did not display any significant differences in terms of G, GxE and E (see Table 13-Annex). Instead, the environment effect explained the variability of the dynamic between Cars related indices against relative chlorophyll content (SPAD). The largest part of the variability for the CARI:SPAD ratio was explained by the environmental factor (*P*= 3.69e-06). Plants under WW showed 33.34% higher ratio compared to those exposed to WD. The only exception was CIMMYT10 with the ratio 17.59% higher under WD. Once again, CIMMYT41, reflecting the dynamic explored in Fig 19-a showed 61.01% of difference between environments (lowest CARI:SPAD under WD, 0.017±0.01, and highest under WW, 0.04±0.002). On the other hand, BOULRAG exhibited the lowest ratio under WW (0.03±0.0007), while CIMMYT23 exhibited the highest mean for CARI:SPAD in response to WD (0.03±0.001). The RARSc:SPAD ratio (Fig20-b) was significantly higher in the plants under WW (*P* = 0.006), compared to those under WD (on average 25% less). However, CIMMYT5, CIMMYT13 and CIMMYT23 showed, for this ratio, values slightly higher under WD (respectively, 0.37%, 1.42% and 6.18%). Interestingly, CIMMYT10 showed 18.01% higher average under WD compared to WW with the lowest RARSc:SPAD in response to WW (0.08±0.004). Despite, no differences in terms of G and GxE, CIMMYT41, showed the highest ratio under WW (0.12±0.01) and the lowest under WD (0.04±0.3). Significant interaction between G and E was determined for CRI:SPAD, explained by CIMMYT41 (*P*= 0.001). CIMMYT10 showed a notable difference (53.95%). Further, similar dynamic likewise for CARI:SPAD were found, with the highest CARI:SPAD under WD (0.08±0.002) and the lowest under WW (0.05±0.002), however it was not depicted by the statistical analysis (*P*= 0.06). For the complete table of mean ± standard error of the indices related to photoprotective traits see Table 16-Annex.

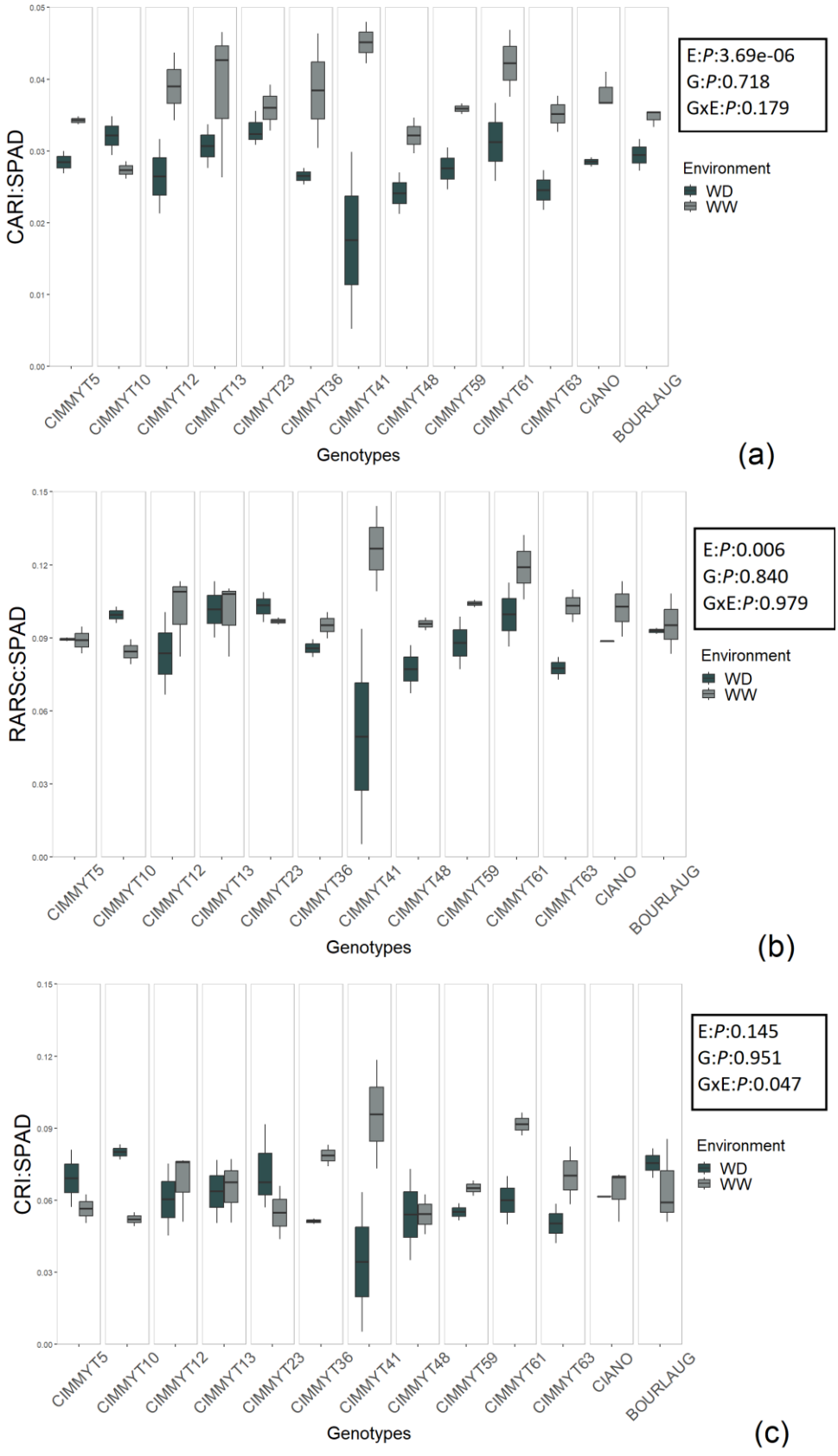


Figure 25:Response of CARI: SPAD (a), RARSc: SPAD (b) and CRI: SPAD (c) for third leaf in reaction to WD (dark grey) and WW (grey).

4.4.3 Correlation with agronomic traits

In both environments (Figure 26,Figure 27), NDVI, RARSc, CARI and CRI showed highly positive significant correlation dynamics. While, ARI showed a highly significant negative correlation with all the indices related to Cars. Under WW, the only dynamic between ARI and the spectral reflectance indices related to Cars were significantly positive associated with RARSc and CARI. Under WD, no correlation was found between spectral reflectance indices measured by the Spectroradiometer (such as RARSc, CARI, CRI) and PRI. Interestingly, under WW, PRI exhibited a significant negative correlation with CRI ad RARSc. Instead, CRI exhibited significant negative correlation with GY and BM. Under WW, this association was not confirmed, however a significant negative correlation between ARI and BM was found. Regarding parameter related to Chl status, under WD, SPAD is the only to display very significant positive correlation with GY (r^2=0.53) and HI (r^2=0.50), while less powerful association (r^2=0.41) was found between SPAD and BM. Under WW, less powerful correlations were found between BM and GY with SPAD (respectively, r^2=0.39 and r^2=0.35).

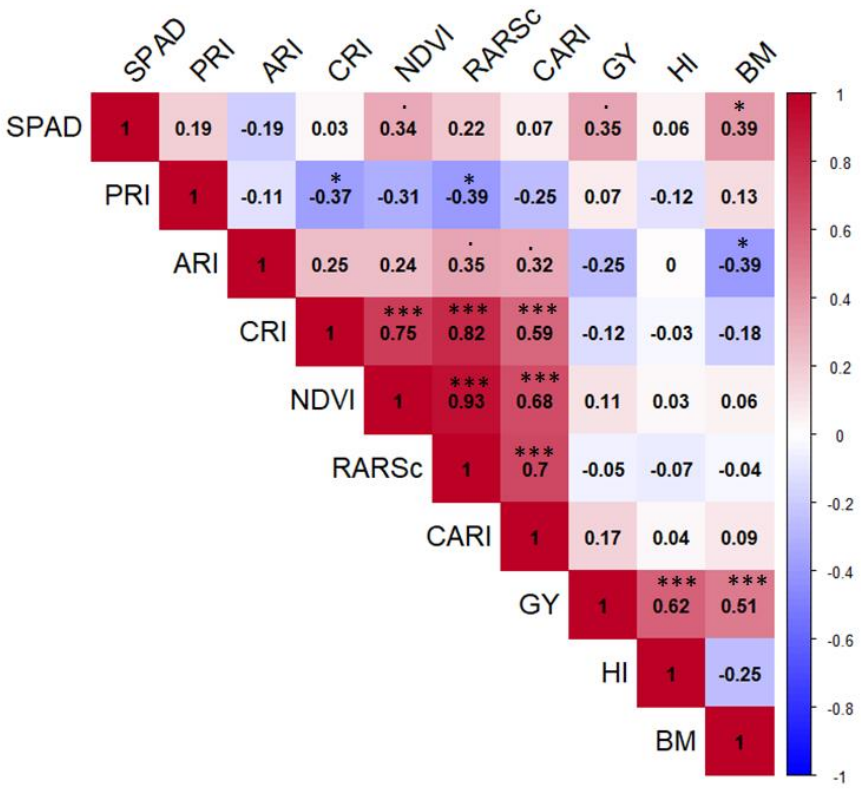


Figure 26:Heatmap displays genotypic correlations among SPAD, PRI, ARI, CRI, CRI, NDVI, RARSc, CARI, GY (g/m2), HI and BM (g/m2) on third leaf in reaction to WW. Significance of the codes: 0.0001´***` 0.001 '**' 0.01 '*' 0.05 '.'

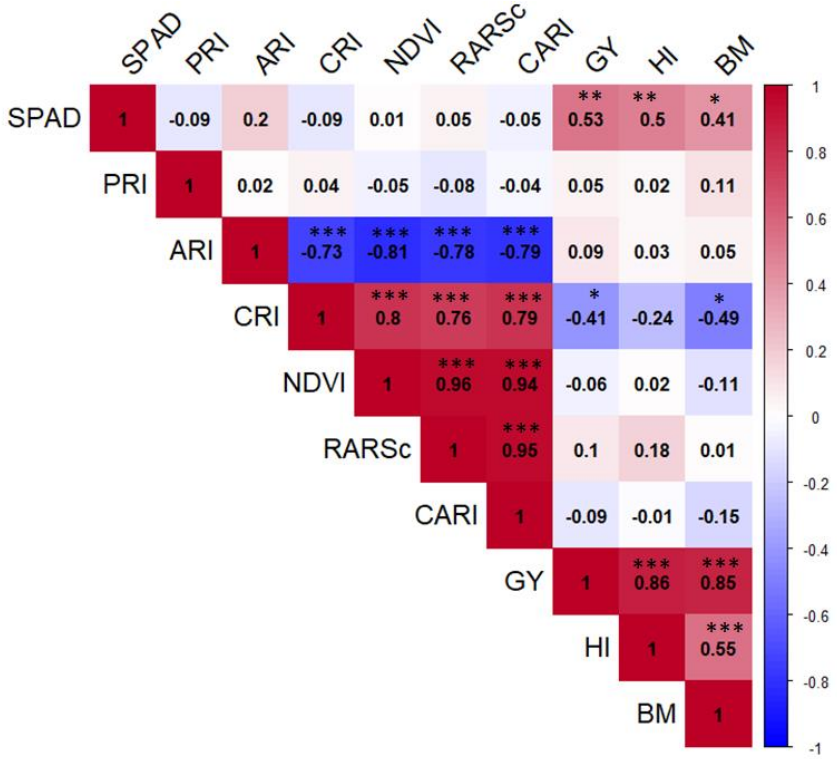


Figure 27:Heatmap displays genotypic correlations among SPAD, PRI, ARI, CRI, CRI, NDVI, RARSc, CARI, GY (g/m2), HI and BM (g/m2) on third leaf in reaction to WD.. Significance of the codes: 0.0001´***` 0.001 '**' 0.01 '*' 0.05 '.

4.5 Third Leaf in response to Heat Stress

4.5.1 Photoprotection related Indices

The analysis on CRI and RARSc measured on the third leaf under WW and HTS did not reveal any significant differences (see Table 18-Annex). The genotypes studied showed significantly higher CARI (Figure 28-a) at the third leaf level for plots under WW compared with the plants subjected to HTS (on average 23.60% higher under WW). The only exception was CIMMYT10, with CARI 11.56% higher under HTS. CIMMYT41, showed, instead the largest difference between environments (44.09% higher under HTS). However, no genotypic-effect or GxE effect was significant. The highest CARI under HTS was found for BOURLAG (1.65±0.08). On the other hand, CIMMYT48, under the same conditions, exhibited the lowest CARI (0.90±0.28). CIMMYT10 outstands for the lowest CARI under WW (1.46±0.12), opposite to CIMMYT41 with the highest value (2.13±0.08). The environment effect on PRI is highly significant, on average (49.15% higher under WW). CIANO showed the largest difference (67.01%), while CIMMMYT36 showed the opposite trend (22.15%). For PRI, similar to the scenario in CARI, no genotypic effect or GxE effect was found to be significant. However, under WW, CIMMYT12 and CIMMYT5 showed an opposite trend (respectively, the lowest 0.04±0.009 and the highest 0.07±0.012). Interestingly, BOURLAG showed the highest PRI under HTS (0.04±0.005), while CIMMYT13 the lowest (0.01±0.001).

Regarding ARI, plants under WW were very significantly different from those under HTS (on average 62.85%). Although, CIMMYT12 and CIMMYT13 are the exceptions with ARI higher for plants of the late-sowing experiment. CIMMYT10 and CIMMYT41 are the most distant genotypes in terms of ARI under WW (respectively, 0.19±0.05 and 0.68±0.09). While CIMMYT23, with negative average ARI (-0.14±0.04) and CIMMYT12 (0.51±0.25) are the most distant genotypes under HTS. However, in both cases the distances were not proofed to be statistically significant. For the complete table of mean ± standard error of the indices related to photoprotective traits see Table 18-Annex.

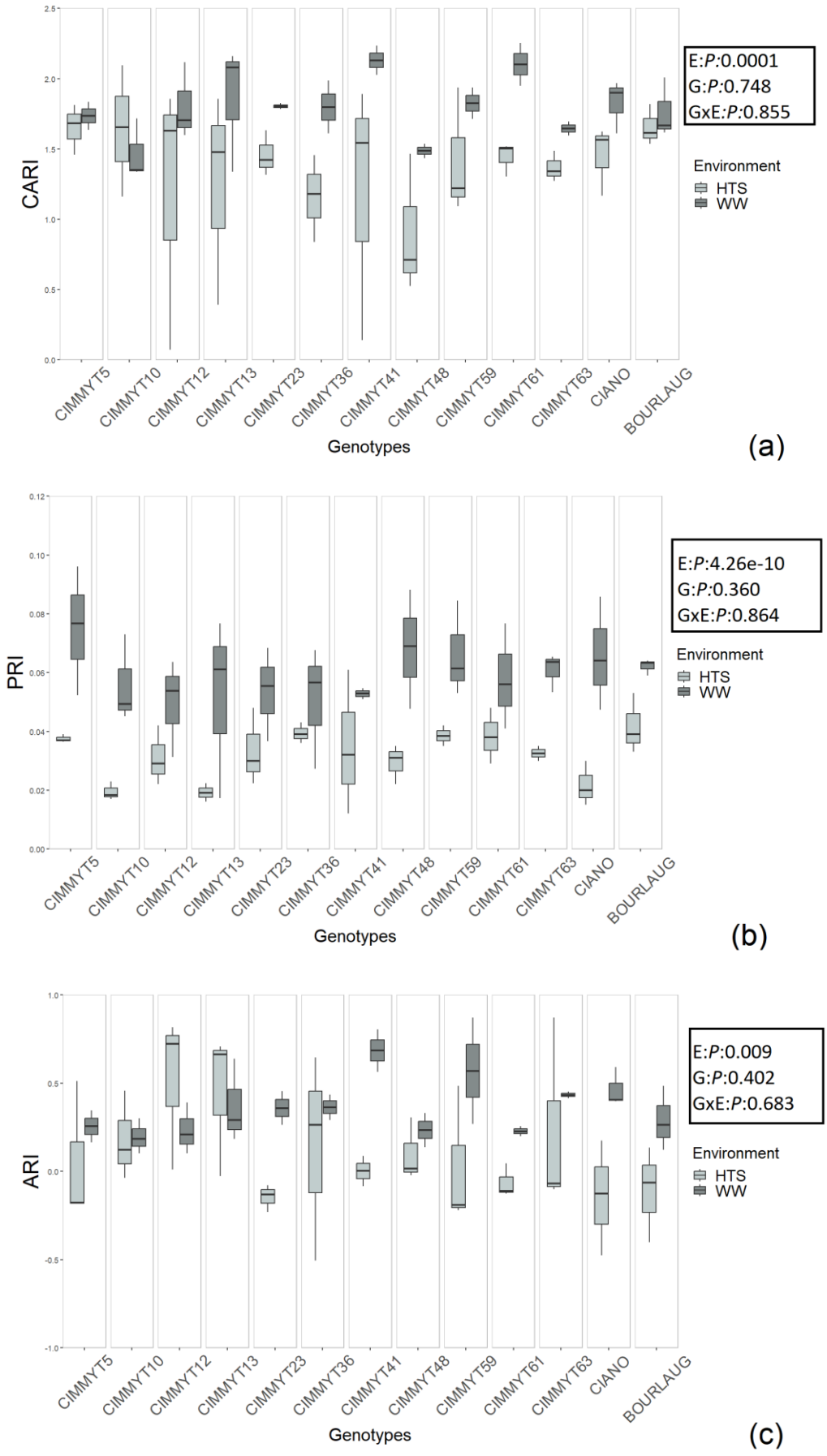


Figure 28:Response of CARI (a), PRI (b) and ARI (c) for third leaf in reaction to HTS (light grey) and WW (grey).

4.5.2 Carotenoid vs Chlorophyll indices relationships

Interestingly, the chlorophyll status of the genotypes at the third leaf level did not showed any statistical significance in terms of G, E and GxE (see Table 19- Annex). The difference between environments was highly significant in case of CARI:SPAD, (33.34%). The only exception was CIMMYT10, within the late sowing environment, CARI:SPAD was 17.58% higher compared to WW. In fact, CIMMYT10 showed the smallest value of CARI:SPAD under WW (0.02±0.0009). CIMMYT41 displayed, instead, the largest difference between environments (47.87% higher under WW). The same line showed, as well, the highest CARI:SPAD under WW (0.04±0.002). Nevertheless, no significant differences were found in terms of G and GxE, however CIMMYT48 and CIMMYT5 were the most contrasting genotypes in response to HTS (respectively, 0.01±0.005 0.03±0.001). For RARSc:SPAD, the environmental factor played a significant role ($P<0.05$), on average RARSc:SPAD was 20% higher under WW, despite BOURLAG (12.14% higher under HTS) , CIMMYT10 (18.88% higher under HTS) and CIMMYT5 (20.12% higher under HTS). Furthermore, BOURLAG showed the highest RARSc:SPAD under HTS (0.10±0.005), contrasting with the lowest value of CIMMYT48 (0.05±0.011). Although there were no significant GxE dynamics, similar to G, CIMMYT41 exhibited the greatest difference between environments, with a magnitude of on average 39.84% higher values observed under WW. This genotype exhibited the highest RARSc:SPAD under WW (0.12±0.014), opposite to CIMMYT10 (0.08±0.004). For the complete table of mean ± standard error of the indices related to the dynamics between Carotenoid indices and SPAD see Table 15-Annex.

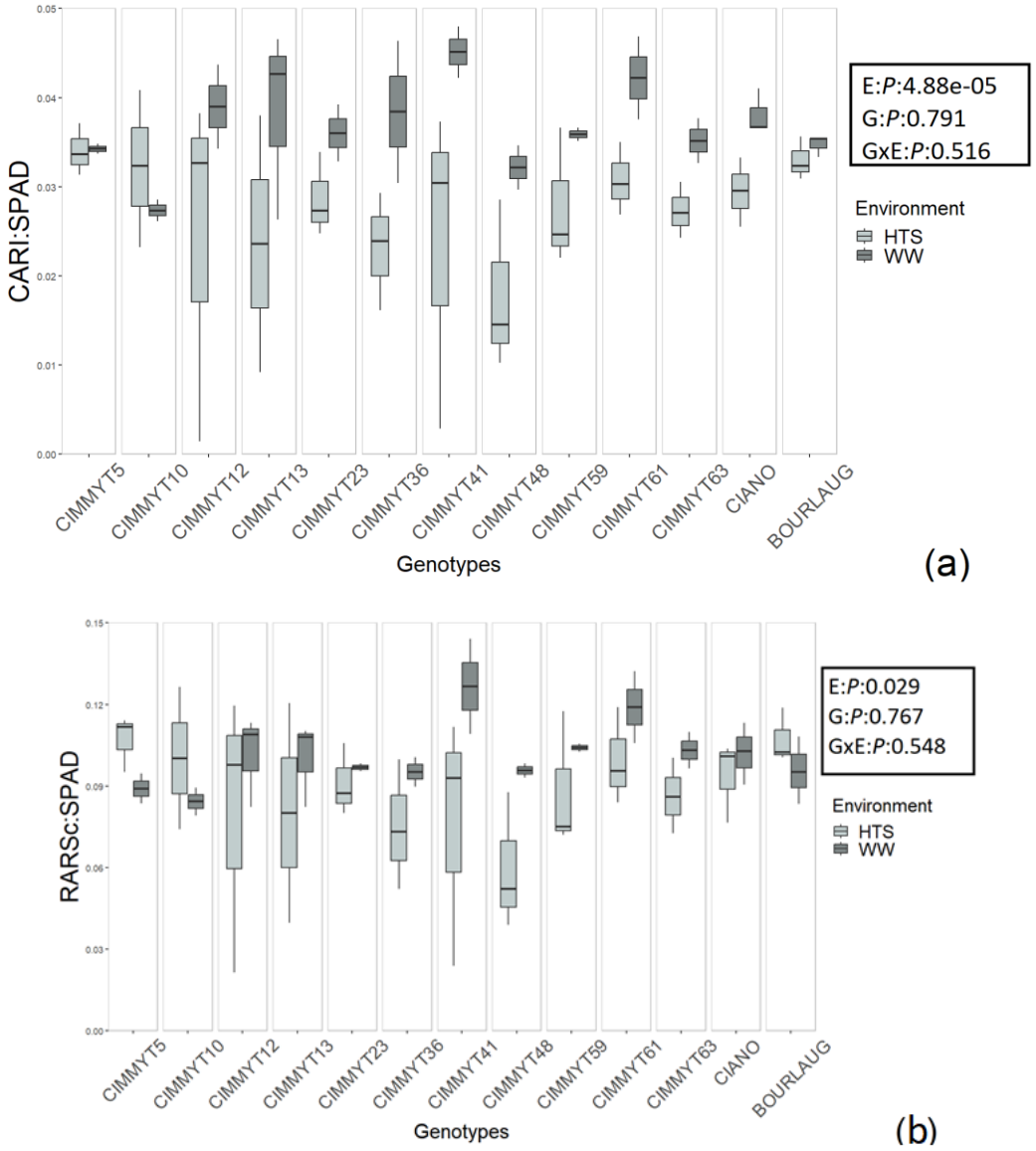


Figure 29:Response for the ratios CARI: SPAD (a), RARSc: SPAD (b) for third leaf in reaction to HTS (light grey) and WW (grey).

4.5.4 Correlation with agronomic traits

In both environments, NDVI, RARSc, CARI and CRI showed highly positive significant correlation dynamics. While, ARI showed a highly significant negative correlation with NDVI and RARSc and significant with CARI. Under WW (Figure 26), the only dynamic between ARI and the spectral reflectance indices related to Cars is a significant positive correlation with RARSc and CARI. Under WD, no correlation was found between spectral reflectance indices measured by the Spectroradiometer (such as RARSc, CARI, CRI) and PRI. Interestingly, under WW, PRI exhibited a significant negative correlation with CRI ad RARSc. Interestingly, ARI showed a significant positive correlation with BM under HTS, while under WW, the same association has a negative verse. In addition, the same correlation was found between GY and ARI. No correlations were found between SPAD and agronomic data, while under WW, significant positive correlations were found between BM and GY with SPAD.

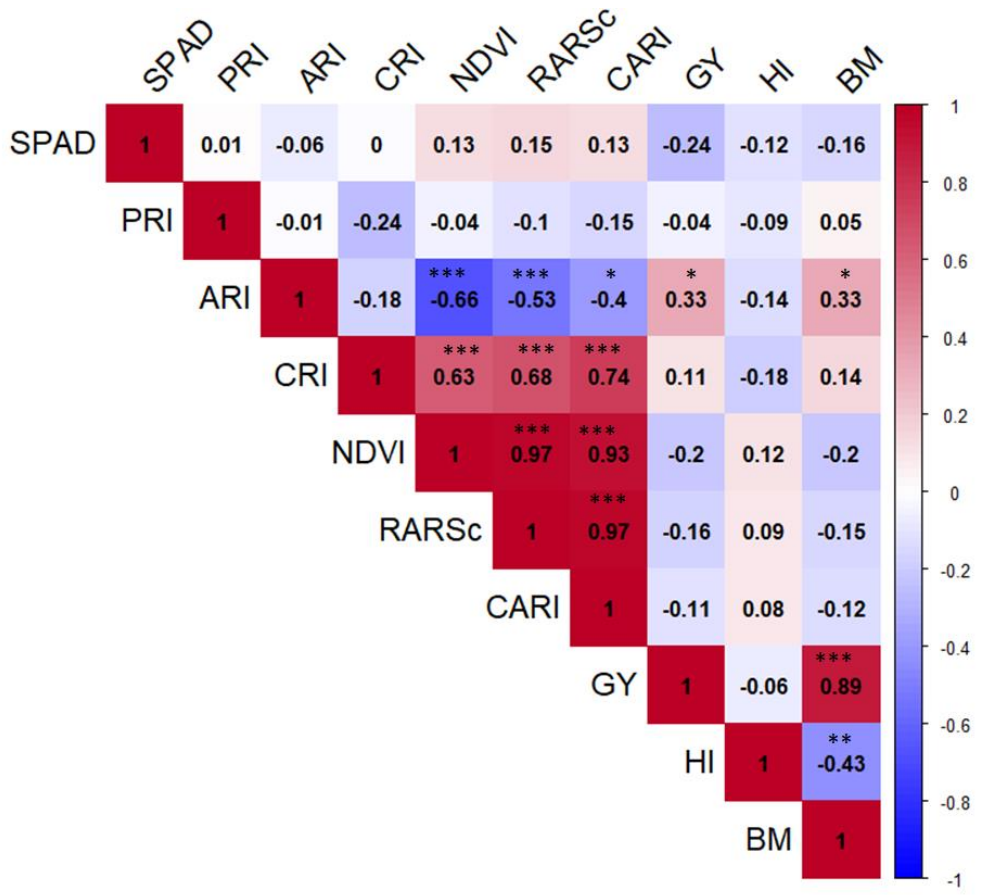


Figure 30:Heatmap displays genotypic correlations for third leaf among SPAD, PRI, ARI, CRI, CRI, NDVI, RARSc, CARI, GY (g/m2), HI and BM (g/m2) on third leaf in reaction to HTS. Significance of the codes: 0.0001´***` 0.001 '**' 0.01 '*' 0.05 '.'

4.6 Multivariate analysis over the three environments on third leaf

In the PCA analysis regarding the third leaf, the combined effect of Dimension 1 (40.5%) and Dimension 2 (28.8%) explains 69.3% of the total variance Dimension 2, clearly distinguishes the ellipse of WD from the one of WW, except for CIMMYT61 within WW. However, several genotypes within HTS ellipse (Figure 32) overlap with the WD ellipse (CIMMYT5, CIMMYT10, BOULRAG, CIMMYT59, CIMMYT23, and CIMMYT63), while CIMMYT61 and CIMMYT5 fall within the WW as well. Instead, Dim1 separates these genotypes within HTS and the other environments from CIMMYT12, CIMMYT13, CIMMYT36, CIMMYT41, CIMMYT48. In addition, these genotpyes, which are placed in the upper left quadrant, may not strongly align with PCA variables representd. Within WD, Dim1 separates CIMMYT10 and CIMMYT23 from the rest. These two lines, along with CIMMYT13 and BOURLAG stand out as especially distant from the primary WD cluster. Carotenoids-related indices cluster in the right-bottom corner of PCA but only CIMMYT61 within WW is in the proximity of CARI. This suggests a ceratin contirbution of CARI on this line. GY, and BM contribute to variability within WW, while PRI, a smaller contributor, may explain variability in CIMMYT5, BOURLAG, and CIMMYT10 within HTS. Interestingly, SPAD, with a small contribution, partially explains variability within WD (Figure 31). In WW conditions, the variability is explained by ARI, GY, and BM, while HI is more shifted towards HTS.

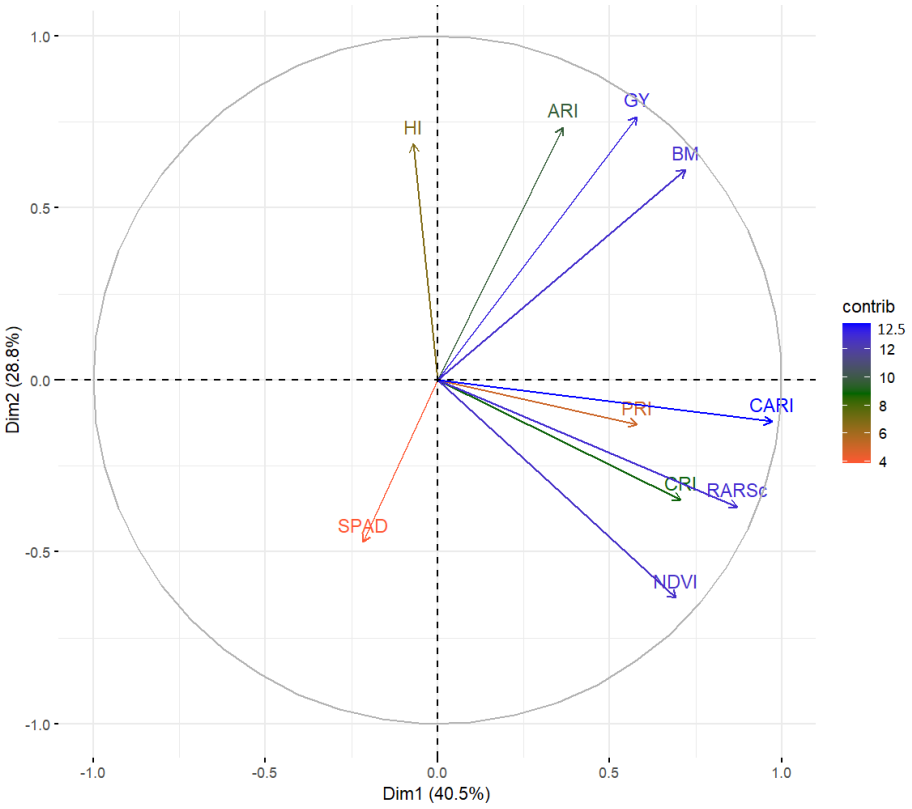


Figure 31:PCA biplot based on third leaf for variables in all the environments and their contribution according to the scale on the right

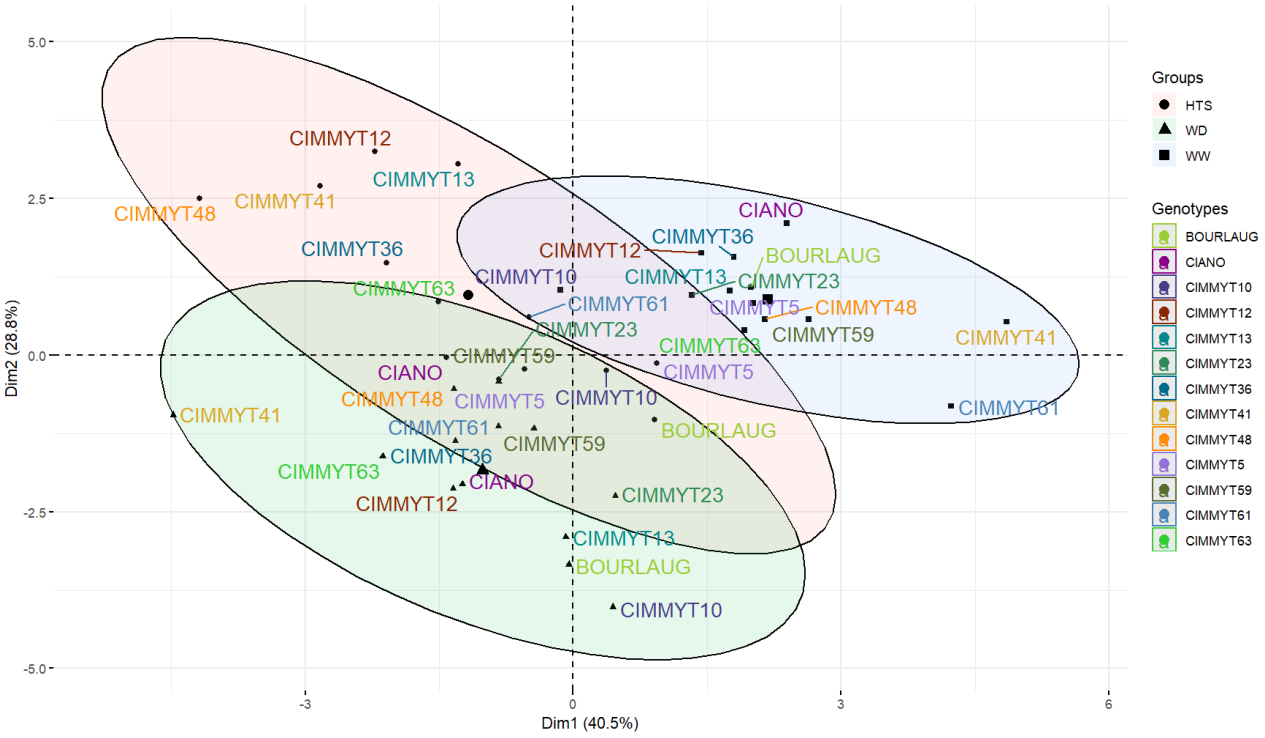


Figure 32:PCA biplot based on third leaf of spatial position of genotypes in relation with variables of Fig. 31

5. Discussion

5.1 Spectral reflectance indices as proxies for Carotenoids and NPQt

In response to abiotic stress, carotenoids are expected to increase in order to quench the excited Chl and avoid damage to the thylakoid membrane (Gitelson et al., 2001). This is not consistent with what has been found in this study. The only exceptions were for CARI for CIMMYT12 and CIMMYT10 in the comparison between WD and WW (Figure 4-a), and CIMMYT12 in the one between HTS and WW (Figure 14-a). Plants show a decrease with regards to Cars estimation to the rise of temperature and to water deficit compared to normalized conditions. Average difference during grain filling between the max mean temperature in HTS and WW was around 8°C, with a maximum temperature of 41°C in HTS. The critical threshold for metabolic and physiological processes in wheat was observed after 30 °C (X. Zhang et al., 2023). However, the findings in our study suggest that Cars synthesis was already stopped under heat and water deficit. Studies conducted by Ramachandran and colleagues (2010), showed that the biosynthesis of Lutein, which is the most abundant carotene in leaf tissue, was limited due to a downregulation of the enzyme responsible during the grain filling stage. Additionally, research has shown that the structure of chloroplast is affected by high temperature and water deficit (Liao et al., 2004; Zuily-Fodil et al., 1990), where Lut and the other carotenes are synthetized. This may contribute to the inactivation of the enzymatic synthesis of Lut and other carotenes. Interestingly, CRI was the only index not able to detect difference in response to WD, while in the comparison between HTS and WW, all the indices show significant differences in term of environments. Further, the pattern of CIMMYT12 which showed higher CARI in response to HTS and WD (Figure 4-a, Figure 14-a) was not reported for the other two indices in all the comparisons. This may suggest an inconsistence in Cars estimation of CARI. In fact, despite CARI was studied on a variety of environments and phenological stage by Zhou and colleagues (2017) using a synthetic dataset, this could not include all the factors implied in a field trial such as the one of this study. Although no GxE significant differences were reported, interestingly, checks show consistent minor differences between HTS and WW and variability among the replications. This may imply that the adaptability to different environments for which these lines were selected as control could be prove by this little variance (Reynolds et al., 2017). All the significant indices show high correlations in both comparisons, to claim which index is better to estimate Cars, corroboration with Cars content detection analysis is needed. Abiotic factors and high light radiation are expected to trigger the de-epoxidation cycle and therefore to increase the heat dissipation(Demmig-Adams et al., 1992.), resulting in a

decreased PRI in response to heat stress (Zhang et al., 2023) and water stress (Liu et al., 2020). This was in line with our findings, in which lower PRI values were recorded in stressed environments compared to WW, this could be associated with higher de-epoxidation activity in WD, and HTS (Figure 4-c, Figure 14-d). The higher NPQt values in response to WD compared to WW (Figure 4-d) confirmed the findings related to PRI, being a major component of NPQ (Demmig-Adams et al., 1992). Furthermore, the importance of NPQt within WD was demonstrated by its substantial contribution in the PCA (Figure 22). However, for HTS, despite the lower PRI, NPQt did not significantly differentiate between environments and it needs to be clarified if this was due to the confounding factor of Chl which may confound the estimation of the pigments related to the de-epoxidation cycle (Yudina et al., 2020). In fact, in this study, despite the significance of the environmental effect, relative chlorophyll content was relatively high in HTS and WD. NPQt measurements were not able to detect any significant difference between HTS and WW, this may be attribute to the significant difference in PAR, which was shown between WW and WD but not for HTS. This difference may be attributed to ´leaf rolling or change in leaf angle due to water stress and high light intensity (Demmig-Adams, 1992.; Sarieva et al., 2010).

5.2 Link between Spectral Reflectance Indices, Carotenoids, and NPQt

Over all, all the indices related to Cars show a tendency to vary together in all the treatments. Positive correlations were highlighted between CRI, RARSc and CARI in all the environments (Figure 9, Figure 10, Figure 19). Although carotenoids and anthocyanins play a similar role against ROS, ARI in our findings on flag leaf indicate a lack of synergy between them. The negative values in the correlation coefficients between ARI and other photoprotective traits may suggest a trade-off. This was consistent in a study by Li and colleagues (2018), in middle- late grain filling, where during late grain filling there was a decrease on Chl and Cars and increase in AnT. Lack of significant correlation between NPQt and PRI in all the environments were counteracted by slightly positive correlations coefficients between NPQt and Cars (within the range 0.20-0.26) within WD and HTS, indicating a slight positive tendency between the two parameters.

The finding about the ratios regarding Cars related indices and Chl (Figure 6, Figure 16) confirmed the trend found for Cars. The expectation that the Cars/Chl ratio would be higher under stressed conditions was not consistently observed across all genotypes. No genotypic and GxE difference could be found. However, there are some differences in genotypes CIMMYT59,

CIMYT61, CIMMYT36 and checks demonstrated less difference in the ratios when comparing HTS and WW (Figure 16). In particular CIMMYT59, together with CIMMYT10, exhibited the same trend when looking at WD and WW. For the other lines, less difference was highlighted between WD and WW in comparison to HTS and WW. Interestingly, in response to water deficit plants show higher Cars/Chl ratio suggesting more need of photoprotection, while under HTS the decreased levels of Cars/Chl suggest a better use of the excitation energy under this environment (Eberhard et al., 2008).

5.3 Spectral reflectance indices as proxies for greenness and ΦPSII

In this study there was a decrease in relative chlorophyll content in response to a premature senescence due to water and high temperature stress compared to WW environment (Figure 5-b, Figure 15-b). This was in line with previous studies for late sowing spring wheat experiments, (Prasad et al., 2011). However, a more contrasting difference was expected due to the abiotic stress and aging, but in this case the genotypes show relatively high Chl content, which is a desirable trait in breeding programs. In Prasad and colleagues study (2011) an average difference in terms of SPAD of 39% was found between control and high temperature environments, established in a grow chamber (day night temperatures 31/18 °C) from heading to maturity. In our study, under late sowing and control trials a difference of 3% on average was found. The trend observed in our study may indicate a certain adaptation to stressed environments in the genetic pool for Chl among the lines studied in all the trials. Although no significant positive correlations were found, NDVI (Figure 5-a, Figure 15-a shows a similar trend for SPAD in all the three environments, confirming as a proxy for the adaptive trait of leaf greenness in spring wheat (Pinto et al., 2016). However, the close linear relationship between NDVI and the indices related to Cars let us think about an overlapping effect of Cars and Chl. In addition Chls are more abundant than Cars (Sytar et al., 2021) and in our case did not cope with a strong degradation. Further, the findings related to Cars indices (lower in response to stressed environments) may be due to Chls overlapping. Under WW, for SPAD a genotypic difference was reported, with CIMMYT63, CIMMYT48 and CIMMYT59 showing higher average (group A), confirmed as well by the spatial distribution in the PCA (Figure 23) and CIMMYT10 the smallest (denoted with letter b), see Fig 6-b. .While in response to WD and HTS, lines show similar trends for relative Chl. High relative Chls content could imply well functionality of the PSII-core and related photosynthetic efficiency (Kitajima & Hogan, 2003).

However, other factors such as photoprotection mechanisms or alterations in the efficiency of energy transfer, could have an influence on the quantum yield independently of chlorophyll levels, especially under high light radiation. The negative phenotypic correlation between NPQt and ΦPSII , in response to WW and HTS, confirmed this assumption, as it was shown in a study by Chen and colleagues (2011) on winter wheat hybrids in response to high light radiation. ΦPSII results are consistent with previous studies in response to water stress (Flagella et al., 1996). Interestingly, a cluster of genotypes can be identified for ΦPSII, with CIMMYT12, CIMMYT23 and CIMMYT63 which showed less variability between WD and WW compared to other lines (Figure 5-c). In the same comparison checks and CIMMYT36 outstand for their photosynthetic efficiency under WW. CIMMYT36 considering the performance across WW and HTS was confirmed significantly to be above the average performance in regards of ΦPSII, being the most contrasting genotypes against CIMMYT59 and CIMMYT61(Figure 5-c). These latter genotypes show a certain proximity as well in the PCA spatial distribution of the genotypes within HTS (Figure 23). Except the findings of these latter two lines, on average ΦPSII was higher for the lines sowed in March, contrasting to our hypothesis and the previous studies (2000). The lack of degradation of green tissue may be a symptom of good functionality of chloroplast (Kong et al., 2015). In addition, a study of Haque and colleagues (Haque et al., 2014) on recovery dynamics of different spring wheat cultivars, show the adaptation of the lines measured by fluorescence measurements, grown at 25°C, exposed at 40°C ΦPSII was higher for the lines coming from regions in the world characterized by hot temperatures. In the same study the scientists identified at 45°C the threshold for irreversible change to the photosynthetic apparatus. The higher values of ΦPSII may be hindered by the adaptation to the high temperatures and light of the late sowing experiment before the grain filling measurements. In addition, our plant material, as well, is coming from international nurseries characterized by high temperature field trials.

Leaf green area confirm our hypothesis. The optimal photosynthetic capacity of plants in response to high temperature is reflected in an increased leaf area index compared to WW environment (Figure 18). Likewise, the increase in PAR and perhaps the amount of radiation intercepted during the late sowing experiment may have influenced the leaf area index result (Reynolds et al., 2005). When comparing WD and WW, a decrease in LAI was noted (Figure 8), however it was not that marked such as in due to the lack of a strong decline in canopy greenness, this was aligned with Anjum and colleagues (2011).

5.4 Response of Yield and Yield components

Results on grain yield in response to WD (Figure 7-b) are aligned with Wang and colleagues (Zheng et al., 2021). According to them, when the stress of water deficit is mostly concentrated after anthesis such as in our study, consequent reduction of fertile spikelet and so number and quality of grains (less build-up of starch and protein) is expected. Despite the reduced time to complete the growth cycle, and the longer and higher photoperiod plants in the heat stress trial(Figure 17-b) were not impacted such as in response to WD (Figure 7-b). A decrease of 23% was noticed between grain yield in HTS and WW in this study. In a similar late sowing experiment, with average temperature of 28 °C, between heading and maturity a reduction of 33% was noticed (Modhej et al., 2008). In our study the average temperature in the same period was of 24 °C, therefore our findings are consistent with the study of Modehej and colleagues. Statistically, only in regards of biomass, we observed genotypes significantly different in both the comparisons, with CIANO, being the average lowest biomass across the environments and CIMMYT48 the largest. Although no significant differences were reported, the lines behave more contrastingly in response to the control environment, in comparison to the response to abiotic stress in terms of grain yield and total above ground biomass. Due to the reduced photosynthesis and leaf area, above ground biomass is expected to be lower in response to high temperature and in order to avoid water loss (Balla et al., 2012; Li et al., 2021). This is confirmed by the findings in WD of this study (Figure 7-a), but not in response to HTS (Figure 17-a), as LAI demonstrated already. Breeders are usually interested in lines with allocation of more resources to grain production than above ground biomass (Reynolds et al., 2005), this is the reason why HI is crucial. Interestingly, despite decreases in overall GY and biomass production in HTS, HI significantly increased overall in HTS treatment compared to the control (Figure 17-c), resulting in a stronger reduction of biomass in HTS compared to WW. Further, looking at HI values found in the literature for spring wheat, where the maximum recorded value is around 0.60, our findings within HTS can be considered high (Reynolds et al., 2005), this was further demonstrated by the high contribution of Hi within HTS in the multivariate analysis (Figure 22). This might indicate a resource allocation shift to grains, in order to ensure seed production. This shift in allocation and the contrasting results for GY and BM within WW contribute to the genotypic and GxE significant difference for HI in the comparison between HTS and WW, with CIANO with the largest average across the environment opposite to CIMMYT48 and CIMMYT59 with the lowest HI. CIMMYT 48 showed better adaptation in response to WD compared to WW, in fact HI is slightly higher in response to WD compared to

WW (Figure 7-c). However, the findings regarding HI indicate a better coping strategy of the lines to high temperature compared to water deficit. Regarding correlations, as carotenoids and xanthophyll related indices increase a negative correlation with yield and yield component parameters was observed in all the environments. Although, we found significant statistical relationships between PRI and Biomass within WW, and between CRI and GY and Biomass within WD (Figure 13). Interestingly, within HTS significant negative correlation coefficients for all the indices related to Cars and Grain yield were showed (Figure 19). We assumed that carotenoid photoprotective role, included Xant, could be coupled with grain yield. Yet, while studies like that of Babar and colleagues (2006) investigate for the relationship between PRI and Grain Yield, there is relatively limited research on carotenoids related indices. Our results may be partially supported by similar one by Blanco and colleague on durum wheat (2011), which proposed that this association may be attributed to the intricate relationship between the genetic marker linked to the yellow pigment and another genetic marker associated with reduced kernel weight.

5.5 Water and Temperature effect on stomatal responses

Stomatal conductance is considered a photosynthetic trait, due to the role in the regulation of CO2 uptake and water evaporation at the leaf level. Stomatal closure is considered a characteristic for drought avoidance and indicative of reduction of grain yield (P. Li et al., 2021). The theory is supported by the consistent observation of low stomatal conductance in response to water deficiency across all lines. Additionally, it was confirmed that within WW, where a significant genotypic difference was highlighted, CIANO, which exhibited higher yield, also had higher stomatal conductance. The cause-and-effect mechanism underlying lower stomatal conductance in drought involves the hormone ABA, contributing to an increase in VPD (Correia et al., 2021). Soil drought conditions result in reduced air humidity, further amplifying VPD (Liu et al., 2023). Moreover, as demonstrated by Correia and colleagues (2021), VPD tends to increase under water deficit due to the increase of CT (Table 2; Figure 11-b) . This is primarily attributed to decreased transpiration resulting from reduced stomatal conductance, leading to a decline in evaporative cooling (Pask et al., 2012). The uniformity of results among the lines in response to water stress does not provide clear indications of genotypes with a cooler canopy temperature and, consequently, a more effective root system for water uptake from deeper soil layers. Additionally, the criteria employed for clustering certain lines in the study did not yield consistent clusters. The criteria, which relied on CT during the vegetative phase for clustering, demonstrated a lack of uniformity when looking at

CT after anthesis. Regarding HTS, there was no significant difference in terms of the environment for VPD (Table 14) and stomatal conductance between HTS and WW (Figure 20), despite a temperature difference of 4°C and 10% in relative humidity. Genotypes were expected to exhibit higher stomatal aperture in response to high temperature , as effect of higher VPD under HTS compared to WW (Liu et al., 2023). However, only few genotypes, such as CIMMYT5, CIMMYT12, CIMMYT36, and CIMMYT41, showed this tendency, though it was not statistically significant. Apart from these non-significant differences, the impact of high temperature on stomatal conductance was not pronounced, indicated by the lack of significant differences between environments (Figure 20-a). It is possible that the lines of our study developed a deeper root system in response to high temperatures, contributing to stomatal openness (McAusland et al., 2023). The observed trend across the lines could also be attributed to the adaptation over several years in field trials characterized by high temperatures, coupled with the high heritability of the stomatal conductance trait (Pask et al., 2012). However, the pattern of VPD and stomatal conductance would expect a lower CT (Liu et al., 2023). In our study, canopy temperature, measured throughout grain filling, was significantly higher in response to heat stress. This suggests an impairment of the transpiration rate through stomatal response, as shown by the lack of significant difference between HTS and WW. However, interestingly, coherence for line CIMMYT63 was shown with the lowest stomatal conductance across the environments of HTS and WW and the largest CT. Further, lower stomatal conductance and high VPD are expected to limit photosynthesis (Rashid et al., 2018). In our case, as reported by results by the finding on spectral reflectance indices as proxies for Chlorophylls and ΦPSII (Figure 15), photosynthetic capacity within heat stress was not limited, therefore this is coherent with the results regarding VPD and stomatal conductance in the comparison between heat stress and control environment. Carotenoid related indices and NPQ did not track a significant causation with VPD, gsw or CT in heat stress trial (Figure 21). The lack of significant differences between HTS and WW for gsw and VPD may be an influencing factor. Within WD (Figure 13), correlations coefficients between NPQt with VPD and CT were slightly positive, as expected, although not significant. As the stomata close due to WD, there is an increase in canopy temperature. Simultaneously, the de-epoxidation cycle shows a slight increase, as does the Non-Photochemical Quenching (NPQt). These observations indicate a form of photoprotection. Interestingly, ARI showed a positive significant correlation with gsw within WD and with VPD within WW. This may be related to the involvement of the hormone ABA in the biosynthesis of anthocyanins and stomatal closure. This was confirmed in a study

on rice, while investigation in wheat needs a more comprehensive understanding(Li et al., 2022).

5.6 Dynamics of spectral reflectance indices as proxies for Carotenoids on third leaf

The findings related to Cars proxy indices for third leaf exhibit partial alignment with our expectations, for which estimated Cars would be higher in response to water or heat stress (Gitelson et al., 2007). In the comparison between WD and WW, differences were mostly dependent on genotypes, although it was not statistically confirmed. Some genotypes, such as CIMMYT 10, consistently show higher Cars estimation in response to WD compared to well-watered WW conditions. CIMMYT13, CIMMYT23, and BOURLAG also align with expectations in terms of RARSc (Figure 24-b) and CRI (Figure 24-c). This was also supported by the PCA distribution of these genotypes towards Cars related indices (Figure 32). In addition, CIMMYT5 and CIMYT48 depict higher CRI in response to WD, therefore CRI, compared to other indices, such as RARSc and CARI, aligns more closely with expectations, although CRI did not show any significant E effect. Moreover, CIMMYT41 and CIMMYT61 show less stability in CRI and RARSc between WD and WW, indicating a significant genotype-by-environment difference. We assumed that observed genotypic variations may be correlated with distinct canopy closure dynamics. Certain genotypes may exhibit a more open canopy structure, reduced leaf area and leaf rolling tendency, facilitating increased light penetration to lower canopy levels. This enhanced light availability, in turn, could contribute to heightened Cars synthesis as a mechanism for photoprotection. Despite leaf rolling is considered an adaptive trait to drought and heat stress in grain filling (Pask et al., 2012), based on our observations plants were more impacted by water deficit in term of height and canopy cover. In fact, only for CIMMYT10 the estimation of Cars was higher in response to the late sowing conditions in the case of HTS and WW comparison. Interestingly, CARI (Figure 28-a) was the only index able to detect differences among high-temperature stress and WW environments. In addition, BOURLAG and CIMMYT5 show similar variability and means among the environments, therefore they show a certain degree of stability. CIMMYT5, as well was clustered within the WW ellipse in the PCA (Figure 32). Results regarding the PRI (Figure 28-b Figure 24-d), showing a decrease in response to abiotic stress in both comparisons, align with findings in the literature and are consistent with the results observed on the flag leaf. However, the increased xanthophyll de-epoxidation is not supported by Chl degradation (Table 20, Table 17), this may

indicate an optimization of the light harvesting through photoprotection mechanisms. Our findings raise questions about the specific photoprotective dynamics of anthocyanin, as described by Zheng and colleagues (2021). An enhancement of AnT was expected to be responsive to abiotic stress, while in this study ARI, as a proxy for AnT, was significantly higher under WW compared to WD and in most of the lines for HTS (Figure 24-e, Figure 28-c). A similar case, was observed on bread wheat under drought, as reported by Rustamova and colleagues (2021), where AnT may compete with Cars in a trade-off. This is supported by the negative correlations observed with ARI and carotenoid-related indices reported within WD and HTS (Figure 30; Figure 27). These findings were consistent with those regarding flag leaf and the study of Li and colleagues (Li et al., 2010).

5.7 Understanding the Lack of Chlorophyll Response to Environmental Stress on the Third Leaf

AnT serves as an attenuator by absorbing excess light energy and safeguarding Chls (Singhet al., 2023), our study on third leaf did not show this protective mechanism. However, examining the PCA, in Fig. 31, reveals an interesting negative relationship between SPAD and ARI, despite the latter's minimal contribution. Chls estimation demonstrated comparable levels in both control and stressed environments, as evidenced by the lack of a significant difference for SPAD and NDVI. This aligns with the theory that shaded leaves exhibit increased photosynthetic efficiency due to elevated chlorophyll levels in response to reductions in the flag leaf (Li et al., 2010). In addition, the results about the ratios regarding Cars related indices and Chl confirmed the trend portrayed in the estimation of Cars and in the genotypic differences. In general, the Cars/ Chl ratio is lower in response to water and heat stress, but the same genotypes which showed higher values in the Cars estimation reflect the same behaviour for Cars/Chl ratio. Despite the lack of significance for SPAD, significant positive correlation factors supported the hypothesis that higher chlorophyll leads to higher grain yield and biomass. This suggests an enhancement of photosynthetic efficiency within WD and WW (Figure 26, Figure 27).

6. Conclusion

In this study, photoprotective and photosynthetic related traits through proximal sensing methods were studied in order to identify wheat lines adapted to stressed environments through response in yield and yield component, as well as water and temperature effect on stomatal responses. In addition, lower leaf level, in the specific the third leaf was evaluated in order to have a broader picture of the plant performance. Cars synthesis was already stopped under heat and water deficit, probably due to a downregulation of the enzyme responsible during the grain filling stage. On the contrary PRI responses to heat and water stress demonstrated a higher de-epoxidation cycle. The higher NPQt values in response to WD compared to WW confirmed the findings related to PRI, being a major component of NPQ in response to water shortage. The leaf adaptation behaviour to high temperature and high light intensity might hinder the response of NPQt, which did not show any difference in response to high temperatures and relative humidity. Interestingly, in response to water deficit plants show higher Cars/Chl ratio in comparison to HTS suggesting more need of photoprotection, while under HTS the decreased levels of Cars/Chl suggest a better use of the excitation energy under this environment (Eberhard et al., 2008). While there was a trend in response for the environments, no genotypic or GxE difference was reported in terms of photoprotective related indices and NPQ. The difference in the canopy structure within WD, may play a role in light availability for lower leaves and heightened Cars synthesis, which acts as a mechanism for photoprotection for some genotypes. While in response to HTS, the trend of third leaf for the Cars related indices follow the trend demonstrated in flag leaf. An increased xanthophyll de-epoxidation was demonstrated in response to abiotic stress for third leaf as well. However, in this case it is not supported by Chl degradation (no significant difference reported for NDVI and SPAD in both comparison in third leaf) this may indicate an optimization of the light harvesting through photoprotection mechanisms. For both leaf levels, negative values in the correlation coefficients. found in all the environments studied, between ARI and other photoprotective traits may suggest a trade-off. In addition this align with the theory of Li and colleagues (2010) that shaded leaves exhibit increased photosynthetic efficiency due to elevated chlorophyll levels in response to reductions in the flag leaf. In fact, NDVI and SPAD decreased in response to abiotic stress at flag leaf level. However, as ΦPSII is following this trend in response to WD, an opposite one was demonstrated in HTS trial. This may confirm an adaptation of the plant genetic material, after several years of trials characterized by high temperatures. The same reason might be behind an increased leaf area index of HTS compared to control and resource allocation shifted to grains,

in order to ensure seed production in HTS as demonstrated by the higher HI compared to WW environment. Another behaviour observed in WD trial, was the reduced photosynthesis, leaf area and above ground biomass were lower compared to WW environment, in order to avoid water loss. Further, the reduction of grain yield was supported by the consistent observation of low stomatal conductance in response to water deficiency across all lines. In this study, the different indices related to Cars respond differently accordingly to environment and leaf and over all show strong correlations between them in all the scenarios. A corroboration with pigment detection analysis is suggested in order to detect the reliability of proximal sensing methods through the detection of Cars related index which can better estimate the content of Cars. As well, the pigment content determination is needed to understand which factor interplays the high positive correlation between NDVI and Cars related index. In general, the lack of significance difference in terms of stomatal conductance and VPD between HTS and WW could also be attributed to the adaptation of plants in response to HTS through deep root system, coupled with the high heritability of the stomatal conductance trait (Pask et al., 2012). The positive association between ARI and stomatal conductance within WD, may be related to the involvement of the hormone ABA in the biosynthesis of anthocyanins as demonstrated in previous studies on rice (Li et al., 2022).

Additional research is required to verify the accuracy of proximal sensing methods and establish their reliability in terms of photoprotection in a field scale. In this study, the lines show higher adaptation to high temperature and high relative humidity compared to WD trial. In general, the lack of significant GxE effect for the majority of the parameters analysed in this study, is indicating a stable genetic pool. It needs to be tested in the following years, if these finding are consistent. Due to growing environments, phenotypic stability is essential to ensure grain yields and answer to the global issue of food security.

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