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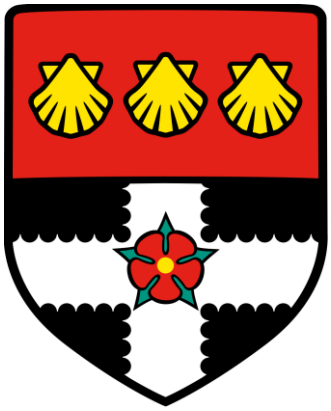

Humidity as a determinant of heat stress tolerance in wheat (*Triticum aestivum L.)*

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Declaration of Original Authorship

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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ABSTRACT

Wheat is one of the most common crops, grown worldwide on about 220 million ha of farmland. However, ongoing climate change has raised concerns about the effects of environmental variation on wheat yields. Studies on grain crops show that high spikelet (or panicle) temperature is the main reason for sterility and can be distinct from ambient air temperature. Transpirational cooling commonly lowers spikelet temperature, but the capacity of plants to cool their tissues may be affected by relative humidity. This thesis reports on findings from three consecutive years of pot and field-based experiments where two wheat cultivars, namely Blasco (bred for warm and dry conditions) and Paragon (bred for wet and cool conditions), were exposed to combinations of temperature (T), relative humidity (RH) and soil moisture treatments. The cooling capacity of Blasco was higher than Paragon under most conditions in controlled environment experiment, while the temperature of the spike was higher than that of the flag leaf in both cultivars in all three experiments.

Even though we have not observed RH effect on tissue temperature in the semi-controlled pot-based and field experiments, controlled environment experiment in the cabinets showed that both RH and air T has an impact on tissue temperature, while the main driver of tissue T in both cultivars was the temperature of surrounding air regardless of the growth stage. Higher RH at low air temperature resulted in a decrease of the cooling capacity in Paragon during early grain filling in controlled climate cabinets, but a complete reversal was observed in Blasco. RH did not modify tissue temperature of the cultivars at higher air T at flowering, but high RH increased spike temperatures in both cultivars during early grain filling. Increased tissue temperatures at flowering triggered lower grain number in Paragon.

Lower grain weight under high RH conditions caused lower grain yield which triggered higher total protein and gluten protein content in both cultivars in the controlled environment and field experiments. In pot-based semi-controlled experiment, higher soil moisture caused higher grain yield in Blasco and Paragon, and protein content tended to be higher at lower soil moisture conditions.

In the controlled environment experiment, we have observed that high tissue temperature under high temperature caused decrease in alpha amylase activity of Paragon. Gluten protein composition in Blasco was affected from high RH and high air temperature as ω-gliadins % glutenin subunits in Blasco increased under these conditions. There was a negative correlation between grain yield and protein content in

both cultivars in all three Experiments. In the semi-controlled field experiment, total nitrogen and gluten protein content in both cultivars increased under high RH and high air temperature conditions, while in pot-based semi-controlled experiment, increase in soil moisture resulted in lower SDS-sedimentation.

Blasco responds to the stress factors with altering its stomata area, while Paragon altered its stomata number. High RH, high air T and low soil caused an increase in stomata are in Blasco, while High RH and high air T resulted in higher stomata number in Paragon. Lower RH caused higher stomatal conductance in Blasco under ambient temperatures. The main finding of the thesis is that atmospheric humidity affects the capacity of wheat plants to regulate tissue temperature which impacts grain quantity and quality.

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Abbreviations and Symbols

ABA	Abscisic acid
ADP	Adenosine Diphosphate
AHDB	Agriculture and Horticulture Development Board
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BN	Billion
BPNPG7	p-nitrophenyl maltoheptaoside
BST	British summer time
C	Carbon
CBP	Chorleywood breadmaking process
cm	Centimetre
CO_2	Carbon dioxide
CTD	Canopy temperature depression
CU	Ceralpha Units

DAA	Days after anthesis
DTT	Dihiothreitol
e.g.	exempli gratia (for example)
ET	Evapotranspiration
et al.	et alia (and others)
FAO	Food and agriculture organization of the United Nations
Fig.	Figure
FLT	Flag leaf temperature
FLTD	Flag leaf temperature depression
g	Gram
GR	Green revolution
GS	Growth stage
Gs (g_s)	Stomatal conductance
h	Hour
HCL	Hydrochloric acid
HMW	High molecular weight
HSPs	Heat shock proteins
i.e.	id est (that is)
IFPRI	International Food Policy Research Institute
IPCC	Intergowernmental Panel on Climate Change
IPT	Internal polytunnel
K	Potassium
kDa	Kilo dalton
kg	Kilogram
LDS	Lithium dodecyl sulphate
LMW	Low molecular weight
m	metre
mg	milligram
Mg	Magnesium
min	Minute
mL	Millilitre

mm	Millimetre
mM	Millimolar
N	Nitrogen
nm	Nanometre
O_2	Oxygen
p	Probability
P	Phosphorus
pH	Potential of hydrogen
RH	Relative humidity
s	Seed
SD	Standard deviation
SDHi	Isopyrazam
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SO_3	Sulphur trioxide
ST	Spike temperature
STD	Spike temperature depression
T	Temperature
T_a	Air temperature
T_c	Canopy temperature
T_f	Flag leaf temperature
TD	Temperature depression
TDR	Time domain reflectrometry
TGW	Thousand grain weight
*T*max	Maximum temperature
*T*min	Minimum temperature
*T*opt	Optimum temperature
T_s	Spike temperature
UK	United Kingdom
USA	United States
VPD	Vapor pressure deficit

v/v	Volume/volume
w/v	Weight/volume
Z	Zadox
βME	2-Mercaptoethanol
%	Percent
°C	Celsius (Degree)
°	Degree
<	Less than
>	Greater than
µl	Microlitre
µm	Micrometre
µmol	Micromole
ΔE	Change in energy
α	Alpha
β	Beta
ϒ	Gamma
ω	Omega

List of Equations

[1] $FLTD = T_a - T_f$

[2] $STD = T_a - T_s$

[3] $CTD = T_a - T_c$

[4] $$\text{Unit(CU)/g flour} = \frac{\Delta E405}{\text{Incubation time}} \times \frac{\text{Total volume in cell}}{\text{Aliquote assayed}} \times \frac{1}{\text{EmM}} \times \frac{\text{Extraction volume}}{\text{Sample weight}} \times \text{Dilution}$$

[5] $\Delta E_{405} = \text{Absorbance(Reaction)} - \text{Absorbance(Blank)}$

[6] $$MC = 100 \times \frac{(P+F) - (P+\text{Dried F})}{(P+F) - P}$$

[7] $Wt = (MC \times 0.0873) + 4.7067$

Chapter 1: Introduction and Literature Review

1.1: Climate Change and Rising Potentiality of Heat Stress

The IPCC 5th assessment report indicates that economic and population growth resulted in increased anthropogenic greenhouse gas emissions since the pre-industrial period and that this fact is quite possibly the main reason of the observed warming since the mid-20th century. Long-term changes in all climate system components and further warming is expected, if greenhouse gas emissions continue. This will raise the possibility of severe effects for ecosystem and people (Barros, 2014). Climatic conditions influence crop performances significantly, causing important food security issues (Reynolds *et al.*, 2002). For example, wheat and maize yields were impacted negatively by climate change in many regions during the period 1960-2013. Without adaptation of agriculture in the temperate and tropical regions, climate change is projected to adversely affect the major crops, namely wheat, maize and rice, between 2030 and 2049 even if global temperature rise is limited to 2°C over the temperature levels in late 20th century. Even more serious yield loss is expected for the period beyond 2050, depending on the warming level (Porter *et al.*, 2014). Besides this, it is expected that the world population will be in the range from 9.15 billion to 9.51 billion (BN) in 2050 (Bremner *et al.*, 2010). According to the estimations of FAO, the number of people undernourished worldwide was 1.02 BN in 2009 (Raney *et al.*, 2009), and the expected rise in the world population will escalate the global demand for food (Raymond Park *et al.*, 2011). Pingali (2012) indicated that 70% increase in food production will be required in 2050. It is predicted that the frequency of hot days and variability in temperature will raise in the future. In order to develop new crop varieties adapting to the future climate, it is essential to understand how crops react to increased temperatures and how they can be improved to resist heat stress (Farooq *et al.*, 2011).

1.2: Green Revolution

Developed countries eliminated the malnutrition threat by sizeable investments in scientific research in agriculture in the 20th century. Improved agronomy, modern plant breeding and modern pesticides and inorganic fertilizer developments helped to achieve sustained food production (IFPRI, 2002). However, population growth at high rates and little investments in food production cause malnutrition and hunger in developing countries to continue, particularly in Asia by the mid-1960`s. Therefore, international agricultural research system established to transfer and adapt scientific

progress to the developing countries. Firstly, research concentrated on developing high-yielding wheat and rice varieties, since they are the mostly used food crops in developing countries. Bioengineered seeds, combined with the use of chemical fertilizers, irrigation and other chemical inputs caused significant yield growth in developing countries (Sebby, 2010; IFPRI, 2002). This growth in agricultural productivity is called “Green Revolution (GR)” which succeeded broader effects over the following years such as developing high-yielding varieties for other food crops crucial to developing countries (Evenson and Gollin, 2003). Adoption rate of new cultivars raised significantly during the GR in developing countries (Pingali, 2012). However, there are still challenges to raising the food production to feed the projected world population which need to be considered. Contribution of the Green Revolution on poverty and hunger reduction in marginal areas was very low, since GR’s original strategy was to concentrate on favourable areas with high rainfall or irrigation (Pingali, 2012). Most of developing countries do not have sufficient agricultural technologies and are located in areas that are under significant threat of climate change (Reynolds *et al.*, 2010). Worryingly, investment in agriculture decreased significantly in the post-GR period, and there is a growing concern about sustainable productivity gains and adaptation to climate change (Pingali, 2012). Therefore, growth in productivity and improvements in the sustainability of agriculture is as crucial today as at the beginning of the GR. A second Green Revolution is aiming to re-establish production system and agricultural innovations with harnessing technological innovations and scientific knowledge in order to meet these complex challenges (Pingali, 2012). Because of the uncertainties in future food production due to climate change and the projections about population growth, it would be unwise to ignore the importance of developing stress-tolerant crop varieties adapting to climate change to meet sustainable development goals (Porter *et al.*, 2014; Raymond Park *et al.*, 2011). Besides this, further enhancements in genetic yield potential will help to prevent over intensive usage of natural landscapes (Reynolds *et al.*, 2009).

1.3: The Wheat Crop

1.3.1: Origin of wheat

Wheat was first cultivated about 10 000 years ago during the `Neolithic Revolution`, and the very early cultivars, which were picked by farmers from wild populations according to their better yield and other features, were in diploid (genome AA) and tetraploid (genome AABB) forms. According to their genetic heritage, they are likely to

originate from south-east of Turkey (Shewry, 2009). Wheat has evolved throughout complex natural crosses which resulted in different ploidy levels, but all belonging to the genus *Triticum* of the family *Gramineae*. Modern wheat is classified as diploid, tetraploid (e.g. durum wheat) and hexaploid (e.g. bread/common wheat) because of the number of chromosomes in the somatic cells (Isidore *et al.*, 2005). Hexaploid wheat (*Triticum aestivum* L.) emerged after hybridization within three diploid species consists of A, B and D genomes (Islam *et al.*, 2003).

1.3.2: Wheat classification and grading

Looking at the type of photosynthesis mechanism, wheat is classified as a C3 plant. Plants can be classified in two categories, C3 (e.g. wheat and rice) and C4 (e.g. corn and sugarcane) plants, due to the essential differences in photosynthetic reactions. The first photosynthetic yield is 3- phosphoglyceric acid (3-C skeleton product) in C3 plants, while the first photosynthetic products are 4-carbon acids in C4 plants. Rubisco, enzyme which triggers the first reaction of the Calvin cycle (series of chemical reaction that occur in chloroplast during photosynthesis), is located in mesophyll cells in C3 plants, while it is in bundle sheath cell in C4 plants. C4 plants produce more yield under high irradiance and temperature, while C3 plants are less efficient when temperature elevates (Markku *et al.*, 2012; Cohen and Waddell, 2009).

Two main types of wheat crops, namely “common or bread wheat” and “durum wheat”, have developed over time, most of the wheat varieties grown nowadays are categorized under these two types. Common or bread wheat (*Triticum aestivum*) accounts for about 95% of world wheat production, while durum wheat (*Triticum turgidum*) accounts around 5% of cultivated wheat varieties (Bushuk and Rasper, 1994; Carver, 2009).

Wheat is generally divided to different grain classes depending on its end-use and agronomic characteristics which are quality, growth habit and colour. According to its bread making quality, wheat for human consumption can be divided into two groups; soft and hard. Kernel of hard wheat is physically hard, and its flour has high gluten content, that is, high protein content. Soft wheat has lower level of protein. While flour of hard wheat is suitable for bread and noodle making that need strong flour, soft wheat flour is used to make biscuits and cakes. Depending on its growth habit, there are two types of wheat; winter and spring wheat. While winter wheat needs vernalisation, this is not necessary for spring wheat (Snape *et al.*, 2001). At the end, wheat is also categorized according to colour of the kernel outer layer as red or white

wheat. Each wheat exporting country has their own grading system in order to meet their specific standards. Moisture content, protein content, grain weight and foreign material content determine the grade of the wheat (Bushuk and Rasper, 1994; Carver, 2009).

1.3.3: Growth stages of wheat

Barber *et al.* (2015) suggested that adaptation of crops to a particular environment and their yield potential in this environment can be understood with phasing of phenological development. Therefore, identifying growth stages of the crops is crucial for an effective crop management. For instance, timing of chemical and fertiliser applications is decided using one of the growth scales. A number of growth scales has been developed. Zadoks growth scale is one of such growth scales with wide international acceptance. It is based on the ten major growth stages in cereals labelled from 0 (germination) to 9 (ripening). Each stage is then sub-divided into ten more growth stages, extending the Zadoks growth scale from 00 (dry seed) to 99 (loss of secondary dormancy). This growth scale was developed by Zadoks *et al.* (1974) and it is used by farmers, researchers, agronomists and other agricultural professionals since when it was first proposed. Growth stages in Zadoks growth scale can be denoted as GS (growth stage) or Z (Zadoks) (Bowden *et al.*, 2008; Zadoks *et al.*, 1974). Planting of a dry seed into a seedbed is the initial stage of the wheat life cycle which ends with formation of dry seed in the head (Klepper *et al.*, 1997). Wheat plants progress through several growth stages from the initial stage to the final stage. These growth stages can be classified as germination leading to seedling, tillering (side shoot formation), jointing (stem elongation), booting (swelling of the flag leaf sheath in which ear expands), heading (the head is pushed out from the flag leaf sheath), flowering/anthesis (extrusion of the anther), grain filling (kernel maturity) and grain ripening (ready to harvest) (Sylvester-Bradley *et al.*, 2015). However, there are few weaknesses of the decimal growth stages especially on account of beginning of meiosis and growing point transition from vegetative to reproductive phases (Barber *et al.*, 2015).

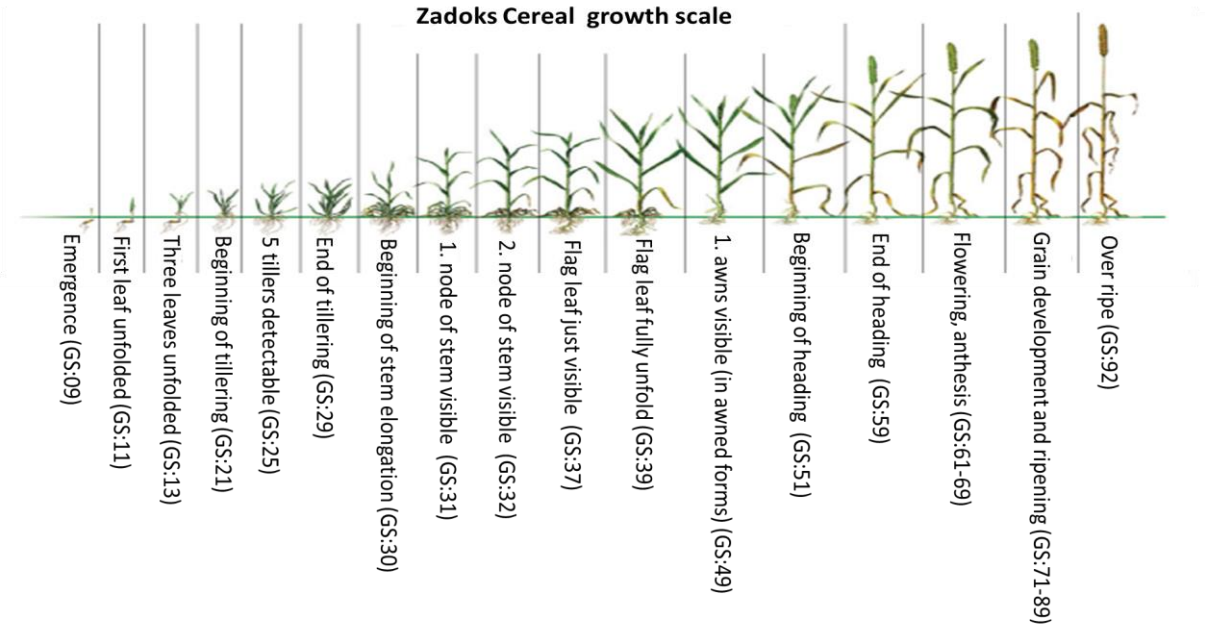


Figure 1.1 Schematic of Zadoks cereal/wheat growth stages (Zadoks *et al.*, 1974).

1.3.4: Temperatures essential for wheat developmental stages

Even though tolerance of each wheat cultivar to extreme temperatures appears to be different, generally it is accepted that the optimum temperature (*T*opt) range of wheat is 17-23°C throughout the entire growing period. Minimum (*T*min) and maximum (*T*max) temperature for wheat are defined as 0°C and 37°C, respectively. Temperatures beyond *T*min and *T*max stop growth of this crop plant (Porter and Gawith, 1999). While *T*opt for wheat during anthesis is between 18 and 24°C, 9 and 31°C considered as *T*min and *T*max, respectively. Super- or sub-optimal temperatures during flowering stage might cause decline in yields due to infertile floret production. If temperature during anthesis is lower than 9°C and higher than 31°C limits the success of anthesis (Porter and Gawith, 1999). There is a linear relationship between plant development and temperature tolerance. Therefore, cardinal temperatures during grain filling are higher than at flowering stage. While *T*min during grain filling stage range from 4.1°C to 8.9°C for spring wheat and 12°C for winter wheat, *T*max values range from 33.4°C to 37.4°C. In addition, *T*opt lies between 19.3 and 22.1°C (Porter and Gawith, 1999).

1.4: Impacts of heat stress on wheat yield and quality

Heat stress generally defined as a duration in that temperatures are high enough for adequate time to induce irreversible damage to plant development or function (A

Hemantaranjan *et al.*, 2014). It usually negatively impacts growth of plants at various developmental stages such as germination, reproduction, growth and yield production. At molecular level, heat stress changes the efficiency of enzymatic reactions, and impacts the continuity of various membranes, RNA species, proteins and cytoskeleton structures. These effects hinder physiological processes and cause metabolic imbalance (Hasanuzzaman *et al.*, 2013). Table 1.1 summarises the common impacts of heat stress during susceptible growth stages in wheat, while sections below (Section 1.4.2 and 1.4.3) and chapters 3,4 and 5 explain these in detail.

Table 1.1: Common impacts of heat stress during susceptible growth stages in wheat

Crop	Treatment	Growth Stage	Main Effects	References
Wheat (Triticum aestivum)	Heat stress	Before and during reproductive stage	Decreased period for days to booting, heading, anthesis and maturity Lower number of grains/spike Smaller grain size Lower yield	Hasanuzzaman *et al.* (2013) FERRIS *et al.* (1998) Barnabás et al. (2008)
Wheat (Triticum aestivum)	Heat stress	Grain filling and maturity stage	Decreased duration of grain filling and maturity Lower kernel weight and yield Lower alpha-amylase activity Higher protein content Higher gliadin synthesis Lower glutenin/gliadin ratio Increase in SDS-sedimentation	Rahman *et al.* (2009) Reddy *et al.* (1984) Rakita *et al.*. (2015) Ashraf, (2014) Castro *et al.* (2007) Corbellini *et al.* (1997) Majoul *et al.* (2003) Farooq *et al.* (2011) Stone and Nicolas, (1998)

It is clear that any adaptation of wheat to a particular environment and optimisation of its yield potential depend on: resource absorption (water, nutrients and light), especially during critical growth stages (Fischer, 1985); increase in resource usage efficiency, such as elevating radiation utilization (Reynolds *et al.*, 2012); and ensuring that specific susceptible growth stages do not coincide with high probability of abiotic stresses such as heat and drought stresses (Worland *et al.*, 1998). Escape, tolerance and decreasing extreme weather risk are the main methods currently used to counteract abiotic stresses in crops.

1.4.1: Critical heat stress incidents affecting wheat yield

Due to disturbance in membrane fluidity, protein conformation, metabolism and assembled cytoskeleton, changes in ambient temperatures are sensed by plants.

Following this, adaptation processes are activated to maintain cellular equilibrium – for example heat shock protein expression. However, temperatures significantly higher than the optimum growth temperatures, commonly called `heat stress`, can cause permanent damage. Magnitude and rate of temperature elevation and exposure duration to the increased temperature determine the intensity of heat stress (Farooq *et al.*, 2011). High temperature, the reason of heat stress in wheat, currently negatively impacts wheat productivity in tropical, subtropical, arid and semiarid regions of the world (Iqbal *et al.*, 2017). Elevated ambient temperature is a significant threat to wheat production, especially when it happens during grain filling and reproductive stages. It has been found that 31°C can dramatically decrease yield in wheat (Wheeler *et al.*, 1996; Porter and Gawith, 1999). However, there is unlikely to be a fixed critical temperature threshold, since the severity of heat stress is affected by its timing and duration of stress, water availability and ambient humidity.

For instance, 2 days heat stress with critical temperature >30°C (Prasad and Djanaguiraman, 2014) would decrease wheat yield significantly, while 4 days heat stress decrease critical temperature of wheat to 30°C (FERRIS *et al.*, 1998) and 5 days decrease critical value to 24°C (Prasad and Djanaguiraman, 2014). Few of existing studies investigated the interaction between temperature and other atmospheric factors affecting plant physiology, such as humidity (Abeysiriwardena *et al.*, 2002; Weerakoon *et al.*, 2008; Van Oort *et al.*, 2014). Tellingly, the amount of work dedicated to studying the interactive effect of temperature and humidity on wheat to date is minimal. Studies have indicated that intensity of yield loss because of heat stress can increase at higher humidity, and higher humidity lowers the critical temperature impacting wheat negatively by heat stress (Dawson and Wardlaw, 1989; Tashiro and Wardlaw, 1990). Under low water availability, critical temperature influencing wheat by heat stress is lower than under high water availability (Wheeler *et al.*, 1996; Porter and Gawith, 1999; Alghabari *et al.*, 2016).

1.4.2: Wheat response to heat stress during reproductive stage

Elevated temperature accelerates wheat growth, thus advancing flowering time and maturity which happen earlier, decreasing the amount of intercepted light over the season and negatively, affecting yields (Barnabás *et al.*, 2008; Stratonovitch and Semenov, 2015). Elevated temperatures from anthesis to maturity accelerate growth, thus shortening the duration of each phenological stage. This decreases the duration to capture resources which causes reduction in grain yield (Farooq *et al.*, 2011).

During the reproductive stage, beginning around the flag leaf appearance (Tottman, 1987), wheat is more vulnerable to temperature than during the vegetative stage (Porter and Gawith, 1999). However, the starting time of meiosis is still not understood and is likely to be determined by an interaction between environment and genotype (Barber *et al.*, 2015). Nevertheless, meiosis within all florets in a single spike happens within about 5 days. Therefore, it is possible to conduct experiments targeting susceptibility of meiosis to abiotic stresses (Barber *et al.*, 2015). It has been found that meiosis is susceptible to biotic and abiotic stresses particularly to heat stress if soil water availability is deficient. Heat stress during meiosis causes grain set failure (Barber *et al.*, 2015; Saini and Aspinall, 1982; Draeger and Moore, 2017). The final phase of reproductive development in wheat is anthesis (Zadoks *et al.*, 1974) which can last number of days via variation in flowering time on spikelets (Lukac *et al.*, 2011). It is highly sensitive to abiotic and biotic stresses, mainly heat stress (Semenov and Halford, 2009), that can dramatically affect yield. Stress around meiosis and anthesis causes yield reduction by decreasing grain fertility and thus lowering grain number (FERRIS *et al.*, 1998; Barnabás *et al.*, 2008), but also due to damaging and shrivelling of pollen under heat stress (Barnabás *et al.*, 2008). A study in rice suggested that pollen dehiscence decreases under heat stress which reduce the number of available pollen reaching to stigma and so lowers the fertility (Prasad *et al.*, 2006). A similar mechanism can be envisaged in wheat (Barnabás *et al.*, 2008). The main reason of pollen and anther sensitivity to heat stress is likely to be connected to the sensitivity of the tapetum, cells in the wall of anther (Dolferus *et al.*, 2011). Heat stress damages or kills tapetum cells, causing decrease in pollen development (Gothandam *et al.*, 2007; Dolferus *et al.*, 2011). Peak heat stress susceptibility happens slightly earlier (GS 61 – early flowering) than peak flowering stage (GS 65 -mid flowering) (Joppa *et al.*, 1968; Porter and Gawith, 1999). Temperatures higher than 30°C during floret formation might results in complete sterility (Farooq *et al.*, 2011).

1.4.3: Wheat response to heat stress during post anthesis

Tissue dehydration and decreased CO_2 assimilation happen under heat stress during the reproductive phase (Farooq *et al.*, 2011). Wheat growth and grain yield are impacted from any decline in photosynthesis which is one of the physiological process most susceptible to increased temperature. Heat stress reduces chlorophyll content and disturbs the function and structure of chloroplasts. This causes oxidative damage to chloroplasts and metabolic limitations. Thus, photosynthetic capacity of plants

decreases under heat stress conditions which results in reduced accumulation of dry matter and so grain yield (Farooq *et al.*, 2011; Ortiz *et al.*, 2008).

Synthesis of starch and its deposition within kernels, availability of photosynthates and their translocation to the kernel are adversely affected during the post-anthesis grain filling stage because of heat stress. This results in altered grain quality and decreased grain weight. Despite the fact that kernel number is not affected by post-anthesis heat stress, it has been shown that grain filling duration and kernel weight decrease (Iqbal *et al.*, 2017). Heat stress induced inhibition of chlorophyll biosynthesis, senescence related metabolic changes and thylakoid components breakdown in wheat triggers leaf senescence, gradual green leaf area loss. Heat stress also cause assimilate scarcity during reproductive development, since crops use assimilates to maintain plant survival under heat stress conditions (Farooq *et al.*, 2011).

Normally, 90-95% of carbon coming from current carbon assimilation is used for grain filling (Farooq *et al.*, 2011). Under heat and water stress, however, pre-anthesis stored stem reserves are remobilized during grain filling to compensate for decreased current (photosynthetic) assimilates. Thus, there is a remarkable rise, between 6 and 100%, in the demand for pre-anthesis stored stem reserves under heat stress, caused by heat-triggered reduction in photosynthesis (Blum, 1997). At temperatures over 30°C, translocation of assimilates from flag leaf to grain is significantly decreased, while assimilate transport from the stem to grain is not affected at temperatures from 1 to 50°C (Farooq *et al.*, 2011).

Exposure to heat stress during grain filling determines grain quality due to the protein accumulation (Porter and Gawith, 1999). Heat stress during this stage might also influence gluten strength and lessening the flour quality of wheat. Even though grain protein percentage increases due to heat stress, protein content per grain is diminished (Stone *et al.*, 1997). The reason is that heat shock genes are activated to produce heat shock proteins (HSPs), responsible for thermo-tolerance acquisition, when heat stress occurs during grain filling. This enhances protein concentration in mature grains and weaken dough production (Iqbal *et al.*, 2017).

1.5: Adaptation of Crops/Wheat to Heat Stress

Crop production is under an increasing threat in many regions because of predicted high temperature events affecting growth, development and productivity of plants. Thus, developing wheat crop cultivars tolerant to high temperature to sustain high

yields is necessary. However, it is a challenge for breeders to improve heat stress tolerance, since it is not easy to identify and confirm the traits conferring heat stress tolerance (Hasanuzzaman *et al.*, 2013). Thus, it is important to screen wheat genotypes under heat stress, and identify genotypes having tolerance to heat stress (Kamrani *et al.*, 2018). Plants are stationary organisms, which cannot escape from heat stress via moving to cooler environments. However, they possess several avoidance, adaptive and acclimation mechanisms (Hasanuzzaman *et al.*, 2013).

1.5.1 Tolerance mechanism

Heat tolerance (thermotolerance) refers to the ability of crops to survive and have good grain yield when they exposure to heat stress (Wahid *et al.*, 2007). One of the thermotolerance mechanism of the crops is the expression of heat shock proteins (HSPs), which is an essential molecular response to heat stress (Rampino *et al.*, 2009). The ability to continue photosynthetic productivity at high temperature is also a tolerance mechanism. In addition, enzymatic tolerance (thermotolerance) to heat stress hinders denaturation of key enzymes (Mahan *et al.*, 1987; Burke, 1990).

1.5.2: Escape mechanisms of crops

Crops are able to shift the time of the developmental stages (Semenov *et al.*, 2014), such as shifting flowering and meiosis (susceptible periods) to an earlier time in the season. Timing of the growth stages is determined by genes sensitive to vernalisation temperature, photoperiod and the accumulation of temperature units (Richards, 1991). This prevents late season heat and drought stress. Agronomic practices, such as altering sowing date, can also help plants to have susceptible stages earlier than stress factors (Richards, 1991; Semenov *et al.*, 2014). Besides this, extended flowering duration, which helps to spread the risk and decrease the amount of spikelets impacted by stress at the same time, is another escape mechanism (Lukac *et al.*, 2011).

1.5.3: Avoidance

Avoidance mechanisms hinder or delay adverse impacts of environmental stress on crops. For instance, wax deposition in the surface of leaf (Clarke and Richards, 1988), elevating stomatal resistance (Oosterhuis and Walker, 1987). In order to decrease heat-induced damage, plants have mechanisms such as leaf shedding, thickening leaves, leaf rolling, leaf size reduction, transpirational cooling and reduction in the duration of growth (Wahid *et al.*, 2007). Wheat genotypes having higher stem reserve

storage capacity and stationary photosynthesis have also better withstand to heat stress (Blum *et al.*, 1994). Regulating stomatal opening, closing and density is other important mechanism of wheat to acclimate and resist stress conditions, since this controls CO_2 assimilation, water use efficiency, canopy cooling and crop yield (Gitz and Baker, 2009).

1.6: Relative Humidity

Relative humidity (RH) is defined as the amount of water vapour in the air compared to what the air can hold if it is saturated at a given temperature (Xie *et al.*, 2011), and in order to cope with the changes in atmospheric temperature, RH plays an important role as it helps plants to modify their water loss via transpiration. Higher relative humidity especially in winter in northern Europe (Niglas *et al.*, 2015; Ruosteenoja and Räisänen, 2013), lower relative humidity primarily in summer in southern Europe, and moister climate for winter in central Europe (Ruosteenoja and Räisänen, 2013) are projected as a result of climate change.

There are few studies conducted on rice about the interactive effect of RH and temperature on grain yield. Yan *et al.* (2010) suggested that genotype, RH and air temperature influence organ temperature of rice, and there is a negative correlation between panicle temperature and grain yield (seed setting and grain filing rate), while Weerakoon *et al.* (2008) indicated that low RH (55-60%) triggers decrease in grain sterility is more due to diminished spikelet temperature than to air temperature, and it is crucial to consider the effect of RH when evaluating the impact of air temperature on grain yield. High air temperature coupled with high RH (85-90%) causes complete grain sterility in rice, while decreasing RH causes decline in high-temperature induce grain sterility (Weerakoon *et al.*, 2008). Higher the RH at a given atmospheric temperature, and an increase in air temperature at a constant RH both elevates the organ temperature in rice (Abeysiriwardena *et al.*, 2002; Yan *et al.*, 2010). At the normal temperature of 30°C day/25°C night, rice at very low RH (34-40%) had significantly higher grain sterility than those at normal RH of 65-70% (Abeysiriwardena *et al.*, 2002). These findings are very informative and give some clues about how RH might modify grain yield in other crops. However, more studies are needed to concentrate the role of RH in grain yield and also in grain quality in other crops. For instance, wheat has not been studied according to our knowledge, and carrying out Experiments to see how RH modifies grain yield and quality under different air temperatures in wheat is very precious.

1.7: Drought Stress

Drought is one of the major abiotic stresses limiting worldwide wheat and food production. Grain filling and flowering stages are the most sensitive growth stages to drought stress, resulting in yield losses. The magnitude of yield loss depends on the duration and severity of stress. The main causes of yield losses are oxidative damage to stomatal closure and chloroplasts, poor development and grain set, pollen sterility, accelerated leaf senescence, decrease in net photosynthesis rate because of metabolic limitations, decrease in assimilate translocation and carbon fixation rate and decreased capacity of sink (Farooq *et al.*, 2014; Farooq *et al.*, 2009; Ahmadi and Baker, 2001). In order to survive and reproduce under drought stress conditions (drought resistance), plants evolved suitable mechanisms. Resistance mechanisms of the plants for drought consist avoidance, escape and tolerance strategies (Barnabás *et al.*, 2008). Most of the times, other environmental stresses like heat stress are accompanied with water deficiency. Therefore, developing cereal cultivars resistant to multiple stresses might be promising viewpoint for the breeders (Barnabás *et al.*, 2008; Farooq *et al.*, 2009).

Meiosis and anthesis stages are very vulnerable to drought stress (Cattivelli *et al.*, 2008) in wheat, there is no documented effect of drought on grain number after anthesis (Plaut *et al.*, 2004). Water stress during meiosis causes pollen sterility since it prevents microsporogenesis, resulting in grain number decline (Cattivelli *et al.*, 2008; Manjarrez-Sandoval *et al.*, 1989). Drought triggers abscisic acid (ABA) biosynthesis (Ji *et al.*, 2011), decrease in invertase activity (Dorion *et al.*, 1996) and a decrease in starch accumulation (Lalonde *et al.*, 1997) which result in pollen sterility. Grain filling duration, which is a starch biosynthesis and accumulation process, decreases under drought conditions causing significant decline in grain dry weight, while grain filling rate is affected slightly. Starch synthase is one of the enzymes regulating the change of sucrose to starch, and thus it plays a crucial role during grain filling (Yang *et al.*, 2004). However, its activity reduces under drought stress (Ahmadi and Baker, 2001).

1.8: Stomata and gas exchange regulation

Stomata are small apertures on the surface of wheat leaves, awns and stems whose role is to regulate gas exchange - mainly CO_2 intake, O_2 release during photosynthesis and water vapor loss via transpiration - between the atmosphere and leaf tissue (Hetherington and Woodward, 2003; Camargo and Marenco, 2011; Laga *et al.*, 2014). Loss of water via stomata opening, i.e. transpiration, helps plants to control

temperature by thermal cooling. Since stomatal opening allows CO_2 intake and O_2 loss, which is necessary for photosynthesis, it plays a crucial role in converting sunlight into energy essential for plant growth (Pallas *et al.*, 1967; Hetherington and Woodward, 2003; Lawson, 2009; Laga *et al.*, 2014). Degree of stomatal opening and stomatal density are both responsible for the resistance to water vapour and CO_2 diffusion between atmosphere and leaf, an alteration in stomatal resistance has a higher influence on transpiration than on photosynthesis. Stomatal density is influenced by environmental factors such as drought, heat stress, increased CO_2 concentration and precipitation change (Teare *et al.*, 1971; Liao *et al.*, 2005; Maghsoudi and Maghsoudi, 2008). Therefore, when studies focus on the effect of abiotic stress on crops, it is crucial to examine the performance and any effects on stomata as well.

1.9: Crucial Knowledge Gaps and Thesis Summary

Many studies focus on the effect of heat stress on wheat. However, most of existing literature which looks at the timing of heat stress does not account for other environmental factors such as high or low relative humidity. Even though the interactive effect of RH and air temperature has been studied in some crops such as rice, there is a paucity of information about their effects on wheat. In order to develop new wheat cultivars resistant to predicted changes in temperature and RH, breeders need to know how wheat cultivars respond to interactions between climatic factors. Therefore, so as to provide a solid basis for our understanding of the role of atmospheric humidity, this project aims to perform manipulative experiments to elucidate interactive effects of humidity and temperature on wheat tissue temperature, grain yield and quality, and suggest potential avenues for using this knowledge in wheat breeding programs. Two varieties, namely Paragon and Blasco, developed for cultivation in the UK and the Mediterranean climates, respectively which adapted to different heat and humidity conditions were selected to be studied during the project. The impact of different combinations of relative humidity and temperature, applied at specific growth stages of wheat (i.e. flowering and 12 DAA), on grain yield and grain processing quality were studied in the two cultivars.

1.10: Summary of the Thesis Structure and Individual Chapter Objectives

Chapter 1 – Introduction and Literature Review

- Summarise climate change and green revolution
- Overview the literature regarding wheat origin, classification and growth stages

- Summarise heat and drought stresses effects of crops/wheat, and adaptation mechanisms of crops
- Specify knowledge gaps about relative humidity effects on wheat
- Summarize research chapters and their aims

Chapter 2 – Experimental Design

- Show the design of the manipulative Experiments in detail performed to elucidate interactive effects of humidity-temperature, and interactive effects of relative humidity-soil moisture on wheat grain yield and its components.

Chapter 3 - Interactive Effects of Manipulated Temperature, Relative Humidity and Soil Moisture on Tissue Temperatures and Grain Yield of Two Spring Wheat (*Triticum aestivum L.*) Cultivars

- Identify self-cooling capacity of ear and flag leaf of different wheat varieties under varying temperature and humidity conditions at flowering and early grain filling stages.
- Determine the relationship between spike/flag leaf temperature depression and grain yield i.e. grain number, grain size and grain weight

Hypothesis (3i)

H_0: Flag leaf/spike cooling capacity at flowering and early grain filling stage is positively correlated with grain yield under abiotic stress conditions.

Chapter 4 - Alpha-amylase and Protein Response of Two Spring Wheat (*Triticum aestivum L.*) cultivars to Temperature, Relative Humidity and Soil Moisture.

- Identify the interactive effect of RH and air temperature on alpha amylase activity
- Identify the interactive effect of RH and air temperature on deposition of storage proteins and on gluten polymer formation and their relationship to the polymerization status of gluten proteins in the mature grains.
- Identify the interactive effect of RH and air temperature on total N content and SDS-sedimentation

Hypothesis (4i)

H_0: High RH causes an increase in alpha-amylase activity, while high air temperature results in lower α-amylase activity during grain filling.

Hypothesis (4ii)

H_0: Elevated air temperature, high RH and low soil moisture result in higher grain protein content and alter protein composition.

Chapter 5 - Response of Spring Wheat (*Triticum aestivum L.)* stomata to changes in Temperature, Relative Humidity and Soil Moisture

- Identify the interactive effect of RH and air temperature on stomata number
- Identify the interactive effect of RH and air temperature on stomata area
- Identify the interactive effect of RH and air temperature on stomatal conductance

Hypothesis (5i)

H_0: Growing conditions characterized by high RH, high air T and low soil moisture result in lower stomatal area and lower stomata density.

Chapter 6 – Discussion

- Overview the thesis
- Discuss the results of the Experiments
- Indicate the future works need to be done
- Conclude the results

Chapter 7 - Genotypic variability enhances the reproducibility of an ecological study

- The chapter demonstrates research outcomes of an initial training experiment carried out at the beginning of the PhD studies
- The paper presents the results of a microcosm experiments in 14 European laboratories, Rahme Seyhun performed one of the repetitions of this experiment in the Harborne laboratory, University of Reading.

1.11: References

A Hemantaranjan, A., Nishant Bhanu, M. S., 2 DK Yadav,1, PK Patel, R. S., 1 Deepmala & Katiyar1 1 Department of Plant Physiology, B. H. (2014). Heat stress responses and thermotolerance **1**(3).

Abeysiriwardena, D. d. Z., Ohba, K. & Maruyama, A. (2002). Influence of temperature and relative humidity on grain sterility in rice. *Journal of the National Science Foundation of Sri Lanka,* **30** (1-2), 33-41.

Ahmadi, A. & Baker, D. A. (2001). The effect of water stress on the activities of key regulatory enzymes of the sucrose to starch pathway in wheat. *Plant Growth Regulation,* **35** (1), 81-91.

Alghabari, F., Ihsan, M. Z., Khaliq, A., Hussain, S., Daur, I., Fahad, S. & Nasim, W. (2016). Gibberellin-sensitive Rht alleles confer tolerance to heat and drought stresses in wheat at booting stage. *Journal of Cereal Science,* **70**, 72-78.

Barber, H., Carney, J., Alghabari, F. & Gooding, M. (2015). Decimal growth stages for precision wheat production in changing environments? *Annals of Applied Biology,* **166** (3), 355-371.

Barnabás, B., Jäger, K. & Fehér, A. (2008). The effect of drought and heat stress on reproductive processes in cereals. *Plant, cell & environment,* **31** (1), 11-38.

Barros, V. R. (2014). *Climate Change 2014–Impacts, Adaptation and Vulnerability: Global and Sectoral Aspects.* Cambridge University Press.

Blum, A. (1997). Improving wheat grain filling under stress by stem reserve mobilisation. *Wheat: Prospects for Global Improvement.* Springer.

Blum, A., Sinmena, B., Mayer, J., Golan, G. & Shpiler, L. (1994). Stem reserve mobilisation supports wheat-grain filling under heat stress. *Functional Plant Biology,* **21** (6), 771-781.

Bowden, P., Edwards, J., Ferguson, N., Mc, Nee, T., Manning, B., Roberts, K., Schipp, A., Schulze, K. & Wilkins, J. (2008). *Wheat growth & development.* State of New South Wales (NSW): State of New South Wales (NSW) Department of Primary Industries.

Bremner, J., Frost, A., Haub, C., Mather, M., Ringheim, K. & Zuehlke, E. (2010). World population highlights: Key findings from PRB's 2010 world population data sheet. *Population Bulletin,* **65** (2), 1-12.

Burke, J. (1990). High temperature stress and adaptations in crops. *Plant biology (USA)*.

Bushuk, W. & Rasper, V. F. (1994). *Wheat: production, properties and quality.* Springer Science & Business Media.

Camargo, M. A. B. & Marenco, R. A. (2011). Density, size and distribution of stomata in 35 rainforest tree species in Central Amazonia. *Acta Amazonica,* **41** (2), 205-212.

Carver, B. F. (2009). *Wheat: science and trade.* John Wiley & Sons.

Cattivelli, L., Rizza, F., Badeck, F.-W., Mazzucotelli, E., Mastrangelo, A. M., Francia, E., Mare, C., Tondelli, A. & Stanca, A. M. (2008). Drought tolerance improvement in crop plants: an integrated view from breeding to genomics. *Field Crops Research,* **105** (1-2), 1-14.

Clarke, J. & Richards, R. (1988). The effects of glaucousness, epicuticular wax, leaf age, plant height, and growth environment on water loss rates of excised wheat leaves. *Canadian Journal of Plant Science,* **68** (4), 975-982.

Cohen, S. J. & Waddell, M. W. (2009). *Climate change in the 21st century.* McGill-Queen's Press-MQUP.

Dawson, I. & Wardlaw, I. (1989). The tolerance of wheat to high temperatures during reproductive growth. III. Booting to anthesis. *Australian Journal of Agricultural Research,* **40** (5), 965-980.

Dolferus, R., Ji, X. & Richards, R. A. (2011). Abiotic stress and control of grain number in cereals. *Plant Science,* **181** (4), 331-341.

Dorion, S., Lalonde, S. & Saini, H. S. (1996). Induction of male sterility in wheat by meiotic-stage water deficit is preceded by a decline in invertase activity and changes in carbohydrate metabolism in anthers. *Plant Physiology,* **111** (1), 137-145.

Draeger, T. & Moore, G. (2017). Short periods of high temperature during meiosis prevent normal meiotic progression and reduce grain number in hexaploid wheat (Triticum aestivum L.). *Theoretical and Applied Genetics,* **130** (9), 1785-1800.

Evenson, R. E. & Gollin, D. (2003). Assessing the impact of the Green Revolution, 1960 to 2000. *science,* **300** (5620), 758-762.

Farooq, M., Bramley, H., Palta, J. A. & Siddique, K. H. (2011). Heat stress in wheat during reproductive and grain-filling phases. *Critical Reviews in Plant Sciences,* **30** (6), 491-507.

Farooq, M., Hussain, M. & Siddique, K. H. (2014). Drought stress in wheat during flowering and grain-filling periods. *Critical Reviews in Plant Sciences,* **33** (4), 331-349.

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D. & Basra, S. (2009). Plant drought stress: effects, mechanisms and management. *Sustainable agriculture.* Springer.

FERRIS, R., Ellis, R., Wheeler, T. & Hadley, P. (1998). Effect of high temperature stress at anthesis on grain yield and biomass of field-grown crops of wheat. *Annals of Botany,* **82** (5), 631-639.

Fischer, R. (1985). Number of kernels in wheat crops and the influence of solar radiation and temperature. *The Journal of Agricultural Science,* **105** (2), 447-461.

Gitz, D. C. & Baker, J. T. (2009). Methods for creating stomatal impressions directly onto archivable slides. *Agronomy journal,* **101** (1), 232-236.

Gothandam, K. M., Kim, E. S. & Chung, Y. Y. (2007). Ultrastructural study of rice tapetum under low-temperature stress. *Journal of Plant Biology,* **50** (4), 396.

Hasanuzzaman, M., Nahar, K., Alam, M. M., Roychowdhury, R. & Fujita, M. (2013). Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. *International Journal of Molecular Sciences,* **14** (5), 9643-9684.

Hetherington, A. M. & Woodward, F. I. (2003). The role of stomata in sensing and driving environmental change. *Nature,* **424** (6951), 901.

IFPRI, G. R. (2002). Curse or Blessing. *International Food Policy Research Institute: Washington, DC, USA*, 4.

Iqbal, M., Raja, N., Yasmeen, F., Hussain, M., Ejaz, M. & Shah, M. (2017). Impacts of heat stress on wheat: A critical review. *Adv. Crop Sci. Technol,* **5**, 1-9.

Isidore, E., Scherrer, B., Chalhoub, B., Feuillet, C. & Keller, B. (2005). Ancient haplotypes resulting from extensive molecular rearrangements in the wheat A genome have been maintained in species of three different ploidy levels. *Genome Research,* **15** (4), 526-536.

Islam, N., Tsujimoto, H. & Hirano, H. (2003). Proteome analysis of diploid, tetraploid and hexaploid wheat: towards understanding genome interaction in protein expression. *Proteomics,* **3** (4), 549-557.

Ji, X., Dong, B., Shiran, B., Talbot, M. J., Edlington, J. E., Hughes, T., White, R. G., Gubler, F. & Dolferus, R. (2011). Control of abscisic acid catabolism and abscisic acid homeostasis is important for reproductive stage stress tolerance in cereals. *Plant physiology,* **156** (2), 647-662.

Joppa, L., McNeal, F. & Berg, M. (1968). Pollen Production and Pollen Shedding of Hard Red Spring (Triticum aestivum L. em Thell.) and Durum (T. durum Desf.) Wheats1. *Crop Science,* **8** (4), 487-490.

Kamrani, M., Hoseini, Y. & Ebadollahi, A. (2018). Evaluation for heat stress tolerance in durum wheat genotypes using stress tolerance indices. *Archives of Agronomy and Soil Science,* **64** (1), 38-45.

Klepper, B., Rickman, R., Waldman, S. & Chevalier, P. (1997). The physiological life cycle of wheat: Its use in breeding and crop management. *Wheat: Prospects for Global Improvement.* Springer.

Laga, H., Shahinnia, F. & Fleury, D. (Year). Image-based plant stornata phenotyping. *In:* Control Automation Robotics & Vision (ICARCV), 2014 13th International Conference on, 2014. IEEE, 217-222.

Lalonde, S., Beebe, D. U. & Saini, H. (1997). Early signs of disruption of wheat anther development associated with the induction of male sterility by meiotic-stage water deficit. *Sexual Plant Reproduction,* **10** (1), 40-48.

Lawson, T. (2009). Guard cell photosynthesis and stomatal function. *New Phytologist,* **181** (1), 13-34.

Liao, J.-X., Chang, J. & Wang, G.-X. (2005). Stomatal density and gas exchange in six wheat cultivars. *Cereal Research Communications,* **33** (4), 719-726.

Lukac, M., Gooding, M. J., Griffiths, S. & Jones, H. E. (2011). Asynchronous flowering and within-plant flowering diversity in wheat and the implications for crop resilience to heat. *Annals of botany,* **109** (4), 843-850.

Maghsoudi, K. & Maghsoudi, M. (2008). Analysis of the effects of stomatal frequency and size on transpiration and yield of wheat (Triticum aestivum L.). *American-Eurasian J Agric Environ Sci,* **3**, 865-72.

Mahan, J., Burke, J. & Orzech, K. (1987). The" thermal kinetic window" as an indicator of optimum plant temperature. *Plant Physiol,* **82**, 518-522.

Manjarrez-Sandoval, P., GONZÁLEZ-HERNÁNDEZ, V. A., MENDOZA-ONOFRE, L. E. & Engleman, E. (1989). Drought stress effects on the grain yield and panicle development of sorghum. *Canadian Journal of Plant Science,* **69** (3), 631-641.

Markku, S., Fradinho, M. J., Santos, A. S. & Miraglia, N. (2012). Forages and grazing in horse nutrition.

Niglas, A., Alber, M., Suur, K., Jasińska, A. K., Kupper, P. & Sellin, A. (2015). Does increased air humidity affect stomatal morphology and functioning in hybrid aspen? *Botany,* **93** (4), 243-250.

Oosterhuis, D. & Walker, S. (1987). Stomatal resistance measurement as an indicator of water deficit stress in wheat and soybeans. *South African Journal of Plant and Soil,* **4** (3), 113-120.

Ortiz, R., Sayre, K. D., Govaerts, B., Gupta, R., Subbarao, G., Ban, T., Hodson, D., Dixon, J. M., Ortiz-Monasterio, J. I. & Reynolds, M. (2008). Climate change: can wheat beat the heat? *Agriculture, Ecosystems & Environment,* **126** (1-2), 46-58.

Pallas, J. E., Michel, B. E. & Harris, D. G. (1967). Photosynthesis, transpiration, leaf temperature, and stomatal activity of cotton plants under varying water potentials. *Plant Physiology,* **42** (1), 76-88.

Pingali, P. L. (2012). Green Revolution: Impacts, limits, and the path ahead. *Proceedings of the National Academy of Sciences,* **109** (31), 12302-12308.

Plaut, Z., Butow, B., Blumenthal, C. & Wrigley, C. (2004). Transport of dry matter into developing wheat kernels and its contribution to grain yield under post-anthesis water deficit and elevated temperature. *Field Crops Research,* **86** (2-3), 185-198.

Porter, J. R. & Gawith, M. (1999). Temperatures and the growth and development of wheat: a review. *European Journal of Agronomy,* **10** (1), 23-36.

Porter, J. R., Xie, L., Challinor, A. J., Cochrane, K., Howden, S. M., Iqbal, M. M., Lobell, D. B., Travasso, M. I., Netra Chhetri, N. C. & Garrett, K. (2014). Food security and food production systems.

Prasad, P., Boote, K., Allen Jr, L., Sheehy, J. & Thomas, J. (2006). Species, ecotype and cultivar differences in spikelet fertility and harvest index of rice in response to high temperature stress. *Field crops research,* **95** (2-3), 398-411.

Prasad, P. V. & Djanaguiraman, M. (2014). Response of floret fertility and individual grain weight of wheat to high temperature stress: sensitive stages and thresholds for temperature and duration. *Functional Plant Biology,* **41** (12), 1261-1269.

Rampino, P., Mita, G., Pataleo, S., De Pascali, M., Di Fonzo, N. & Perrotta, C. (2009). Acquisition of thermotolerance and HSP gene expression in durum wheat (Triticum durum Desf.) cultivars. *Environmental and experimental Botany,* **66** (2), 257-264.

Raney, T., Gerosa, S., Khwaja, Y., Skoet, J., Steinfeld, H., McLeod, A., Opio, C. & Cluff, M. (2009). The state of food and agriculture 2009: livestock in the balance. *Food and Agriculture Organization of the United Nations, Rome, Italy*.

Raymond Park, J., McFarlane, I., Hartley Phipps, R. & Ceddia, G. (2011). The role of transgenic crops in sustainable development. *Plant Biotechnology Journal,* **9** (1), 2-21.

Reynolds, M., Bonnett, D., Chapman, S. C., Furbank, R. T., Manes, Y., Mather, D. E. & Parry, M. A. (2010). Raising yield potential of wheat. I. Overview of a consortium approach and breeding strategies. *Journal of experimental botany,* **62** (2), 439-452.

Reynolds, M., Foulkes, M. J., Slafer, G. A., Berry, P., Parry, M. A., Snape, J. W. & Angus, W. J. (2009). Raising yield potential in wheat. *Journal of Experimental Botany,* **60** (7), 1899-1918.

Reynolds, M., Pask, A. & Mullan, D. (2012). *Physiological breeding I: interdisciplinary approaches to improve crop adaptation.* CIMMYT.

Reynolds, M., Trethowan, R., Crossa, J., Vargas, M. & Sayre, K. (2002). Physiological factors associated with genotype by environment interaction in wheat. *Field crops research,* **75** (2-3), 139-160.

Richards, R. (1991). Crop improvement for temperate Australia: future opportunities. *Field Crops Research,* **26** (2), 141-169.

Ruosteenoja, K. & Räisänen, P. (2013). Seasonal changes in solar radiation and relative humidity in Europe in response to global warming. *Journal of Climate,* **26** (8), 2467-2481.

Saini, H. & Aspinall, D. (1982). Abnormal sporogenesis in wheat (Triticum aestivum L.) induced by short periods of high temperature. *Annals of Botany,* **49** (6), 835-846.

Sebby, K. (2010). The Green Revolution of the 1960's and Its Impact on Small Farmers in India.

Semenov, M., Stratonovitch, P., Alghabari, F. & Gooding, M. (2014). Adapting wheat in Europe for climate change. *Journal of Cereal Science,* **59** (3), 245-256.

Semenov, M. A. & Halford, N. G. (2009). Identifying target traits and molecular mechanisms for wheat breeding under a changing climate. *Journal of experimental botany,* **60** (10), 2791-2804.

Shewry, P. R. (2009). Wheat. *Journal of experimental botany,* **60** (6), 1537-1553.

Snape, J., Butterworth, K., Whitechurch, E. & Worland, A. (2001). Waiting for fine times: genetics of flowering time in wheat. *Wheat in a global environment.* Springer.

Stone, P., Gras, P. & Nicolas, M. (1997). The influence of recovery temperature on the effects of a brief heat shock on wheat. III. Grain protein composition and dough properties. *Journal of cereal science,* **25** (2), 129-141.

Stratonovitch, P. & Semenov, M. A. (2015). Heat tolerance around flowering in wheat identified as a key trait for increased yield potential in Europe under climate change. *Journal of experimental botany,* **66** (12), 3599-3609.

Sylvester-Bradley, R., Berry, P. B., Blake, J., Kindred, D., Spink, J., Bingham, I., McVittie, J., Foulkes, J., Boys, E. & Geary, F. (2015). Wheat growth guide Stoneleigh Park Warwickshire: AHDB Cereals & Oilseeds.

Tashiro, T. & Wardlaw, I. (1990). The response to high temperature shock and humidity changes prior to and during the early stages of grain development in wheat. *Functional Plant Biology,* **17** (5), 551-561.

Teare, I., Peterson, C. & Law, A. (1971). Size and Frequency of Leaf Stomata in Cultivars of Triticum aestivum and Other Triticum Species1. *Crop Science,* **11** (4), 496-498.

Tottman, D. (1987). The decimal code for the growth stages of cereals, with illustrations. *Annals of applied biology,* **110** (2), 441-454.

Van Oort, P., Saito, K., Zwart, S. & Shrestha, S. (2014). A simple model for simulating heat induced sterility in rice as a function of flowering time and transpirational cooling. *Field Crops Research,* **156**, 303-312.

Wahid, A., Gelani, S., Ashraf, M. & Foolad, M. R. (2007). Heat tolerance in plants: an overview. *Environmental and experimental botany,* **61** (3), 199-223.

Weerakoon, W., Maruyama, A. & Ohba, K. (2008). Impact of Humidity on Temperature-Induced Grain Sterility in Rice (Oryza sativa L). *Journal of Agronomy and Crop Science,* **194** (2), 135-140.

Wheeler, T., Hong, T., Ellis, R., Batts, G., Morison, J. & Hadley, P. (1996). The duration and rate of grain growth, and harvest index, of wheat (Triticum aestivum L.) in response to temperature and CO2. *Journal of experimental botany,* **47** (5), 623-630.

Worland, A., Korzun, V., Röder, M., Ganal, M. & Law, C. (1998). Genetic analysis of the dwarfing gene Rht8 in wheat. Part II. The distribution and adaptive significance of allelic variants at the Rht8 locus of wheat as revealed by microsatellite screening. *Theoretical and Applied Genetics,* **96** (8), 1110-1120.

Xie, B., Zhang, Q. & Ying, Y. (2011). Trends in precipitable water and relative humidity in China: 1979–2005. *Journal of Applied Meteorology and Climatology,* **50** (10), 1985-1994.

Yan, C., Ding, Y., Wang, Q., Liu, Z., Li, G., Muhammad, I. & Wang, S. (2010). The impact of relative humidity, genotypes and fertilizer application rates on panicle,

leaf temperature, fertility and seed setting of rice. *The Journal of Agricultural Science,* **148** (3), 329-339.

Yang, J., Zhang, J., Wang, Z., Xu, G. & Zhu, Q. (2004). Activities of key enzymes in sucrose-to-starch conversion in wheat grains subjected to water deficit during grain filling. *Plant Physiology,* **135** (3), 1621-1629.

Zadoks, J. C., Chang, T. T. & Konzak, C. F. (1974). A decimal code for the growth stages of cereals. *Weed research,* **14** (6), 415-421.

Chapter 2: Experimental Design

This study performed manipulative experiments to elucidate interactive effects of humidity, soil moisture and atmospheric temperature on wheat grain yield and its components. The main motivation was to provide a solid basis for our understanding of the role of atmospheric humidity in crop plant physiology and make suggestions for wheat breeding programmes about how and to what extend does humidity determine yield and yield quality in wheat. Two wheat varieties, namely Paragon and Blasco, respectively developed for cultivation in the UK and the Mediterranean climates, and thus adapted to different heat, humidity and soil moisture conditions were studied during the project. Two controlled environment experiments and one field-based polytunnel study were conducted. This chapter describes all three experiments in detail, while photos illustrating experimental setup are included in the Appendix section.

2.1: Control Cabinet Experiment – Experiment 1

2.1.1: Experimental design and growing conditions

A controlled-environment experiment was conducted in $1.37 \times 1.47\ m^2$ Saxil growth cabinets during the 2014–2015 winter growing season at the Plant Environment Laboratory, University of Reading, UK (51° 27`N latitude, 00° 56` W longitude). 42 plastic pots (180 mm diameter; 4 l volume) were prepared contained 2.8 kg of growing media comprising 4:4:2:1 of 6mm gravel: medium vermiculite: sharp sand: a peat based compost mixed with 2 kg/m^3 of Osmocote slow release granules containing a ratio of 15:11:13:2 of $N:P_2O_5:K_2O$:MgO (Barber *et al.*, 2017). Four `Paragon` seeds per pot were sown into half of the pots, while four `Blasco` seeds per pot were sown to the other half of the pots. The pots were kept in a single polytunnel section until the first green leaf appeared at the end of coleoptiles (Zadok`s Growth Stage 11, (Zadoks *et al.*, 1974)).

The pots were then transferred to a growth cabinet (control cabinet) set to provide 20°C day (18h)/16°C night (6h) diurnal cycle, with relative humidity (RH) of 77% set for the duration of the experiment. Position of the pots inside of the cabinet was randomised three times until plants reach to flowering stage (from Zadok`s growth stage 14 to Zadok`s growth stage 61, (Zadoks *et al.*, 1974)). Each pot inside of this cabinet was restricted to two plants at Growth Stage 13 and each plant was reduced to three tillers per plant at Growth Stage 29 (Zadoks *et al.*, 1974) to simplify plant structure. Pots

were initially watered by hand, after restricting the number of plants in the pots an automatic drip-irrigation system was installed to water pots to full holding capacity once a day. Temperature and relative humidity inside of the growth cabinets was measured by thermocouples and humidity sensors, respectively, and recorded in every 30 min (Fig. 2.2).

2.1.2: Temperature and relative humidity treatment

Three additional growth cabinets were prepared prior to plants reaching the flowering stage (Zadok`s growth stage 61 (Zadoks *et al.*, 1974)). The first of the 'treatment' cabinets was set up to provide 32°C day (18h)/24°C night (6h) temperature regime with relative humidity (RH) of 45%, the second provided temperature 32°C day (18h)/24°C night (6h) with relative humidity (RH) of 86%, while the third cabinet provided temperature 20°C day (18h)/16°C night (6h) with relative humidity (RH) of 45%. The experiment thus represented a factorial setup with two temperature and two humidity levels. When plants reached flowering - Growth Stage 61, three randomly selected pots per cultivar were transferred to treatment cabinets and plants exposed to different temperature and humidity conditions for three consecutive days. After three days they transferred back to the control cabinet until harvest (Experiment 1A). At early grain filling stage (12 DAA (Days after anthesis, Growth Stage 71), a different set of three randomly selected pots per cultivar were transferred to the treatment cabinets and exposed to the same combination of temperature and humidity three days as in Experiment 1A. After the three days exposure, these pots were also transferred back to the control cabinet to stay there until they get matured (Experiment 1B) (Fig. 2.1).

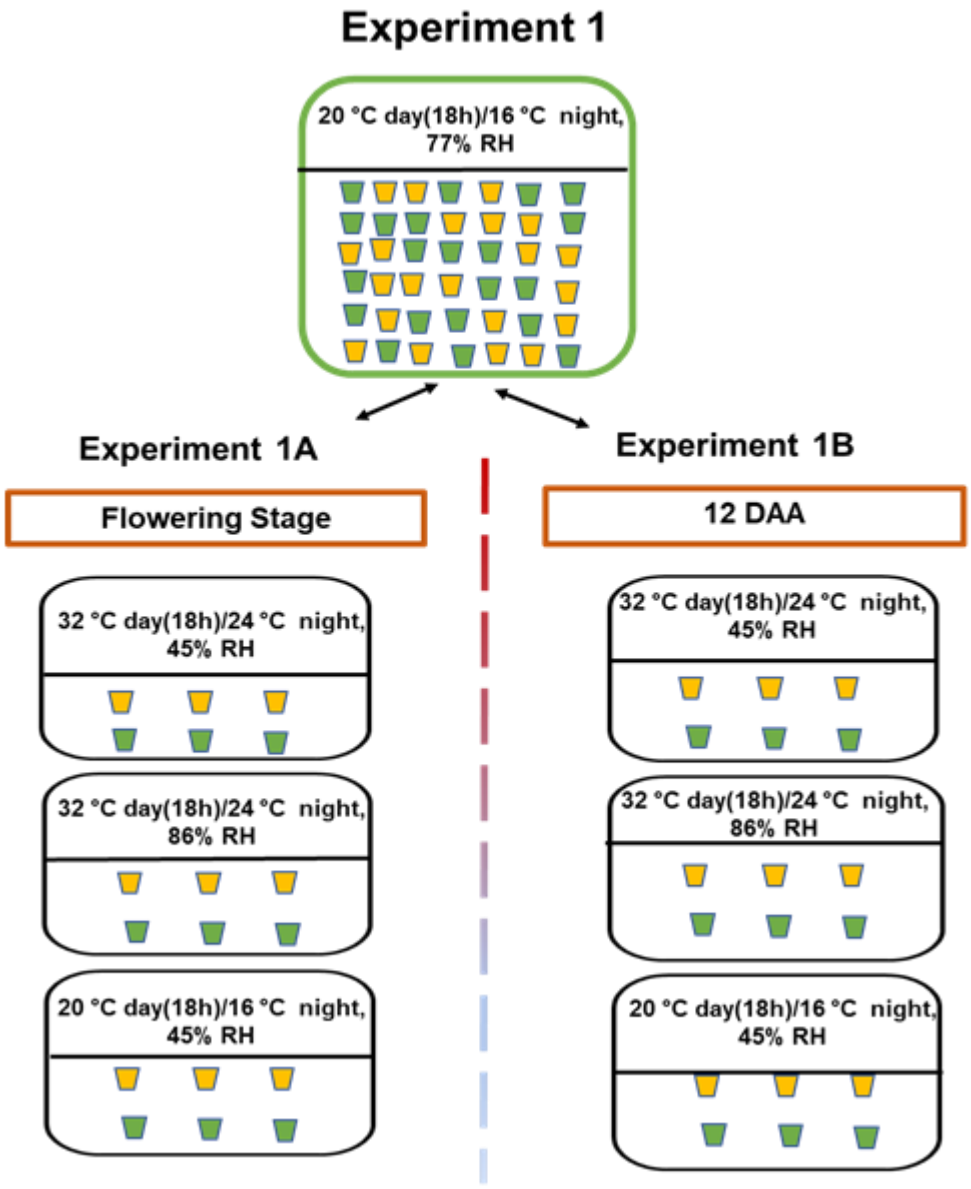


Figure 2.1: Design of Experiment 1. Green rectangle shows the growth cabinet (control), while black rectangles show treatment cabinets. In Experiment 1A, three different combination temperature and humidity treatments applied at flowering stage to the cultivars in the treatment cabinets, while in Experiment 1B, the same treatments applied 12 DAA. Temperature and RH values for each control cabinet were shown inside the rectangles. Yellow colour pots in the rectangles (control cabinets) represent `Blasco`, while green colour shows `Paragon`.

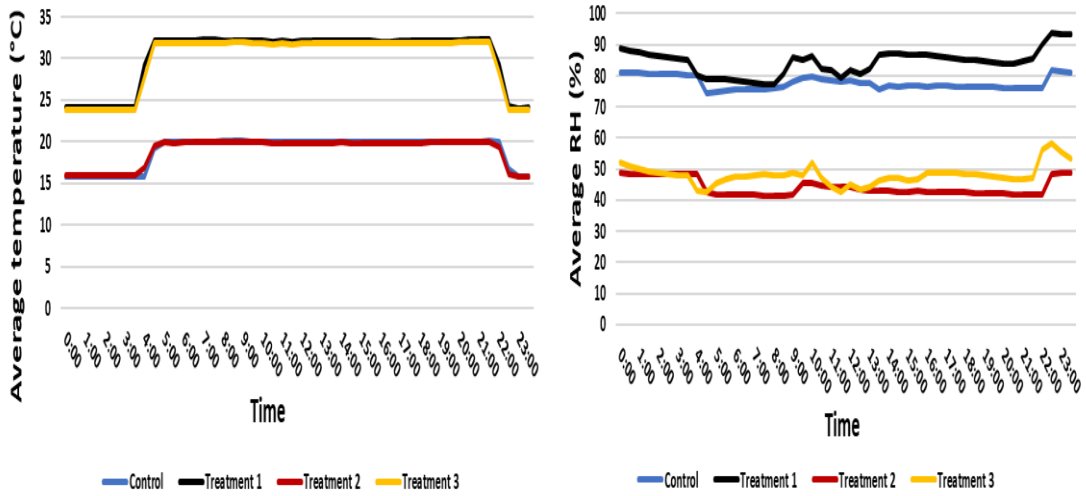


Figure 2.2: Average air temperature (a) and average relative humidity (b) within the growth cabinets for each treatment recorded during the three consecutive days at the flowering and early grain filling stages of Blasco and Paragon wheat varieties in Experiment 1.

2.2: Polytunnel Experiment - Experiment 2

2.2.1: Experimental design and growing conditions

A controlled-environment experiment was conducted in a polytunnel during the 2015 growing season at the Plant Environment Laboratory, University of Reading, UK (51° 27`N latitude, 00° 56`W longitude). 144 plastic pots (180 mm diameter; 4 l volume) were prepared to contain 2.8 kg of growing media comprising 4:4:2:1 of 6mm gravel: medium vermiculite: sharp sand: a peat based compost mixed with 2 kg/m^3 of Osmocote slow release granules containing a ratio of 15:11:13:2 of N:P_2O_5:K_2O:MgO (Barber *et al.*, 2017). Four `Paragon` seeds per pot were sown into half of the pots, while four `Blasco` seeds per pot were sown to the other half of the pots. The pots were kept in a single polytunnel section until the first green leaf appeared at the end of coleoptiles Growth Stage 11, (Zadoks *et al.*, 1974). Then, they were transferred to eight plastic chambers, which were constructed within the polytunnel where they remained until harvest. Each pot was restricted to two plants Growth Stage 13 and each plant was reduced to three tillers Growth Stage 29 to simplify plant structure. When it was required fungicide was applied.

2.2.2: Temperature and soil moisture treatment

The eight chambers were arranged in four blocks, one chamber in each block randomly allocated to ambient RH, while the other chamber was equipped with

dehumidifiers to achieve low RH. In order to equalize air circulation regime between the chambers caused by working of the dehumidifiers, small fans were inserted into ambient RH chambers. In addition to RH treatments, the experiment was carried out with three different soil moisture levels (high, medium, low) to investigate the effect of soil moisture on flag leaf and ear temperatures (Fig. 2.3). Soil moisture content in the pots was continuously measured every 2 hours by an array of Theta TDR (Time Domain Reflectrometry) probes (Delta-T), while temperature and relative humidity within the chambers were continuously measured every half an hour by iButtons (Maxim Integrated, California, USA). These data were then transferred to a computer at weekly intervals (Fig. 2.4).

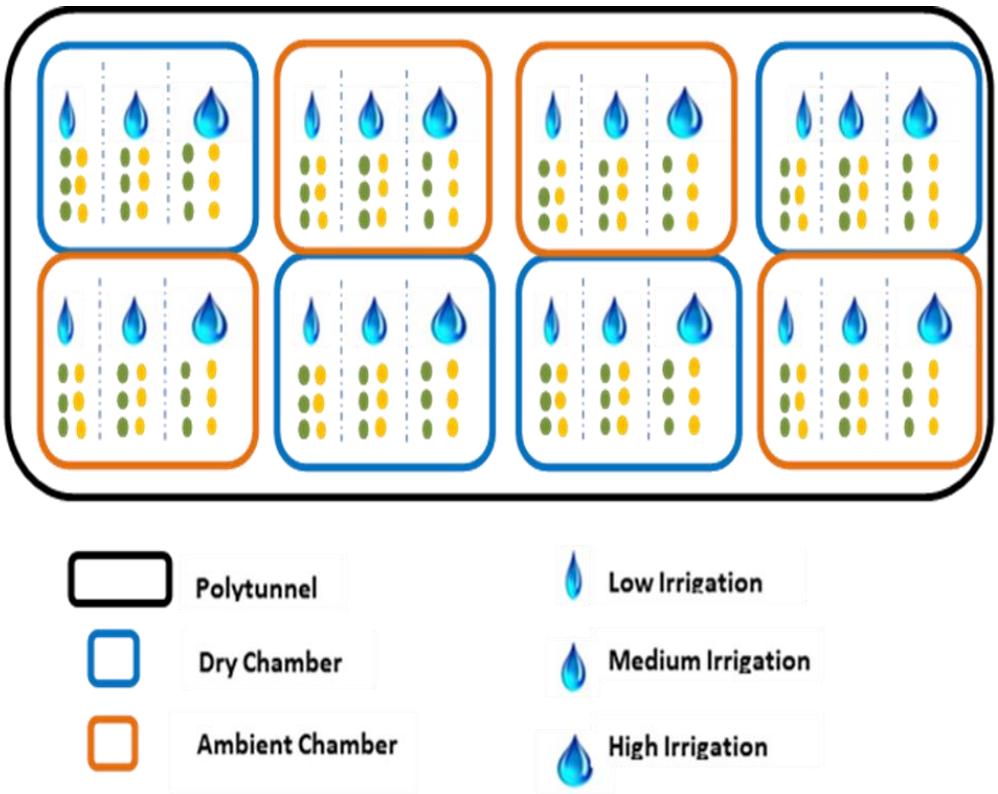


Figure 2.3: Design of the Polytunnel Experiment. Black rectangle shows the polytunnel in which the experiment was carried out. Blue and orange squares represent chambers allocated to low RH and ambient RH, respectively. While yellow filled circles in the chambers show `Blasco`, green filled circles show `Paragon`. Blue drops represent low, medium and high irrigations according to their dimensions.

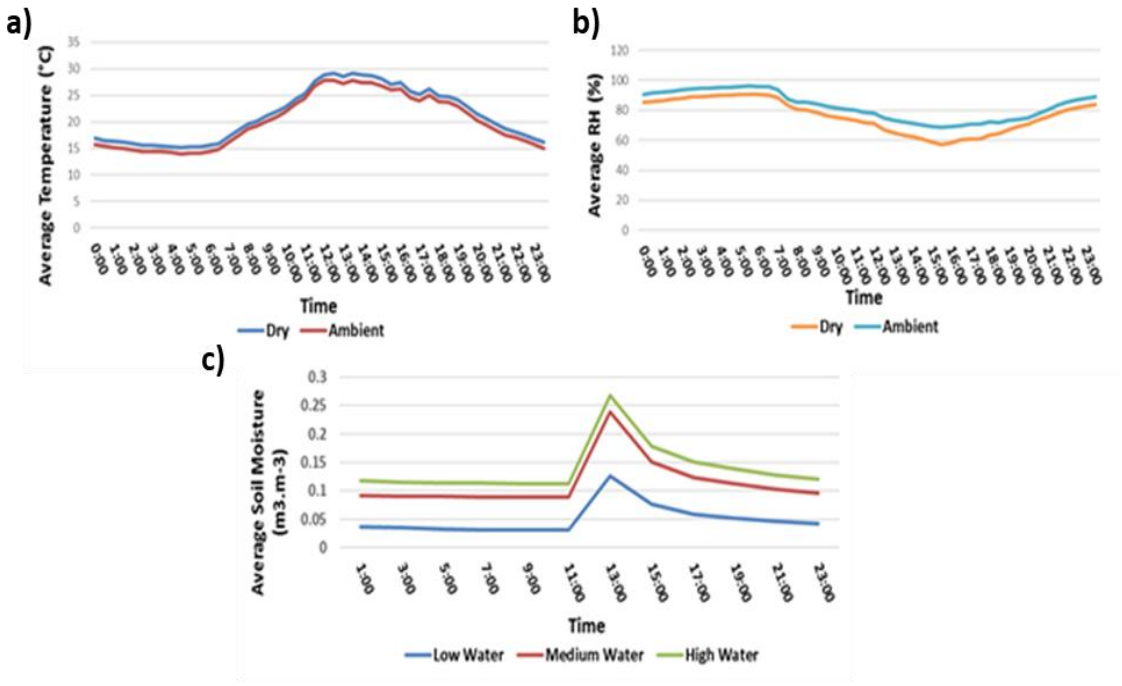


Figure 2.4: Average air temperature (a), average relative humidity (b) and average soil moisture (c) within the dry and ambient chambers over the course of the experiment 2.

2.3: Field Experiment – Experiment 3

2.3.1: Experimental design and growing conditions

A field-based experiment including eight different half-covered polytunnels, measuring 3 x 12m each, was conducted during 2016 growing season at Reading University Crop Research Unit, Sonning, UK (0°54`W longitude, 51°29`N latitude). Experimental design was composed of four polytunnels with ambient relative humidity and four polytunnels with high relative humidity. In order to provide high RH, misting units were attached to the ceilings of high RH polytunnels. The soil type in the polytunnels was a thin sandy loam over gravel. Half of each polytunnel was drilled with `Blasco`, while other half was drilled with `Paragon` (seed rate was 400s/m^2). At Zadock's stage 13, fertilizer consisting 100kg N and 501 g SO_3 applied per hectare by hand and watered in. At Zadock's growth stage 16-17 crops sprayed with herbicide and fungicide; Harmony M SX, Amistar Opti and Proline, while two days later they sprayed with T2 fungicide; Flexity (metrafenone) and Seguris (isopyrazam (SDHi)+ epoxiconazole). At Zadock's stage 26 crops sprayed with mildewcide; Flaxity (metrafenone).

2.3.2: Temperature and relative humidity treatment

Plants were watered for one hour on Mondays, Wednesdays and Fridays. When plants had reached to Zadock`s Growth Stage 22 (Zadoks *et al.*, 1974) misting systems were turned on. We gained advantage of direct sunshine during flowering and early grain filling stages to apply heat stress. Middle section of the polytunnels, covering half of `Blasco` and `Paragon` sub-plots, were closed using plastics, while remaining parts stayed open in which plants were not exposed to raised temperature (Appendix 2.3.1). The middle section of the polytunnels remained closed for three consecutive days during flowering stage, and three consecutive days 12 days after anthesis. Therefore, there were four sections in each tunnel (Fig. 2.5). iButtons (Maxim Integrated, California, USA) were used to record internal polytunnel temperature (IPT) and relative humidity every 10 min. TDR probes (Delta-T, Cambridge, UK) were used to record soil moisture level every 30 min (Fig. 2.6). Fifteen ears per section in each tunnel were tagged for data collection when plants reached to flowering stage (Zadok`s Growth Stage 61 (Zadoks *et al.*, 1974)). Ears that were damaged or positioned within about 20 cm of the plot margin were not selected to be tagged. In addition, care was taken to select the ears which flowered on the same day.

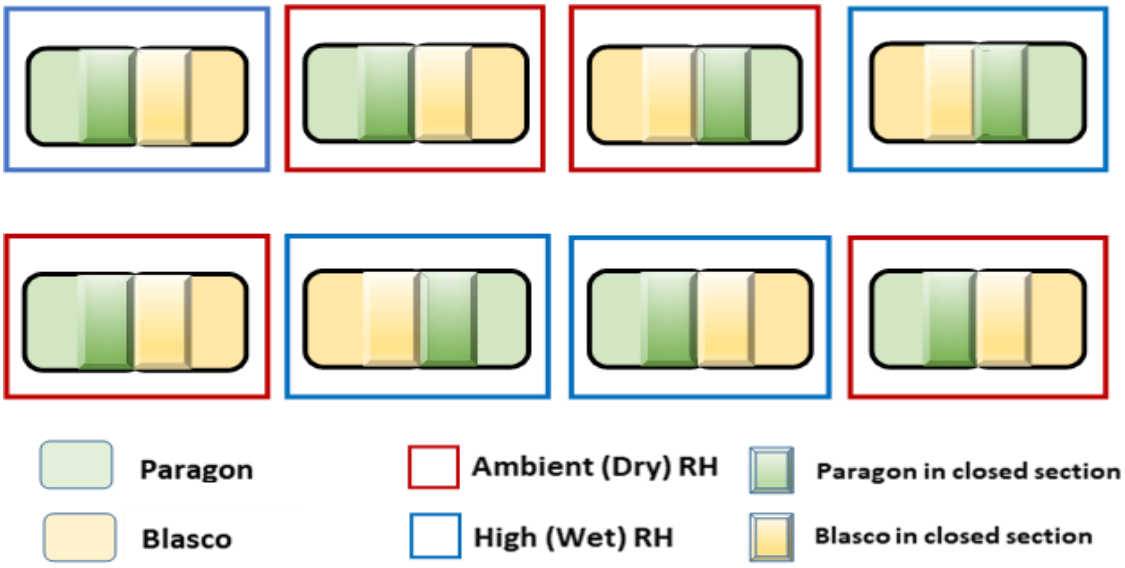


Figure 2.5: Design of the Field experiment. Red and blue rectangles represent the polytunnels allocated to ambient (dry) and high (wet) RH, respectively. While yellow sections in the tunnels show `Blasco`, green sections represent `Paragon`. Yellow and green rectangles in the middle of the tunnels, including `Blasco` and `Paragon`, respectively, shows the sections closed 3 days during flowering and early grain filling stage.

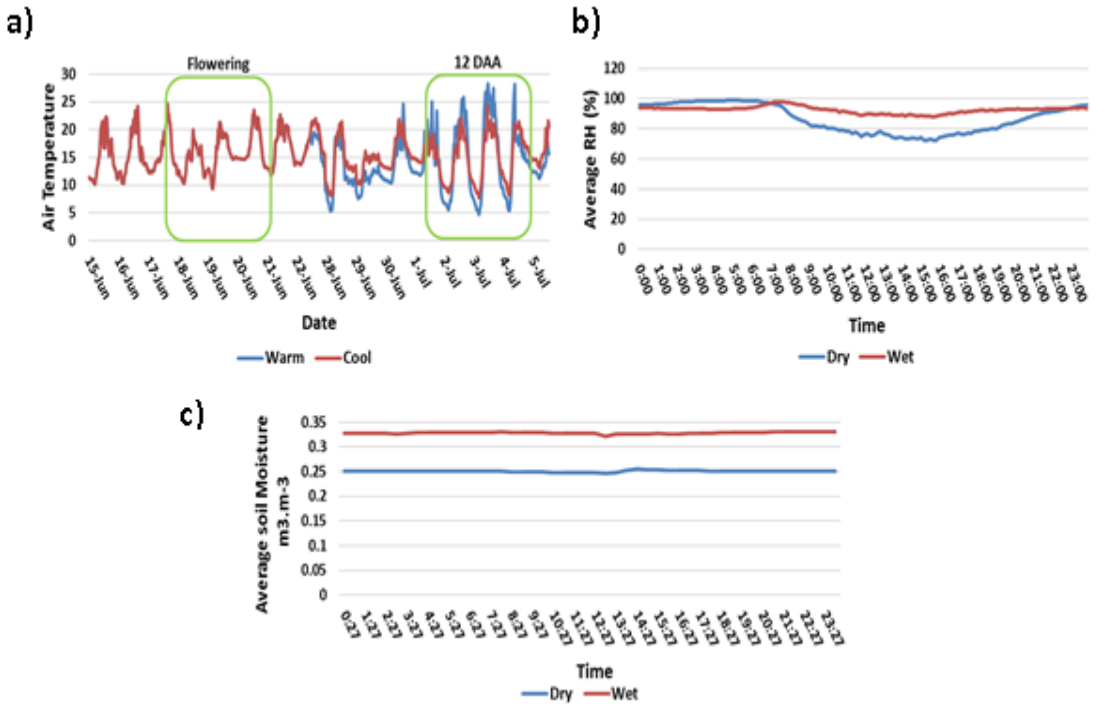


Figure 2.6: Air temperature at the flowering stage and during early grain filling stage (12 DAA), while the average air T during the treatments 12DAA in warm and cool sections of the tunnels were 17.2 °C and 14.1, respectively **(a)**, average relative humidity **(b)** and average soil moisture **(c)** in the dry and wet tunnels recorded during the life time of Blasco and Paragon wheat varieties in experiment 3.

2.4: References

Barber, H. M., Lukac, M., Simmonds, J., Semenov, M. A. & Gooding, M. J. (2017). Temporally and genetically discrete periods of wheat sensitivity to high temperature. *Frontiers in plant science,* **8**.

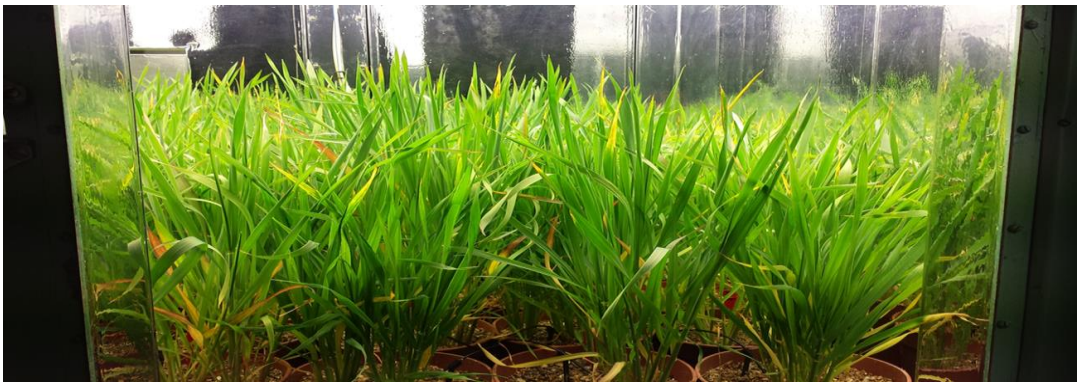
Zadoks, J. C., Chang, T. T. & Konzak, C. F. (1974). A decimal code for the growth stages of cereals. *Weed research,* **14** (6), 415-421.

2.5: Chapter 2 – Appendix

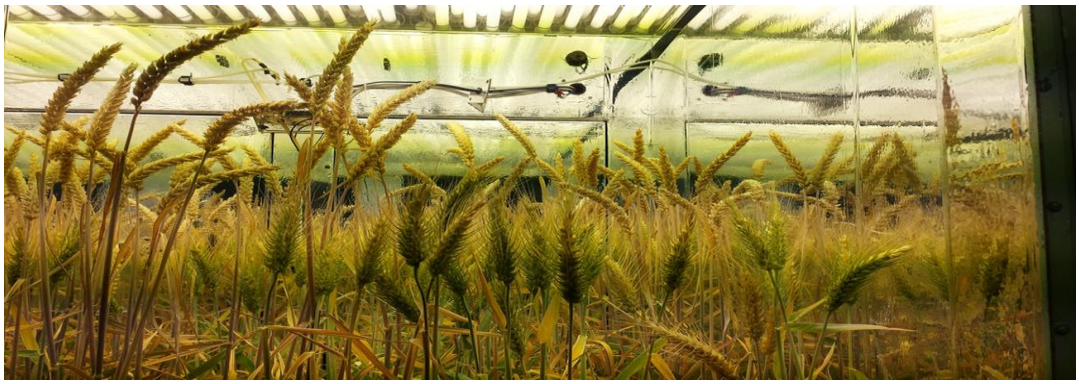
Appendix 2.1 - Pictures from Experiment 1

Appendix 2.1.1: Crops in the control cabinet

a)



b)

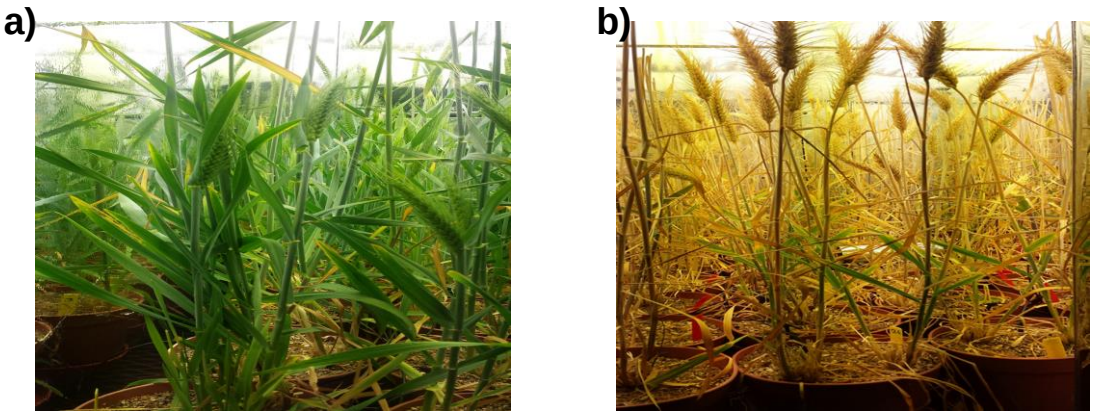


Appendix 2.1: Blasco and Paragon wheat cultivars in the control cabinet at Zadoks growth stage 23/24 (a) and at ripening growth stage (GS 75-99) (b) in Experiment 1.

Appendix 2.1.2: Automatic drip-irrigation

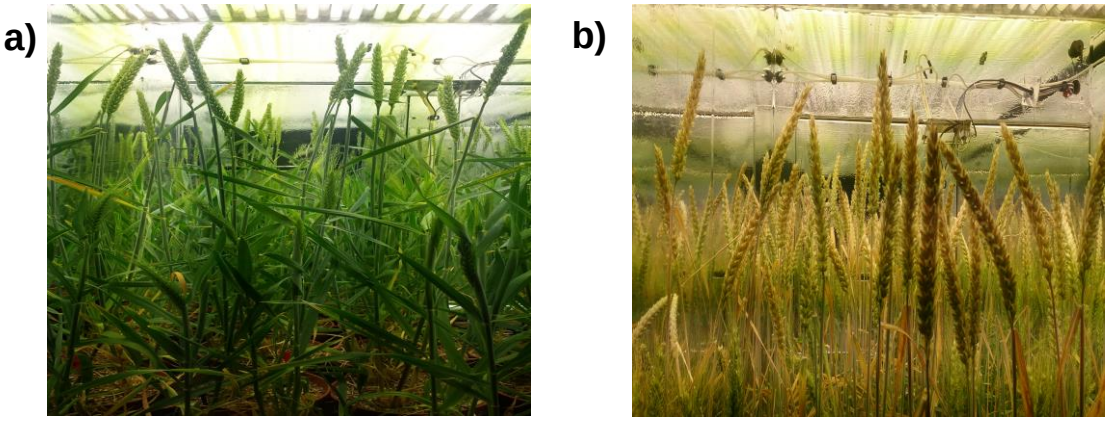
Appendix 2.2: An example for automatic drip-irrigation in Experiment 1.

Appendix 2.1.3: Blasco wheat cultivar



Appendix 2.3: Blasco wheat cultivar at Zadoks growth stage 59 (a) and at ripening growth stage (GS 75-99) (b).

Appendix 2.1.4: Paragon wheat cultivar



Appendix 2.4: Paragon wheat cultivar at Zadoks growth stage 59 (a) and at ripening growth stage (GS 75-99) (b).

Appendix 2.2: Pictures from Experiment 2

Appendix 2.2.1: Polytunnel and plastic chambers

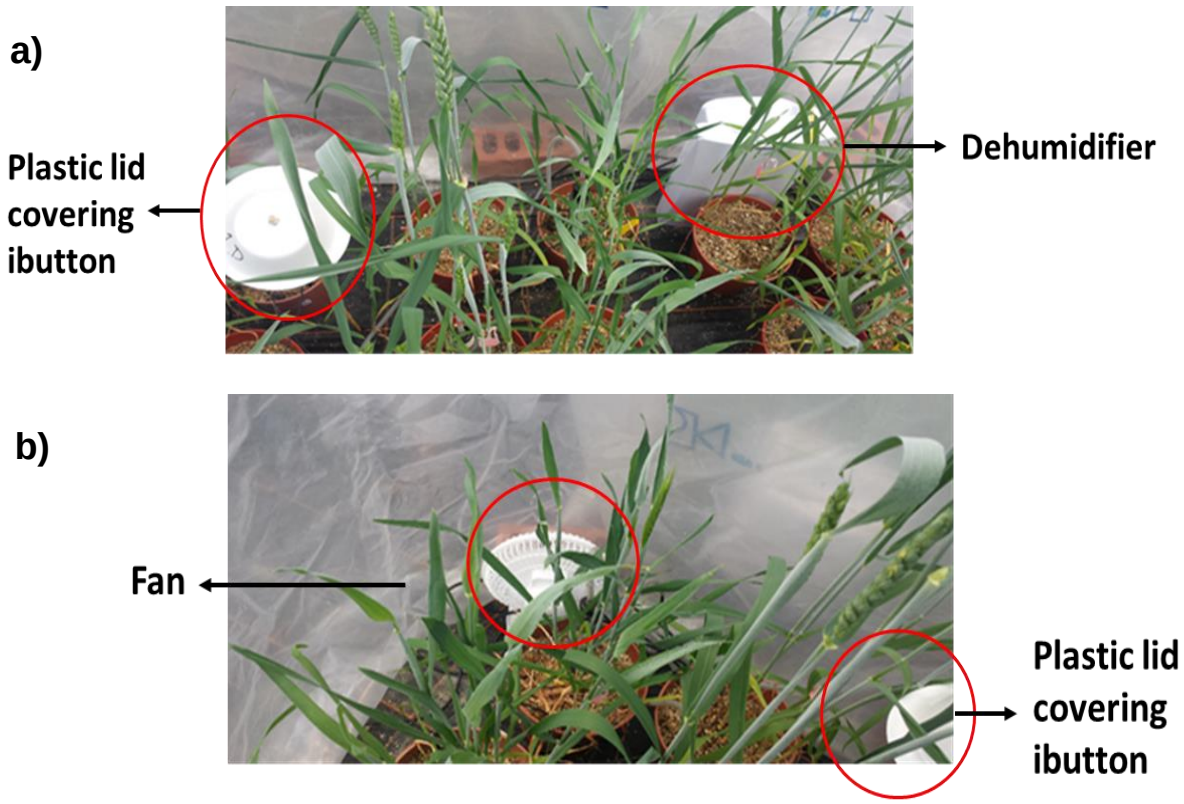
a)

b)

c)

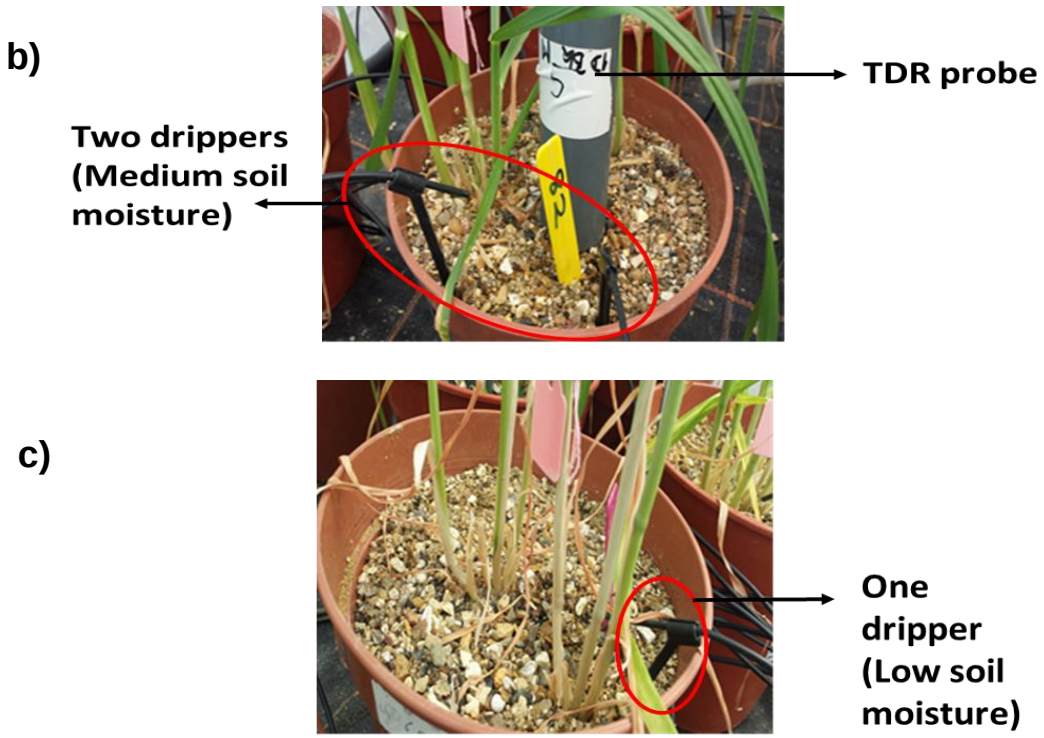
Appendix 2.5: Polytunnel in which Experiment 2 was conducted (a). Plastic pots having Blasco and Paragon wheat cultivars at growth stage 11, and plastics before (b) and after forming the chambers (c).

Appendix 2.2.2: Inside of the plastic chambers



Appendix 2.6: Inside of a chamber having a dehumidifier to lower the RH and a plastic lid covering an ibutton (a), and inside of a chamber having a fan for air circulation equalization and a plastic lid covering an ibutton (b).

Appendix 2.2.3: Automatic drip-irrigation for three different soil moisture levels

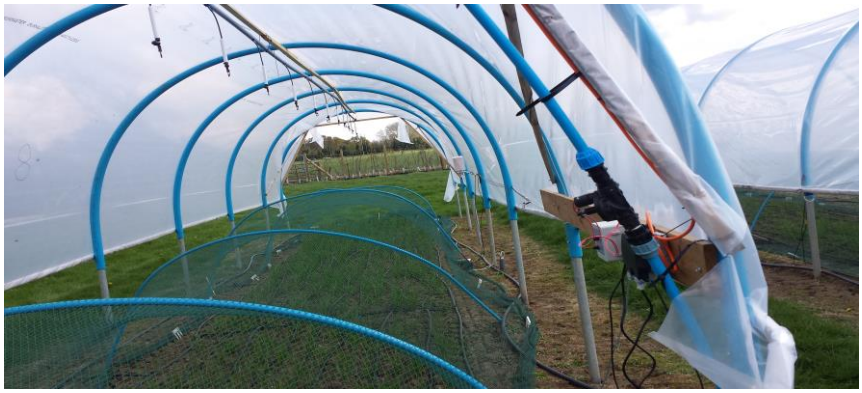


Appendix 2.7: Automatic drip-irrigation for three different soil moisture levels, low soil moisture (a), medium soil moisture (b) and high soil moisture (c) in Experiment 2.

Appendix 2.3: Pictures from Experiment 3

Appendix 2.3.1: Polytunnel and Misting Units

a)



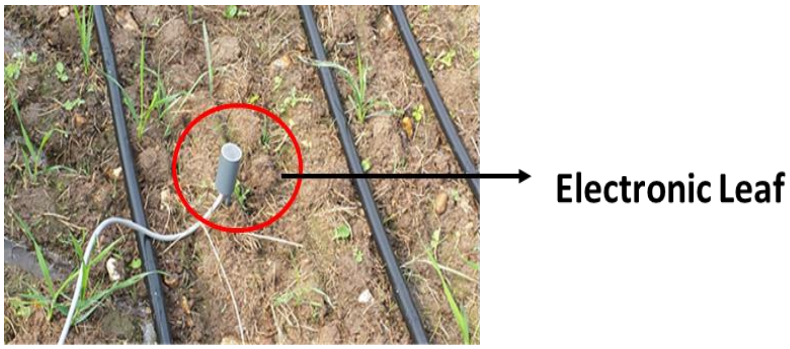
b)

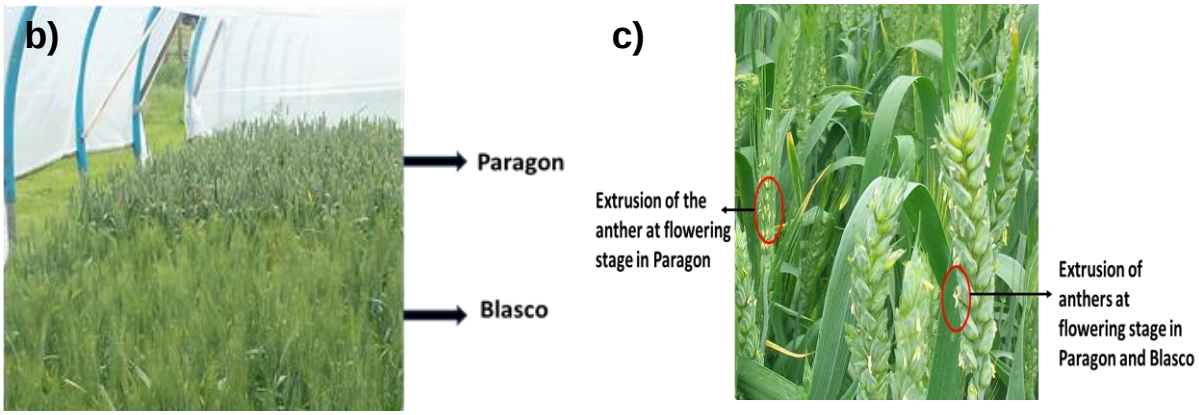
c)

Appendix 2.8: A polytunnel having crops covered with nests and misting units off (a) and on (b), and a tunnel with a heat stress section (c).

Appendix 2.3.2: Drip-Irrigation and crops in the tunnels

a)





Appendix 2.9: Drip irrigation system used in the tunnels and electronic leaf (moisture sensor) determining the frequency of misting (a), and Blasco and Paragon wheat cultivars in a tunnel (b). Extrusion of anthers at flowering stage of Blasco and Paragon (c).

Appendix 2.3.3: Ants on the data logger

Appendix 2.10: One of the data loggers covered with ants preventing recording of the atmospheric temperature.

Chapter 3: Interactive Effects of Manipulated Temperature, Relative Humidity and Soil Moisture on Tissue Temperatures and Grain Yield of Two Spring Wheat (*Triticum aestivum L.*) Cultivars

3.1: Introduction and Literature

Wheat (*Triticum aestivum*) is grown on 200 million farmland hectares globally and traded internationally, it is responsible for around 21% of the world's food (Ortiz et al., 2008). It is, being used as human and livestock food, one of the `big three` cereal crops with over 700 million tonnes global wheat production. Food and agriculture organization of the United Nations (FAO - http://www.fao.org/home/en/) reports that global total wheat production in 2017 was about 750 million tonnes (Shewry, 2009). Interestingly, its consumption increases in several countries where wheat yields are decreasing, especially in Eastern Europe. In the UK, winter and spring wheat is the dominant crop (AHBD, 2015). In some countries, such as Peru, Afghanistan and Iraq, overall wheat production is too low to satisfy local demand, necessitating imports (Ray *et al.*, 2013). Therefore, increases in wheat production are necessary to improve food security, especially for poor households. Farooq *et al.* (2014) suggested that wheat production rise relies on higher yield instead of an elevation in cropping area. One estimate has it that a doubling global crop production is required by 2050, in order to meet the expected demands due to increasing human population. Even though production of wheat rise 0.9% every year, it is less than the annual 2.4% increase rate needed to double global wheat production by 2050 (Ray *et al.*, 2013). Because the direct influence of changes in precipitation, temperature and carbon dioxide (CO_2) concentration, and indirect impact through changes in the rate of infestation by diseases and pests and soil moisture, the vulnerability of agricultural productivity to climate change is high (Abeysingha *et al.*, 2016). Therefore, to develop feasible adaptation strategies for wheat production, understanding the effects of climate change on it is tremendously important.

3.1.1: Heat stress and wheat yield

Heat and drought abiotic stress are the two main stress factors limiting crop yields (Araus *et al.*, 2002). In some of the important wheat growing areas around the world, crops are commonly exposed to heat stress during their life cycle, and generally these peak temperature levels coincide with susceptible growth stages (e.g. anthesis), which can dramatically influence grain yield (Wheeler *et al.*, 1996; Porter and Semenov, 2005; Farooq *et al.*, 2011; Semenov and Shewry, 2011). Therefore, the impact of heat

stress on grain yield is becoming a growing concern (Porter and Gawith, 1999; Battisti and Naylor, 2009; Semenov and Shewry, 2011). Even though elevated temperature is advantageous for the production of crops in several cooler areas of the world, its general effect on global food production is negative (Fahad *et al.*, 2017; Challinor *et al.*, 2014). It is expected that worldwide wheat production will decrease of 6% for each degree Celsius increase in temperature. Unfavourable influences of elevated temperature on cereal crops change with duration, timing and severity of the heat stress (Asseng *et al.*, 2015; Fahad *et al.*, 2017).

Heat stress influence grain development of wheat, since changes in ambient temperature directly affect assimilate translocation, grain filling rate and grain filling duration. Grain number of wheat per spike is affected dramatically by heat stress. Spike development is accelerated by heat stress which results in lower spikelet number and therefore lower grain number per spike. Elevated temperature during the developmental stage determines grain weight and number (Farooq *et al.*, 2011; FERRIS *et al.*, 1998; Wheeler *et al.*, 1996). Since spikelets appear the spikes at the double ridge stage, the period between the double ridge appear on the shoot apex and flag leaf is the most sensitive to heat stress. Thus, reduction in any shortening of the period between emerge and anthesis results in decreased spikelet and grain number (Rawson and Bagga, 1979; Farooq *et al.*, 2011; Saini and Aspinall, 1982). Temperatures higher than 20°C between Spike initiation and anthesis lessen grain number per spike, since spikelet initiation, differentiation of floral organ, sporogenesis, pollination and fertilization during this phenostage are affected negatively from temperature increase (Farooq *et al.*, 2011; Saini and Aspinall, 1982). Heat stress during floral initiation also causes significant impacts on grain number, and temperatures higher than 30°C around floret development may result in complete sterility (Saini and Aspinall, 1982; Fischer, 1985). Carbohydrate availability is an important factor affecting grain number, since insufficient assimilate availability may induce complete floret sterility (Farooq *et al.*, 2011; Kirby, 1988). If heat stress occurs when pollen mother cells divide, grain set and thus grain yield can be significantly reduced (Farooq *et al.*, 2011; Saini and Aspinall, 1982). Heat stress damages pollen and anther viability causing poor fertilization. The susceptibility of pollen to heat stress is related to insufficiency of pollen to synthesize heat shock proteins (HSPs) (Mascarenhas and Crone, 1996). Pre-anthesis heat stress especially during growth of ovaries and meiosis also cause decreases in grain number (Savin *et al.*, 1999; Farooq *et al.*, 2011).

The length of the period between anthesis and grain maturity decreases under elevated temperature and often results in a decrease in grain weight (Warrington *et al.*, 1977; Farooq *et al.*, 2011). The aleurone layer has large cells and surrounds a starchy endosperm in a wheat grain under ambient temperatures. However, higher temperatures may lead grain shrinkage because of ultrastructural changes in the endosperm cells and aleurone layer (Dias *et al.*, 2008; Farooq *et al.*, 2011). The major cause of decline in grain weight is decrease in starch deposition, while starch is in charge of about 70% of wheat dry grain weight (Bhullar and Jenner, 1985; Farooq *et al.*, 2011). Temperature above 18-22°C results in starch deposition reduction since enzymes in developing kernels, corresponding to starch biosynthesis, are affected negatively (Spiertz *et al.*, 2006). Grain filling rate accelerates because of heat stress which shortened the duration of grain-filling (Dias and Lidon, 2009). (Streck, 2005) indicated that every 1°C increase in optimal growing temperatures causes 2.8 days reduction in the grain-filling duration. Even though it would be expected that shorter period of grain filling could be compensated by elevated grain filling rate, this does not happen under heat stress conditions at temperatures over 30°C. Heat stress causes decrease in both rate and duration of grain growth (Sofield *et al.*, 1977; Stone *et al.*, 1995; Viswanathan and Khanna-Chopra, 2001). Studies have indicated that wheat grain size is positively correlated with thousand grain weight (TGW) which in turn with flour yield and agronomic yield (Addis, 2000; Abdipour *et al.*, 2016). Martinez-Carrasco (1979) has suggested that grain yield is determined by photosynthetic efficiency, grain size and carbohydrate transfer from other organs to the grain. Heat and droughts stress during grain filling stage reduce the grain size since grain filling period and grain filling rate decreases. Even though, grain filling rate might slightly increase under heat stress, it does not compensate the shortened filling duration which still end smaller grain size and yield (Farooq *et al.*, 2011; Prasad *et al.*, 2008).

Reynolds *et al.* (2000), recommended that breeding for heat-tolerance might help to address the problem. Some of the studies suggested selecting for elevated spike photosynthesis can dramatically help to maximize grain filling under stress conditions (Tambussi *et al.*, 2005; Tambussi *et al.*, 2007; Parry *et al.*, 2010; Sanchez-Bragado *et al.*, 2014).

3.1.2: Relative humidity

Relative humidity (RH) is defined as the amount of water vapour, expressed in percentage, in the air compared to what the air can hold if it is saturated at a given temperature (Xie *et al.*, 2011). In northern Europe, darker and moister climate

especially in winter is simulated as precipitation and RH are elevating and solar radiation is decreasing (Niglas *et al.*, 2015; Ruosteenoja and Räisänen, 2013). In southern Europe, more arid climate is expected primarily in summer as insolation increases and RH and precipitation decline, while raising aridity for summer and moister climate for winter are simulated for central Europe (Ruosteenoja and Räisänen, 2013). In order to cope with the changes in atmospheric temperature, RH plays an important role as it helps plants to modify their water loss via transpiration. However, water holding capacity of air can be varied under different air temperatures, as the amount of water that warmer air holds is greater than what cooler air can hold. Therefore, in order to explain the propulsive power of water loss from leaves vapor pressure deficit (VPD) values is also used as VPD is temperature independent (Rundel *et al.*, 1989). VPD is vapor pressure difference between leaf and air, and there is a negative correlation between atmospheric relative humidity and vapor pressure deficit (Pantin and Blatt, 2018; Yong *et al.*, 1997).

3.1.3: Transpiration

Transpiration is usually occurring at leaf surface and has a cooling influence on plants. It provides the necessary force to absorb nutrients and water from soil solution and transport them through the plant body. Transpiration is responsible the loss of above 95% of the water in the plant (Maghsoudi and Maghsoudi, 2008). Water first evaporates from the mesophyll cell walls, and then moves across the gaps inside the mesophyll. Following this, it moves through the stomata and travels from the outer surface of the leaf to the air (Forbes and Watson, 1992; Ramulu, 1998; Roberts *et al.*, 2000; Roberts, 1986). Transpiration is affected by environmental factors and functioning and structure of the plant. Because of solar radiation leaf temperature increases, and this elevates the energy of movement of water molecules in the mesophyll which in turn raises the proportion of evaporation from the cell surfaces. Water potential of the atmosphere is another important factor influencing transpiration. The capacity of the air to hold more water vapour determines the water potential of the air, which is in turn impacted both from temperature and relative humidity of the air. The water vapour holding capacity of air increases with decreasing relative humidity (RH), while air at 100% RH cannot hold any more water vapour since it is saturated. When air temperature elevates, the absolute water vapour holding capacity of the air increases (Forbes and Watson, 1992; Ramulu, 1998; Roberts *et al.*, 2000; Roberts, 1986).

However, it should be considered that these effects of water potential of the atmosphere and solar radiation on transpiration are dramatically altered by the response and structure of the plant. For instance, transpiration elevates when solar radiation increases but not just because leaf temperature rise. Stomata also respond to light and temperature. Stomata usually open in the light especially in well-watered plants while they close in the dark. Increase in solar radiation induce transpiration to increase since it warms the atmosphere which increase the water holding capacity of the air. At temperature up to around 30°C, stomata pores sizes increase. Stomata in most species response to changes in relative humidity, as stomata inclined to open when RH is high and close when it is low. This RH effects on the way of water potential and stomata working in opposite directions, helps to balance the transpiration rate. However, the effect of solar radiation on transpiration, encouraging stomata to open by decreasing atmospheric RH and increasing leaf temperature, is significantly altered if soil moisture depletion and water stress within the plant happen which in turn stomata close (Forbes and Watson, 1992; Ramulu, 1998; Roberts *et al.*, 2000; Roberts, 1986). Boundary layers, form due to friction between air molecules and the surface, on leaf surfaces cause decrease in water transpiration from the leaf since transpiring water inclined to accumulate within the layers. Therefore, air flows across the leaf as turbulent eddy flux which thins out the boundary layers and raises the transpiration rate. However, when the leaf temperatures are above ambient temperature in bright sunlight, wind has a cooling effect on leaves which declines transpiration (Forbes and Watson, 1992; Ramulu, 1998; Roberts *et al.*, 2000; Roberts, 1986). When water evaporates from plants, it absorbs the latent heat from the surface and keeps leaves cool. Minerals and organic products of root metabolism is delivered to the leaves via water flow up the xylem via transpiration. Therefore, it appears that transpiration is very crucial in many species (Forbes and Watson, 1992; Ramulu, 1998; Roberts *et al.*, 2000; Roberts, 1986).

3.1.4: Evapotranspiration

Water loss from the soil in the form of water vapor to the atmosphere is called evaporation during which transportation of water happens through the Soil-Plant-Atmosphere system (SPAS). Evaporation process is influenced by environmental interactions, meteorological factors (e.g. humidity and temperature), tillage and soil characteristic (Lal and Shukla, 2004; Teh, 2006; Hillel, 2012). Transpiration is responsible the loss of above 95% of the water in the plant (Maghsoudi and Maghsoudi, 2008). The capacity of the air to hold more water vapour determines the water potential of the air, which is in turn impacted both from temperature and relative

humidity of the air.as explained in detail in section 3.1.3. Evapotranspiration (ET) represents the simultaneous water loss by plant transpiration and by soil evaporation (Teh, 2006).

3.1.5: Tissue temperature and temperature depression

Wheat floret temperature plays a significantly crucial role in controlling the impact of heat stress, and relative humidity determines crop so floret cooling response to heat stress via transpiration (Suzuki *et al.*, 2016; Steinmeyer *et al.*, 2013). Leaf-canopy temperature is an indicator of plant water status and help to evaluate plant responses to environmental stresses. It shows the interactions between the energy influx and energy dissipation occurring within the tissue (Yan *et al.*, 2010). Environmental factors via stomatal transpiration (solar radiation, air temperature, relative humidity and tissue water status) and internal (physiological) factors affect Leaf-canopy temperature of plants (Oerke *et al.*, 2006; Bahar *et al.*, 2008). Difference between air temperature and Leaf-canopy temperature determines Leaf-canopy temperature depression. It is important to measure tissue temperature and temperature depressions, since this helps to understand the capacity of the crops to avoid dehydration under stress conditions like drought (Ayeneh *et al.*, 2002; Hossain *et al.*, 2016). Canopy temperature depression is being used as a selection criterion in breeding programmes for cereal crops resistance to heat and drought stress (Reynolds *et al.*, 1997; Steinmeyer *et al.*, 2013; Romano *et al.*, 2011).

We have hypothesized that flag leaf/spike cooling capacity at flowering and early grain filling stage is positively correlated with grain yield under abiotic stress conditions. Therefore, grain yield and tissue temperature analyses have been done. Methods which were used during the analyses and results were represented below.

3.2: Methodology

3.2.1: Evapotranspiratory loss of water

Pots with wheat plats utilised for Experiment 1A and 1B were used for an assessment of evapotranspiratory loss of water from the plat-soil system. The pots were irrigated every morning at 10:00 with excessive amount of water until exceeding soil water holding capacity. As soon as the water stopped dripping from the pots, they were weighted and exposed to the allocated temperature and relative humidity treatments in the growth cabinets. Two hours later, the pots were weighted again and the difference between the first and second weigh measurement recorded as an indicator of the

evapotranspiration rate. Each weight measurement took about 1 minute (to move the pots and record the data).

3.2.2: Flag leaf, spike and canopy temperature

Hand-held/tripod mounted infrared camera (FLIR model T-335, FLIR Systems, Wilsonville, OR, USA) was used every day during the treatments between 11:00 and 11:30 h (BST) in Experiments 1A and 1B , while In Experiment 2, the camera was used twice in every week, generally between 11:00 and 12:30 h (BST) during the life cycle of the crops to record flag leaf and spike infrared images of `Paragon` and `Blasco` plants. In Experiment 3, the camera was used once a week, generally between 11:00 and 15:00 h (BST), to record infrared images of tagged flag leaves and of the canopy.

It took about 40 seconds for each sample to align the camera, adjust the spike, flag leaf and canopy and take an image. The images of the desired part of the plants were taken horizontally at a distance of approximately 30-35 cm. The temperatures inside of the growth cabinets, chambers and tunnels were recorded while the images of flag leaves, spikes and canopies were being taken. Following this, the images were analysed using FILIR infrared camera software to record tissue temperatures.

3.2.3: Flag leaf, Spike and canopy temperature depression

Following the measurement of flag leaf, spike, canopy and atmospheric temperatures, flag leaf, spike and canopy temperature depressions were analysed. Flag leaf temperature depression (FLTD) signifies the difference between the air temperature and flag leaf tissue temperature which is expressed as:

$$FLTD = T_a - T_f$$

Spike temperature depression (STD) signifies the difference between the air temperature and spike tissue temperature which is expressed as:

$$STD = T_a - T_S$$

Canopy temperature depression, signifies the difference between the air temperature and canopy tissue temperature which is expressed as:

$$CTD = T_a - T_c$$

Where T_a, T_f, T_s and $T_{c,}$ represent the air temperature, flag leaf temperature, spike temperature and canopy temperature, respectively (Ayeneh *et al.*, 2002; Balota *et al.*, 2007; Steinmeyer *et al.*, 2013).

3.2.4: Yield data collection

In Experiments 1A, 1B and 2, all wheat plants were hand harvested at maturity (Zadox`s Growth Stage 90 (Zadoks *et al.*, 1974), and spikes were threshed. Dry weight and grain number were established for each spike separately. Average grain size was then obtained by dividing grain mass by the grain number. Results were recorded to per spike, pot and whole plant level. In Experiment 3, spikes in two circle areas (0.1 m^2) and the 15 tagged spikes per section at maturity were hand harvested. While tagged spikes were threshed using a plastic cylinder, spikes in the circles were threshed using thresher (Wintersteiger, Hege 16). Dry grain weight, grain number and grain size were recorded to per tagged spike and circle area (0.1 m^2). Grain weights were measured by an analytical balance (Precisa, 165 BJ), while seed counter (Elmor C3 seed counter) was used to count the grains.

3.2.5: Statistical analysis

All statistical analyses were performed using GenStat software (version 17.1.0.13780, VSNI international Ltd). Relative humidity, temperature and variety were considered as categorical predictors in the control cabinet and field experiments, while relative humidity, soil moisture and variety were considered as categorical predictors for polytunnel experiment. Because of dehumidifiers in half of the chambers in the polytunnel experiment, temperature differences were observed between dry and ambient chambers. Tissue temperature data describing the dry chambers were corrected using temperature differences measured at 20 minute intervals to correspond to the time of infrared observation.

ANOVA was used to assess differences between treatments, a complete randomised block design was used for all experiments. Treatment effects were considered significant at $p<0.05$. When significant differences were observed, pair-wise multiple comparisons were performed using Tukey test ($p<0.05$) and T-test ($p<0.05$) to discriminate differences among the treatments and between the cultivars. Shapiro-Wilk normality test ($p<0.05$) and Bartlett`s test for homogeneity of variances ($p<0.05$) were carried out prior to each ANOVA. Data found not to satisfy these assumptions were transformed to attain homoscedasticity and normal distribution (McDonald, 2009; Rasmussen, 1985; Bland and Altman, 1996). Transformation was performed by mainly with an equation: x^3.

3.3: Results

Three Experiments were conducted over three successive growing seasons to investigate the effect of manipulated air temperature (T), relative humidity (RH) and soil moisture on grain yield and tissue temperature of two spring wheat cultivars, Blasco and Paragon. Experiments 1, 2 and 3 represent the control cabinet Experiment, polytunnel Experiment and field Experiment, respectively. While air T and RH treatments were applied to the cultivars in Experiment 1 and 3, soil moisture and RH treatments were applied in the Experiment 2. In Experiment 1A treatments applied at flowering stage, while in Experiment 1B, treatments applied 12 days after anthesis.

Significant effects on measured variables as a result of experimentally applied treatments have been observed in all three Experiments and results represented in the sections above. Summary tables showing the mean and standard deviation values, and Analysis of Variance of the grain yield and tissue temperature parameters are shown in Appendix section of this chapter.

3.3.1: Effects of air temperature and relative humidity on evapotranspiration

Higher air temperature at low RH condition (p=0.018) and low RH at high air temperature condition (p=0.001) triggered higher evapotranspiration rate in Blasco at flowering (Fig. 3.1 a). Interaction between RH and air temperature also affected evapotranspiration rate of Blasco, where it had higher evapotranspiration rate at low RH/high air T than at high RH/low air T (p=0.001). Regardless of what was the air temperature at flowering stage, evapotranspiration rate of Paragon was higher at low RH than those at high RH (p=0.002), and high air temperature triggered higher evapotranspiration rate at both high RH and low RH (p< 0.001), as shown in Fig. 3.1 b.

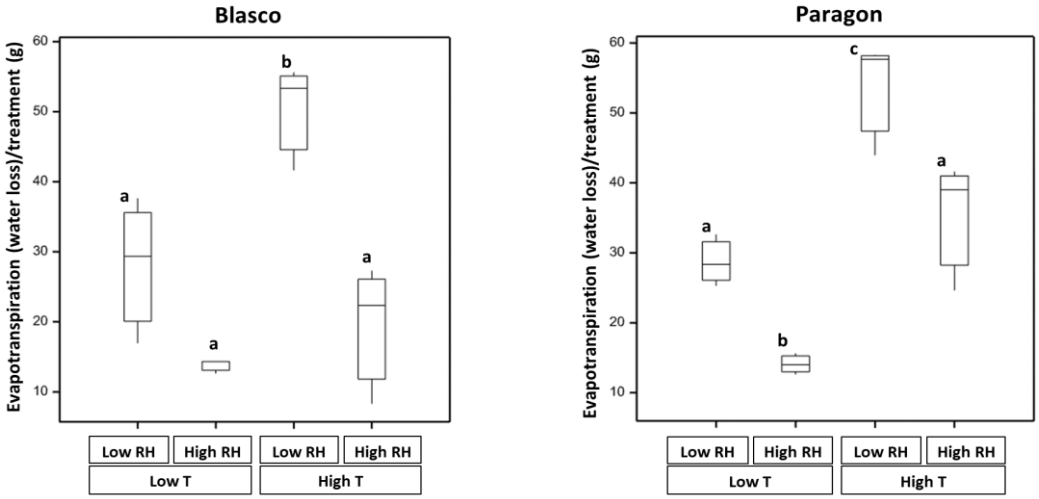


Figure 3.1: Evapotranspiration rate of Blasco (a) and Paragon (b) wheat varieties as a response to temperature and humidity manipulation at the flowering stage (Experiment 1A). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

At early grain filling stage, high air temperature in comparison to low air temperature has resulted in elevated evapotranspiration rate in Paragon at high RH conditions (p=0.004). We also observed an interactive RH and air temperature effect on the evapotranspiration rate in Paragon (p =0.01) where it had higher rate at low RH/high air T than at high RH/low air T (Fig. 3.2 b). Besides this, low RH at both high and low air T conditions caused higher evapotranspiration rate in Blasco (p<0.001). Even though there was not an air temperature at high RH, we have observed that Blasco had higher evapotranspiration rate at high air T than at low air T under low RH condition (p=0.003), as shown in Fig. 3.2 a.

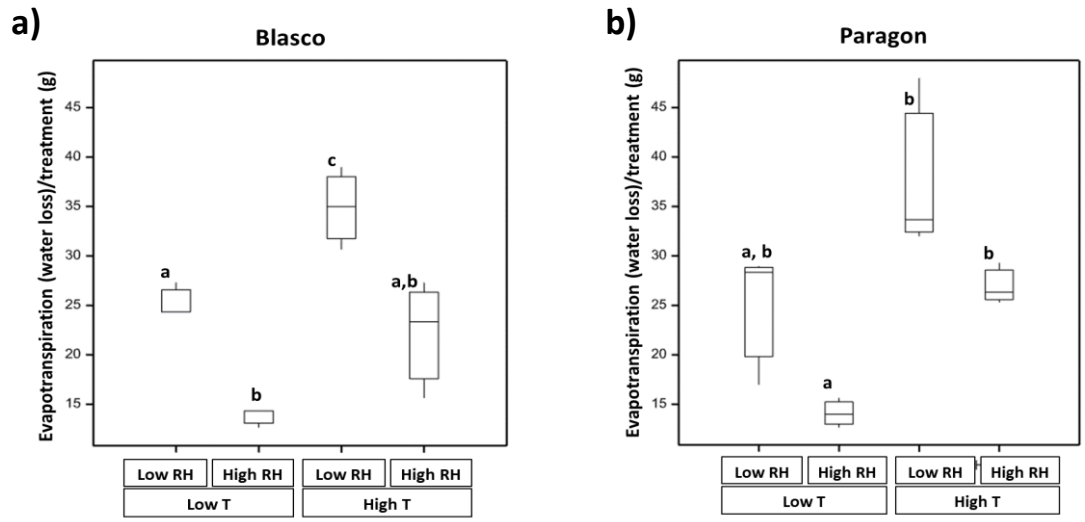


Figure 3.2: Effect of temperature and humidity manipulation, applied for three consecutive days during early grain filling stage, on evapotranspiration rate of Blasco (a) and Paragon (b) wheat varieties (Experiment 1B). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

3.3.2: Effects of air temperature, relative humidity and soil moisture on tissue temperature

3.3.2.1: Spike temperature

As expected, tissue temperature of wheat Spikes was primarily affected by ambient temperature in Experiment 1A and Experiment 1B. In both cultivars, the plants were not able to regulate spike temperature at the physiological optimum during early

flowering and higher spike temperature was observed in high T treatments ($p<0.001$, Fig. 3.3 a and 3.3 b). We did not see any modification of this effect by RH treatments at this plant development stage apart from low T treatment in Blasco where high RH significantly lowered the spike leaf temperature ($p=0.036$, Fig. 3.3 a).

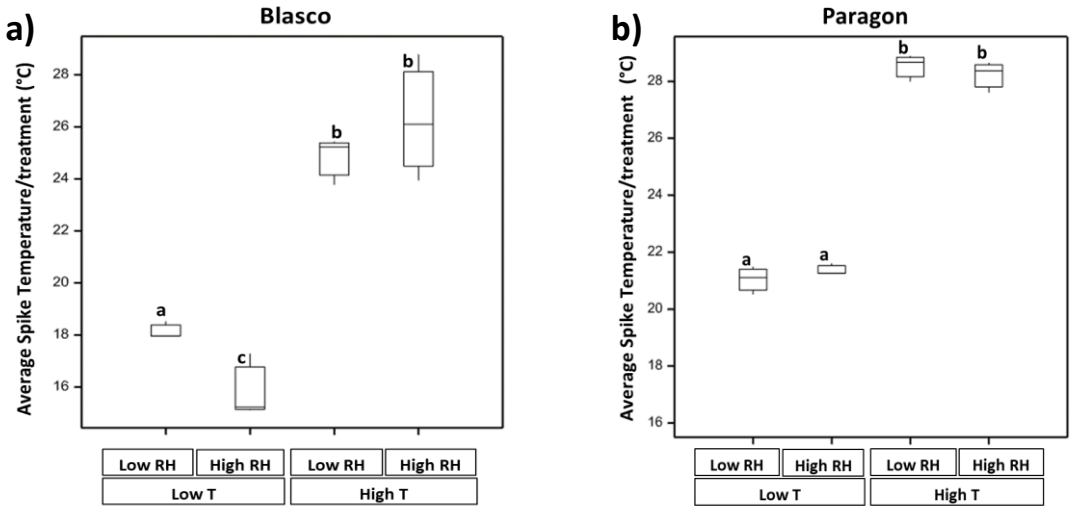


Figure 3.3: Spike temperature of Blasco (a) and Paragon (b) wheat varieties exposed to different temperature and humidity manipulation at the flowering stage (Experiment 1A). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

At early grain filling in Experiment 1, Blasco again had higher spike temperature at low RH than those at high RH ($p<0.001$, Fig. 3.4 a), however, a contrasting effect of RH was observed in Paragon where at low T, high RH increased its spike temperature ($p<0.001$, Fig. 3.4 b). At high T, high RH increased surface temperature of spikes both in Blasco and in Paragon at early grain filling ($p<0.001$, Fig. 3.4 a and 3.4 b).

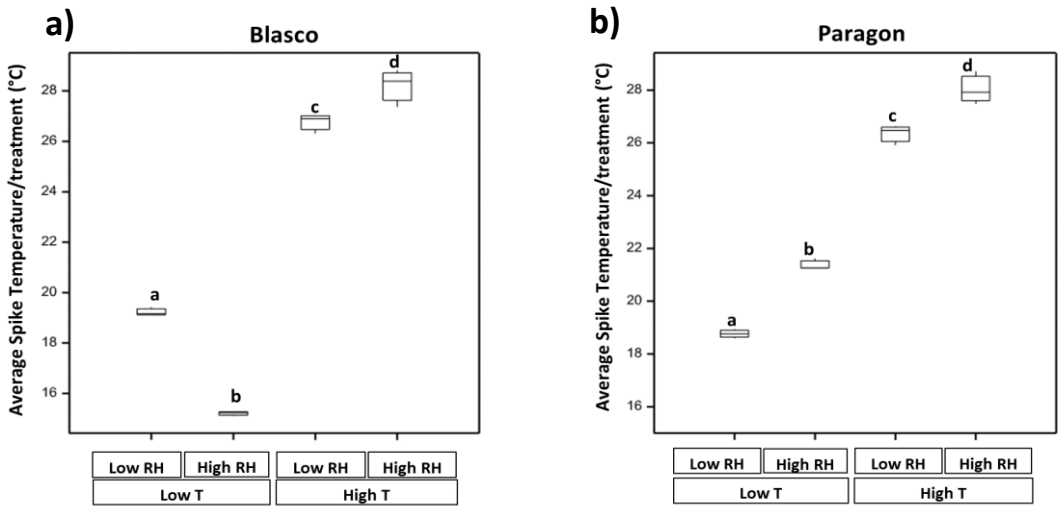


Figure 3.4: Effect of temperature and humidity manipulation, applied for three consecutive days during early grain filling stage, on spike temperature of Blasco (a) and Paragon (b) wheat varieties (Experiment 1B). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

There were no significant effects of RH, air T or soil moisture on average spike temperature in Experiments 2 and Experiment 3 (Appendix 3.2).

3.3.2.2: Flag leaf temperature

Similar to spike temperature, infrared imaging of flag leaves of the two wheat varieties revealed that the main driver of tissue temperature at flowering is the temperature of surrounding air in Experiment 1A. RH did not modify this effect, apart from low T treatment in Blasco where high RH significantly lowered the flag leaf temperature ($P<0.001$, Fig. 3.5 a and 3.5 b).

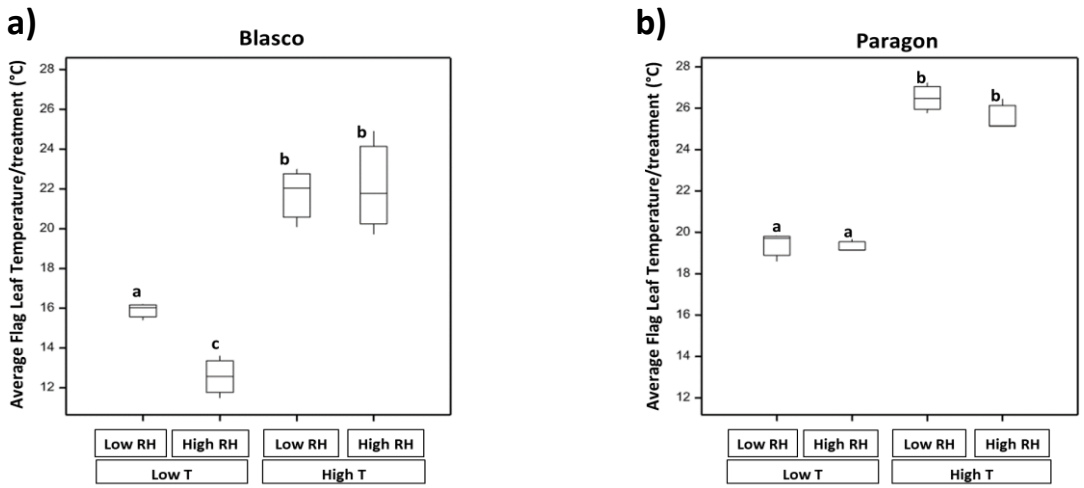


Figure 3.5: Flag leaf temperature of Blasco (a) and Paragon (b) wheat varieties exposed to different temperature and humidity manipulation at the flowering stage (Experiment 1A). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

Intriguingly, when the same modification of the environment was applied at early grain filling stage in Experiment 1, we saw a result identical to that observed on spikes. High air temperature still increased flag leaf temperature, but this effect was modified by RH and there was a contrasting effect between the two varieties. High RH decreased flag leaf temperature in Blasco but increased it in Paragon (Fig. 3.6 a and 3.6 b).

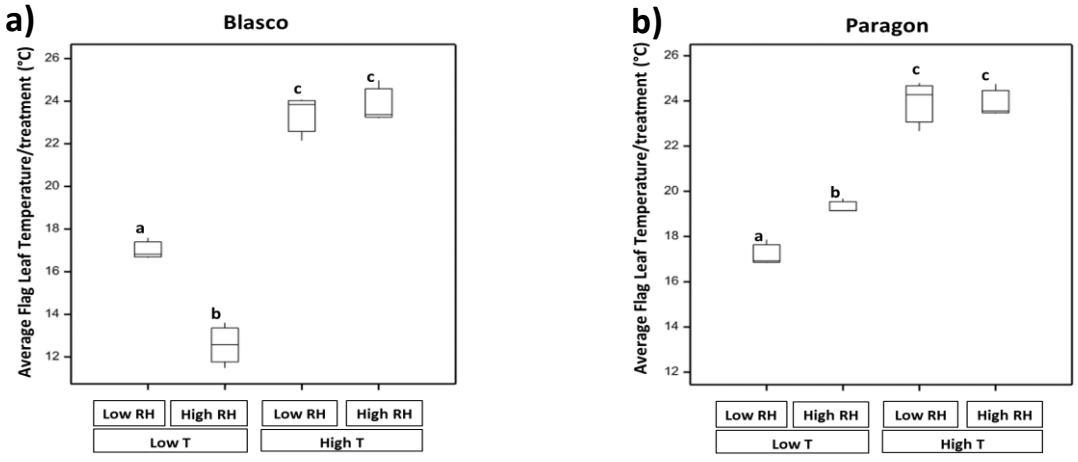


Figure 3.6: Flag leaf temperature of Blasco (a) and Paragon (b) wheat varieties exposed to different temperature and humidity manipulation during Early grain filling stage (Experiment 1B). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

There were no significant effects of RH, air T or soil moisture on flag leaf or canopy temperature in either of the wheat varieties in Experiment 2 and Experiment 3 (Appendix 3.2).

3.3.2.3: Spike temperature depression

Significantly higher spike temperature depression (STD) was found in both cultivars at high air T than those at low air T in Experiment 1A. This effect was present at both plant developmental stages at which environmental modification was applied ($p<0.001$). We saw an effect of humidity on STD at flowering stage in Blasco, as STD was higher at high RH than at low RH at lower T conditions and vice versa at higher T (Fig. 3.7 a). Interestingly, Paragon spikes under low T regardless of what was the RH were warmer than surrounding air, as evidenced by negative STD values ($p<0.001$, Fig 3.7 b).

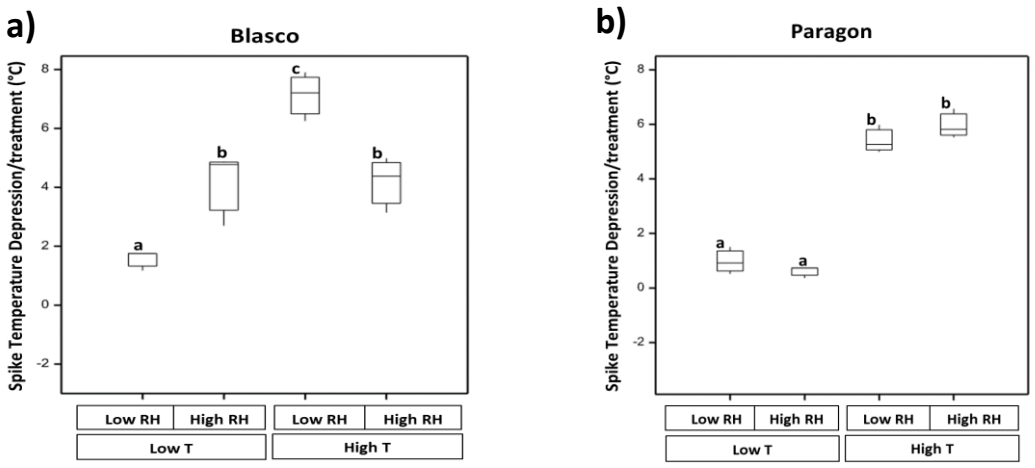


Figure 3.7: Effect of temperature and humidity manipulation, applied for three consecutive days at flowering stage, on spike temperature depression of Blasco (a) and Paragon (b) wheat varieties (Experiment 1A). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

At early grain filling stage in Experiment 1, STD was modified by RH at both low T and high T. Increasing RH in Blasco while keeping air temperature low and high results in higher and lower STD, respectively but in lower STD in Paragon under both higher and lower air temperature ($p<0.001$, Fig. 3.8 a and 3.8 b). We did not observe any air T, RH or soil moisture effect on STD in Experiments 2 and 3 (Appendix 3.3).

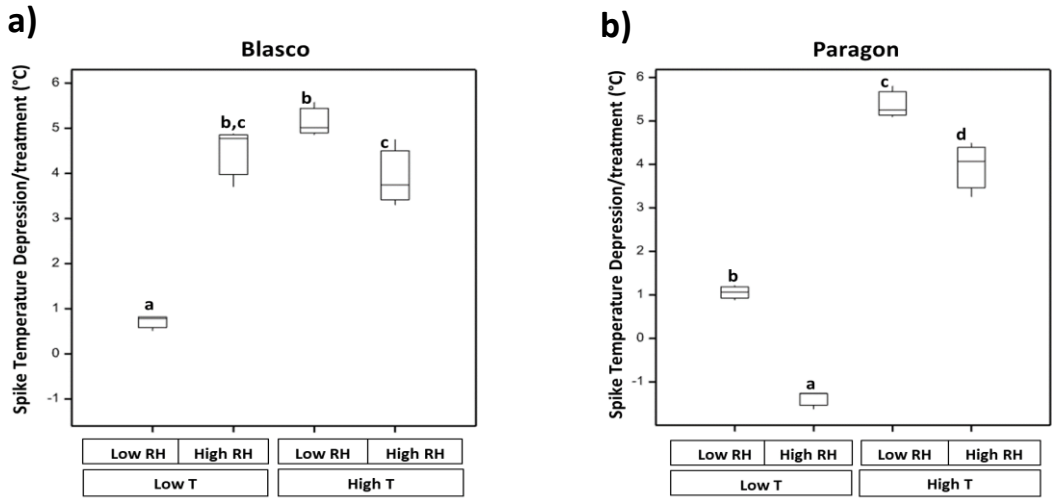


Figure 3.8: Effect of temperature and humidity manipulation, applied for three consecutive days during early grain filling stage, on spike temperature depression of Blasco (a) and Paragon (b) wheat varieties (Experiment 1B). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

A very strong effect of RH on STD was observed in Experiment 2. Both varieties had higher STD in low humidity compartments than in those where higher atmospheric humidity was maintained. This effect was not modified by soil water availability (p<0.001, Fig 3.9 a and 3.9 b).

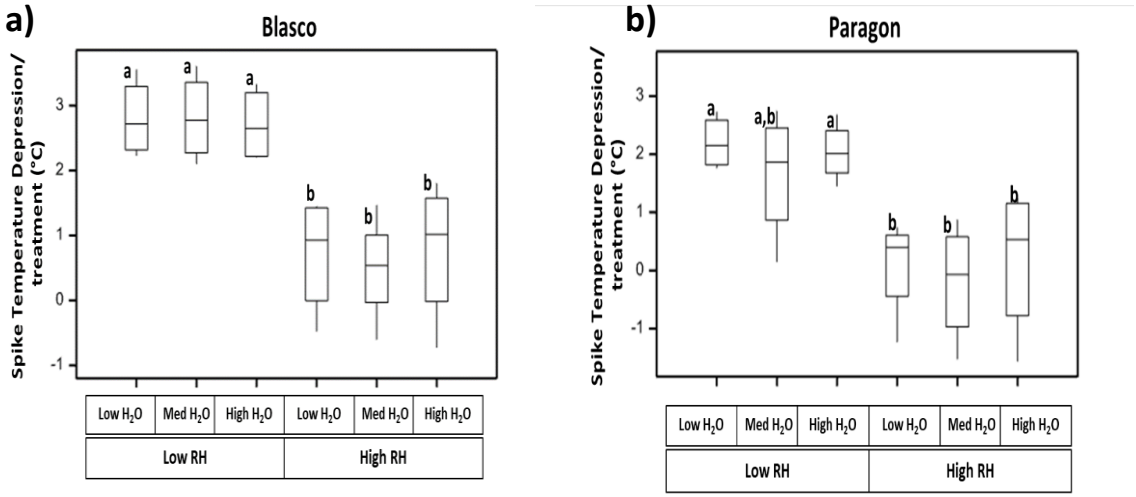


Figure 3.9: Spike temperature depression of Blasco (a) and Paragon (b) wheat varieties exposed to different soil moisture and humidity manipulation (Experiment 2). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

3.3.2.4: Flag leaf temperature depression

Flag leaf temperature depression is indicative of the capacity of wheat plants to maintain photosynthesising tissues at optimum temperature and thus to maintain carbohydrate supply to carbon sinks. In Experiment 1 where wheat plants were grown in fully waters pots, we saw more than doubling of FTD between the low and high T treatments at flowering (Fig. 3.10 a and 3.10 b). This effect was not modified by RH in Paragon, but there was a significant increase of FTD under low T and significant decrease under high T in Blasco as a result of increasing RH ($p<0.001$, Fig. 3.10 a).

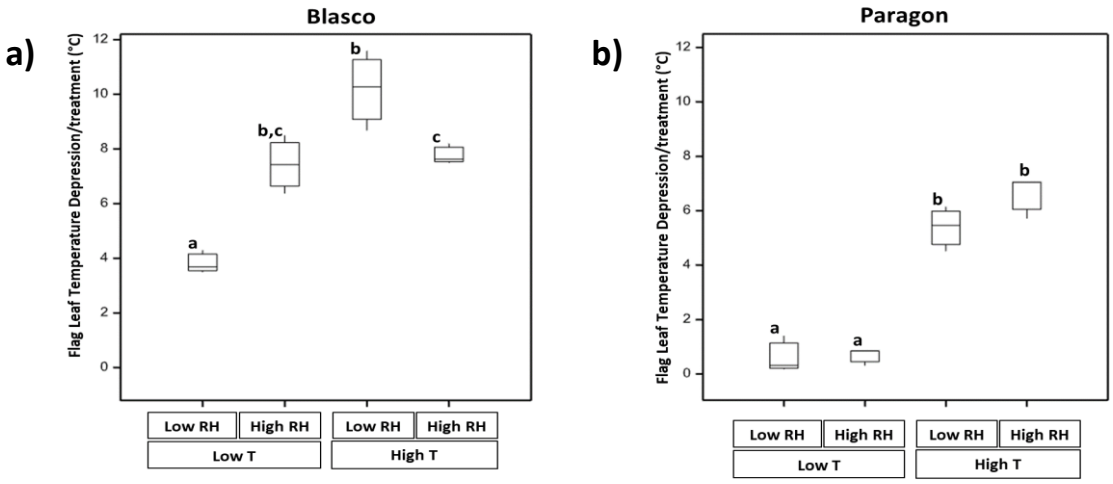


Figure 3.10: Effect of temperature and humidity manipulation, applied for three consecutive days at flowering stage, on flag leaf temperature depression of Blasco (a) and Paragon (b) wheat varieties (Experiment 1A). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

During early grain filling stage, we saw more than doubling of FTD between the low and high T treatments in Paragon, while relative humidity effect has also been observed under low T, as Paragon had higher FTD at low RH than those at high RH ($p<0.001$, Fig. 3.11 b). FTD of Blasco was modified by both air T and relative humidity while higher RH and air T resulted in higher STD in Blasco ($p<0.001$, Fig. 3.11 a).

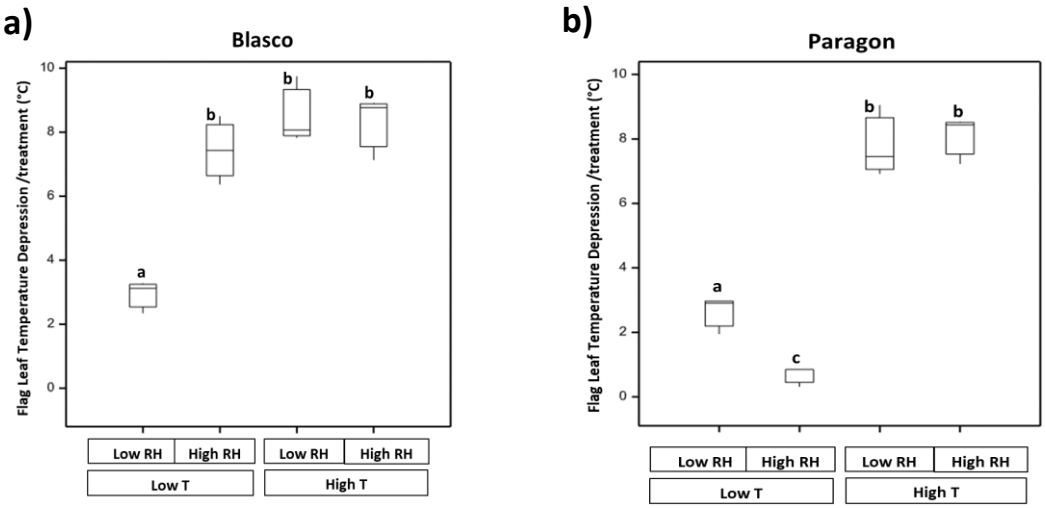


Figure 3.11: Impact of temperature and humidity manipulation, applied for three consecutive during early grain filling stage, on flag leaf temperature depression of Blasco (a) and Paragon (b) wheat varieties (Experiment 1B). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

We did not observe any effect of RH on spike and canopy temperature depression in Experiment 3 (Appendix 3.3).

When we compared tissue temperature and tissue temperature depression data of Blasco with the data of Paragon, we have observed that Blasco had statistically higher cooling capacity than Paragon under about all treatments, and thus it had lower tissue temperatures than the tissue temperatures of Paragon in Experiment 1A and Experiment 1B. We have also compared spike temperature and flag leaf temperature or canopy temperature in the Experiments. Spike temperature found to be significantly lower than flag leaf and canopy temperatures in both cultivars under about all treatments apart from the temperatures in Experiment 2 at low RH where there was not any significant difference between spike and flag leaf temperatures.

3.3.3: Effects of air temperature, relative humidity and soil moisture on yield

3.3.3.1: Dry grain weight

In Experiment 1A, we observed that RH and air T did not affect grain weight of the cultivars (Appendix 3.4). However, grain weight of the cultivars in Experiment 1B were significantly affected from RH, air T and interactions between RH and air T (Fig. 3.12 a and 3.12 b). At lower air T, grain weight in Blasco was significantly higher at low RH than at high RH ($p=0.005$). At low RH, variety Blasco had higher grain weight at lower air T than those at higher air T ($p=0.034$). It also had higher grain weight at low RH/low air T than those at high RH/high air T ($p=0.049$), as shown in Fig. 3.12 a. At higher air

T, Paragon had higher grain weight at low RH than those at high RH (p=0.002). In complete contrast to Blasco, it had higher grain weight at higher air T than those at lower air T under low RH conditions (p=0.012). However, similar to Blasco, it had higher grain weight at low RH/low air T than those at high RH/high air T (p=0.01), as shown in Fig. 3.12 b.

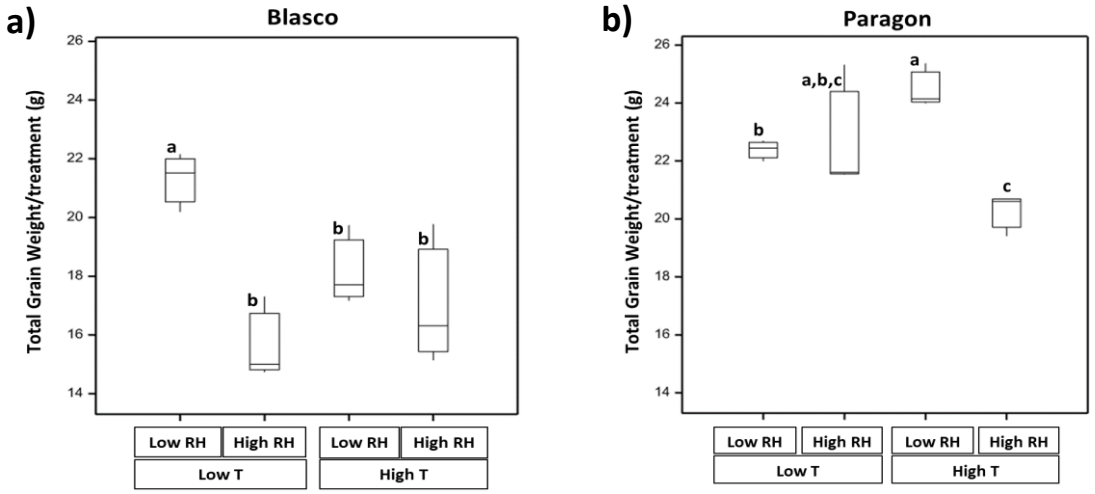


Figure 3.12: Effect of temperature and humidity manipulation, applied for three consecutive days during early grain filling stage, on grain weight of Blasco (a) and Paragon (b) wheat varieties (Experiment 1B). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

At low RH, soil moisture also had a significant influence on grain weight in Blasco in Experiment 2. It had higher grain weight at higher soil moisture than those at lower soil moisture (p<0.001), as shown in Fig. 3.13 a. Grain weight per pot in Paragon was also affected by an interaction between RH and soil moisture. At high RH/medium soil moisture, Paragon achieved higher grain weight per pot than those at high RH/low soil moisture and low RH/low soil moisture (p=0.011, Fig. 3.13 b).

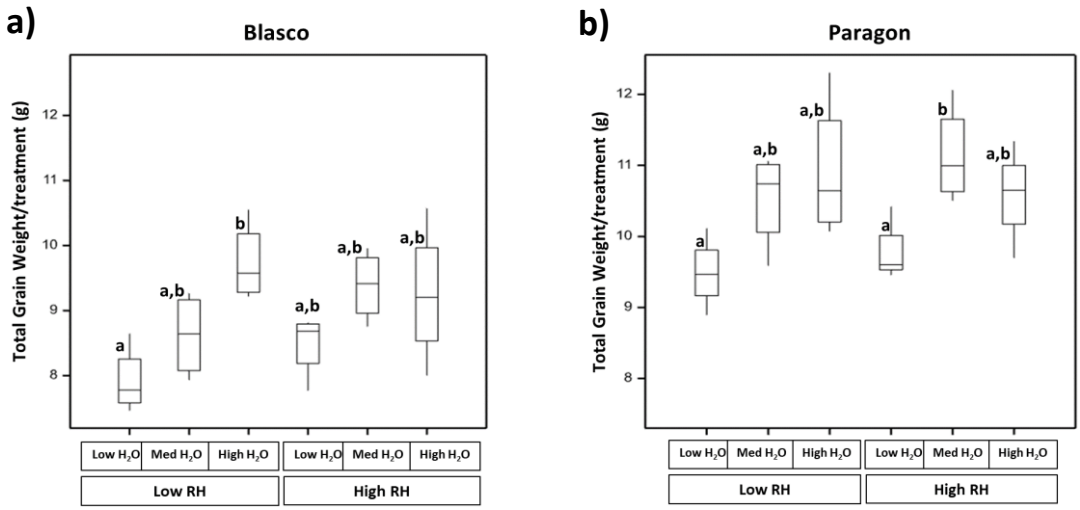


Figure 3.13: Grain weight of Blasco (a) and Paragon (b) wheat varieties exposed to humidity and soil moisture manipulation during their life cycle (Experiment 2). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

At both lower and higher air T, Paragon had higher grain weight/spike/treatment at low RH than at high RH in Experiment 3 ($p=0.004$). We also saw that grain weight/spike of Paragon was lower at high RH/high air T than that at low RH/low air T ($p=0.029$), as shown in Fig. 3.14. We did not see any direct RH or air T effect on grain weight per circular area of the cultivars in Experiment 3 (Appendix 3.4).

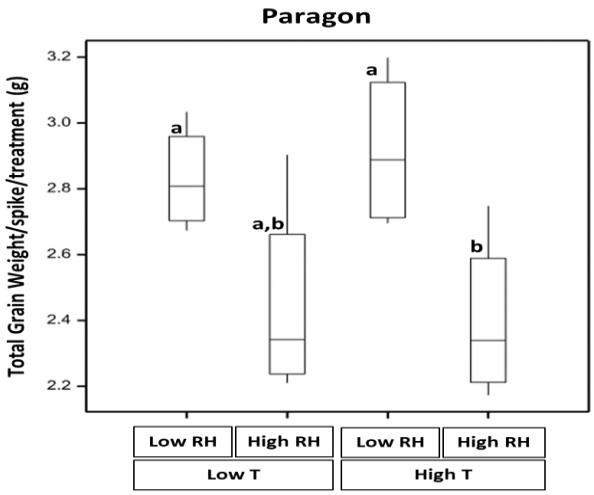


Figure 3.14: Effect of temperature manipulation, applied three consecutive days at flowering and during grain filling stages, and humidity manipulation, applied during the life cycle of the varieties, on grain weight of Paragon wheat variety (Experiment 3). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

3.3.3.2: Grain number

In Experiment 1A, total grain number in Paragon was higher at low RH/low air T than that at high RH/high air T (p=0.041, Fig. 3.15), but there was no effect on total grain number in Blasco (Appendix 3.4).

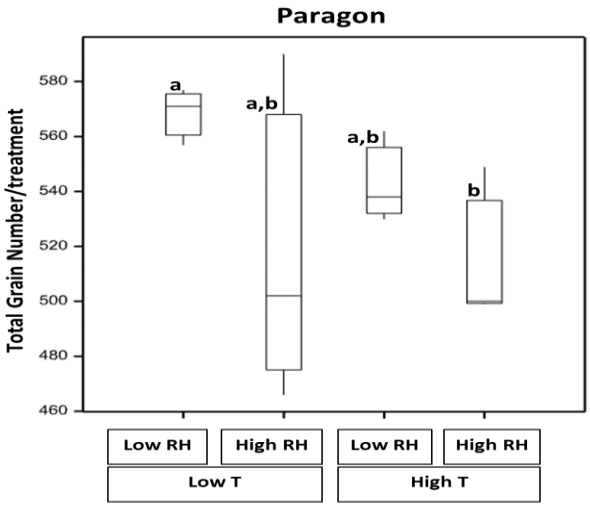


Figure 3.15: Grain number of Paragon wheat variety exposed to temperature and humidity manipulation, three consecutive days at the flowering stage (Experiment 1A). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

We observed a much wider variety of effects in Experiment 1B, for example an interactive effect of humidity and air temperature on total grain number was seen in Blasco (p=0.016, Fig. 3.16 a). Grain number was higher at low RH than at high RH under lower ait T conditions. Similar interactive observed in Paragon (p=0.037, Fig. 3.16 b), at higher air T Paragon had higher total grain number at a low RH than those at a high RH, while it had higher values under higher air T than those at lower air T under low RH conditions.

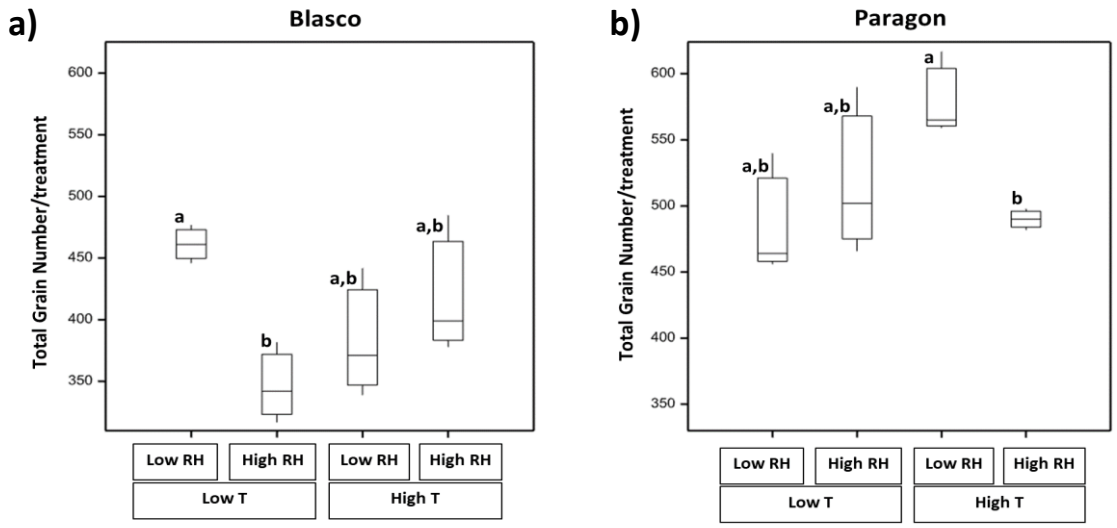


Figure 3.16: Effect of temperature and humidity manipulation, applied for three consecutive days during early grain filling stage, on grain number of Blasco (a) and Paragon (b) wheat varieties (Experiment 1B). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

At lower soil moisture in Experiment 2, an interactive effect of humidity and soil moisture on grain number in Blasco was found where grain number in Blasco was higher at high RH than those at low RH (p=0.02). Besides this, Blasco had higher grain number at higher soil moisture than those at lower soil moisture regardless of what was the RH (p<0.001, Fig. 3.17 a). Although there was no RH effect on the grain number of Paragon, it had higher values at higher soil moisture than those at lower soil moisture under both high and low RH conditions (p<0.001, Fig. 3.17 b).

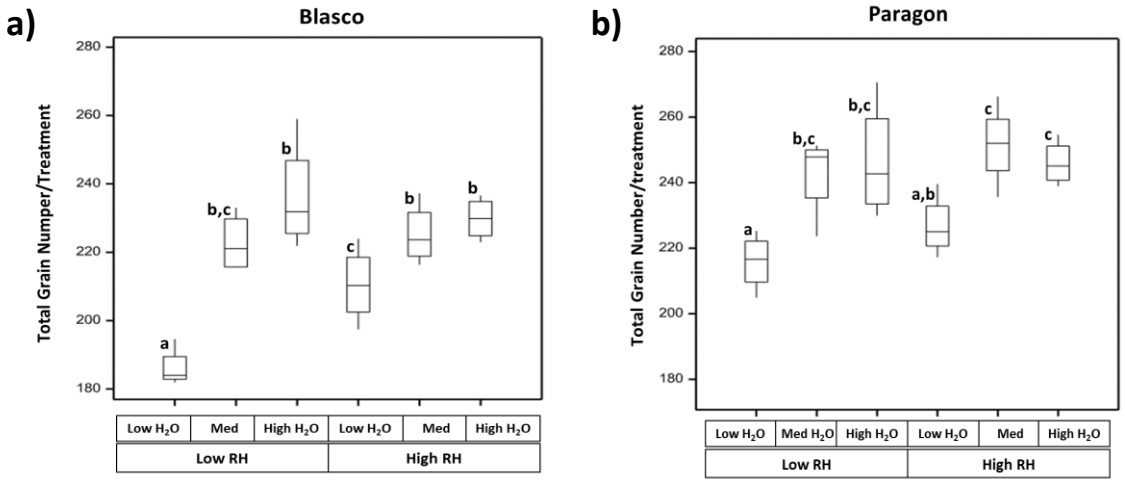


Figure 3.17: Effect of humidity and soil moisture manipulation, applied during the life cycle of the varieties, on grain number of Blasco (a) and Paragon (b) wheat varieties (Experiment 2). Top and

bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

There were no significant effects of RH or air T on total grain number in Experiments 3 (Appendix 3.4).

3.3.3.3: Grain size

In Experiment 1A, we saw that Paragon had larger grain size at high RH than at low RH at higher T (P=0.027, Fig. 3.18 a), while there was no effect on Blasco. In Experiment 1B, however, environmental manipulation resulted in larger grain size for Blasco at low RH than at high RH (p=0.031) under higher air T conditions, as shown in Fig. 3.18 b.

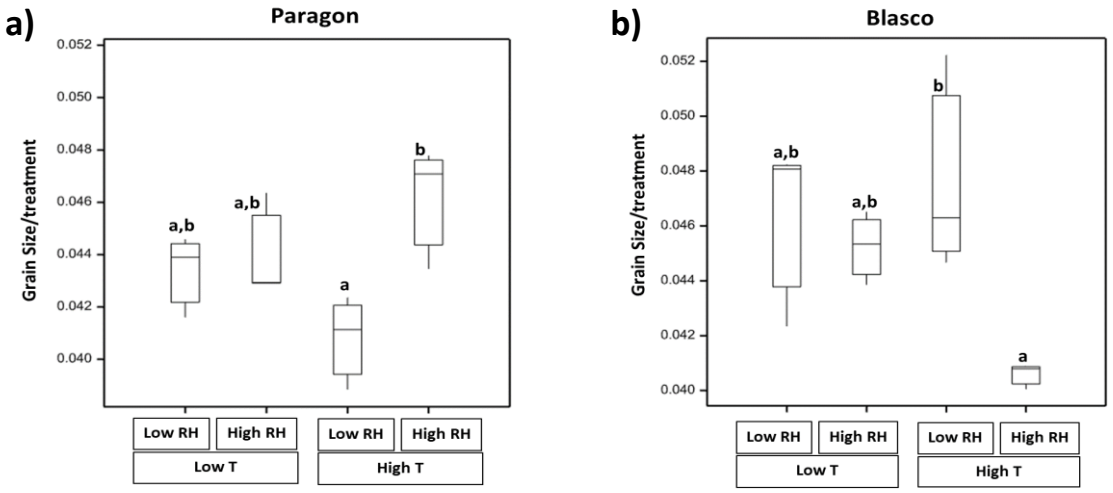


Figure 3.18: Grain size of Paragon (a) and Blasco (b) wheat varieties, exposed to temperature and humidity manipulation for three consecutive days at the flowering (Experiment 1A) and during Early grain filling stages (Experiment 1B), respectively. Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

There was no significant effect of RH or soil moisture on grain size in Experiment 2 (Appendix 3.4). In Experiment 3 however, we saw larger grain size/spike at low RH than at high RH under both high and low air T conditions in Paragon (p<0.001, Fig, 3.19 and 3.19 b). In Blasco, grain size/area was also affected, while we found that larger grains were produced at low RH than at high RH under low air T conditions (p=0.011, Fig. 3.20 a). An effect of humidity and air temperature on grain size was seen in Paragon, where larger grain size was found at low RH/low air T than at high RH/high air T but lower grain size at high RH/low air T than those at low RH/high air T (p<0.001, Fig. 3.19 and 3.20 b).

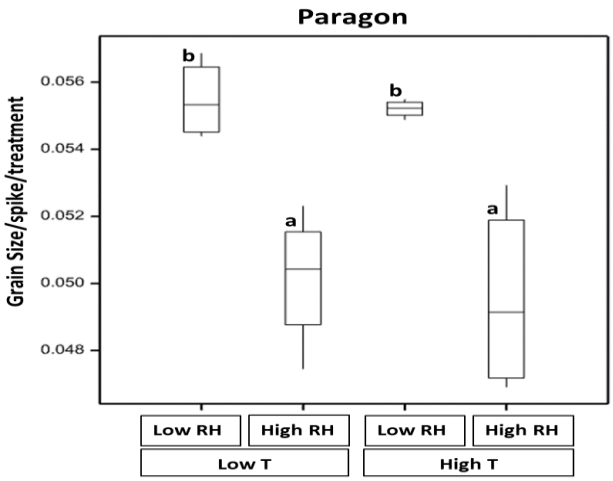


Figure 3.19: Grain size per Spike of Paragon wheat variety after exposing to the humidity manipulation during the life cycle and temperature manipulation three consecutive days at the flowering stage and during early grain filling stage (Experiment 3). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

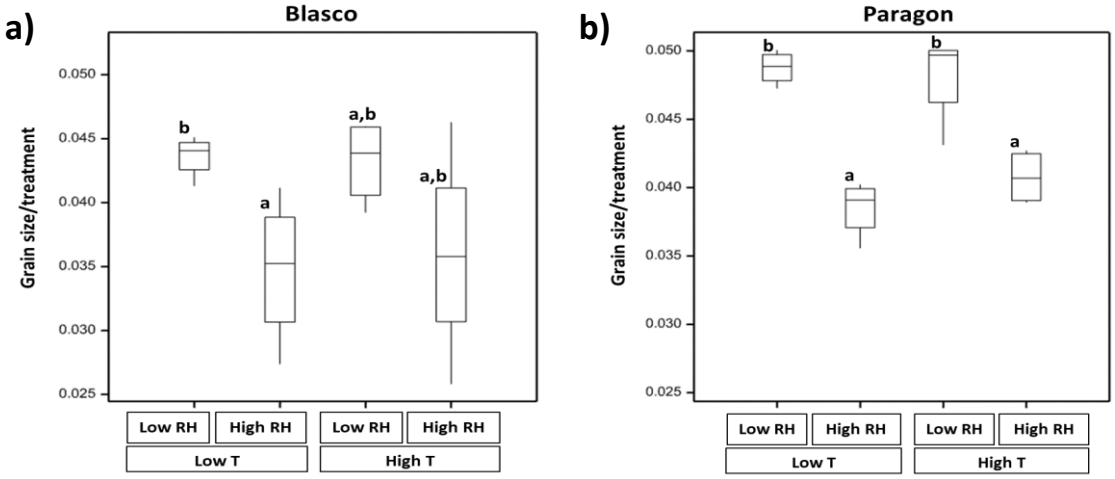


Figure 3.20: Effect of temperature manipulation, applied three consecutive days at the flowering stage and during Early grain filling stage, and humidity manipulation, applied during the life cycle of the cultivars, on grain size of Blasco (a) and Paragon (b) wheat varieties (Experiment 3). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

3.4: Discussion

3.4.1: Tissue temperature, evapotranspiration and tissue temperature depression

Cooling capacity of Blasco was higher than Paragon under all of the treatments in Experiment 1A and under high RH/low air temperature in Experiment 1B, which makes it to have lower tissue temperature than Paragon at these conditions. This might be because of differences on ear morphology. While Paragon is an awnless cultivar, Blasco has awns, and these a crucial transpiration and photosynthetic organ (Li *et al.*, 2010; Grundbacher, 1963; Rebetzke *et al.*, 2016). Awn, needle-like structure extending from lemma, raise the surface area of the ear (Blum, 1985), and it is speculated that awns promote to higher temperature tolerance than leaf tissue (Blum, 1985). Ferguson *et al.* (1973) found that awns provide a surface for additional transpiration resulting in greater cooling. Besides this, Rebetzke *et al.* (2016) observed that awnless wheat cultivars are cooler under cooler air temperatures, while wheat cultivars having awns are cooler at warmer canopy temperatures of over 23 °C. The reason of the contradictions about the effect of awns on cooling of the cultivars is not yet clear. Therefore, it is highly possible that the reason of lower tissue temperature in Blasco compare to tissue temperature in Paragon might be awns which might have allowed Blasco to have higher surface for transpirational cooling.

Temperature depression in plants represents a function of water availability for transpiration (Amani *et al.*, 1996). Blum (1985) indicated that even though spike has a larger surface area than of the flag leaves, the spike has lower transpiration rate. Therefore, the spikes have lower TD than flag leaves. Our results in Experiments 1, 2 and 3 have shown that the cooling capacity of the flag leaf of both cultivars was higher than that of the spike under all combinations of treatments. Since spike position relative to flag leaf is higher, possibly resulting in smaller water availability for transpiration in spikes and subsequently in higher tissue temperature.

The main driver of tissue temperature, in Blasco and Paragon at both flowering and early grain filling, was the temperature of surrounding air. Even though the cooling capacity (STD and FLTD) of the crops and their evapotranspiration rate at high air temperature was higher than that at low air T, high atmospheric temperature still resulted in higher tissue temperatures. In Experiment 1, at low air temperature, high RH significantly lowered the tissue temperature in Blasco at both flowering and early grain filling since it increased the cooling capacity, while high RH decreased the cooling capacity thus increased the tissue temperatures in Paragon at early grain filling. Higher evapotranspiration rate at low RH than at high RH at low air temperature

in the cultivars might have caused lower soil moisture and thus might have triggered Blasco to decrease stomatal area in order to prevent excessive water loss since it adapted to water stress conditions, as observed in rice (Abeysiriwardena *et al.*, 2002). Since Paragon was not bred to tolerate drought conditions, it might have smaller capacity to modify its stomata opening when compared to Blasco. In Experiment 2 and 3 (Chapter 5) we observed that Paragon responds to unfavourable conditions via changing its stomata number, but short-term unfavourable conditions probably was not enough to modify the stomata number in Experiment 1. RH did not modify tissue temperatures of the cultivars at higher air T at flowering, but at early grain filling high RH lowered the cooling capacity and increased ear temperatures. Similar results in rice has been seen in the other studies (Abeysiriwardena *et al.*, 2002; Weerakoon *et al.*, 2008; Yan *et al.*, 2010).

In Experiment 1A and 1B, the highest cooling capacity of tissues in Blasco was at low RH/high air T, while the lowest was at low RH/low air T. Low RH probably allowed Blasco to increase transpiration under high air temperature and full soil water availability to cool itself, therefore tissue temperature of Blasco was lower than air temperature. However, stomatal closure might have happened at low RH/low air temperature to prevent excessive water loss. Besides this, tissue TD of Paragon was the lowest at high RH/low air T low air temperatures and highest at low RH/high air T. Stomatal closure probably happened under high RH/low air temperature since there was not any increase in temperature and atmosphere had high moisture, at low RH/high air T, lower RH allowed it to transpire water and cool itself under high air temperature.

In Experiment 1, spike temperature depression of Paragon under cool conditions was negative – meaning tissue temperature higher than ambient air – a similar situation to all spike TD values of both cultivars in Experiment 3. However, FLTD values of both cultivars were positive in all experiments. This might be because of photosynthetic, respiratory and tissue composition differences between the spike and the flag leaf. Spike has a complex geometry compare to flag leaf, which might be the reason of higher tissue temperature in the spike. In Experiment 1, spike of the crops under cool conditions emitted radiation from the lambs because of their position, this may have increased their tissue temperature and resulted in negative STD (Thimijan and Heins, 1983; Tibbitts *et al.*, 1983). In the Experiment 3, there was a soil moisture difference between the tunnels having high RH and low RH. Tunnels having low RH had lower soil moisture, therefore there may not be enough water to reach to the spikes and this

might have prevented transpiration which caused higher spike tissue temperature. Stomatal closure might have happened in the spikes causing lower transpiration.

TD values in Experiment 1 was higher than the values in Experiment 2 and 3. Because of the lambs on the roof of the control cabinets in the Experiment 1 which emit a dramatic amount of far-infrared radiating, plants exposed to high radiative heating (Thimijan and Heins, 1983; Tibbitts *et al.*, 1983) resulting in higher tissue temperatures. This might have caused the TD difference between Experiment 1 and the other two experiments.

We have not found any effect of RH on tissue temperatures in the cultivars in Experiment 2 and Experiment 3. This might be because of high RH in humid tunnels and lower soil moisture in dry tunnels, as stomatal closure might have happened because of high RH and insufficient water for transpiration in Experiment 3. Secondly, weather conditions such as wind, daily changes in temperature, and rainfall in Experiment 3 might have covered the actual effects on the tissue temperatures in the cultivars. Thirdly, during Experiment 3, 28 days were classified as rainy (169mm rainfall total) with increased the atmospheric humidity. Rainy weather resulted in ambient atmospheric humidity higher that 85%, limiting any potential for observing any effects due to high RH in treatment tunnels. In Experiment 2, better dehumidifier with higher power and better materials to form the chambers to isolate the conditions in the chambers from the conditions in the polytunnel would have increased the difference between the two humidity treatments.

3.4.2: Grain yield

In Experiment 1A, Paragon had higher grain number at low RH/low air temperature than those at high RH/high air T, which is probably because of higher ear and flag leaf temperature at high RH/high air T. Sporogenesis, pollination and fertilization might be affected negatively from high temperature causing lower grain number in Blasco (Farooq *et al.*, 2011; Saini and Aspinall, 1982). There are other possibility as carbohydrate level might be decreased because of reduced transpiration rate under wet conditions inducing floret sterility and thus influencing grain number negatively (Farooq *et al.*, 2011; Kirby, 1988). However, higher cooling capacity in Paragon at high RH/high air temperature is not supporting this possibility. We also have observed that grain number of Blasco was lower at high RH than at low RH at low air temperatures in Experiment 1B. Low temperature (lower than optimal temperature for starch synthesis in Blasco) might have caused decrease in the reaction of enzymes (Bettelheim *et al.*, 2012) responsible for starch synthesis in Blasco and some of the grains might not be

able to developed at all after flowering stage. At high air temperature in Experiment 1B, Paragon had lower grain number at high RH than those at low RH. At high RH/high air temperature, some of the grains in Paragon might not be able to continue to develop after anthesis due to higher tissue temperature than the optimal tissue temperature. There is another possibility as heat stress might have shortened grain filling period in Paragon during early grain filling which might have caused grain loss (Dias and Lidon, 2009). However, short-term treatments might not have triggered this mechanism.

Grain weight in both cultivars was not affected by the treatments in Experiment 1A, however, in Experiment 1B, grain weight of Blasco was influenced where it had higher grain weight per pot and spike at low RH/low air temperature condition than at all other conditions. Grain weight in Blasco in Experiment 1B was higher at low RH than those at high RH at low air temperature. These results show that the most suitable tissue temperature for starch synthesis in Blasco was at low RH/low air T, and lower and higher tissue temperature than this tissue temperature during grain filling triggered lower grain yield. Lower tissue temperature in Blasco at high RH/low air temperature was probably lower than its optimal temperature for starch synthesis (Bettelheim *et al.*, 2012; Spiertz *et al.*, 2006) which resulted in lower grain weight. On the other hand, the most suitable tissue temperature in Paragon was at low RH/high air temperature where it also had the highest cooling capacity (highest photosynthetic activity), and lower and higher tissue temperatures than this tissue temperature caused lower grain weight. The highest photosynthetic activity probably helped Paragon to have higher grain yield at low RH/high air temperature. The highest ear temperature at high RH/high air temperature at early grain filling resulted in the lowest grain weight. Blasco and Paragon with higher spike temperature might have had shorter period between anthesis and maturity (Warrington *et al.*, 1977; Farooq *et al.*, 2011), and reduced enzyme activity responsible for starch biosynthesis (Spiertz *et al.*, 2006) which resulted in lower starch accumulation and grain weight.

Grain size in Paragon in Experiment 1A and grain size in Blasco in Experiment 1B was affected from RH at high air temperature. While Paragon had larger grain size at high RH than at low RH when treatments applied at flowering, we observed opposite results for Blasco when it exposed to the treatments 12 DAA where it had larger grain size at low RH than those at high RH. Paragon and Blasco might have compensated the lower grain number with increasing the size of the grains which is positively correlated with grain weight (Rebetzke *et al.*, 2016).

In Experiment 2, when soil moisture gets higher at low RH, grain weight of Blasco increased, while higher soil moisture increased grain weight of Paragon at high RH. The reason of this is probably decrease in grain filling duration and the activity of starch synthase under water stress (Farooq *et al.*, 2011; Ahmadi and Baker, 2001). Soil moisture effect has also been observed on grain number in the cultivars where lower grain number at low soil moisture than at medium and high soil moisture under both dry and wet conditions in both cultivars. Water stress during meiosis probably prevented microsporogenesis and during anthesis affected fertilization negatively resulting in reduced grain number (Cattivelli *et al.*, 2008; Manjarrez-Sandoval *et al.*, 1989). The other possibility is ABA biosynthesis (Ji *et al.*, 2011) might be happened, or invertase activity (Dorion *et al.*, 1996) and starch accumulation (Lalonde *et al.*, 1997) might be decreased which resulted in grain sterility.

There was not any effect on grain weight and grain number in the cultivars in Experiment 3, but high RH triggered decrease in the grain size in Blasco at low air temperature, and high RH and high air temperature caused decrease in grain size in Paragon. Even though there was not any significant effect on grain weight and grain number, grain number of Paragon tended to be lower at low RH than at high RH conditions regardless what was the air temperature, thus Paragon might have compensated the lower grain number with increasing the size of the grains (Rebetzke *et al.*, 2016).

In conclusion, the cooling capacity of the flag leaf of both cultivars was higher than that of the spike under about all treatment in the Experiments, which is probably due to lower transpiration rate in spikes than in flag leaves. The main driver of tissue temperatures in the cultivars was the temperature of surrounding air where higher the air temperature triggered higher tissue temperatures in Experiment 1 at flowering and early grain filling. While higher RH at low air temperature decreased the cooling capacity of Paragon during early grain filling as it was hypothesised, but complete opposite result observed in Blasco where higher RH at low air temperature increased the cooling capacity of it at flowering and early grain filling. RH did not modify tissue temperatures in the cultivars at higher air T at flowering but did at early grain filling when high RH lowered the cooling capacity and increased ear temperatures in both cultivars. We have not observed any effect on tissue temperature and cooling capacity in the cultivars in Experiment 2 and Experiment 3.

Increased tissue temperatures at flowering triggered lower grain number in Paragon, while we have not observed this in Blasco. The most suitable tissue temperature at

grain filling in Blasco and Paragon was at low RH/low air T, and low RH/high air temperature, respectively, and decrease or increase in these tissue temperatures triggered lower grain yield. RH manipulation on grain number in Blasco in Experiment 1B observed at low air temperature where higher the RH decreased the grain number. At high air temperature in Experiment 1B, higher the RH lowered the grain number in Paragon. In Experiment 3, we have not observed any effect on grain yield, however in Experiment 2 soil moisture was found to significantly affect grain yield. Higher the soil moisture increased the grain weight in Blasco and grain number in both Blasco and Paragon.

In conclusion, controlled environment experiment in the cabinets showed that both RH and air T has an impact on tissue temperature. The main driver of tissue T in both cultivars was the temperature of surrounding air regardless of the growth stage. Besides this, we observed both air T and RH effect on grain yield in the controlled environment experiment when the treatments applied during early grain filling (Experiment 1B), and soil moisture effect on grain yield in the pot based semi-controlled environment experiment (Experiment 2). However, we have not observed any concrete impact of the treatments on grain yield or on tissue temperature in the semi-controlled field experiment (Experiment 3). Therefore, we conclude that tissue temperatures and grain yield in both cultivars are not affected by RH in the field.

3.5: References

Abdipour, M., Ebrahimi, M., Izadi-Darbandi, A., Mastrangelo, A. M., Najafian, G., Arshad, Y. & Mirniyam, G. (2016). Association between Grain Size and Shape and Quality Traits, and Path Analysis of Thousand Grain Weight in Iranian Bread Wheat Landraces from Different Geographic Regions. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca,* **44** (1), 228.

Abeysingha, N., Singh, M., Islam, A. & Sehgal, V. (2016). Climate change impacts on irrigated rice and wheat production in Gomti River basin of India: a case study. *SpringerPlus,* **5** (1), 1250.

Abeysiriwardena, D. d. Z., Ohba, K. & Maruyama, A. (2002). Influence of temperature and relative humidity on grain sterility in rice. *Journal of the National Science Foundation of Sri Lanka,* **30** (1-2), 33-41.

Addis, A. (2000). *The Eleventh Regional Wheat Workshop For Eastern, Central and Southern Africa.* CIMMYT.

Ahmadi, A. & Baker, D. A. (2001). The effect of water stress on the activities of key regulatory enzymes of the sucrose to starch pathway in wheat. *Plant Growth Regulation,* **35** (1), 81-91.

Amani, I., Fischer, R. & Reynolds, M. (1996). Canopy temperature depression association with yield of irrigated spring wheat cultivars in a hot climate. *Journal of Agronomy and Crop Science,* **176** (2), 119-129.

Araus, J., Slafer, G., Reynolds, M. & Royo, C. (2002). Plant breeding and drought in C3 cereals: what should we breed for? *Annals of botany,* **89** (7), 925-940.

Asseng, S., Ewert, F., Martre, P., Rötter, R. P., Lobell, D., Cammarano, D., Kimball, B., Ottman, M., Wall, G. & White, J. W. (2015). Rising temperatures reduce global wheat production. *Nature Climate Change,* **5** (2), 143.

Ayeneh, A., Van Ginkel, M., Reynolds, M. & Ammar, K. (2002). Comparison of leaf, spike, peduncle and canopy temperature depression in wheat under heat stress. *Field Crops Research,* **79** (2-3), 173-184.

Bahar, B., Yildirim, M., Barutcular, C. & Ibrahim, G. (2008). Effect of canopy temperature depression on grain yield and yield components in bread and durum wheat. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca,* **36** (1), 34.

Balota, M., Payne, W. A., Evett, S. R. & Lazar, M. D. (2007). Canopy temperature depression sampling to assess grain yield and genotypic differentiation in winter wheat. *Crop Science,* **47** (4), 1518-1529.

Battisti, D. S. & Naylor, R. L. (2009). Historical warnings of future food insecurity with unprecedented seasonal heat. *Science,* **323** (5911), 240-244.

Bettelheim, F., Brown, W., Campbell, M., Farrell, S. & Torres, O. (2012). *Introduction to general, organic and biochemistry.* Nelson Education.

Bhullar, S. & Jenner, C. (1985). Differential responses to high temperatures of starch and nitrogen accumulation in the grain of four cultivars of wheat. *Functional Plant Biology,* **12** (4), 363-375.

Bland, J. M. & Altman, D. G. (1996). Statistics notes: Transforming data. *Bmj,* **312** (7033), 770.

Blum, A. (1985). Photosynthesis and transpiration in leaves and ears of wheat and barley varieties. *Journal of experimental botany,* **36** (3), 432-440.

Cattivelli, L., Rizza, F., Badeck, F.-W., Mazzucotelli, E., Mastrangelo, A. M., Francia, E., Mare, C., Tondelli, A. & Stanca, A. M. (2008). Drought tolerance improvement in crop plants: an integrated view from breeding to genomics. *Field Crops Research,* **105** (1-2), 1-14.

Challinor, A. J., Watson, J., Lobell, D., Howden, S., Smith, D. & Chhetri, N. (2014). A meta-analysis of crop yield under climate change and adaptation. *Nature Climate Change,* **4** (4), 287.

Dias, A. & Lidon, F. (2009). Evaluation of grain filling rate and duration in bread and durum wheat, under heat stress after anthesis. *Journal of Agronomy and Crop Science,* **195** (2), 137-147.

Dias, A. S., Bagulho, A. S. & Lidon, F. C. (2008). Ultrastructure and biochemical traits of bread and durum wheat grains under heat stress. *Brazilian Journal of Plant Physiology,* **20** (4), 323-333.

Dorion, S., Lalonde, S. & Saini, H. S. (1996). Induction of male sterility in wheat by meiotic-stage water deficit is preceded by a decline in invertase activity and changes in carbohydrate metabolism in anthers. *Plant Physiology,* **111** (1), 137-145.

Fahad, S., Bajwa, A. A., Nazir, U., Anjum, S. A., Farooq, A., Zohaib, A., Sadia, S., Nasim, W., Adkins, S. & Saud, S. (2017). Crop production under drought and heat stress: plant responses and management options. *Frontiers in plant science,* **8**, 1147.

FAO. (2018). *FAO Cereal Supply and Demand Brief.*

Farooq, M., Bramley, H., Palta, J. A. & Siddique, K. H. (2011). Heat stress in wheat during reproductive and grain-filling phases. *Critical Reviews in Plant Sciences,* **30** (6), 491-507.

Farooq, M., Hussain, M. & Siddique, K. H. (2014). Drought stress in wheat during flowering and grain-filling periods. *Critical Reviews in Plant Sciences,* **33** (4), 331-349.

Ferguson, H., Eslick, R. & Aase, J. (1973). Canopy Temperatures of Barley as Influenced by Morphological Characteristics 1. *Agronomy Journal,* **65** (3), 425-428.

FERRIS, R., Ellis, R., Wheeler, T. & Hadley, P. (1998). Effect of high temperature stress at anthesis on grain yield and biomass of field-grown crops of wheat. *Annals of Botany,* **82** (5), 631-639.

Fischer, R. (1985). Number of kernels in wheat crops and the influence of solar radiation and temperature. *The Journal of Agricultural Science,* **105** (2), 447-461.

Forbes, J. C. & Watson, D. (1992). *Plants in agriculture.* Cambridge University Press.

Grundbacher, F. (1963). The physiological function of the cereal awn. *The Botanical Review,* **29** (3), 366-381.

Hillel, D. (2012). *Applications of soil physics.* Elsevier.

Hossain, M. A., Wani, S. H., Bhattacharjee, S., Burritt, D. J. & Tran, L.-S. P. (2016). *Drought stress tolerance in plants, Vol 1: Physiology and biochemistry.* Springer.

Ji, X., Dong, B., Shiran, B., Talbot, M. J., Edlington, J. E., Hughes, T., White, R. G., Gubler, F. & Dolferus, R. (2011). Control of abscisic acid catabolism and abscisic acid homeostasis is important for reproductive stage stress tolerance in cereals. *Plant physiology,* **156** (2), 647-662.

Kirby, E. (1988). Analysis of leaf, stem and ear growth in wheat from terminal spikelet stage to anthesis. *Field Crops Research,* **18** (2-3), 127-140.

Lal, R. & Shukla, M. K. (2004). *Principles of soil physics.* CRC Press.

Lalonde, S., Beebe, D. U. & Saini, H. (1997). Early signs of disruption of wheat anther development associated with the induction of male sterility by meiotic-stage water deficit. *Sexual Plant Reproduction,* **10** (1), 40-48.

Li, X.-f., Bin, D. & Hong-Gang, W. (2010). Awn anatomy of common wheat (Triticum aestivum L.) and its relatives. *Caryologia,* **63** (4), 391-397.

Maghsoudi, K. & Maghsoudi, M. (2008). Analysis of the effects of stomatal frequency and size on transpiration and yield of wheat (Triticum aestivum L.). *American-Eurasian J Agric Environ Sci,* **3**, 865-72.

Manjarrez-Sandoval, P., GONZÁLEZ-HERNÁNDEZ, V. A., MENDOZA-ONOFRE, L. E. & Engleman, E. (1989). Drought stress effects on the grain yield and panicle development of sorghum. *Canadian Journal of Plant Science,* **69** (3), 631-641.

Martinez-Carrasco, R. & THORNE, G. N. (1979). Physiological factors limiting grain size in wheat. *Journal of Experimental Botany,* **30** (4), 669-679.

Mascarenhas, J. P. & Crone, D. E. (1996). Pollen and the heat shock response. *Sexual Plant Reproduction,* **9** (6), 370-374.

McDonald, J. H. (2009). *Handbook of biological statistics.* Sparky House Publishing Baltimore, MD.

Niglas, A., Alber, M., Suur, K., Jasińska, A. K., Kupper, P. & Sellin, A. (2015). Does increased air humidity affect stomatal morphology and functioning in hybrid aspen? *Botany,* **93** (4), 243-250.

Oerke, E., Steiner, U., Dehne, H. & Lindenthal, M. (2006). Thermal imaging of cucumber leaves affected by downy mildew and environmental conditions. *Journal of experimental botany,* **57** (9), 2121-2132.

Pantin, F. & Blatt, M. R. (2018). Stomatal Response to Humidity: Blurring the Boundary between Active and Passive Movement. *Plant physiology,* **176** (1), 485-488.

Parry, M. A., Reynolds, M., Salvucci, M. E., Raines, C., Andralojc, P. J., Zhu, X.-G., Price, G. D., Condon, A. G. & Furbank, R. T. (2010). Raising yield potential of

wheat. II. Increasing photosynthetic capacity and efficiency. *Journal of experimental botany,* **62** (2), 453-467.

Porter, J. R. & Gawith, M. (1999). Temperatures and the growth and development of wheat: a review. *European Journal of Agronomy,* **10** (1), 23-36.

Porter, J. R. & Semenov, M. A. (2005). Crop responses to climatic variation. *Philosophical Transactions of the Royal Society B: Biological Sciences,* **360** (1463), 2021-2035.

Prasad, P., Staggenborg, S. & Ristic, Z. (2008). Impacts of drought and/or heat stress on physiological, developmental, growth, and yield processes of crop plants. *Response of crops to limited water: Understanding and modeling water stress effects on plant growth processes,* (responseofcrops), 301-355.

Ramulu, U. S. (1998). *Management of water resources in agriculture.* New Age International.

Rasmussen, J. L. (1985). Data transformation maximizing homoscedasticity and within-group normality. *Behavior Research Methods,* **17** (3), 411-412.

Rawson, H. & Bagga, A. (1979). Influence of temperature between floral initiation and flag leaf emergence on grain number in wheat. *Functional Plant Biology,* **6** (3), 391-400.

Ray, D. K., Mueller, N. D., West, P. C. & Foley, J. A. (2013). Yield trends are insufficient to double global crop production by 2050. *PloS one,* **8** (6), e66428.

Rebetzke, G., Bonnett, D. & Reynolds, M. (2016). Awns reduce grain number to increase grain size and harvestable yield in irrigated and rainfed spring wheat. *Journal of experimental botany,* **67** (9), 2573-2586.

Reynolds, M., Gutiérrez-Rodríguez, M. & Larqué-Saavedra, A. (2000). Photosynthesis of wheat in a warm, irrigated environment: I: genetic diversity and crop productivity. *Field Crops Research,* **66** (1), 37-50.

Reynolds, M., Nagarajan, S., Razzaque, M. & Ageeb, O. (1997). *Using canopy temperature depression to select for yield potential of wheat in heat-stressed environments.* CIMMYT.

Roberts, M., Reiss, M. J. & Monger, G. (2000). *Advanced biology.* Nelson Thornes.

Roberts, M. B. V. (1986). *Biology: A functional approach.* Nelson Thornes.

Romano, G., Zia, S., Spreer, W., Sanchez, C., Cairns, J., Araus, J. L. & Müller, J. (2011). Use of thermography for high throughput phenotyping of tropical maize adaptation in water stress. *Computers and Electronics in Agriculture,* **79** (1), 67-74.

Rundel, P., JARREL, W., Pearcy, R., Ehleringer, J. & Mooney, H. (1989). Plant physiological ecology-field methods and instrumentation. *Plant physiological ecology: field methods and instrumentation*.

Ruosteenoja, K. & Räisänen, P. (2013). Seasonal changes in solar radiation and relative humidity in Europe in response to global warming. *Journal of Climate,* **26** (8), 2467-2481.

Saini, H. & Aspinall, D. (1982). Abnormal sporogenesis in wheat (Triticum aestivum L.) induced by short periods of high temperature. *Annals of Botany,* **49** (6), 835-846.

Sanchez-Bragado, R., Molero, G., Reynolds, M. P. & Araus, J. L. (2014). Relative contribution of shoot and ear photosynthesis to grain filling in wheat under good agronomical conditions assessed by differential organ δ13C. *Journal of experimental botany,* **65** (18), 5401-5413.

Savin, R., Calderini, D. F., Slafer, G. A. & Abeledo, L. G. (1999). Final grain weight in wheat as affected by short periods of high temperature during pre-and post-anthesis under field conditions. *Functional Plant Biology,* **26** (5), 453-458.

Semenov, M. A. & Shewry, P. R. (2011). Modelling predicts that heat stress, not drought, will increase vulnerability of wheat in Europe. *Scientific reports,* **1**, 66.

Shewry, P. R. (2009). Wheat. *Journal of experimental botany,* **60** (6), 1537-1553.

Sofield, I., Evans, L., Cook, M. & Wardlaw, I. F. (1977). Factors influencing the rate and duration of grain filling in wheat. *Functional Plant Biology,* **4** (5), 785-797.

Spiertz, J., Hamer, R., Xu, H., Primo-Martin, C., Don, C. & Van Der Putten, P. (2006). Heat stress in wheat (Triticum aestivum L.): Effects on grain growth and quality traits. *European Journal of Agronomy,* **25** (2), 89-95.

Steinmeyer, F. T., Lukac, M., Reynolds, M. P. & Jones, H. E. (2013). Quantifying the relationship between temperature regulation in the ear and floret development stage in wheat (Triticum aestivum L.) under heat and drought stress. *Functional Plant Biology,* **40** (7), 700-707.

Stone, P., Savin, R., Wardlaw, I. & Nicolas, M. (1995). The influence of recovery temperature on the effects of a brief heat shock on wheat. I. Grain growth. *Functional Plant Biology,* **22** (6), 945-954.

Streck, N. A. (2005). Climate change and agroecosystems: the effect of elevated atmospheric CO2 and temperature on crop growth, development, and yield. *Ciência Rural,* **35** (3), 730-740.

Suzuki, N., Bassil, E., Hamilton, J. S., Inupakutika, M. A., Zandalinas, S. I., Tripathy, D., Luo, Y., Dion, E., Fukui, G. & Kumazaki, A. (2016). ABA is required for plant

acclimation to a combination of salt and heat stress. *PLoS One,* **11** (1), e0147625.

Tambussi, E. A., Bort, J., Guiamet, J. J., Nogués, S. & Araus, J. L. (2007). The photosynthetic role of ears in C3 cereals: metabolism, water use efficiency and contribution to grain yield. *Critical Reviews in Plant Sciences,* **26** (1), 1-16.

Tambussi, E. A., Nogués, S. & Araus, J. L. (2005). Ear of durum wheat under water stress: water relations and photosynthetic metabolism. *Planta,* **221** (3), 446-458.

Teh, C. (2006). *Introduction to mathematical modeling of crop growth.* Dissertation. com.

Thimijan, R. W. & Heins, R. D. (1983). Photometric, radiometric, and quantum light units of measure: a review of procedures for interconversion. *HortScience,* **18** (6), 818-822.

Tibbitts, T., Morgan, D. & Warrington, I. (1983). Growth of lettuce, spinach, mustard, and wheat plants under four combinations of high-pressure sodium, metal halide, and tungsten halogen lamps at equal PPFD. *J. Amer. Soc. Hort. Sci,* **108** (4), 622-630.

Viswanathan, C. & Khanna-Chopra, R. (2001). Effect of heat stress on grain growth, starch synthesis and protein synthesis in grains of wheat (Triticum aestivum L.) varieties differing in grain weight stability. *Journal of agronomy and crop science,* **186** (1), 1-7.

Warrington, I., Dunstone, R. & Green, L. (1977). Temperature effects at three development stages on the yield of the wheat ear. *Australian Journal of Agricultural Research,* **28** (1), 11-27.

Weerakoon, W., Maruyama, A. & Ohba, K. (2008). Impact of Humidity on Temperature-Induced Grain Sterility in Rice (Oryza sativa L). *Journal of Agronomy and Crop Science,* **194** (2), 135-140.

Wheeler, T., Batts, G., Ellis, R., Hadley, P. & Morison, J. (1996). Growth and yield of winter wheat (Triticum aestivum) crops in response to CO 2 and temperature. *The Journal of Agricultural Science,* **127** (1), 37-48.

Xie, B., Zhang, Q. & Ying, Y. (2011). Trends in precipitable water and relative humidity in China: 1979–2005. *Journal of Applied Meteorology and Climatology,* **50** (10), 1985-1994.

Yan, C., Ding, Y., Wang, Q., Liu, Z., Li, G., Muhammad, I. & Wang, S. (2010). The impact of relative humidity, genotypes and fertilizer application rates on panicle, leaf temperature, fertility and seed setting of rice. *The Journal of Agricultural Science,* **148** (3), 329-339.

Yong, J., Wong, S. & Farquhar, G. (1997). Stomatal responses to changes in vapour pressure difference between leaf and air. *Plant, Cell & Environment,* **20** (10), 1213-1216.

Zadoks, J. C., Chang, T. T. & Konzak, C. F. (1974). A decimal code for the growth stages of cereals. *Weed research,* **14** (6), 415-421.

3.6: Chapter 3 – Appendix

Appendix 3.1: Summary of evapotranspiration scores of Blasco and Paragon wheat cultivars prior to temperature, relative humidity treatments.

	Experiment 1A Evapotranspiration/ Pot (g); mean ± SD		Experiment 1B Evapotranspiration / Pot (g); mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Cool	28±10.4 **a**	28.78±3.69 **a**	25.33±1.73 **a**	24.78±6.7 ab
Dry/Warm	50.22±7.5 **b**	53.33±8.09 **c**	34.89±4.17 **c**	37.89±8.8 **b**
Wet/Cool	13.78±0.96 **a**	14.11±1.5 **b**	13.78±0.96 **b**	14.11±1.5 **a**
Wet/Warm	19.33±9.85 **a**	35.11±9.14 **a**	22.11±5.93 **ab**	27±2.08 **b**

Appendix 3.2: Summary of tissue temperature scores of Blasco and Paragon wheat cultivars prior to temperature, relative humidity and soil moisture treatments.

	Experiment 1A Spike T/ Pot (°C); mean ± SD		Experiment 1B Spike T/ Pot (°C); mean ± SD		Experiment 3 Spike T/ plot (°C); mean ± SD			Experiment 2 Normilized Spike T/ Pot (°C); mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Treatment**	**Blasco**	**Paragon**
Dry/Cool	18.15±0.33 **(a)**	21.04±0.49 **(a)**	19.23±0.17 **(a)**	18.77±0.17 **(a)**	21.50±0.83 (a)	21.39±0.88 (a)	**Dry/Low**	28.19±0.5 (a)	28.8±0.46 (a)
Dry/Warm	24.81±0.9 **(c)**	28.52±0.47 **(b)**	26.76±0.38 **(b)**	26.34±0.38 **(b)**	21.69±0.7 (a)	21.89±1.01 (a)	**Dry/Med**	28.18±0.54 (a)	29.35±1.55 (a)
Wet/Cool	15.88±1.23 **(b)**	21.38±0.21 **(a)**	15.88±1.23 **(c)**	21.38±0.21 **(c)**	21.73±0.69 (a)	21.74±0.48 (a)	**Dry/High**	28.29±0.5 (a)	28.96±0.41 (a)
Wet/Warm	26.28±2.43 **(c)**	28.21±0.54 **(b)**	28.2±0.74 **(d)**	28.04±0.63 **(d)**	21.82±0.16 (a)	21.75±0.48 (a)	**Wet/Low**	28.66±0.84 (a)	29.29±0.61 (a)
							Wet/Med	28.74±0.85 (a)	29.57±0.75 (a)
							Wet/High	28.59±1 (a)	29.18±0.99

	Experiment 1A Flag Leaf T/ Pot (°C); mean ± SD		Experiment 1B Flag Leaf T / Pot (°C); mean ± SD		Experiment 3 Canopy T/ m^2 (°C); mean ± SD			Experiment 2 Normilized Flag Leaf T / Pot (°C); mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Treatment**	**Blasco**	**Paragon**
Dry/Cool	15.88±0.42 **(a)**	19.39±0.68 **(a)**	17.02±0.5 **(a)**	17.21±0.57 **(a)**	19.55±0.56 (a)	19.53±0.62 (a)	**Dry/Low**	26.42±0.9 (a)	26.25±0.9 (a)
Dry/Warm	21.71±1.48 **(c)**	26.5±0.73 **(b)**	23.36±1.05 **(b)**	23.91±1.11 **(b)**	19.5±0.5 (a)	19.88±0.67 (a)	**Dry/Med**	26.34±0.8 (a)	26.58±0.67 (a)
Wet/Cool	12.56±1.06 **(b)**	19.32±0.31 **(a)**	12.56±1.06 **(c)**	19.32±0.31 **(c)**	19.88±0.77 (a)	19.68±0.61 (a)	**Dry/High**	26.47±0.71 (a)	26.41±0.82 (a)
Wet/Warm	22.15±2.62 **(c)**	25.58±0.77 **(b)**	23.85±0.99 **(b)**	23.92±0.73 **(b)**	20.18±0.45 (a)	20±0.51 (a)	**Wet/Low**	26.65±1 (a)	26.87±0.89 (a)
							Wet/Med	26.69±0.77 (a)	26.99±1.09 (a)
							Wet/High	26.6±1.1 (a)	27.01±0.91 (a)

Appendix 3.3: Summary of tissue temperature depression scores of Blasco and Paragon wheat cultivars prior to temperature, relative humidity and soil moisture treatments.

	Experiment 1A Spike TD/ Pot (°C); mean ± SD		Experiment 1B Spike TD/ Pot (°C); mean ± SD		Experiment 3 Spike TD/ plot (°C); mean ± SD			Experiment 2 Spike TD/ Pot (°C); mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Treatment**	**Blasco**	**Paragon**
Dry/Cool	1.56±0.33 **(a)**	-1.02±0.49 **(a)**	0.71±0.17 **(a)**	1.06±0.17 **(a)**	-0.29±0.97 (a)	-0.84±1.03 (a)	**Dry/Low**	2.8±0.6 **(a)**	2.2±0.5 **(a)**
Dry/Warm	7.13±0.83 **(b)**	3.4±0.5 **(b)**	5.15±0.38 **(b)**	5.39±0.38 **(b)**	-0.46±1.2 (a)	-0.21±0.96 (a)	**Dry/Med**	2.8±0.7 **(a)**	1.66±1.1 (ab)
Wet/Cool	4.5±0.65 **(c)**	-1.39±0.21 **(a)**	4.45±0.65 **(bc)**	-1.39±0.21 **(c)**	-0.27±0.93 (a)	-0.23±0.41 (a)	**Dry/High**	2.7±0.6 **(a)**	2±0.5 **(a)**
Wet/Warm	4.17±0.94 **(c)**	3.98±0.54 **(b)**	3.93±0.74 **(c)**	3.94±0.63 **(d)**	-0.12±1.46 (a)	-0.18±0.66 (a)	**Wet/Low**	0.71±0.91 **(b)**	0.08±0.89 **(b)**
							Wet/Med	0.48±0.85 **(b)**	0.19±1.03 **(b)**
							Wet/High	0.78±1.1 **(b)**	0.19±1.29 **(b)**

	Experiment 1A Flag Leaf TD/ Pot (°C); mean ± SD		Experiment 1B Flag Leaf TD/ Pot (°C); mean ± SD		Experiment 3 Canopy TD/ m^2 (°C); mean ± SD			Experiment 2 Flag Leaf TD / Pot (°C); mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Treatment**	**Blasco**	**Paragon**
Dry/Cool	3.83±0.42 **(a)**	0.64±0.68 **(a)**	2.92±0.51 **(a)**	2.62±0.57 **(a)**	1.85±0.79 (a)	1.254±0.74 (a)	**Dry/Low**	1.16±0.7 **(a)**	1.02±0.68 **(a)**
Dry/Warm	10.19±1.46 **(b)**	5.38±0.82 **(b)**	8.55±1.05 **(b)**	7.81±1.11 **(b)**	1.56±0.91 (a)	1.6±0.83 (a)	**Dry/Med**	1.23±0.8 **(a)**	0.84±0.88 **(a)**
Wet/Cool	7.44±1.06 **(bc)**	0.67±0.31 **(a)**	7.44±1.06 **(b)**	0.67±0.31 **(c)**	1.35±1.1 (a)	1.5±0.63 (a)	**Dry/High**	1.14±1.07 **(a)**	0.94±0.69 **(a)**
Wet/Warm	7.78±0.38 **(c)**	6.6±0.77 **(b)**	8.28±0.99 **(b)**	8.07±0.73 **(b)**	1.67±1.4 (a)	1.77±0.77 (a)	**Wet/Low**	2.9±0.70 **(b)**	2.53±0.29 **(b)**
							Wet/Med	2.43±0.1 **(ab)**	2.47±0.18 **(b)**
							Wet/High	3±0.66 **(b)**	2.45±0.3 **(b)**

Appendix 3.4: Summary of grain yield/pot/treatment in Experiment 1A, 1B and 2, and per m^2 in Experiment 3 scores of Blasco and Paragon wheat cultivars prior to temperature, relative humidity and soil moisture treatments.

	Experiment 1A Total Grain Weight / pot (g); mean ± SD		Experiment 1B Total Grain Weight / pot (g); mean ± SD		Experiment 3 Total Grain Weight / m^2 (g); mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Cool	17.39±2.2 (a)	24.64±0.6 (a)	21.29±1 **(a)**	22.38±0.4 **(a)**	68.39±6 (a)	89.95±6 (a)
Dry/Warm	16.53±2.8 (a)	22.17±1.5 (a)	18.21±1.4 **(b)**	24.51±0.8 **(b)**	65.3±6 (a)	88.46±7.9 (a)
Wet/Cool	15.69±1.4 (a)	22.82±2.2 (a)	15.69±1.4 **(b)**	22.82±2.2 (abc)	53.55±17.9 (a)	76.32±10.8(a)
Wet/Warm	17.39±1.1 (a)	23.75±0.2 (a)	17.08±2.4 **(b)**	20.24±0.7 **(c)**	49.18±17 (a)	77.42±14 (a)

	Experiment 2 Total Grain Weight / pot (g); mean ± SD	
Treatment	**Blasco**	**Paragon**
Dry/Low	7.92±0.5 **(a)**	9.49±0.5 **(a)**
Dry/Med	8.62±0.7 (ab)	10.54±0.7 (ab)
Dry/High	9.73±0.6 **(b)**	10.92±1 (ab)
Wet/Low	8.49±0.5 (ab)	9.77±0.4 **(a)**
Wet/Med	9.39±0.5 (ab)	11.14±0.7 **(b)**
Wet/High	9.25±1 (ab)	10.59±0.7 (ab)

	Experiment 1A Total Grain Number / pot; mean ± SD		Experiment 1B Total Grain Number /pot; mean ± SD		Experiment 3 Total Grain Number / m^2; mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Cool	397.7±62.5 (a)	568±10.3 **(a)**	461.3±15.5 **(a)**	486.7±46.4 (ab)	1569±140.6 (a)	1956±214 (a)
Dry/Warm	396±43.9 (a)	543±16.7 (ab)	384±52.7 (ab)	580.3±31.9 **(a)**	1508±78.5 (a)	1838±271 (a)
Wet/Cool	347±32.8 (a)	519.3±63.8 (ab)	347±32.8 **(b)**	519.3±63.8 (ab)	1497±411.7 (a)	1958±314.8 (a)
Wet/Warm	401±11.5 (a)	516±28.6 **(b)**	420.7±56.7 (ab)	490±8 **(b)**	1236±226.3 (a)	2120±658.6 (a)

	Experiment 2 Total Grain Number / pot; mean ± SD	
Treatment	**Blasco**	**Paragon**
Dry/Low	186.2±5.8 **(a)**	215.9±8.6 **(a)**
Dry/Med	222.7±8.6 **(bc)**	242.7±12.8 **(bc)**
Dry/High	236.2±16 **(b)**	246.5±17.8 **(bc)**
Wet/Low	210.5±11 **(c)**	226.8±9.4 **(ab)**
Wet/Med	225.2±9 **(b)**	251.5±12.5 **(c)**
Wet/High	229.8±6.2 **(b)**	246±6.8 **(c)**

	Experiment 1A		Experiment 1B		Experiment 3			Experiment 2	
	Grain Size/ pot; mean ± SD		Grain Size / pot; mean ± SD		Grain Size / m^2; mean ± SD			Grain Size / pot; mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Treatment**	**Blasco**	**Paragon**
Dry/Cool	0.044±0.002 (a)	0.043±0.002 (ab)	0.046±0.003 (ab)	0.046±0.004 (a)	0.044±0.002 **(a)**	0.049±0.001 **(a)**	**Dry/Low**	0.04±0.002 (a)	0.044±0.001 (a)
Dry/Warm	0.042±0.004 (a)	0.041±0.0018 **(a)**	0.048±0.004 **(a)**	0.042±0.001 (a)	0.043±0.003 (ab)	0.048±0.003 **(a)**	**Dry/Med**	0.04±0.003 (a)	0.043±0.001 (a)
Wet/Cool	0.045±0.001 (a)	0.044±0.002 (ab)	0.05±0.001 **(a)**	0.044±0.002 (a)	0.035±0.006 **(b)**	0.039±0.002 **(b)**	**Dry/High**	0.04±0.002 (a)	0.04±0.001 (a)
Wet/Warm	0.04±0.0016 (a)	0.046±0.0023 **(b)**	0.041±0.001 **(b)**	0.041±0.001 (a)	0.036±0.008 (ab)	0.041±0.002 **(b)**	**Wet/Low**	0.04±0.002 (a)	0.043±0.001 (a)
							Wet/Med	0.04±0.002 (a)	0.044±0.001 (a)
							Wet/High	0.04±0.003 (a)	0.043±0.003 (a)

Appendix 3.5: Summary of grain yield/spike/treatment scores of Blasco and Paragon wheat cultivars prior to temperature, relative humidity and soil moisture treatments in Experiment 1A, 1B and 2, and per m^2 in Experiment 3.

	Experiment 1A Total Grain Weight / spike (g); mean ± SD		Experiment 1B Total Grain Weight / spike (g); mean ± SD		Experiment 3 Total Grain Weight / spike (g); mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Cool	2.94±0.5 (a)	4.4±0.3 (a)	3.74±0.2 **(c)**	3.78±0.8 (a)	2.43±0.2 (a)	2.83±0.2 **(b)**
Dry/Warm	2.85±0.4 (a)	4±0.2 (a)	3.27±0.8 **(b)**	3.93±0.7 (a)	2.35±0.1 (a)	2.92±0.25 **(b)**
Wet/Cool	2.39±0.4 (a)	4±0.2 (a)	2.39±0.4 **(a)**	3.95±0.4 (a)	2.25±0.2 (a)	2.45±0.32 (ab)
Wet/Warm	2.62±1.4 (a)	4.1±0.3 (a)	2.9±0.4 **(ba)**	3.64±0.1 (a)	2.19±0.2 (a)	2.4±0.26 **(a)**

	Experiment 2 Total Grain Weight / spike (g); mean ± SD	
Treatment	**Blasco**	**Paragon**
Dry/Low	1.58±0.2 (ab)	1.8±0.1 **(a)**
Dry/Med	1.48±0.1 **(a)**	1.95±0.1 (ab)
Dry/High	1.84±0.1 **(b)**	1.97±0.2 (ab)
Wet/Low	1.55±0.1 (ab)	1.8±0.3 **(a)**
Wet/Med	1.76±0.2 (ab)	2.1±0.3 **(b)**
Wet/High	1.73±0.2 (ab)	1.96±0.1 (ab)

	Experiment 1A Total Grain Number / spike; mean ± SD		Experiment 1B Total Grain Number / spike; mean ± SD		Experiment 3 Total Grain Number / spike; mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Cool	65.67±11.8 (a)	103.67±0.6 **(a)**	77.3±7.8 (a)	83.5±10.6 (a)	47.92±3 (a)	51.63±4.1 (a)
Dry/Warm	68±6.1 (a)	99.5±4.8 **(ac)**	71.17±2.4 (a)	99.2±9.4 (a)	46.07±1.9 (a)	53.15±4.3 (a)
Wet/Cool	58.5±9.4 (a)	96.3±11.6 (abc)	58.5±9.4 (a)	96.3±11.6 (a)	44.71±6.9 (a)	45.27±10 (a)
Wet/Warm	74.17±8.2 (a)	89±1.32 **(b)**	71.67±9.8 (a)	87.3±4.9 (a)	41.68±6.7 (a)	47.38±10 (a)

	Experiment 2 Total Grain Number / spike; mean ± SD	
Treatment	**Blasco**	**Paragon**
Dry/Low	37.17±4.2 (a)	39.96±2.2 (a)
Dry/Med	36.21±2 (a)	43±1.6 (a)
Dry/High	43.33±3 (a)	44.04±3.3 (a)
Wet/Low	36.21±3.4 (a)	42.17±2.6 (a)
Wet/Med	41.92±3.9 (a)	46.54±3.6 (a)
Wet/High	41.29 ±2.6 (a)	44.92±3.7 (a)

	Experiment 1A		Experiment 1B		Experiment 3		Experiment 2		
	Grain Size/ spike; mean ± SD		Grain Size / spike; mean ± SD		Grain Size / spike; mean ± SD			Grain Size / spike; mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Blasco**	**Paragon**	**Treatment**	**Blasco**	**Paragon**
Dry/Cool	0.45±0.001 (a)	0.042±0.002 (ab)	0.049±0.002 **(a)**	0.045±0.004 (a)	0.05±0.001 (a)	0.056±0.001 (a)	**Dry/Low**	0.042±0.001 (a)	0.045±0.001 (a)
Dry/Warm	0.04±0.004 (a)	0.04±0.001 **(a)**	0.046±0.0004 **(a)**	0.04±0.005 (a)	0.05±0.001 (a)	0.055±0.0003 (a)	**Dry/Med**	0.041±0.003 (a)	0.045±0.002 (a)
Wet/Cool	0.045±0.005 (a)	0.041±0.003 (ab)	0.045±0.005 (ab)	0.04±0.003 (a)	0.48±0.002 (a)	0.05±0.002 (b)	**Dry/High**	0.043±0.002 (a)	0.045±0.001 (a)
Wet/Warm	0.04±0.005 (a)	0.046±0.003 **(b)**	0.04±0.001 **(b)**	0.042±0.002 (a)	0.05±0.002 (a)	0.05±0.003 (b)	**Wet/Low**	0.043±0.002 (a)	0.043±0.004 (a)
							Wet/Med	0.042±0.02 (a)	0.045±0.003 (a)
							Wet/High	0.042±0.003 (a)	0.044±0.002 (a)

Analysis Variance of Evapotranspiration

Appendix 3.6: Analysis of Variance of evapotranspiration/treatment of Blasco in Experiment 1A.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	1526.26	1526.26	23.28	0.001
Temperature	1	578.7	578.7	8.83	0.018
Humidity.Temperature	1	208.33	208.33	3.18	0.113
Residual	8	524.59	65.57		
Total	11	2837.89			

Appendix 3.7: Analysis of Variance of evapotranspiration/treatment of Blasco in between the results at low RH/high air T and at high RH/low air T in Experiment 1A.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	1992.3	1992.3	69.68	0.001
Residual	4	114.37	28.59		
Total	5	2106.67			

Appendix 3.8: Analysis of Variance of evapotranspiration/treatment of Paragon in Experiment 1A.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	811.26	811.26	19.68	0.002
Temperature	1	1556.48	1556.48	37.76	<.001
Humidity.Temperature	1	9.48	9.48	0.23	0.644
Residual	8	329.78	41.22		
Total	11	2707			

Appendix 3.9: Analysis of Variance of evapotranspiration/treatment of Blasco in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	444.08	444.08	31.47	<.001
Temperature	1	240.01	240.01	17.01	0.003
Humidity.Temperature	1	1.12	1.12	0.08	0.785
Residual	8	112.89	14.11		
Total	11	798.1			

Appendix 3.10: Analysis of Variance of evapotranspiration/treatment of Paragon in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	348.48	348.48	10.77	0.011
Temperature	1	507	507	15.67	0.004
Humidity.Temperature	1	0.04	0.04	0	0.974
Residual	8	258.89	32.36		
Total	11	1114.41			

Appendix 3.11: Analysis of Variance of evapotranspiration/treatment of Paragon between the results at low RH/high air T and at high RH/low air T in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	848.07	848.07	21.3	0.01
Residual	4	159.26	39.81		
Total	5	1007.33			

Analysis Variance of grain weight

Appendix 3.12: Analysis of Variance of total grain weight/treatment in Blasco in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	33.99	33.99	12.74	0.007
Temperature	1	2.147	2.147	0.8	0.396
Humidity.Temperature	1	15.03	15.03	5.63	0.045
Residual	8	21.344	2.668		
Total	11	72.512			

Appendix 3.13: Analysis of Variance of total grain weight/treatment in Paragon in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	10.96	10.96	7.4	0.026
Temperature	1	0.155	0.155	0.1	0.755
Humidity.Temperature	1	16.605	16.605	11.21	0.01
Residual	8	11.854	1.482		
Total	11	39.574			

Appendix 3.14: Analysis of Variance of total grain weight/treatment in Blasco in Experiment 2.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	0.4883	0.4883	1.09	0.31
Soil Moisture	2	6.747	3.3735	7.55	0.004
Humidity.Soil moisture	2	1.802	0.901	2.02	0.162
Residual	18	8.0477	0.4471		
Total	23	17.0851			

Appendix 3.15: Analysis of Variance of total grain weight/treatment in Paragon in Experiment 2.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	0.2091	0.2091	0.45	0.513
Soil Moisture	2	7.2706	3.6353	7.75	0.004
Humidity.Soil moisture	2	0.9068	0.4534	0.97	0.399
Residual	18	8.4447	0.4692		
Total	23	16.8312			

Appendix 3.16: Analysis of Variance of total grain weight/spike/treatment in Paragon in Experiment 3.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	0.80773	0.80773	12.85	0.004
Temperature	1	0.00139	0.00139	0.02	0.884
Humidity.Temperature	1	0.01849	0.01849	0.29	0.598
Residual	12	0.75427	0.06286		
Total	15	1.58189			

Analysis Variance of Grain Number

Appendix 3.17: Analysis of Variance of total grain number/treatment in Paragon between the results at low RH/low air T and at high RH/high air T in Experiment 1A.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	4108.2	4108.2	8.91	0.041
Residual	4	1844.7	461.2		
Total	5	5952.8			

Appendix 3.18: Analysis of Variance of total grain number/treatment in Blasco in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	4524	4524	2.48	0.154
Temperature	1	10	10	0.01	0.943
Humidity.Temperature	1	17101	17101	9.36	0.016
Residual	8	14617	1827		
Total	11	36252			

Appendix 3.19: Analysis of Variance of total grain number/treatment in Paragon in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	2494	2494	1.37	0.276
Temperature	1	3104	3104	1.7	0.228
Humidity.Temperature	1	11347	11347	6.22	0.037
Residual	8	14600	1825		
Total	11	31545			

Appendix 3.20: Analysis of Variance of total grain number/treatment in Blasco in Experiment 2.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	281.3	281.3	2.79	0.112
Soil Moisture	2	5175.6	2587.8	25.65	<.001
Humidity.Soil Moisture	2	996.1	498	4.94	0.02
Residual	18	1815.7	100.9		
Total	23	8268.7			

Appendix 3.21: Analysis of Variance of total grain number/treatment in Paragon in Experiment 2.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	243.8	243.8	1.73	0.205
Soil Moisture	2	3422.9	1711.5	12.15	<.001
Humidity.Soil Moisture	2	147.5	73.8	0.52	0.601
Residual	18	2534.9	140.8		
Total	23	6349.1			

Analysis Variance of grain size

Appendix 3.22: Analysis of Variance of grain size/treatment in Paragon in Experiment 1A.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	2.73E-05	2.73E-05	7.29	0.027
Temperature	1	2.15E-07	2.15E-07	0.06	0.817
Humidity.Temperature	1	1.61E-05	1.61E-05	4.29	0.072
Residual	8	2.99E-05	3.74E-06		
Total	11	7.35E-05			

Appendix 3.23: Analysis of Variance of grain size/treatment in Blasco in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	4.96E-05	4.96E-05	6.82	0.031
Temperature	1	7.43E-06	7.43E-06	1.02	0.342
Humidity.Temperature	1	2.85E-05	2.85E-05	3.92	0.083
Residual	8	5.82E-05	7.28E-06		
Total	11	1.44E-04			

Appendix 3.24: Analysis of Variance of grain size/spike/treatment in Paragon in Experiment 3.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	1.21E-04	1.21E-04	35.28	<.001
Temperature	1	8.01E-07	8.01E-07	0.23	0.638
Humidity.Temperature	1	1.21E-07	1.21E-07	0.04	0.854
Residual	12	4.12E-05	3.43E-06		
Total	15	1.63E-04			

Appendix 3.25: Analysis of Variance of grain size per area in Blasco in Experiment 3.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	5.8E-07	5.8E-07	0.02	0.89
Temperature	1	0.000263	0.000263	9.03	0.011
Humidity.Temperature	1	2.44E-06	2.44E-06	0.08	0.777
Residual	12	0.000349	2.91E-05		
Total	15	0.000615			

Appendix 3.26: Analysis of Variance of grain size per area in Paragon in Experiment 3.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	2.67E-06	2.67E-06	0.51	0.489
Temperature	1	3.12E-04	3.12E-04	59.52	<.001
Humidity.Temperature	1	8.42E-06	8.42E-06	1.61	0.229
Residual	12	6.28E-05	5.23E-06		
Total	15	3.85E-04			

Analysis of Variance of Spike Temperature

Appendix 3.27: Analysis of Variance of Spike temperature/treatment in Blasco in Experiment 1A.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	0.485	0.485	0.23	0.642
Temperature	1	218.567	218.567	105.24	<.001
Humidity.Temperature	1	10.528	10.528	5.07	0.054
Residual	8	16.615	2.077		
Total	11	246.196			

Appendix 3.28: Analysis of Variance of spike temperature/treatment in Blasco between the results at high RH/low air T and at low RH/low air T in Experiment 1A.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	7.7672	7.7672	9.64	0.036
Residual	4	3.2224	0.8056		
Total	5	10.9896			

Appendix 3.29: Analysis of Variance of spike temperature/treatment in Paragon in Experiment 1A.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	0.0005	0.0005	0	0.962
Temperature	1	153.6345	153.6345	769.13	<.001
Humidity.Temperature	1	0.3123	0.3123	1.56	0.246
Residual	8	1.598	0.1998		
Total	11	155.5454			

Appendix 3.30: Analysis of Variance of spike temperature/treatment in Blasco in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	2.7552	2.7552	4.94	0.057
Temperature	1	295.4507	295.4507	529.79	<.001
Humidity.Temperature	1	17.24	17.24	30.91	<.001
Residual	8	4.4614	0.5577		
Total	11	319.9074			

Appendix 3.31: Analysis of Variance of spike temperature/treatment in Paragon in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	13.9378	13.9378	91.23	<.001
Temperature	1	151.993	151.993	994.84	<.001
Humidity.Temperature	1	0.6162	0.6162	4.03	0.079
Residual	8	1.2223	0.1528		
Total	11	167.7693			

Analysis of Variance of flag leaf temperature

Appendix 3.32: Analysis of Variance of flag leaf temperature/treatment in Blasco in Experiment 1A.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	6.247	6.247	2.41	0.159
Temperature	1	178.217	178.217	68.79	<.001
Humidity.Temperature	1	10.581	10.581	4.08	0.078
Residual	8	20.727	2.591		
Total	11	215.772			

Appendix 3.33: Analysis of Variance of flag leaf temperature/treatment in Paragon in Experiment 1A.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	0.7264	0.7264	1.73	0.225
Temperature	1	133.9507	133.9507	319.08	<.001
Humidity.Temperature	1	0.5408	0.5408	1.29	0.289
Residual	8	3.3584	0.4198		
Total	11	138.5764			

Appendix 3.34: Analysis of Variance of flag leaf temperature/treatment in Blasco in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	11.8074	11.8074	13.66	0.006
Temperature	1	233.1127	233.1127	269.71	<.001
Humidity.Temperature	1	18.4264	18.4264	21.32	0.002
Residual	8	6.9144	0.8643		
Total	11	270.2609			

Appendix 3.35: Analysis of Variance of flag leaf temperature/treatment in Paragon in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	3.3646	3.3646	6.14	0.038
Temperature	1	95.8381	95.8381	174.94	<.001
Humidity.Temperature	1	3.3224	3.3224	6.06	0.039
Residual	8	4.3828	0.5478		
Total	11	106.9079			

Appendix 3.36: Analysis of Variance of flag leaf temperature/treatment in Blasco in Experiment 2.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	4.7482	4.7482	9.93	0.006
Soil Moisture	2	0.0296	0.0148	0.03	0.97
Humidity.Soil Moisture	2	0.0223	0.0112	0.02	0.977
Residual	18	8.6056	0.4781		
Total	23	13.4057			

Analysis of Variance of spike temperature depression

Appendix 3.37: Analysis of Variance of spike temperature depression/treatment in Blasco in Experiment 1A.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	0.0028	0.0028	0.01	0.944
Temperature	1	20.9176	20.9176	39.83	<.001
Humidity.Temperature	1	25.6279	25.6279	48.8	<.001
Residual	8	4.2013	0.5252		
Total	11	50.7496			

Appendix 3.38: Analysis of Variance of spike temperature depression/treatment in Paragon in Experiment 1A.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	0.0276	0.0276	0.13	0.726
Temperature	1	71.888	71.888	343.63	<.001
Humidity.Temperature	1	0.6476	0.6476	3.1	0.117
Residual	8	1.6736	0.2092		
Total	11	74.2368			

Appendix 3.39: Analysis of Variance of spike temperature depression/treatment in Blasco in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	4.788	4.788	16.66	0.004
Temperature	1	11.5379	11.5379	40.15	<.001
Humidity.Temperature	1	18.4843	18.4843	64.32	<.001
Residual	8	2.2992	0.2874		
Total	11	37.1094			

Appendix 3.40: Analysis of Variance of spike temperature depression/treatment in Paragon in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	11.3154	11.3154	74.06	<.001
Temperature	1	69.9513	69.9513	457.85	<.001
Humidity.Temperature	1	0.7497	0.7497	4.91	0.058
Residual	8	1.2223	0.1528		
Total	11	83.2386			

Analysis of Variance of flag leaf temperature depression

Appendix 3.41: Analysis of Variance of flag leaf temperature depression/treatment in Blasco in Experiment 1A.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	1.077	1.077	1.2	0.305
Temperature	1	33.6508	33.6508	37.61	<.001
Humidity.Temperature	1	27.0951	27.0951	30.28	<.001
Residual	8	7.158	0.8948		
Total	11	68.9809			

Appendix 3.42: Analysis of Variance of flag leaf temperature depression/treatment in Paragon in Experiment 1A.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	1.1912	1.1912	2.63	0.144
Temperature	1	85.5424	85.5424	188.63	<.001
Humidity.Temperature	1	1.0655	1.0655	2.35	0.164
Residual	8	3.628	0.4535		
Total	11	91.4271			

Appendix 3.43: Analysis of Variance of flag leaf temperature depression/treatment in Blasco in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	13.5115	13.5115	15.63	0.004
Temperature	1	31.428	31.428	36.36	<.001
Humidity.Temperature	1	17.232	17.232	19.94	0.002
Residual	8	6.9144	0.8643		
Total	11	69.0859			

Appendix 3.44: Analysis of Variance of flag leaf temperature depression/treatment in Paragon in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	2.1456	2.1456	3.92	0.083
Temperature	1	118.8653	118.8653	216.97	<.001
Humidity.Temperature	1	3.6236	3.6236	6.61	0.033
Residual	8	4.3828	0.5478		
Total	11	129.0173			

Image 3.1: Infrared camera used to take infrared pictures (a) and an example for the recorded flag leaf and spike infrared images (b).

Chapter 4: Effects of Temperature, Relative Humidity and Soil Moisture on Breadmaking Quality of Two Spring Wheat (*Triticum aestivum* L.) Cultivars

4.1: Introduction and Literature

Bread is the major wheat product in most parts of the world. Even though wheat is being adapted to a broad range of weather conditions and geographical areas, bread making and other wheat end products quality can be adversely affected by growth conditions (Koga *et al.*, 2015). Storage proteins and starch are the primary component of the mature wheat grain and accumulate in the endosperm (Bhullar and Jenner, 1985; Csiszár *et al.*, 2010). Composition, quantity, and extent of polymerization of storage proteins determine the bread-making quality of wheat flour (Finney and Barmore, 1948; Payne *et al.*, 1987; Shewry *et al.*, 1992; Csiszár *et al.*, 2010; He *et al.*, 2013; Žilić, 2013) and are affected from the interaction of genotypes and environmental factors, such as water, nutrient availability (especially nitrogen and sulphur) and temperature (Randall and Moss, 1990; Blumenthal *et al.*, 1993; Stone and Nicolas, 1994; Zhao *et al.*, 1997; Johansson *et al.*, 2002; Flæte *et al.*, 2005; Pompa *et al.*, 2013; Koga *et al.*, 2015; Koga *et al.*, 2016). It has been suggested that drought and even more so high temperature during and post anthesis (Ottman *et al.*, 2000; Rharrabti *et al.*, 2003; DuPont *et al.*, 2006; Dupont *et al.*, 2006; Pompa *et al.*, 2013; Aslani *et al.*, 2013; Ashraf, 2014; Koga *et al.*, 2015) are the main factors limiting wheat grain quality. Nevertheless, different kinds of stress and their severity cause differential impacts on grain quality and composition. For instance, while drought stress causes increase in dough strength and decrease in bread loaf volume and dough extensibility, heat stress causes a small decrease in dough strength and an increase in bread loaf volume and dough extensibility (Aslani *et al.*, 2013; Li *et al.*, 2013). The opposite impact on dough rheological properties affecting bread-making quality is likely due to different impact on specific subtypes of gluten protein and glutenin polymers composition and size distribution (Li *et al.*, 2013). Alpha-amylase activity of flour, which degrades starch granules, is also an important parameter for bread making process and present both a genetic and an environmental component (McCleary *et al.*, 2002; Csiszár *et al.*, 2010; Tipples, 1969). The aim of our study was to examine the impact of relative humidity, temperature, soil moisture and their interaction on the breadmaking quality of two different wheat cultivars, Paragon and Blasco, developed for cultivation in the UK and the Mediterranean climate, respectively.

4.1.1: Wheat grain

The botanical term for wheat grain/kernel, one seeded fruit, is caryopsis. Each grain consists of the seed coat (testa), endosperm and germ (embryo). Endosperm is surrounded by a nucellar epidermis, while the seed is enclosed by a fruit coat (pericarp) which adheres to the testa (Eliasson and Larsson, 1993). Primary storage tissue of the grain is the starchy endosperm, comprising over 80% of the grain dry weight. The starchy endosperm consists of protein, starch and cell wall polysaccharides, representing 8-12%, 70-80% and 2-3% of the starchy endosperm, respectively (Halford and Karp, 2010; Tosi *et al.*, 2011). Gluten proteins are restricted to the starchy endosperm cells of the grain and interact to form large polymers during grain development (Shewry *et al.*, 2002; Tosi *et al.*, 2011). Proteins in the starchy endosperm are not distributed homogeneously, with two or three layers of sub-aleurone cells containing fewer starch granules and higher protein than the cells in the central starchy endosperm (He *et al.*, 2013).

4.1.2: Wheat gluten Proteins

Work by Žilić *et al.* (2010) indicated that total protein content ranges from 11.46 to 16.53% in durum wheat and from 10.87 to 13.04% in bread wheat genotypes. Variation in the protein content is largely due to environmental rather than to genetic factors (Graybosch *et al.*, 1996; Huebner *et al.*, 1997; Zhu and Khan, 2001; Žilić, 2013). Because of the large complexity of wheat proteins and their various interactions with each other, their characterisation is not straightforward. Wheat storage proteins are comprised of gluten proteins and non-gluten proteins. Gluten proteins are classically separated into two major fractions: polymeric glutenins and monomeric gliadins. Glutenin and gliadins, major wheat storage proteins, together form 80-85% of the total grain protein content in bread wheat (common wheat) with a ratio of about 1:1 (Abdel-Aal *et al.*, 1996). Non-gluten proteins are classified into globulins (8% of the total kernel protein) and albumins (12% of the total kernel protein) (van den Broeck *et al.*, 2009; He *et al.*, 2013; Žilić, 2013; Koga *et al.*, 2015). Globulins (salt soluble) and albumins (water soluble) have little impact on the dough quality, however, they have good nutritional properties due to their balanced amino acid profile (Ashraf, 2014). Gliadins are soluble in alcohol-water mixtures, exhibit strong non-covalent protein-protein hydrophobic interaction mostly via hydrogen bonds and determine viscosity and extensibility of the dough. They can be classified into α-, β-, Ɣ-, and ω-gliadins having a molecular weight in the range of 30 to 80 kDa, depending on the mobility on SDS-PAGE. Glutenins are stabilized by interchain disulphide bonds, are alcohol soluble only under reducing conditions and they provide elasticity/strength to the dough (Shewry and Gutteridge,

1992; He *et al.*, 2013; Žilić, 2013; Koga *et al.*, 2015). Glutenins can be divided into the low molecular weight (LMW) and the high molecular weight (HMW) subunits having molecular weight in the range 30 to 74kDa and 75 to 120 kDa, respectively (Žilić, 2013). HMW glutenin subunits promote the formation of large glutenin polymers, thus play an important role in dough strength (i.e. elasticity) (Shewry *et al.*, 2002; He *et al.*, 2013). The exact balance between the glutenin (elasticity) and gliadin (viscosity) is essential for bread making (Primard *et al.*, 1991; Žilić, 2013). Therefore, the amounts and combinations of glutenin and gliadins subunits can be fixed in breeding programs to improve the end use quality of wheats (Primard *et al.*, 1991).

4.1.3: Starch and alpha-amylase activity

Starch, the main carbohydrate in the endosperm, is deposited in the form of A-type granules (diameter>9.9µm) and B-type granules (diameter <9.9µm), during grain development (Geera *et al.*, 2006; Torbica *et al.*, 2010; Zeng *et al.*, 2011). Starch represents 70-80% of the endosperm dry weight and 65-75% of the dry grain weight (Zhang *et al.*, 2017; Torbica *et al.*, 2010; DuPont and Altenbach, 2003). It is comprised of two kinds of polysaccharide molecules namely amylopectin (75% of dried mass) and amylose (25% of dried mass). The ratio of these two polymers (amylose to amylopectin) determines functionality and physical properties of starch (Csiszár *et al.*, 2010; Whan *et al.*, 2014; Zeng *et al.*, 2011). There are two types of starch which accumulate in cereal plants, transitory and reserve starch. Transitory starch piles up within the day in the chloroplast of leaf cells as a result of photosynthesis and is degraded to sugar during the night to be used in the other part of the plants. Reserve starch forms in amyloplasts, primarily inside the storage tissues of plants e.g. seeds, and is used as a fuel for the growth of seedling upon germination and sprouting (Whan *et al.*, 2014; Marston *et al.*, 2015).

Alpha-amylase is an essential enzyme to initiate starch-degradation in the grain endosperm of cereals. It hydrolyses the α-1,4-glucosidic linkages of starch, linking glucose residues to form amylose linear chain, for degradation to produce dextrins, maltose and glucose. The dextrins and maltose are then degraded by β-amylase, phosphorylase and glucosidases which in turn produce glucose-6-phosphate and glucose. The products of the degradation processes supply energy source and substrates for embryo during germination (Reddy *et al.*, 1984; Csiszár *et al.*, 2010). During wheat grain development, α-amylase activity increases, reaching its peak 16 days after heading, to decrease then to close to zero during ripening phase (Csiszár *et al.*, 2010). Following the initiation of germination, α-amylase is produced at an

increasing rate in the embryo and layers covering the endosperm and breaks down starch into sugars in the sprouting seed. Excessive α-amylase amount is correlated with preharvest sprouting and grain germination before harvest in the ear.

4.1.4: Breadmaking quality

There are four main features of the wheat grain considered by breeders when developing wheat crops having better grain quality. These features are grain milling texture, storage protein content, alpha amylase content and protein quality (Griffin, 1983). Protein content (%) is an important parameter to determine development time of dough and water absorption of flour, however it is not enough to test the breadmaking quality (Lindeque *et al.*, 2017). Therefore, in order to understand molecular basis of bread making quality and the effect of environmental factors on wheat quality properly, both protein quantity (percent grain protein content) and protein quality (glutenin, gliadin and albumin/globulin concentration) need to be examined (Zhu and Khan, 2001; Lindeque *et al.*, 2017).

Grain processing quality is determined by gluten, glutenins and gliadins proteins, which are recognised as the major storage proteins in the mature wheat grain (Tosi *et al.*, 2011). Once wheat flour (white flour, milled from endosperm) is mixed with water and kneaded to form dough, these storage proteins constitute a network called gluten, a cohesive mass. Gluten has two physical properties, elasticity and extensibility, which allow dough to expand (i.e. viscous flow) by fermentation and give leavened bread and other products. (Shewry and Gutteridge, 1992; Shewry *et al.*, 2002; Tosi *et al.*, 2011). While highly extensible doughs are necessary to make biscuits and cakes, very elastic doughs are needed for breadmaking (Shewry *et al.*, 2002). In countries using the Chorleywood Breadmaking Process (CBP), such as the UK, a minimum of 13% grain protein content is required for breadmaking, while 12 % is needed as a minimum for other breadmaking processes (He *et al.*, 2013). There are many tests to assess protein quality and in this study we have used SDS-sedimentation in conjunction with SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Zhu and Khan, 2001).

Starch granules in wheat flour are broken down to fermentable sugars by α-amylase during bread-making process when mixed with water. However, if α-amylase is too high, excessive starch break down can occur through the bread baking process, resulting in sticky crumb and bread slicing problems (McCleary *et al.*, 2002; Csiszár *et al.*, 2010). Since alpha amylase activity is associated with flour quality and presprout damage, the impacts of abiotic stresses on alpha-amylase activity during germination

and maturation stages of wheat are object of intense research. There are many tests, which are classified as nephelometric (particle concentration in a liquid), viscometric and colorimetric, to assess either directly or indirectly the *alpha*-amylase activity. In this study we have used CERALPHA (Megazyme) method to assess the *alpha*-amylase activity.

4.1.3.1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of flour protein allows their separation into high-molecular-weight (HMW) glutenins, low-molecular-weight glutenins and gliadins (Murphy and Peterson, 2000) on the basis of their molecular weight and allows quantitative analyse of separated gluten protein fractions (Rédei, 1998; Wan *et al.*, 2013). In this method, proteins are treated with sodium dodecyl sulfate (SDS), an anionic detergent which breaks the non-covalent bonds of the proteins, while the addition of a reducing agents such as 2-mercaptoethanol and Dihiothreitol (DTT) allows the disruption of the disulphide bonds in peptides and proteins. Therefore, tertiary structure of proteins is disturbed, and folded proteins turn to linear molecules. In addition to this, proteins are coated with a uniform negative charge because of SDS binding. Following this, proteins are separated by polyacrylamide gel electrophoresis (PAGE) according to their molecular weight instead (Hames, 1998).

4.1.3.2: SDS-sedimentation test

SDS-Sedimentation tests have been developed to predict breadmaking quality of wheat flour and meals on the basis of the polymeric protein formation (Morris *et al.*, 2007; Rittau *et al.*, 2005). There is in fact a significant positive statistical correlation between HMW glutenin subunits, gluten strength analysed in terms of SDS-Sediment and breadmaking quality (Czuchajowska *et al.*, 1996; Schuster *et al.*, 1997; Dhaka and Khatkar, 2015). Lactic acid solution including isopropyl alcohol (Zeleny sedimentation) or detergent sodium dodecyl sulphate (SDS sedimentation) is used in sedimentation techniques to hydrate small flour samples. The hydrated flour particles sink as a form of sediment and the level of the sediment volume correlates with the amount of glutenin (strength of the gluten). Higher sedimentation volume demonstrates that the gluten is stronger (Rittau *et al.*, 2005).

4.1.3.3: CERALPHA (Megazyme) method

There are many tests to assess, either directly or indirectly, *alpha*-amylase activity of flour. Ceralpha (Megazyme) is a direct colorimetric method. Amylase HR reagent,

consisting of non-reducing end blocked p-nitrophenyl maltoheptaoside (BPNPG7) and α-glucosidase, is used in the Ceralpha procedure (BHANDARI, 2002). Because of endo-acting α-amylase, hydrolysis of the oligosaccharide occurs. Excess amount of α-glucosidase causes hydrolysis of the *p*-nitrophenyl maltosaccharide fragment to glucose and free *p*-nitrophenol. Weak alkaline solution addition terminates the reaction, and the absorbance value observed at 400nm directly shows the α-amylase in the sample analysed (Megazyme, 2007).

4.1.3.4: Quantitative protein and nitrogen analyses

We have used Direct Detect Spectrometer to measure the concentration of the extracted total protein and gluten protein. The spectrometer examines protein and peptide concentration with quantifying peak high (intensity) of the Amide 1 band associated with 80% C=O stretching vibration and 20% C-N stretching vibration of the peptide bond. Analysis method 2 (AM2) in the spectrometer was used, since it allows quantitation of protein/peptide solubilized in Tris buffer regardless of the interference caused by the buffer. Besides this we have used the LECO CHN628 Elemental Analyser to measure total nitrogen concentration of the samples. This instrument utilizes temperatures up to 1050 °C to combust the organic samples in pure oxygen. Following the combustion of the samples with oxygen, the combustion gas (nitrogen oxide) is converted to nitrogen using chemical reagents, and then nitrogen is detected by thermal conductivity sensors. LECO CHN628 makes it possible to obtain fast results with low cost per analysis (less usage of chemical reagents) and it is easy to use it.

4.2: Methodology

4.2.1: Harvesting

Wheat plants grown in pots in Experiments 1 and Experiment 2 and tagged wheat ears in the field in Experiment 3 were hand harvested at maturity (Zadox`s Growth Stage 90). Following this, spikes of the crops were threshed using a plastic cylinder. Crops in selected circle areas (0.1 m^2)/treatment in Experiment 3 were also hand harvested at maturity, but spikes were threshed using a mechanical thresher (Wintersteiger, Hege 16).

4.2.2: Alpha-amylase activity

4.2.2.1: Assessing alpha-amylase activity

Alpha-Amylase activity within the wholemeal samples of wheat was estimated using the α-amylase kit (Ceralpha, Megazyme Co., Ltd., Wicklow, Ireland). 1 g wholemeal

wheat sample per pot (bulked grains) was extracted with 6 ml of extraction buffer solution (pH 5.4). 0.2 mL Aliquots of extract (including α-amylase) were incubated for 20 min with 0.2 mL Amylase HR Reagent (substrate mixture) under manufacturer-defined temperature and pH conditions. The reaction was then terminated by adding 3 mL Stopping Reagent (weak alkaline solution) which developed colour. The absorbance values of the solution and reaction blank were measured against distilled water at 405 nm using a spectrophotometer (Multiskan ascent, Thermo Labsystems). The same procedure was followed for the reaction blank solution but without using flour sample. Absorbance value is directly related to the α-amylase activity in the analysed sample. One unit of *α-amylase* activity represents the amount of enzyme needed to release 1 µmol of p-nitrophenol per minute under defined assay conditions. Results were shown in Ceralpha Units (CU) on a dry basis.

4.2.2.2: Preparation of extraction buffer, substrate (Amylase HR Reagent) and stopping reagent

Concentrated enclosed extraction buffer (50 mL, pH 5.4), containing 1M sodium malate, 1M sodium chloride, 40 mM calcium chloride and 0.1% sodium azide, was diluted to 1000 mL with distilled water. Enclosed substrate, containing nonreducing end-block p-nitrophenyl maltoheptaoside (BPNPG7) in the presence of excess amount of thermostable alpha-glucosidase was dissolved in 10 mL of distilled water. Concentrated enclosed stopping reagent (25 mL), contains 20% [w/v] tri-sodium phosphate solution, pH ~11), was diluted to 500 mL with distilled water.

4.2.2.3: Calculation of the *α*-amylase activity in ceralpha unit

The Ceralpha unit was calculated using the following equation:

$$\text{Unit(CU)/g flour} = \frac{\Delta E405}{\text{Incubation time}} \times \frac{\text{Total volume in cell}}{\text{Aliquote assayed}} \times \frac{1}{\text{EmM}} \times \frac{\text{Extraction volume}}{\text{Sample weight}} \times \text{Dilution}$$

$$\Delta E_{405} = \text{Absorbance(Reaction)} - \text{Absorbance(Blank)}$$

Incubation Time = 20 min (wheat extract)
Total Volume in Cell = 0.2 mL
Aliquot Assayed = 0.2 mL
E_{mM} of p-nitrophenol (at 400 nm) in 1% tri-sodium phosphate = 18.1
Extraction Volume = 6 ml per gram wheat flour

4.2.3: Total grain protein content

Total protein in the carefully weighted (0.10 -0.15 g) wholemeal flour samples was extracted by adding 25µL extraction buffer (combination of Tris-HCL, SDS and DTT) per mg of flour. Samples were denatured by incubating at 95°C for 5 min and were then centrifuged for 10 min at 14,000 rpm during the extraction process. Extracts were diluted 1:1 with distilled water, and their protein concentration then was measured using Direct Detect Spectrometer (EMD Millipore Corporation, Billerica, MA, U.S.A.). Analysis method 2 (AM2) in the spectrometer was used, since it allows quantitation of protein/peptide solubilized in Tris buffer regardless of the interference caused by the buffer. Protein content of the grains was measured on a dry matter basis.

4.2.3.1: Materials

Total protein extraction Buffer: 50 mM Tris-HCL (pH 6.8), 4 % (w/v) sodium dodecylsulphate (SDS) and 1.5% dithiothreitol (DTT).

4.2.4: Gluten protein content: SDS-page

4.2.4.1: 50% 1-Propanol protein extraction

50% 1-Propanol was used to extract gluten subunits which were then separated by SDS-PAGE (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis) for densitometric analysis. The protocol (Fig. 4.1) to exclusively extract gluten protein and produce clear bands on acrylamide gels was modified from a two-step gluten extraction procedure described by Broeck (van den Broeck *et al.*, 2009). A combination of 1-Propanol and 2-Mercaptoethanol (βME) was used for the gluten protein extraction buffer. Firstly, high molecular weight subunits and gliadin proteins were extracted twice from each flour sample/pot. The extracts were pooled to collect the extracted gluten proteins in one sample, dried in a concentrator (Eppendorf^{TM} concentrator^{TM} plus, Hamburg, Germany) to evaporate 1- propanol and then resuspended in 1x LDS (Lithium dodecyl sulfate) sample loading buffer. Following this, proteins remaining in the pellet were extracted by applying total protein extraction buffer containing the same substances as in the loading buffer. A ratio of 1 mg flour: 25 µl extraction buffer was used for each extraction. Since considerable amount of HMW gluten subunits (10-20%) remained in the residual pellet, the residual pellet extracts were also examined in the SDS-PAGE.

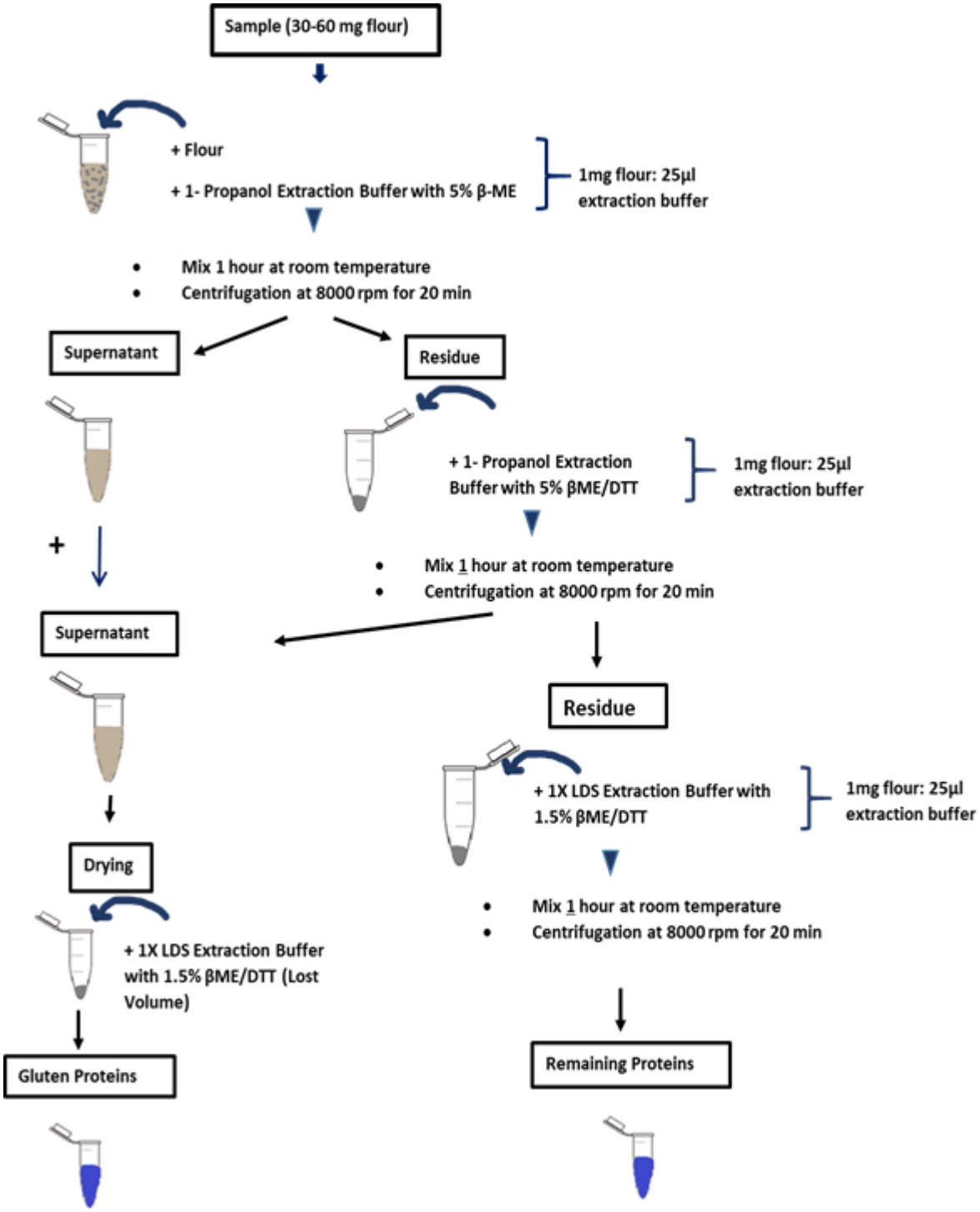


Figure 4.1: 50% 1-Propanol protein extraction protocol

4.2.4.2: Total protein extraction

A combination of 1X LDS Buffer and 2-mercaptoethanol (1mg flour: 25µl extraction buffer) was used to extract total protein from each flour sample/pot as shown in Fig. 4.2. Total protein extracts were also examined in the SDS-PAGE.

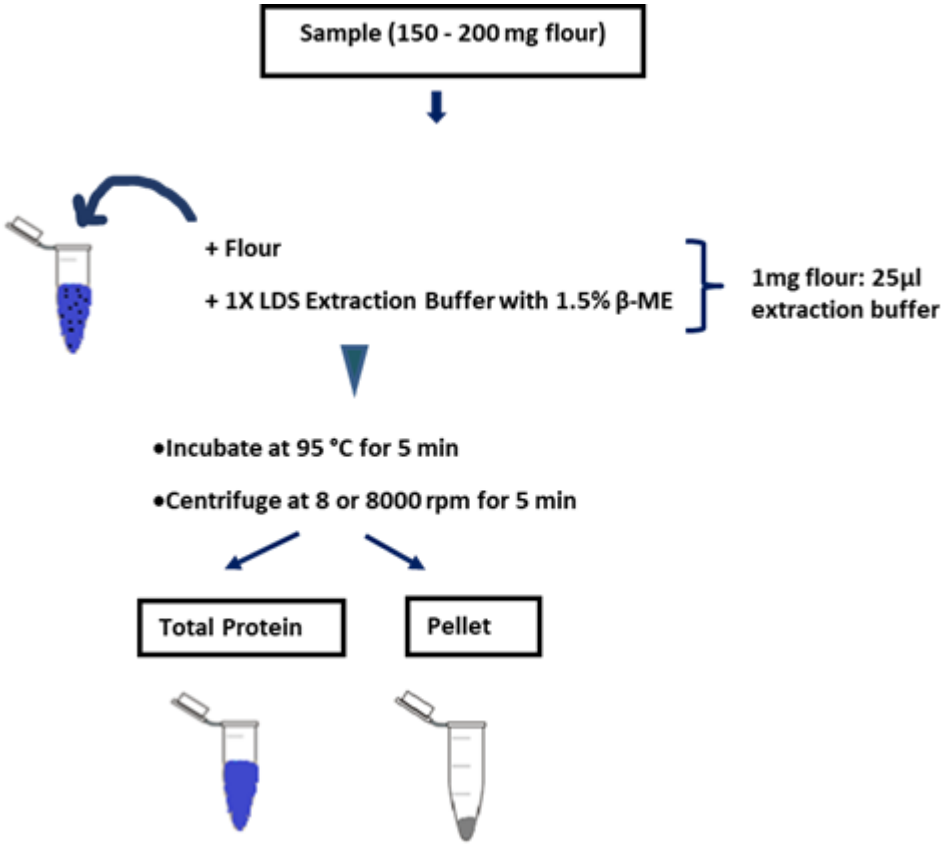


Figure 4.2: Total protein extraction protocol

4.2.4.3: SDS-PAGE gel scanning

Protein extracts (total protein, propanol and residual pellet extracts) were separated on acrylamide gels (10%) (ThermoFisher, Invitrogen™, USA) using SDS-PAGE technique. The gels were run at 200V until the two lowest protein bands run off the gel for better separation. The bands were then stained 90 min using PageBlue™ (ThermoFisher, Invitrogen™, USA) and de-stained 5 min with distilled water. Gels were scanned straight away after de-staining with an imager (GelDoc-It^{TS2} Imager, Germany) and proteins were quantified using Image J software (Wayne Rasband, National Institutes of health (NIH)).

4.2.4.4: Densitometric analysis

Protein bands corresponding to the extracts were divided into three main subgroups: HMW-GS, ω-gliadins and LMW-GS+ α-/γ-gliadins (Image 4.1). Volume of protein bands (total intensity of pixels within a band/area) was read by Image J software. Volume of the bands in residual pellet extracts was added to the volume of the bands in the propanol extracts. The percentage of each protein subgroup was calculated using the following formula:

$$\%\ \text{gluten protein subgroup} = \frac{\text{Gluten protein subgroup}}{\text{Total gluten protein}} \times 100$$

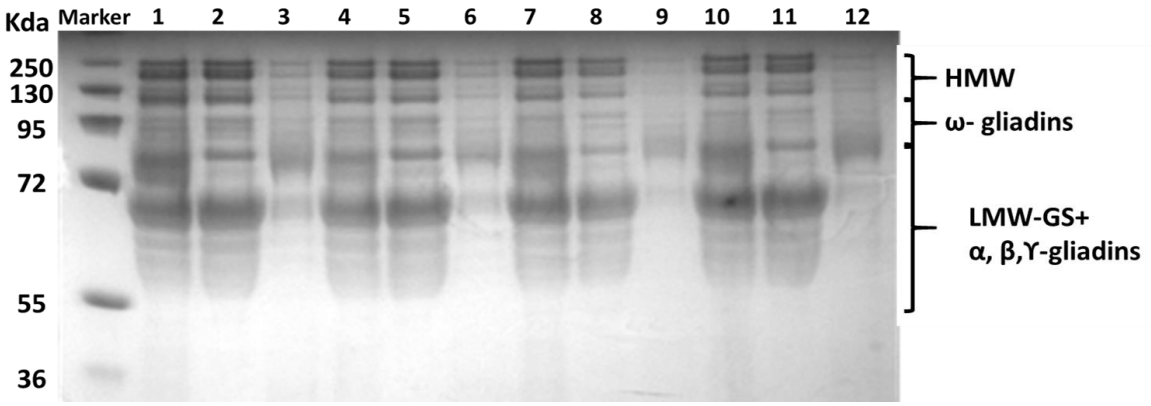


Image 4.1: SDS-PAGE of protein extracts from wholemeal flour. First well shows pre-stained protein ladder. 1,4,7,10 represent total protein extracts, 2,5,8,11 represent propanol extracts while 3,6,9,12 represent residual pellet extracts. The amount of loaded protein in each line is the same. The position on the gel of the main gluten protein subgroups are shown on the right position of the gel.

4.2.4.5: Materials

Gels: ThermoFisher, Invitrogen Bolt^{TM}10% Bis-Tris gels, 1.0 mmx15 wells

Running buffer: 1X Bolt™ MES SDS Running Buffer

Total protein extraction buffer: 1X LDS buffer, 5% 2- Mercaptoethanol

Gluten protein extraction buffer: 50% 1-propanol and 5% 2-Mecaptoethanol

Loading buffer: 1x LDS buffer and 1.5% 2-Mercaptoethanol

4.2.5: Sodium dodecyl sulfate (SDS) sedimentation

Gluten strength and baking quality of wheat wholemeal samples were examined using SDS-sedimentation test. Sedimentation value is determined by the swelling capacity of gluten proteins in a flour sample suspended in an SDS medium. Prior to SDS sedimentation test, SDS medium was prepared by adding 20 g of pure SDS powder to 1 litre purified water and then adding 20 ml diluted lactic acid solution after the powder dissolved completely. In order to prepare dilute lactic acid solution 10 ml of 88%, lactic acid was diluted in 80 ml purified water. Moisture content (*MC*) of flour samples was calculated in advance using the following AACC International approved formula:

$$MC\,(\%) = 100 \times \frac{(P+F) - (P + \text{Dried}\quad F)}{(P+F) - P}$$

Where ***P***, ***F***, ***Dried F*** represent empty container weight, flour sample weight and flour sample weight after 48 hours drying, respectively.

A flour sample weighing 6 g with 15% moisture content is required for SDS Sedimentation test. Since the moisture content of each sample was different the formula below was used to calculate the equivalent amount of flour per sample (*Wt)*.

$$Wt = (MC \times 0.0873) + 4.7067$$

Prescribed weight of four was added to 50 ml of purified water in a 100ml cylinder, which was shaken rapidly for 15 min to disperse the flour. The solution in the cylinder was shaken (for fifteen seconds) 2 min and 4 min after addition of water. 50 ml of SDS-lactic acid reagent was added immediately after the last shake using an automatic dispenser and mixed in by inverting the cylinder four times. Inversion (four times) was repeated 8 min, 10 min and 12 min after addition of the reagent, the sedimentation volume was read after 20 min.

4.2.6: Total nitrogen concentration

The LECO CHN628 series Elemental Analyser was used to assess total nitrogen. Wholemeal wheat samples weighing 0.2 g with 6-14% moisture content, were encapsulated as small balls with tin foil cups and then loaded into the LECO autoloader. Resulting nitrogen % content was converted to protein by multiplying nitrogen content by factor 5.7 and results then reported as a protein concentration.

4.2.7: Total gluten protein concentration

Wholemeal wheat samples (100 mg) were extracted twice with 1.0 mL of solvent (0.4 mol/L NaCl) at room temperature. Each extraction step began with 2 min vortexing at room temperature which continued with 10 min magnetic stirring at 60 rpm, and then samples were centrifuged for 15 min at 7,000 rpm. The supernatant from both extractions was discarded.

The resulting pellet was then extracted twice with 2 mL extraction buffer containing 62.5 mM Tris-HCL (pH 6.8), 2% SDS and 5% 2-Mercaptoethanol. Each extraction step began with 2 min vortexing at room temperature, which continued with 10 min magnetic stirring at 60 rpm, and then samples were centrifuged for 15 min at 7,000 rpm. Corresponding supernatants containing gluten proteins were combined at the end and their gluten concentration was measured with using Direct Detect Spectrometer (EMD Millipore Corporation, Billerica, MA, U.S.A.). Analysis method 2 (AM2) in the spectrometer was selected to be used, since it allows quantitation of protein/peptide

solubilized in Tris buffer regardless of the interference caused by the buffer. Gluten protein content of the grains was expressed on a dry matter basis.

4.2.8: Statistical analysis

All statistical analyses were performed using GenStat software (version 17.1.0.13780, VSNI international Ltd). Relative humidity, temperature and variety were considered as categorical predictors in the control cabinet and field experiments, while relative humidity, soil moisture and variety were considered as categorical predictors for polytunnel experiment. An ANOVA was used to assess differences between treatments, a complete randomised block design was used for all experiments. Treatment effects were considered significant at $p<0.05$. When significant differences were observed, pair-wise multiple comparisons were performed using Tukey test ($p<0.05$) and T-test ($p<0.05$) to discriminate differences among the treatments and between the cultivars. Shapiro-Wilk normality test ($p<0.05$) and Bartlett`s test for homogeneity of variances ($p<0.05$) were carried out prior to each ANOVA. Data found not to satisfy these assumptions were transformed to attain homoscedasticity and normal distribution (Rasmussen, 1985; Bland and Altman, 1996; McDonald, 2009).

4.3: Results

The three experiments were conducted over three successive growing seasons to investigate the effect of manipulated air temperature (T), relative humidity (RH) and soil moisture on grain quality of two spring wheat cultivars, Blasco and Paragon. Experiments 1, 2 and 3 represent control cabinet Experiment, polytunnel Experiment and field Experiment, respectively. While air T and RH treatments were applied to the cultivars in Experiment 1 and Experiment 3, soil moisture and RH treatments were applied in the Experiment 2. In Experiment 1A treatments were applied at flowering stage, while in Experiment 1B, treatments were applied 12 days after anthesis.

Significant impacts of Experimentally applied RH, air T and soil moisture treatments have been noticed in all three experiments and are showed in the following sections. Summary tables showing the mean and standard deviation values and Analysis of Variance of the grain quality parameters are shown in Appendix section of this chapter.

4.3.1: Effects of relative humidity and air temperature on alpha-amylase activity and total protein content

4.3.1.1: Alpha amylase activity

Although we have not observed any effect on α-amylase activity in Experiment 1A for any of the two cultivars (Appendix 4.1) at different RH conditions, α-amylase activity of Paragon at low RH/high air T was significantly lower than those at low RH/low air T ($p<0.001$) and high RH/low air T ($p=0.001$) in Experiment 1B, as shown in Fig. 4.3.

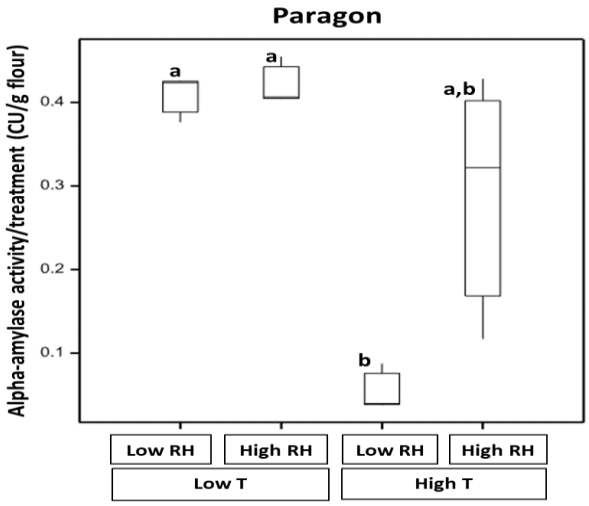


Figure 4.3: Effect of temperature and humidity manipulations, applied for three consecutive days during early grain filling stage, on α-amylase activity in Paragon wheat cultivar (Experiment 1B). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

4.3.1.2: Total protein content – Direct Detect Spectrometer

In Experiment 1A, we observed that total protein content in Blasco was higher at high RH/low air T than at low RH/high air T ($p=0.041$, Fig. 4.4a). In Experiment 1B, total protein content in Blasco was affected by RH, as at lower air T, it had higher total protein content at high RH than at low RH ($p=0.004$, Fig. 4.4b).

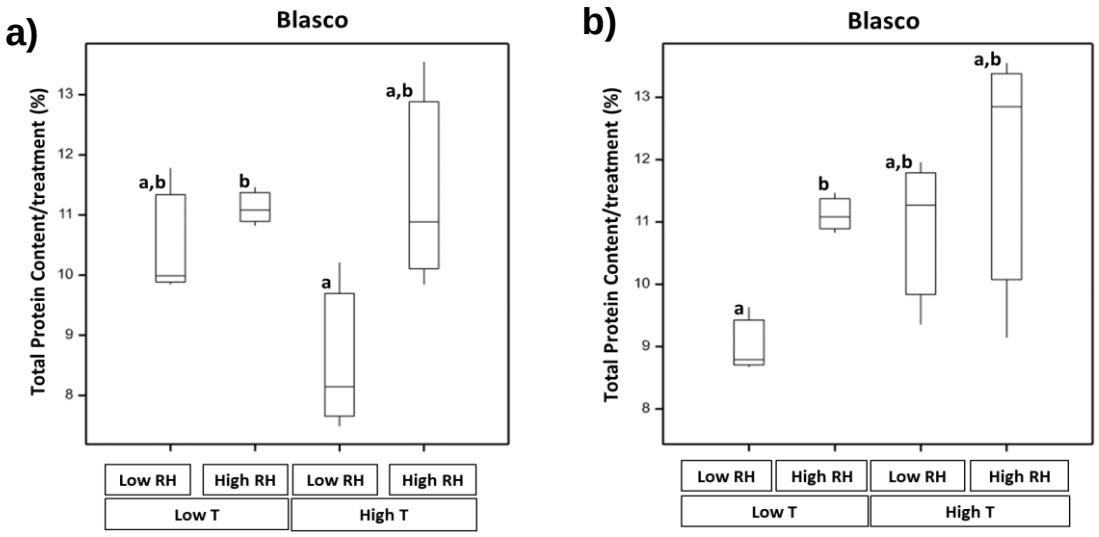


Figure 4.4: Total protein content in Blasco wheat cultivar following the three consecutive days of temperature and humidity treatments at the flowering stage (a) (Experiment 1A), and during early grain filling stage (b) (Experiment 1B). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

4.3.2: Effects of relative humidity, air temperature and soil moisture on total nitrogen and gluten protein content

4.3.2.1: Total nitrogen content - Elemental Analyser

In Experiment 1A, we have not observed any significant effect of the treatments on grain N content for any of the two cultivars (Appendix 4.1). However, both cultivars had higher total nitrogen content at high RH/high air T than those at low RH/low air T (Blasco, $p=0.027$, Fig. 4.5 a; Paragon, $p=0.024$, 4.5 b) in Experiment 1B. Besides this, Paragon had higher N content at higher air T than those at lower air T regardless what was the RH ($p<0.001$).

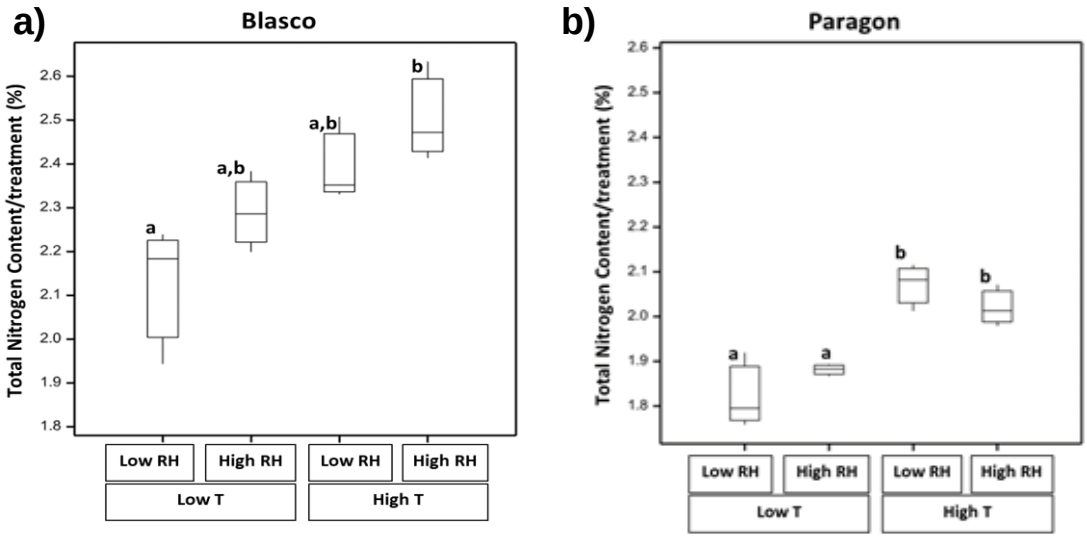


Figure 4.5: Total nitrogen content in Blasco (a) and Paragon (b) wheat cultivars following the exposure to the temperature and RH treatments three consecutive days at early grain filling stage (Experiment 1B). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

In Experiment 2, Blasco had higher total nitrogen content at low RH/low soil moisture than at high RH/medium soil moisture and high RH/high soil moisture (p=0.031, Fig. 4.6 a), while Paragon had higher total nitrogen content at high RH/low soil moisture than at low RH/high soil moisture and low RH/medium soil moisture (P=0.0016, Fig. 4.6 b). Besides this, Paragon had higher total nitrogen content at low soil moisture than at medium soil moisture (p=0.0016, Fig. 4.6 b).

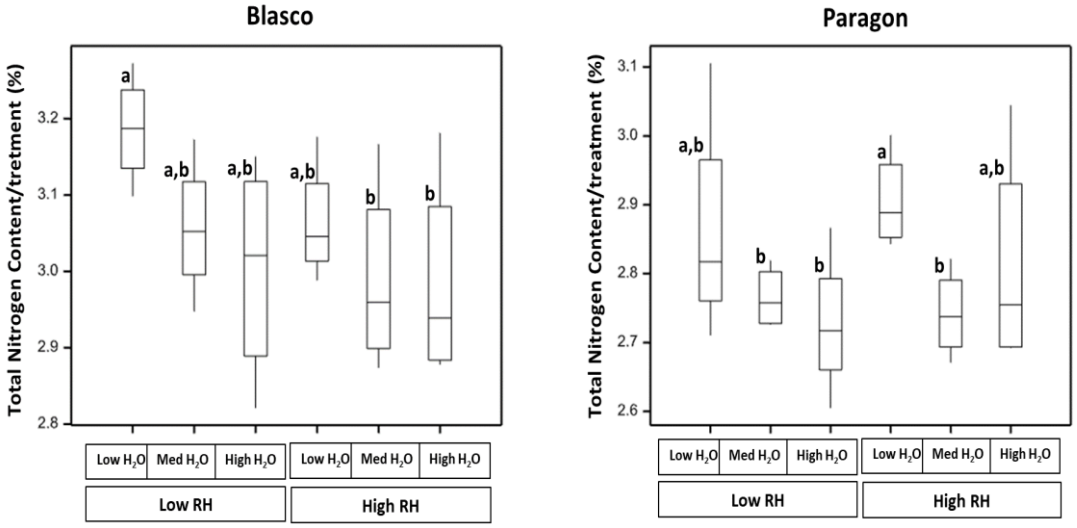


Figure 4.6: Effect of soil moisture and humidity manipulations, applied during the life cycle of the cultivars, on total nitrogen content in Blasco (a) and Paragon (b) wheat cultivars (Experiment 2). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

As shown in Fig 4.7 a and 4.7 b, total nitrogen content of Blasco (p=0.009) and Paragon (p=0.007) was significantly higher at high RH/high air T than at low RH/low air T in Experiment 3.

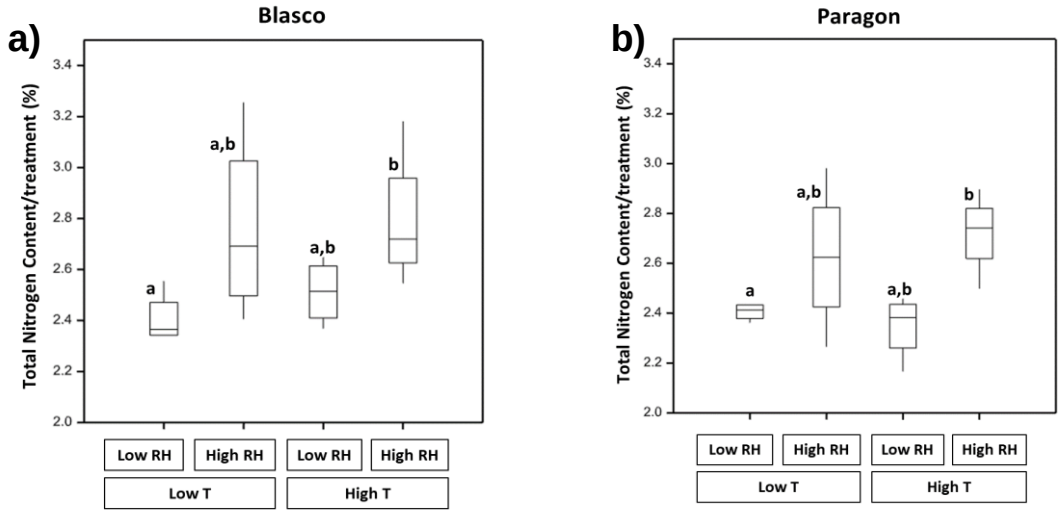


Figure 4.7: Total nitrogen content in Blasco (a) and Paragon (b) wheat cultivars following the exposure to the temperature manipulations three consecutive days at the flowering and early grain filling stage and humidity manipulations during their life cycles (Experiment 3). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

4.3.2.2: Gluten protein content - Direct Detect Spectrometer

Gluten protein content of Blasco was affected from RH (p=0.005) in Experiment 2. As shown in Fig. 4.8, under low soil moisture conditions, Blasco had lower gluten protein content at high RH than at low RH. It also had lower gluten protein content at high RH/high soil moisture than those at low RH/low soil moisture.

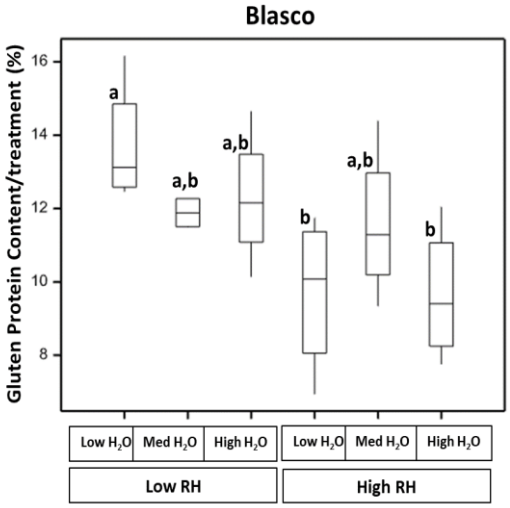


Figure 4.8: Gluten protein content in Blasco wheat cultivar after exposing to the humidity and soil moisture manipulations during its life cycle (Experiment 2). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

In experiment 3, at lower air T, Blasco had higher gluten protein content at high RH than at low RH ($p=0.022$, Fig. 4.9 a), while an interactive effect of RH and air T on gluten protein content of Paragon has been observed. Paragon had lower gluten protein content at low RH/high air T than at high RH/low air T ($p=0.037$, Fig. 4.9 b).

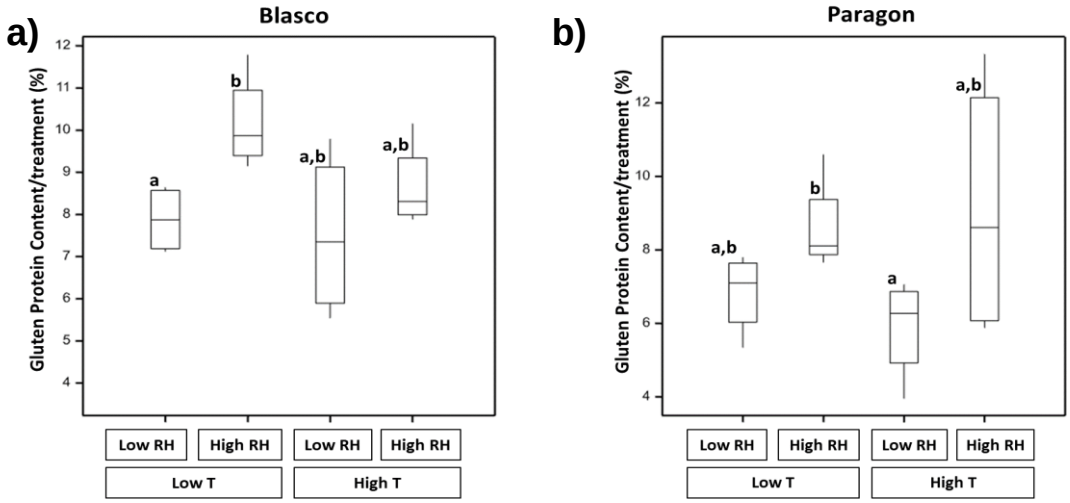


Figure 4.9: Effect of temperature manipulations, applied three consecutive days at the flowering stage and during early grain filling stage, and humidity manipulations, applied during the life cycle of the cultivars, on gluten protein content in Blasco (a) and Paragon (b) wheat cultivars (Experiment 3). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

4.3.2.3: Gluten protein content- SDS-PAGE

In Experiment 1B, we found RH effect on gluten protein content both in Blasco (p=0.015) and Paragon (p=0.038) as they had lower gluten content at low RH/low air T than at high RH/low air T, as shown in Fig. 4.10 a. Gluten content in Blasco was also impacted from temperature (p=0.015), with higher temperature at low RH resulting in higher gluten content (Fig. 4.10 b).

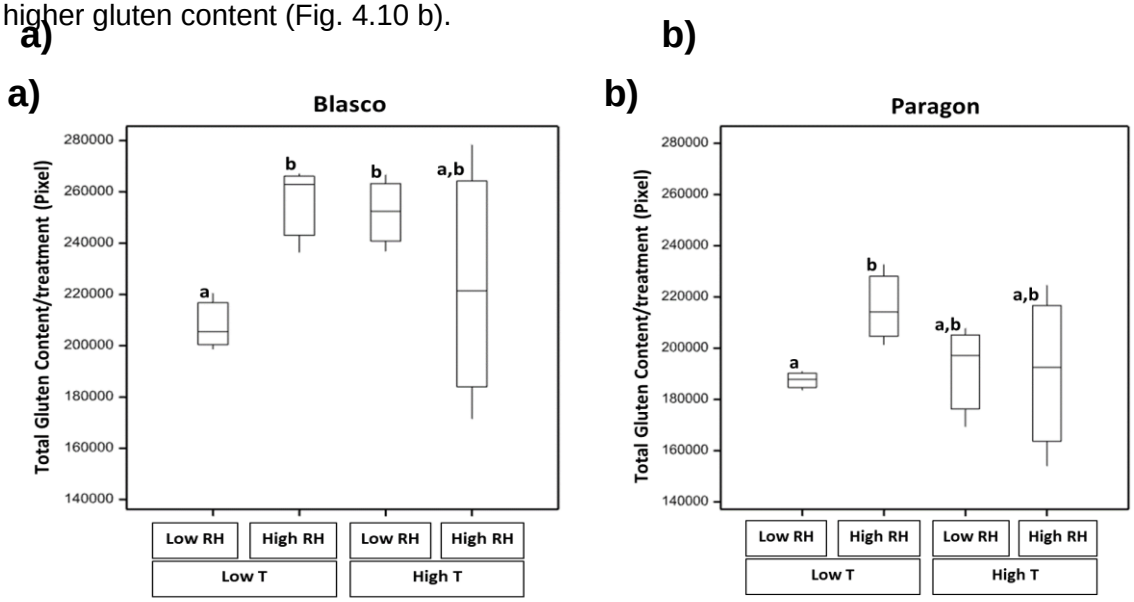


Figure 4.10: Gluten content in Blasco (a) and Paragon (b) wheat cultivars exposed to temperature and humidity manipulations for three consecutive days during early grain filling stage (Experiment 1B). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

In Experiment 1A (p=0.026, Fig. 4.11), we found a relative humidity effect on LMW + ω,β,ϒ-gliadins in Blasco, as it had higher level of LMW + ω,β,ϒ-gliadins at low RH/low air T than those at high RH/high air T.

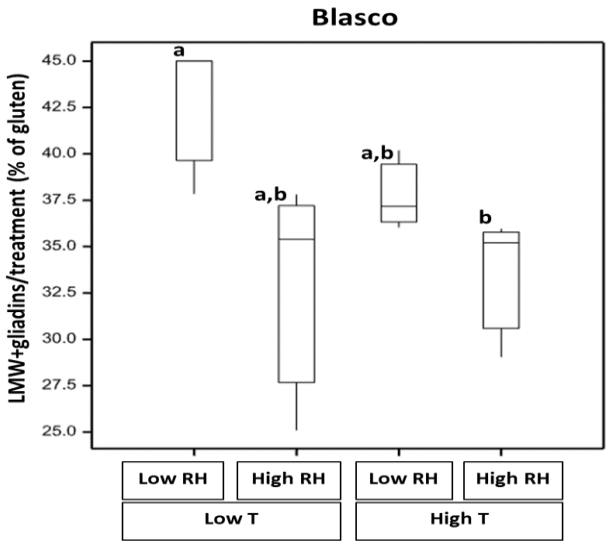


Figure 4.11: LMW+gliadins subunits in Blasco wheat variety exposed to temperature and humidity manipulation applied for three consecutive days at the flowering stage (Experiment 1A). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

ω-gliadins in Blasco in Experiment 1B were affected by RH ($p=0.049$) and an interaction between RH and air temperature ($p=0.011$) where ω-gliadins in Blasco was lower at low RH/low air T than those at high RH/low air T, low RH/high air T, high RH/high air T (Fig. 4.12 a).

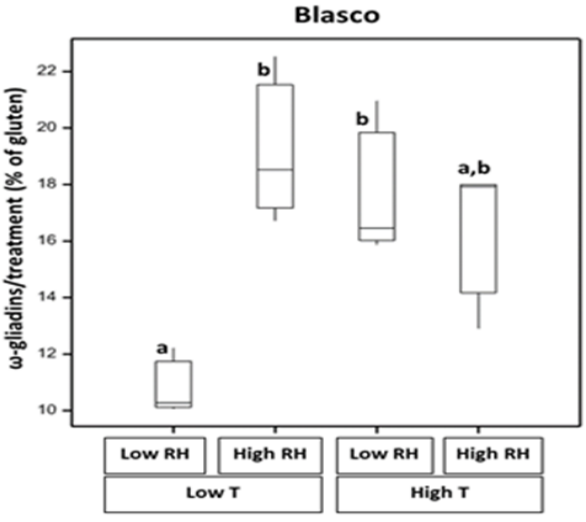


Figure 4.12: Effect of temperature and humidity manipulations, applied for three consecutive days during early grain filling stage of the cultivars, on ω-gliandins subunits in Blasco wheat cultivar (Experiment 1B). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

4.3.3: Effects of relative humidity, air temperature and soil moisture on SDS-sedimentation

4.3.3.1: SDS-sedimentation

SDS-sedimentation of Paragon was impacted by soil moisture and RH in Experiment 2. At high RH Paragon had higher SDS-sedimentation at medium soil moisture than at high soil moisture (p=0.003), while at high soil moisture, it had higher SDS-sedimentation at low RH than at high RH (p=0.022). Besides this, interactive effects of soil moisture and RH on SDS-sedimentation of Paragon was observed. Paragon had lower SDS-sedimentation at high RH/high soil moisture than at low RH/low soil moisture and low RH/medium soil moisture conditions (p= 0.022, Fig. 4.13).

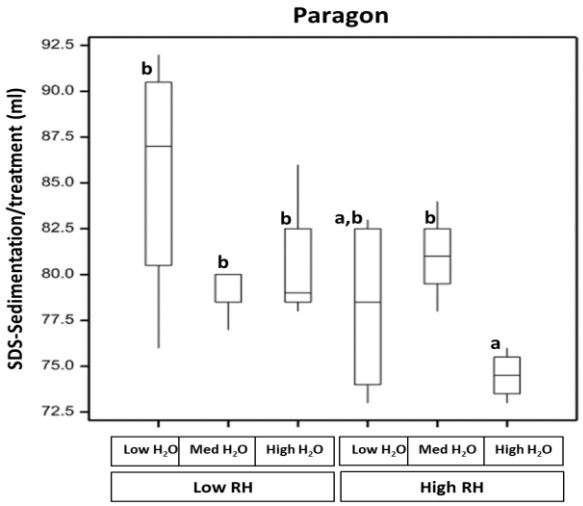


Figure 4.13: SDS-sedimentation in Paragon wheat variety after exposing to the humidity and soil moisture manipulations during its life cycle (Experiment 2). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

We did not see any direct RH or air T effect on SDS-sedimentation volume for any of the two cultivars in Experiment 3 (Appendix 4.3).

4.4: Discussion

4.4.1: Alpha amylase activity

Variation of climatic factors such as temperature, RH and rainfall have an impact on flour properties and bread making quality of wheat. Prolonged rainfall and high humidity following grain maturation but preceding harvesting can result in pre-harvest sprouting (PHS) i.e. premature germination, where wheat kernels in the ear absorb

moisture and swell, resulting in degradation of starch by α-amylase. Even though a certain amount of starch degradation is desirable during breadmaking, germination of seeds on ear cause decrease in flour quality due to excessive α-amylase activity i.e. higher starch breakdown of flour (Thomason *et al.*, 2009). Increase in temperatures from about 15°C to 30 °C (Fadeel *et al.*, 1980; Reddy *et al.*, 1984), prolonged rainfall and high humidity during grain filling may dramatically elevates the α-amylase level (Thomason *et al.*, 2009; Mares and Mrva, 2014; Rakita *et al.*, 2015). However, temperatures higher than 30°C during ripening cause decrease in α-amylase activity (Reddy *et al.*, 1984; Rakita *et al.*, 2015). These studies also suggested that lower activity at temperatures higher than 30°C might be the result of a higher inactivation rate, or lower enzyme synthesis, or both. Confirming earlier observations (Reddy *et al.*, 1984; Rakita *et al.*, 2015), we also observed climate effect on alpha amylase activity, as Paragon in Experiment 1B had lower alpha amylase activity at low RH/high air T (0.1 CU/g flour) than at low RH/low air T (0.41 CU/g flour) and high RH/low air T (0.42 CU/g flour). We have also observed in Chapter 3 that Paragon had higher spike temperature (ST) and flag leaf temperature (FLT) at low RH/high air T (26.3°C ST, 23.9°C FLT) than at low RH/low air T (18.7°C ST, 17.2 FLT) and high RH/low air T (21.4°C ST, 19.3°C FLT) in Experiment 1B. Besides this, Blasco and Paragon had similar spike and flag leaf temperatures values at low RH/high air temperature (Appendix 3.2). Therefore, it is possible that optimal temperature for alpha amylase synthesis in Paragon might be lower than in Blasco, and this might have caused decrease in alpha amylase activity (~0.1CU/g flour) in Paragon at low RH/high air temperature.

Reddy *et al.* (1984) suggested that lower alpha amylase activity resulted in higher kernel weight under higher temperature conditions, since lower enzyme activity means lower starch degradation and thus higher starch deposition. We have also observed this, as it can be seen in Chapter 3 Paragon had significantly higher grain weight at dry/warm than at dry/cool (Fig. 3.12 b). Even though it was not significant, Paragon tended to have higher grain weight at dry/warm than at wet/cool. Results of a linear recreation analysis showed that grain weight tended to be negatively correlated with alpha amylase activity in Paragon in Experiment 1B (Appendix 4.23).

4.4.2: Total nitrogen, total protein and total gluten content

High temperatures during the grain-filling stage cause a decrease in starch deposition and since protein deposition remain mostly unaffected, this result in higher grain protein concentration (Stone and Nicolas, 1998; Farooq *et al.*, 2011). Grain quality in

wheat is mainly determined by the protein content, which is derived from nitrogen concentration of grain (N%) (Kato, 2012). Since photosynthetic process is inhibited, and leaf senescence is accelerated under heat stress from anthesis to maturity (Al-Khatib and Paulsen, 1990), pre-anthesis stores of nitrogen and carbohydrates in the stem become a particularly important source of nutrients under stress conditions (Tahir and Nakata, 2005). Indeed a series of studies indicated that more than 80% of the total N content accumulates before flowering stage, and that pre anthesis N is responsible for around 50-100% of final wheat grain N content (Daigger *et al.*, 1976; Simpson *et al.*, 1983; Papakosta and Gagianas, 1991; Tahir and Nakata, 2005). Tahir and Nakata (2005) suggested that accelerated leaf senescence and loss of chlorophyll under heat stress during grain filling is linked with N and carbohydrate remobilization from stems. Water deficit conditions decrease enzyme activities, which play role in starch synthesis and accumulation (Bing *et al.*, 2014). Ahmadi and Baker (2001) suggested that this reduced enzyme activity causes reduction in grain filling (starch deposition), while other researchers Rharrabti *et al.* (2003) found that drought stress resulted in a dramatic rise in protein content. It is usually suggested that drought-induced decrease in grain yield of crops is correlated with an elevate in protein content (Ashraf, 2014).

Our results also supported some of these findings, as in Experiment 1B we observed higher total protein, total N and gluten protein content in Blasco and Paragon under high RH (77% and 86%) and high air T (32 °C day/24 °C night) conditions (Appendix 3.1 and 3.2). When we compared these results with the results of grain yield in chapter 3 (Appendix 3.4), we found that Blasco and Paragon had lower grain yield under high RH and high temperature conditions. We have observed similar results in Experiment 3, where high RH and high air temperature (higher than optimal RH and air temperature) caused increase in total nitrogen and gluten protein content in Blasco and Paragon. Even though there was not any significant effect on grain yield in the cultivars, grain yield tended to be lower at high RH and high air temperature conditions (Appendix 3.4). In Experiment 2, higher soil moisture under wet condition resulted in lower nitrogen content in Paragon, while higher RH under low soil moisture condition resulted in lower gluten protein content in Blasco. Results in Chapter 3 showed that Paragon had higher grain yield at higher soil moisture conditions, while Blasco had higher grain yield at higher RH and higher soil moisture conditions. These results show a negative correlation between grain weight and grain protein content for both cultivars (Appendix 4.20, 4.21 and 4.22) broadly consistent with existing observations (Stone and Nicolas, 1998; Farooq *et al.*, 2011). Tissue temperature and soil moisture outside of the optimum values might have caused lower enzyme activity involved in starch

biosynthesis, resulting in more nitrogen per unit of starch in Blasco and Paragon similar to the observations in the previous studies (Bazzaz and Sombroek, 1996; Stone and Nicolas, 1998; Rharrabti *et al.*, 2003; Tahir and Nakata, 2005; Farooq *et al.*, 2011; Ashraf, 2014).

4.4.3: Gluten protein subunits

Even though grain protein content rises, glutenin/gliadin ratio decreases under heat stress, which impacts flour quality negatively (Corbellini *et al.*, 1997; Majoul *et al.*, 2003; Castro *et al.*, 2007; Ashraf, 2014). Furthermore, heat stress during grain filling can adversely influence the level of gluten protein aggregation, as it generally causes significant increase in ω-gliadins synthesis and decrease in glutenin synthesis (Majoul *et al.*, 2003; Ashraf, 2014; Koga *et al.*, 2015; Koga *et al.*, 2016). Altered gluten protein composition is considered as a contributor to poor breadmaking quality (BeNCze and VeiSz, 2011; Ashraf, 2014; Koga *et al.*, 2015; Koga *et al.*, 2016). It has been noted that HMW glutenins represents only 10% of the total storage proteins in comparison to 40% for LMW, nevertheless HMW glutenin subunits have the biggest impact on breadmaking quality (Appels, 2008) (Acton, 2013).

Following the quantitative analysis of separated gluten protein fractions in SDS-PAGE in Experiment 1, we found that ω-gliadins and LMW + gliadins subunits of gluten in Blasco are affected by increase in air temperature and increase in RH. In Experiment 1B, high RH and high T in Blasco caused increase in ω-gliadins, as % of gluten, similarly to what reported by other studies (Majoul *et al.*, 2003; Ashraf, 2014; Koga *et al.*, 2015; Koga *et al.*, 2016), while higher RH in Blasco, in Experiment 1A, resulted in lower LMW + gliadins subunits of gluten. Since the effect of humidity on wheat grain quality has not been studied before, we could not compare our result on the effect of RH on LMW + α,β,Ɣ-gliadins % of gluten with other studies. (Fido *et al.*, 1997; Malalgoda *et al.*, 2017) suggested that ω-gliadins cause decrease in loaf height, thus dough strength is negatively affected by an increase in ω-gliadins. Therefore, we can say that dough strength in Blasco was affected negatively in Experiment 1B at high RH and high temperature conditions. Quantitative analysis of separated gluten protein fractions by SDS-PAGE for Experiment 1A and 1B did not show significant differences within the treatments in terms of HMW glutenin subunits

Several studies (Aslani et al., 2013; Li et al., 2013; ZHANG et al., 2014) have indicated that heat stress causes increase in SDS-sedimentation volume. Drought stress, particularly at the grain filling stage, dramatically affects grain protein quality. (Panozzo *et al.*, 2001) found that polymeric protein fragments increased under drought stress,

but gliadin/glutenin ratio was not affected from the stress. Lan *et al.* (2004; Dai *et al.*, 2006) found an increase in glutenin/gliadin ratio, while others (Hajheidari *et al.*, 2007; Ashraf, 2014; ZHANG *et al.*, 2014) suggested that gliadin proteins increase under drought stress. It has been also reported that water stress causes increase in SDS-sedimentation volume (Aslani *et al.*, 2013; Li *et al.*, 2013; ZHANG *et al.*, 2014), however Gooding et al. (2003) found that drought stress during grain filling stage significantly decrease the SDS-sedimentation volume. Even though we did not carry out SDS-PAGE in Experiment 2 and Experiment 3, we analysed SDS-sedimentation volumes which is positively correlated with polymerisation and therefore, indirectly, with HMW glutenin subunits content. In Experiment 2, we found that Paragon had lower SDS-sedimentation volume at high RH/high soil moisture than at other RH and soil moisture conditions. This is consistent with the previous results as SDS-sedimentation volume of Paragon is negatively correlated with soil moisture (Aslani *et al.*, 2013; Li *et al.*, 2013; ZHANG *et al.*, 2014). In Experiment 3, there was not any difference within the treatments in terms of SDS-sedimentation volume for any of the two cultivars.

In conclusion, in Experiment 1B, we have observed that high tissue temperature under high air temperature caused decrease in alpha amylase activity of Paragon but not in Blasco. Increase in atmospheric temperature and increase in RH and decrease in soil moisture resulted in higher protein content, while there was a negative correlation between grain yield and grain total protein and gluten protein content in both cultivars. Gluten protein composition in Blasco was affected from the treatments, as ω-gliadins as % gluten in Blasco increased under high RH and high air T conditions. In Experiment 3, total nitrogen and gluten protein content in both cultivars increased under high RH and high air temperature conditions, while grain yield tended to decrease. In Experiment 2, increase in soil moisture resulted in lower SDS-sedimentation, i.e. HMW glutenin subunits at humid conditions. These findings suggest that projected higher atmospheric temperature and more intense drought stresses and possible change in RH due to climate change is likely to cause many changes in wheat grain quality in the future.

4.5: References

Abdel-Aal, E.-S., Salama, D., Hucl, P., Sosulski, F. & Cao, W. (1996). Electrophoretic characterization of spring spelt wheat gliadins. *Journal of agricultural and food chemistry,* **44** (8), 2117-2123.

Acton, Q. A. (2013). *Issues in Agricultural Research.* PhD.

Ahmadi, A. & Baker, D. A. (2001). The effect of water stress on the activities of key regulatory enzymes of the sucrose to starch pathway in wheat. *Plant Growth Regulation,* **35** (1), 81-91.

Al-Khatib, K. & Paulsen, G. M. (1990). Photosynthesis and productivity during high-temperature stress of wheat genotypes from major world regions. *Crop Science,* **30** (5), 1127-1132.

Appels, R. (2008). *Proceedings of the 11th International Wheat Genetics Symposium, 24-29 August 2008, Brisbane, Qld., Australia.* Sydney University Press.

Ashraf, M. (2014). Stress-induced changes in wheat grain composition and quality. *Critical reviews in food science and nutrition,* **54** (12), 1576-1583.

Aslani, F., Mehrvar, M. R., Nazeri, A. & Juraimi, A. S. (2013). Investigation of wheat grain quality characteristics under water deficit condition during post-anthesis stage. *ARPN J Agr Biol Sci,* **8** (4), 273-278.

Bazzaz, F. A. & Sombroek, W. G. (1996). *Global climate change and agricultural production: direct and indirect effects of changing hydrological, pedological and plant physiological processes.* Food & Agriculture Org.

BeNCze, S. & VeiSz, O. (2011). Quality of winter wheat in relation to heat and drought shock after anthesis. *Czech J. Food Sci. Vol,* **29** (2), 117-128.

BHANDARI, D. (2002). DEVELOPMENT OF A BLUE STARCH/AGAROSE GEL METHOD FOR RAPID INTAKE AND FIELD TESTING FOR CEREAL ALPHA-AMYLASE ACTIVITY. Campden & Chorleywood Food Research Association: HGCA.

Bhullar, S. & Jenner, C. (1985). Differential responses to high temperatures of starch and nitrogen accumulation in the grain of four cultivars of wheat. *Functional Plant Biology,* **12** (4), 363-375.

Bing, Y., ZHOU, Y.-f., GAO, M.-y., ZHANG, Z., Yi, H., YANG, G.-d., Wenjuan, X. & HUANG, R.-d. (2014). Effect of drought stress during flowering stage on starch accumulation and starch synthesis enzymes in sorghum grains. *Journal of Integrative Agriculture,* **13** (11), 2399-2406.

Bland, J. M. & Altman, D. G. (1996). Statistics notes: Transforming data. *Bmj,* **312** (7033), 770.

Blumenthal, C., Barlow, E. & Wrigley, C. (1993). Growth environment and wheat quality: the effect of heat stress on dough properties and gluten proteins. *Journal of Cereal Science,* **18** (1), 3-21.

Castro, M., Peterson, C., Dalla Rizza, M., Dellavalle, P. D., Vázquez, D., Ibáñez, V. & Ross, A. (2007). Influence of heat stress on wheat grain characteristics and

protein molecular weight distribution. *Wheat production in stressed environments.* Springer.

Corbellini, M., Canevar, M., Mazza, L., Ciaffi, M., Lafiandra, D. & Borghi, B. (1997). Effect of the duration and intensity of heat shock during grain filling on dry matter and protein accumulation, technological quality and protein composition in bread and durum wheat. *Functional Plant Biology,* **24** (2), 245-260.

Csiszár, J., Guóth, A., Kolbert, Z., Gallé, Á., Tari, I., Ciulca, S., Madosa, E. & Erdei, L. (2010). Starch to protein ratio and α-amylase activities in grains of different wheat cultivars. *Acta Biologica Szegediensis,* **54** (1), 19-23.

Czuchajowska, Z., Lin, P. & Smolinski, S. (1996). Role in dough rheology of high molecular weight glutenin subunits of soft white winter and club wheats. *Cereal chemistry,* **73** (3), 338-345.

Dai, T., Zhao, H., Jing, Q., Jiang, D. & Cao, W. (2006). Effects of high temperature and water stress during grain filling on grain protein and starch formation in winter wheat. *Acta Ecologica Sinica,* **26** (11), 3670-3676.

Daigger, L., Sander, D. & Peterson, G. (1976). Nitrogen Content of Winter Wheat During Growth and Maturation 1. *Agronomy Journal,* **68** (5), 815-818.

Dhaka, V. & Khatkar, B. (2015). Effects of gliadin/glutenin and HMW-GS/LMW-GS ratio on dough rheological properties and bread-making potential of wheat varieties. *Journal of Food Quality,* **38** (2), 71-82.

DuPont, F., Hurkman, W., Vensel, W., Chan, R., Lopez, R., Tanaka, C. & Altenbach, S. (2006). Differential accumulation of sulfur-rich and sulfur-poor wheat flour proteins is affected by temperature and mineral nutrition during grain development. *Journal of Cereal Science,* **44** (1), 101-112.

DuPont, F. M. & Altenbach, S. B. (2003). Molecular and biochemical impacts of environmental factors on wheat grain development and protein synthesis. *Journal of cereal science,* **38** (2), 133-146.

Dupont, F. M., Hurkman, W. J., Vensel, W. H., Tanaka, C., Kothari, K. M., Chung, O. K. & Altenbach, S. B. (2006). Protein accumulation and composition in wheat grains: effects of mineral nutrients and high temperature. *European Journal of Agronomy,* **25** (2), 96-107.

Eliasson, A.-C. & Larsson, K. (1993). *Cereals in breadmaking: a molecular colloidal approach.* Marcel Dekker.

Fadeel, A., Moll, B. A. & Jones, R. L. (1980). Effect of temperature on the synthesis and secretion of α-amylase in barley aleurone layers. *Plant physiology,* **66** (3), 466-470.

Farooq, M., Bramley, H., Palta, J. A. & Siddique, K. H. (2011). Heat stress in wheat during reproductive and grain-filling phases. *Critical Reviews in Plant Sciences,* **30** (6), 491-507.

Fido, R., Bekes, F., Gras, P. & Tatham, A. (1997). Effects of α-, β-, γ-and ω-gliadins on the dough mixing properties of wheat flour. *Journal of Cereal Science,* **26** (3), 271-277.

Finney, K. F. & Barmore, M. A. (1948). Loaf volume and protein content of hard winter and spring wheats. *Cereal Chem.,* **25**, 291-312.

Flæte, N., Hollung, K., Ruud, L., Sogn, T., Færgestad, E., Skarpeid, H., Magnus, E. & Uhlen, A. (2005). Combined nitrogen and sulphur fertilisation and its effect on wheat quality and protein composition measured by SE-FPLC and proteomics. *Journal of Cereal Science,* **41** (3), 357-369.

Geera, B., Nelson, J., Souza, E. & Huber, K. (2006). Composition and properties of A- and B-type starch granules of wild-type, partial waxy, and waxy soft wheat. *Cereal Chemistry,* **83** (5), 551-557.

Gooding, M., Ellis, R., Shewry, P. & Schofield, J. (2003). Effects of restricted water availability and increased temperature on the grain filling, drying and quality of winter wheat. *Journal of Cereal Science,* **37** (3), 295-309.

Graybosch, R. A., Peterson, C. J., Shelton, D. R. & Baenziger, P. S. (1996). Genotypic and environmental modification of wheat flour protein composition in relation to end-use quality. *Crop Science,* **36** (2), 296-300.

Griffin, W. (Year). Screening for breadbaking quality by the SDS-sedimentation test and SDS-polyacrylamide gel electrophoresis. *In:* Proceedings of the Agronomy Society of New Zealand, 1983. 111-115.

Hajheidari, M., Eivazi, A., Buchanan, B. B., Wong, J. H., Majidi, I. & Salekdeh, G. H. (2007). Proteomics uncovers a role for redox in drought tolerance in wheat. *Journal of Proteome Research,* **6** (4), 1451-1460.

Halford, N. G. & Karp, A. (2010). *Energy crops.* Royal Society of Chemistry.

Hames, B. D. (1998). *Gel electrophoresis of proteins: a practical approach.* OUP Oxford.

He, J., Penson, S., Powers, S. J., Hawes, C., Shewry, P. R. & Tosi, P. (2013). Spatial patterns of gluten protein and polymer distribution in wheat grain. *Journal of agricultural and food chemistry,* **61** (26), 6207-6215.

Huebner, F., Nelsen, T., Chung, O. & Bietz, J. (1997). Protein distributions among hard red winter wheat varieties as related to environment and baking quality. *Cereal chemistry,* **74** (2), 123-128.

Johansson, E., Nilsson, H., Mazhar, H., Skerritt, J., MacRitchie, F. & Svensson, G. (2002). Seasonal effects on storage proteins and gluten strength in four Swedish wheat cultivars. *Journal of the Science of Food and Agriculture,* **82** (11), 1305-1311.

Kato, Y. (2012). Grain nitrogen concentration in wheat grown under intensive organic manure application on andosols in central Japan. *Plant Production Science,* **15** (1), 40-47.

Koga, S., Böcker, U., Moldestad, A., Tosi, P., Shewry, P. R., Mosleth, E. F. & Uhlen, A. K. (2015). Influence of temperature on the composition and polymerization of gluten proteins during grain filling in spring wheat (Triticum aestivum L.). *Journal of Cereal Science,* **65**, 1-8.

Koga, S., Böcker, U., Moldestad, A., Tosi, P., Shewry, P. R., Mosleth, E. F. & Uhlen, A. K. (2016). Influence of temperature during grain filling on gluten viscoelastic properties and gluten protein composition. *Journal of the Science of Food and Agriculture,* **96** (1), 122-130.

Lan, T., Jiang, D., Xie, Z., Dai, T., Jing, Q. & Cao, W. (2004). Effects of post-anthesis drought and waterlogging on grain quality traits in different specialty wheat varieties. *Journal of Soil and Water Conservation,* **18** (1), 193-196.

Li, Y., Wu, Y., Hernandez-Espinosa, N. & Peña, R. J. (2013). The influence of drought and heat stress on the expression of end-use quality parameters of common wheat. *Journal of Cereal Science,* **57** (1), 73-78.

Lindeque, R., Labuschagne, M. & van Biljon, A. (2017). Can Protein Quantity and Quality Predict the Breadmaking Quality of South African Wheat? *Cereal Foods World,* **62** (5), 196-201.

Majoul, T., Bancel, E., Triboï, E., Ben Hamida, J. & Branlard, G. (2003). Proteomic analysis of the effect of heat stress on hexaploid wheat grain: Characterization of heat-responsive proteins from total endosperm. *Proteomics,* **3** (2), 175-183.

Malalgoda, Maneka, Ohm, J. B., Meinhardt, Steven, Simsek & Senay (2017). Association between gluten protein composition and breadmaking quality characteristics in historical and modern spring wheat. . *Cereal Chemistry*.

Mares, D. J. & Mrva, K. (2014). Wheat grain preharvest sprouting and late maturity alpha-amylase. *Planta,* **240** (6), 1167-1178.

Marston, J. M., Guedes, J. D. a. & Warinner, C. (2015). *Method and theory in paleoethnobotany.* University Press of Colorado.

McCleary, B. V., McNally, M., Monaghan, D. & Mugford, D. C. (2002). Measurement of α-amylase activity in white wheat flour, milled malt, and microbial enzyme

preparations, using the Ceralpha assay: collaborative study. *Journal of AOAC international,* **85** (5), 1096-1102.

McDonald, J. H. (2009). *Handbook of biological statistics.* Sparky House Publishing Baltimore, MD.

Megazyme (2007). MICROPLATE FORMAT ASSAY PROCEDURE For ALPHA AMYLASE (CERALPHA METHOD). Megazyme International Ireland Ltd.

Morris, C. F., Paszczynska, B., Bettge, A. D. & King, G. E. (2007). A critical examination of the sodium dodecyl sulfate (SDS) sedimentation test for wheat meals. *Journal of the Science of Food and Agriculture,* **87** (4), 607-615.

Murphy, C. F. & Peterson, D. M. (2000). *Designing crops for added value.* American Society of Agronomy.

Ottman, M. J., Doerge, T. A. & Martin, E. C. (2000). Durum grain quality as affected by nitrogen fertilization near anthesis and irrigation during grain fill. *Agronomy Journal,* **92** (5), 1035-1041.

Panozzo, J., Eagles, H. & Wootton, M. (2001). Changes in protein composition during grain development in wheat. *Australian Journal of Agricultural Research,* **52** (4), 485-493.

Papakosta, D. K. & Gagianas, A. (1991). Nitrogen and dry matter accumulation, remobilization, and losses for Mediterranean wheat during grain filling. *Agronomy Journal,* **83** (5), 864-870.

Payne, P., Seekings, J., Worland, A., Jarvis, M. & Holt, L. (1987). Allelic variation of glutenin subunits and gliadins and its effect on breadmaking quality in wheat: Analysis of F5 progeny from Chinese Spring× Chinese Spring (Hope 1A). *Journal of Cereal Science,* **6** (2), 103-118.

Pompa, M., Giuliani, M. M., Palermo, C., Agriesti, F., Centonze, D. & Flagella, Z. (2013). Comparative analysis of gluten proteins in three durum wheat cultivars by a proteomic approach. *Journal of agricultural and food chemistry,* **61** (11), 2606-2617.

Primard, S., Graybosch, R., Peterson, C. J. & Lee, J. (1991). Relationships between gluten protein composition and quality characteristics in four populations of high-protein, hard red winter wheat. *Cereal Chem,* **68** (3), 305-312.

Rakita, S. M., Torbica, A. M., Dokić, L. P., Tomić, J. M., Pojić, M. M., Hadnađev, M. S. & Hadnađev-Dapčević, T. R. (2015). Alpha-amylase activity in wheat flour and breadmaking properties in relation to different climatic conditions. *Food and Feed Research,* **42** (2), 91-99.

Randall, P. & Moss, H. (1990). Some effects of temperature regime during grain filling on wheat quality. *Australian journal of agricultural research,* **41** (4), 603-617.

Rasmussen, J. L. (1985). Data transformation maximizing homoscedasticity and within-group normality. *Behavior Research Methods,* **17** (3), 411-412.

Reddy, L., Ching, T. & Metzger, R. (1984). Alpha-amylase activity in wheat kernels matured and germinated under different temperature conditions. *Cereal chemistry*.

Rharrabti, Y., Villegas, D., Royo, C., Martos-Núñez, V. & Del Moral, L. G. (2003). Durum wheat quality in Mediterranean environments: II. Influence of climatic variables and relationships between quality parameters. *Field Crops Research,* **80** (2), 133-140.

Rittau, A., Batey, I., Copeland, L., Miskelly, D. & Wrigley, C. (2005). Factors affecting the results of SDS sedimentation tests of flour and wholemeal.

Rédei, G. P. (1998). *Genetics manual: Current theory, concepts, terms.* World Scientific.

Schuster, I., Souza, M. A. d., Cardoso, A. A., Sediyama, C. S. & Moreira, M. A. (1997). Correlation between high molecular weight gluten subunits composition and bread-making quality in Brazilian wheat. *Brazilian Journal of Genetics,* **20** (4).

Shewry, P., Halford, N. & Tatham, A. (1992). High molecular weight subunits of wheat glutenin. *Journal of cereal science,* **15** (2), 105-120.

Shewry, P. R. & Gutteridge, S. (1992). *Plant protein engineering.* Cambridge University Press.

Shewry, P. R., Halford, N. G., Belton, P. S. & Tatham, A. S. (2002). The structure and properties of gluten: an elastic protein from wheat grain. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* **357** (1418), 133-142.

Simpson, R. J., Lambers, H. & Dalling, M. J. (1983). Nitrogen redistribution during grain growth in wheat (Triticum aestivum L.): IV. Development of a quantitative model of the translocation of nitrogen to the grain. *Plant Physiology,* **71** (1), 7-14.

Stone, P. & Nicolas, M. (1994). Wheat cultivars vary widely in their responses of grain yield and quality to short periods of post-anthesis heat stress. *Functional Plant Biology,* **21** (6), 887-900.

Stone, P. & Nicolas, M. (1998). Comparison of sudden heat stress with gradual exposure to high temperature during grain filling in two wheat varieties differing in heat tolerance. II. Fractional protein accumulation. *Functional Plant Biology,* **25** (1), 1-11.

Tahir, I. & Nakata, N. (2005). Remobilization of nitrogen and carbohydrate from stems of bread wheat in response to heat stress during grain filling. *Journal of Agronomy and Crop Science,* **191** (2), 106-115.

Thomason, W. E., Hughes, K., Griffey, C. A., Parrish, D. J. & Barbeau, W. (2009). Understanding pre-harvest sprouting of wheat.

Tipples, K. (1969). The relation of starch damage to the baking performance of flour. *Bakers' Dig.,* **43**, 28.

Torbica, A., Živančev, D., Hadnađev, M. & Mastilović, J. (Year). Influence of heat stress on wheat grain quality. *In:* Proceedings of the 45th Croatian & 5th International Symposium on Agriculture, 2010. 940-944.

Tosi, P., Gritsch, C. S., He, J. & Shewry, P. R. (2011). Distribution of gluten proteins in bread wheat (Triticum aestivum) grain. *Annals of botany,* **108** (1), 23-35.

van den Broeck, H. C., America, A. H., Smulders, M. J., Bosch, D., Hamer, R. J., Gilissen, L. J. & van der Meer, I. M. (2009). A modified extraction protocol enables detection and quantification of celiac disease-related gluten proteins from wheat. *Journal of Chromatography B,* **877** (10), 975-982.

Wan, Y., Gritsch, C. S., Hawkesford, M. J. & Shewry, P. R. (2013). Effects of nitrogen nutrition on the synthesis and deposition of the ω-gliadins of wheat. *Annals of botany,* **113** (4), 607-615.

Whan, A., Dielen, A.-S., Mieog, J., Bowerman, A. F., Robinson, H. M., Byrne, K., Colgrave, M., Larkin, P. J., Howitt, C. A. & Morell, M. K. (2014). Engineering α-amylase levels in wheat grain suggests a highly sophisticated level of carbohydrate regulation during development. *Journal of experimental botany,* **65** (18), 5443-5457.

Zeng, J., Li, G., Gao, H. & Ru, Z. (2011). Comparison of A and B starch granules from three wheat varieties. *Molecules,* **16** (12), 10570-10591.

Zhang, W., Gu, J., Wang, Z., Wei, C., Yang, J. & Zhang, J. (2017). Comparison of Structural and Functional Properties of Wheat Starch Under Different Soil Drought Conditions. *Scientific reports,* **7** (1), 12312.

ZHANG, Y.-f., HUANG, X.-w., WANG, L.-l., Liu, W., WU, Z.-h., YOU, M.-s. & LI, B.-y. (2014). Proteomic analysis of wheat seed in response to drought stress. *Journal of Integrative Agriculture,* **13** (5), 919-925.

Zhao, F., Withers, P., Evans, E., Monaghan, J., Salmon, S., Shewry, P. & McGrath, S. (1997). Sulphur nutrition: an important factor for the quality of wheat and rapeseed. *Soil Science and Plant Nutrition,* **43** (sup1), 1137-1142.

Zhu, J. & Khan, K. (2001). Effects of genotype and environment on glutenin polymers and breadmaking quality. *Cereal Chemistry,* **78** (2), 125-130.

Žilić, S. (2013). Wheat Gluten: Composition and Health Effects. *Gluten*, 71-86.

Žilić, S., Dodig, D., Šukalović, V. H. T., Maksimović, M., Saratlić, G. & Škrbić, B. (2010). Bread and durum wheat compared for antioxidants contents, and lipoxygenase and peroxidase activities. *International journal of food science & technology,* **45** (7), 1360-1367.

4.6: Chapter 4 - Appendix

Appendix 4.1: Summary of alpha-amylase activity, total protein content and total N content scores of Blasco and Paragon wheat cultivars prior to temperature and relative humidity in Experiment 1A and 1B.

	Experiment 1A α-amylase/treatment (CU/ g flour); mean±SD		Experiment 1B α-amylase/treatment (CU/ g flour); mean±SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Cool	0.32±0.064 (a)	0.39±0.07 (a)	0.13±0.08 (a)	0.41±0.03 **(a)**
Dry/Warm	0.26±0.08 (a)	0.43±0.013 (a)	0.27±0.16 (a)	0.1±0.066 **(b)**
Wet/Cool	0.26±0.05 (a)	0.42±0.029 (a)	0.26±0.05 (a)	0.42±0.029 **(a)**
Wet/Warm	0.29±0.059 (a)	0.43±0.02 (a)	0.25±0.08(a)	0.29±0.16 (ab)

	Experiment 1A Total Protein Content/treatment (%); mean±SD		Experiment 1B Total Protein Content/treatment (%); mean±SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Cool	10.54±1 (ab)	9.3±3.1 (a)	9.04±0.5 **(a)**	9.7±2.1 (a)
Dry/Warm	8.62±1.4 **(a)**	9.5±1.6 (a)	10.9±1.35 (ab)	11.34±3.23 (ab)
Wet/Cool	11.13±0.3 **(b)**	9.23±1.8 (a)	11.1±0.3 **(b)**	9.23±1.75 (b)
Wet/Warm	11.43±1.9 (ab)	10.95±3.1 (a)	11.85±2.4(ab)	10.98±2.7(ab)

	Experiment 1A Total Nitrogen/treatment (%); mean±SD		Experiment 1B Total Nitrogen/treatment (%); mean±SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Cool	2.41±0.38 (a)	1.95±0.15 (a)	2.12±0.16 **(a)**	1.83±0.09 **(a)**
Dry/Warm	2.22±0.07 (a)	1.96±0.1 (a)	2.4±0.1 (ab)	2.07±0.05 **(b)**
Wet/Cool	2.29±0.09 (a)	1.88±0.01 (a)	2.29±0.09 (ab)	1.88±0.01 **(a)**
Wet/Warm	2.29±0.17 (a)	2.04±0.2 (a)	2.51±0.11 **(b)**	2.01±0.05 **(b)**

Appendix 4.2: Summary of total gluten protein content and gluten subunit scores of Blasco and Paragon wheat cultivars prior to temperature and relative humidity in Experiment 1A and 1B.

	Experiment 1A Total Gluten Content/treatment (pixel); mean±SD		Experiment 1B Total Gluten Content/treatment (pixel); mean±SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Cool	230200±32367 (a)	196610±31578 (a)	208236±11185 **(a)**	187504±3716 **(a)**
Dry/Warm	238788±29895 (a)	189792±42920 (a)	252038±14947 **(b)**	191452±19856 (ab)
Wet/Cool	255504±16654 (a)	216115±15739 (a)	255504±16654 **(b)**	216115±15739 **(b)**
Wet/Warm	262414±28255 (a)	173897±22811 (a)	223797±53521 (ab)	190426±35378 (ab)

	Experiment 1A HMW % of gluten/treatment (pixel); mean±SD		Experiment 1B HMW % of gluten/treatment (pixel); mean±SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Cool	33.01±4.1 (a)	37±3.36 (a)	36.66±5.48 (a)	33.65±6.29 (a)
Dry/Warm	35.24±2.36 (a)	36.75±4 (a)	37.48±2.55 (a)	37.44±4.95 (a)
Wet/Cool	39.42±2.35 (a)	37.29±5.02 (a)	39.42±2.35 (a)	37.29±5.03 (a)
Wet/Warm	39.21±4.46 (a)	37.03±6.7 (a)	41.17±5.36 (a)	37.93±4 (a)

	Experiment 1A ω-gliadins % of gluten/treatment (pixel); mean±SD		Experiment 1B ω-gliadins % of gluten/treatment (pixel); mean±SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Cool	14.61±1.1 (a)	12.76±0.4 (a)	10.86±1.2 **(a)**	12.98±2.16 (a)
Dry/Warm	17.32±1.95 (a)	14.26±4.6 (a)	17.77±2.8 **(b)**	14.09±1.55 (a)
Wet/Cool	19.26±2.98 (a)	12.91±3.3 (a)	19.26±12.91 **(b)**	12.9±3.25 (a)
Wet/Warm	17.72±1.13 (a)	11.96±2.6 (a)	16.28±2.9 **(ab)**	12.39±4.15 (a)

	Experiment 1A LMW+gliadins % of gluten/treatment (pixel); mean±SD		Experiment 1B LMW+gliadins % of gluten/treatment (pixel); mean±SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Cool	42.63±4.1 **(a)**	37.19±2.1 (a)	39.44±4.**3** (a)	41.39±6.3 (a)
Dry/Warm	37.81±2.15 (ab)	37.66±1.5 (a)	35.14±0.2 (a)	35.76±1.97 (a)
Wet/Cool	32.77±6.8 **(b)**	39.89±0.7 (a)	32.77±6.75 (a)	39.89±0.7 (a)
Wet/Warm	33.41±3.8 (ab)	39.11±5.5 (a)	33.61±3.7 (a)	36.69±1.4 (a)

Appendix 4.3: Summary of total gluten protein content, total N content and SDS-sedimentation volume scores of Blasco and Paragon wheat cultivars prior to temperature, relative humidity and soil moisture in Experiment 2 and 3.

Experiment 2
Total Nitrogen Content/treatment (%); mean±SD

Treatment	Blasco	Paragon
Dry/Low	3.19±0.07 **(a)**	2.86±0.17 (ab)
Dry/Med	3.06±0.09 (ab)	2.77±0.05 **(a)**
Dry/High	3±0.15 (ab)	2.73±0.11 **(a)**
Wet/Low	3.06±0.08 (ab)	2.9±0.07 **(b)**
Wet/Med	2.99±0.13 **(b)**	2.74±0.06 **(a)**
Wet/High	2.98±0.14 **(b)**	2.8±0.17 (ab)

Experiment 3
Total Nitrogen Content/treatment (%); mean±SD

Treatment	Blasco	Paragon
Dry/Cool	2.4±0.1 **(a)**	2.4±0.03 **(a)**
Dry/Warm	2.51±0.13 (ab)	2.35±0.13 (ab)
Wet/Cool	2.76±0.37 (ab)	2.62±0.29 (ab)
Wet/Warm	2.79±0.27 **(b)**	2.72±0.16 **(b)**

Experiment 2
Total Gluten Protein Content/treatment (%); mean±SD

Treatment	Blasco	Paragon
Dry/Low	13.75±1.7 **(a)**	9.14±1.4 (a)
Dry/Med	11.89±0.4 (ab)	9.51±3.1 (a)
Dry/High	12.28±1.9 (ab)	8.6±3.1 (a)
Wet/Low	9.7±2.1 **(b)**	10.7±0.5 (a)
Wet/Med	11.6±2.1 (ab)	10.5±1.85 (a)
Wet/High	9.7±2.1 (ab)	10±2.5 (a)

Experiment 3
Total Gluten Protein Content/treatment (%); mean±SD

Treatment	Blasco	Paragon
Dry/Cool	7.9±0.8 **(a)**	6.8±1.1 (ab)
Dry/Warm	7.5±1.96 (ab)	5.9±1.4 **(b)**
Wet/Cool	10.2±1.2 **(b)**	8.6±1.3 **(a)**
Wet/Warm	8.7±1.03 (ab)	9.1±3.6 (ab)

Experiment 2
SDS- Sedimentation/treatment (ml); mean±SD

Treatment	Blasco	Paragon
Dry/Low	72±2.45 (a)	85.5±6.95 **(a)**
Dry/Med	68.75±3.3 (a)	79.25±1.5 **(a)**
Dry/High	67.75±2.06 (a)	80.5±3.7 **(a)**
Wet/Low	69.5±3.3 (a)	78.25±4.99 (ab)
Wet/Med	73.75±7.04 (a)	81±2.45 **(a)**
Wet/High	69.75±2.63 (a)	74.5±1.29 **(b)**

Experiment 3
SDS-Sedimentation/treatment (ml); mean±SD

Treatment	Blasco	Paragon
Dry/Cool	80.5±7.14 (a)	78±12.87 (a)
Dry/Warm	86.38±2.29 (a)	81.25±9.97 (a)
Wet/Cool	78.75±3.3 (a)	79.38±9.76 (a)
Wet/Warm	78.88±10.16 (a)	86.5±6.75 (a)

Analysis of Variance of alpha-amylase activity

Appendix 4.4: Analysis of Variance of alpha amylase activity per treatment of Paragon in Experiment 1B

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	0.030998	0.030998	4.01	0.08
Temperature	1	0.146553	0.146553	18.96	0.002
Humidity.Temperature	1	0.02352	0.02352	3.04	0.119
Residual	8	0.061835	0.007729		
Total	11	0.262906			

Analysis of Variance of total protein content

Appendix 4.5: Analysis of Variance of total protein content per treatment of Blasco between the results at high RH/low air T and at low RH/high air T in Experiment 1A

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	9.453	9.453	8.9	0.041
Residual	4	4.249	1.062		
Total	5	13.702			

Appendix 4.6: Analysis of Variance of total protein content per treatment of Blasco between the results at low RH/low air T and at high RH/low air T in Experiment 1B

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	6.5536	6.5536	34.63	0.004
Residual	4	0.7569	0.1892		
Total	5	7.3105			

Analysis of Variance of total nitrogen content

Appendix 4.7: Analysis of Variance of total nitrogen content per treatment of Blasco in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	0.05773	0.05773	4.16	0.076
Temperature	1	0.18123	0.18123	13.05	0.007
Humidity.Temperature	1	0.0025	0.0025	0.18	0.683
Residual	8	0.11107	0.01388		
Total	11	0.35253			

Appendix 4.8: Analysis of Variance of total nitrogen content per treatment of Paragon in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	0.000047	0.000047	0.02	0.905
Temperature	1	0.111612	0.111612	36.44	<.001
Humidity.Temperature	1	0.00839	0.00839	2.74	0.136
Residual	8	0.024502	0.003063		
Total	11	0.14455			

Appendix 4.9: Analysis of Variance of total nitrogen content per treatment of Blasco in Experiment 3.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	0.16779	0.16779	9.86	0.009
Temperature	1	0.02584	0.02584	1.52	0.241
Humidity.Temperature	1	0.00245	0.00245	0.14	0.711
Residual	12	0.2042	0.01702		
Total	15	0.40029			

Appendix 4.10: Analysis of Variance of total nitrogen content per treatment of Paragon in Experiment 3.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	0.3486	0.3486	10.63	0.007
Temperature	1	0.0014	0.0014	0.04	0.84
Humidity.Temperature	1	0.02354	0.02354	0.72	0.413
Residual	12	0.39342	0.03279		
Total	15	0.76696			

Analysis of Variance of total gluten protein content

Appendix 4.11: Analysis of Variance of total gluten protein content per treatment of Blasco between the results at low RH/low air T and at high RH/low air T in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	3.35E+09	3.35E+09	16.65	0.015
Residual	4	8.05E+08	2.01E+08		
Total	5	4.16E+09			

Appendix 4.12: Analysis of Variance of total gluten protein content per treatment of Blasco between the results at low RH/low air T and at low RH/high air T in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Temperature	1	2.88E+09	2.88E+09	16.51	0.015
Residual	4	6.97E+08	1.74E+08		
Total	5	3.58E+09			

Appendix 4.13: Analysis of Variance of total gluten protein content per treatment of Paragon between the results at low RH/low air T and at high RH/low air T in Experiment 1B.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	1.23E+09	1.23E+09	9.39	0.038
Residual	4	5.23E+08	1.31E+08		
Total	5	1.75E+09			

Appendix 4.14: Analysis of Variance of total gluten content per treatment of Blasco in Experiment 2.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	2	3.044	1.522	0.48	0.625
Soil moisture	1	32.041	32.041	10.14	0.005
Humidity.Soil moisture	2	13.982	6.991	2.21	0.138
Residual	18	56.859	3.159		
Total	23	105.926			

Appendix 4.15: Analysis of Variance of total gluten content per treatment of Blasco in Experiment 3.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	11.885	11.885	6.91	0.022
Temperature	1	3.506	3.506	2.04	0.179
Humidity.Temperature	1	1.291	1.291	0.75	0.403
Residual	12	20.65	1.721		
Total	15	37.332			

Appendix 4.16: Analysis of Variance of total gluten content per treatment of Paragon in Experiment 3.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	24.983	24.983	5.51	0.037
Temperatrue	1	0.207	0.207	0.05	0.834
Humidity.Temperature	1	2.049	2.049	0.45	0.514
Residual	12	54.418	4.535		
Total	15	81.657			

Appendix 4.17: Analysis of Variance of LMW+gliadins per treatment of Blasco in Experiment 1A.

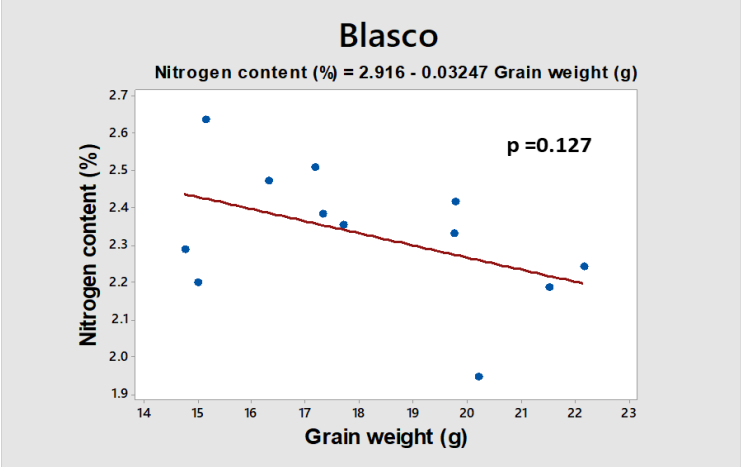
Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	152.34	152.34	7.46	0.026
Temperatrue	1	13.12	13.12	0.64	0.446
Humidity.Temperature	1	22.38	22.38	1.1	0.326
Residual	8	163.31	20.41		
Total	11	351.14			

Appendix 4.18: Analysis of Variance of ω-gliadins per treatment in Blasco in Experiment 1B.

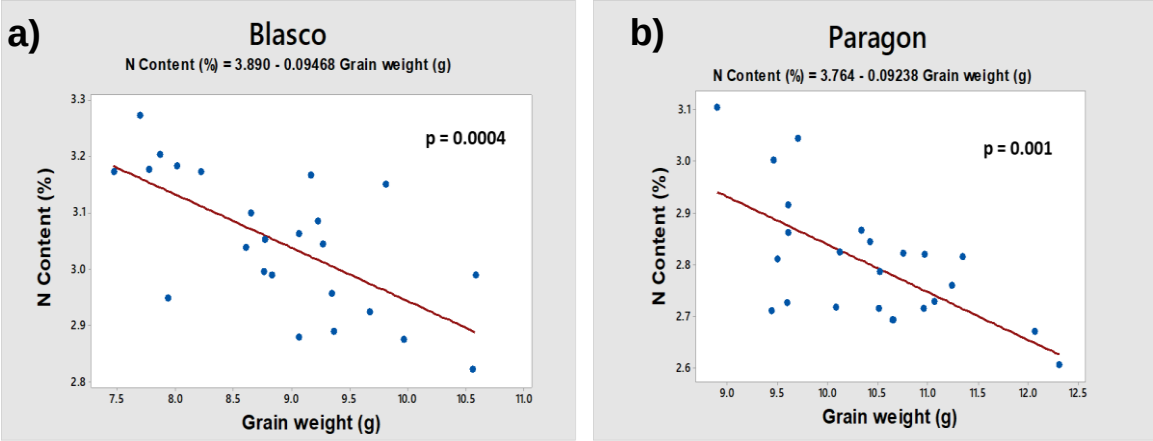
Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	35.883	35.883	5.4	0.049
Temperatrue	1	11.61	11.61	1.75	0.223
Humidity.Temperature	1	73.219	73.219	11.03	0.011
Residual	8	53.122	6.64		
Total	11	173.834			

Appendix 4.19: Analysis of Variance of SDS-sedimentation per treatment of Blasco in Experiment 2.

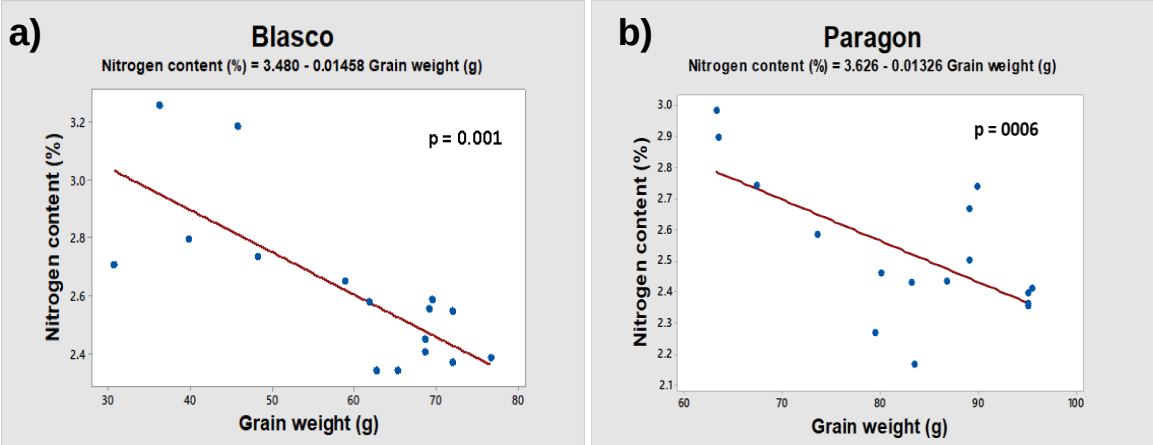
Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	88.17	88.17	5.46	0.031
Soil moisture	2	77.58	38.79	2.4	0.119
Humidity.Soil moisture	2	95.08	47.54	2.95	0.078
Residual	18	290.5	16.14		
Total	23	551.33			



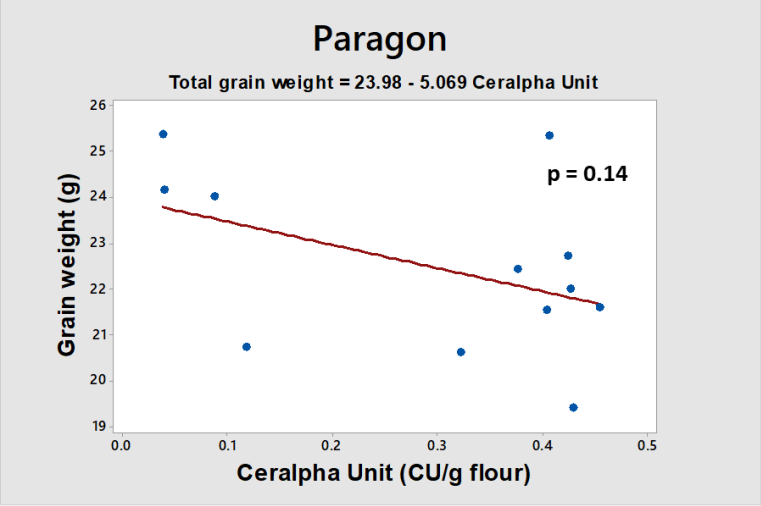
Appendix 4.20: Linear regression between nitrogen content and grain weight in Blasco in Experiment 1B.



Appendix 4.21: Linear regression between nitrogen content and grain weight in Blasco (a) and Paragon (b) in Experiment 2.



Appendix 4.22: Linear regression between nitrogen content and grain weight in Blasco (a) and Paragon (b) in Experiment 3.



Appendix 4.23: Linear regression between ceralpha unit (alpha amylase activity) and grain weight in Paragon in Experiment 1B.

Chapter 5: Response of Spring Wheat (*Triticum aestivum L.)* stomata to changes in Temperature, Relative Humidity and Soil Moisture

5.1: Introduction and Literature

Stomata are small apertures on the surface of leaves and stems whose role is to regulate gas exchange - mainly CO_2 intake, O_2 release during photosynthesis and water vapor loss via transpiration - between the atmosphere and leaf tissue (Hetherington and Woodward, 2003; Camargo and Marenco, 2011; Laga *et al.*, 2014). In general, between 0.5%-5% of the leaf epidermis is occupied by stomata. Wheat leaves are amphistomatous, which means having stomata on both sides of leaves. However, stomatal frequency of wheat is higher on the adaxial (lower side) than abaxial (upper side) surface (Teare *et al.*, 1971; Baldocchi, 1994; Maghsoudi and Maghsoudi, 2008).

5.1.1: Stomatal opening and closure

Gas exchange rate in plants is regulated by the opening and closing of the stomata which is triggered and controlled by two guard cells surrounding stomatal pore (Hetherington and Woodward, 2003; Laga *et al.*, 2014). Stomatal movement requires energy which is supplied from oxidative phosphorylation in the dark (uses enzymes for nutrient oxidization to produce ATP) and photophosphorylation in the light (uses light energy to produce ATP from ADP) (Raghavendra, 1981; Zeiger *et al.*, 1987; Willmer and Fricker, 1996; Setty, 2002). Loss of water via stomatal opening, i.e. transpiration, helps plants to control temperature by thermal cooling. Since stomatal opening allows CO_2 intake and O_2 loss, which is necessary for photosynthesis, it plays a crucial role in converting sunlight into energy essential for plant growth (Pallas *et al.*, 1967; Hetherington and Woodward, 2003; Lawson, 2009; Laga *et al.*, 2014). In order to regulate the stomatal pore and gas exchange, guard cells integrate endogenous hormonal signals (e.g. Abscisic acid) and environmental signals (e.g. Biotic and abiotic stresses, and CO_2 concentration) (Lawson, 2009; Daszkowska-Golec and Szarejko, 2013). These signals are then converted by guard cells into suitable turgor pressure which triggers stomatal closure (shrinking of the guard cells) or opening (swelling of the guard cells). Stomatal opening occurs when guard cell volume elevates due to accumulation of K^+ ions and or/ solutes in guard cells which raises turgor pressure and widen stomata. Since guard cells contain chloroplasts they can raise their sugar level that in turn results in water absorption and swelling (Maghsoudi and Maghsoudi, 2008).

In contrast, stomatal closure occurs via decrease in turgor pressure in guard cells because of ion, solute and water loss (Lawson *et al.*, 2002; Lawson, 2009; Kim *et al.*, 2010; Daszkowska-Golec and Szarejko, 2013).

Stomatal opening is triggered by illumination with blue light and other photosynthetically active waveband, low CO_2 concentration and high air humidity, while stomata closure is stimulated by high temperature, darkness, low air humidity, abscisic acid (ABC), high CO_2 concentration (Assmann, 1993; Weyers and Paterson, 2001; Lawson *et al.*, 2002; Lawson, 2009). Elevated CO_2 concentration in the atmosphere triggers stomatal closure, since less opening is required for CO_2 influx. Even though rise in ambient CO_2 concentration causes an increase in net CO_2 assimilation, this is accompanied by reduction in stomatal pore, stomatal size and stomatal conductance. The decrease in stomatal conductance, which is a function of stomatal opening, size and density, may increase water-use efficiency for plans, however lower transpiration rate triggers heat stress since evaporative cooling of leaves is restricted (Jarvis *et al.*, 1999; Kim *et al.*, 2010; Xu *et al.*, 2016).

5.1.2: Stomatal density and area/size

Stomatal density, stomatal number per unit leaf area, is an essential ecophysiological parameter impacting gas exchange (Gitz and Baker, 2009). Degree of stomatal opening and stomatal density are both responsible for the resistance to water vapour and CO_2 diffusion between atmosphere and leaf, and an alteration in stomatal resistance has a higher influence on transpiration than on photosynthesis. Stomatal density is influenced from environmental factors such as drought, heat stress, increased CO_2 concentration and precipitation change (Teare *et al.*, 1971; Liao *et al.*, 2005; Maghsoudi and Maghsoudi, 2008). Lower stomatal density increases stomatal resistance and decreases transpiration rate. However, photosynthesis rate is not affected from changes in stomata number. Wheat cultivars have higher grain yield with no more water consumption when they have wider stomatal pore. Therefore, selection of wheat varieties having fewer stomata can decrease transpiration rate, water loss, without changing photosynthesis rate (Teare *et al.*, 1971; Liao *et al.*, 2005; Maghsoudi and Maghsoudi, 2008; Shahinnia *et al.*, 2016).

5.2: Methodology

5.2.1: Stomatal imprint and analyse

One plant per pot was tagged for data collection (epidermal imprints) in Experiment 2, while three plants per plot were tagged in Experiment 3. An epidermal imprint of the flag leaf of each tagged plant at flowering and at early grain filling stage (12 DAA (Days after anthesis) (Zadok`s Growth Stage 71 (Zadoks *et al.*, 1974)) was collected. In order to prepare epidermal imprints, the abaxial surface of the flag leaves was coated with clear nail polish. Once the nail polish had dried (within few minutes), a piece of clear tape was attached to the part on the leaf containing the dried nail polish to peel off the epidermis under the nail polish and then glued onto a microscope slide and stored until analysis. The abaxial epidermis imprints were then analysed at 10x magnification under microscope (Leitz Wetzlar, Germany), and then the images (e.g. Image 5.1) of the stomata viewed by the microscope were taken using a Canon camera (Canon Eds 600D) which has EOS utility (connects with the camera).

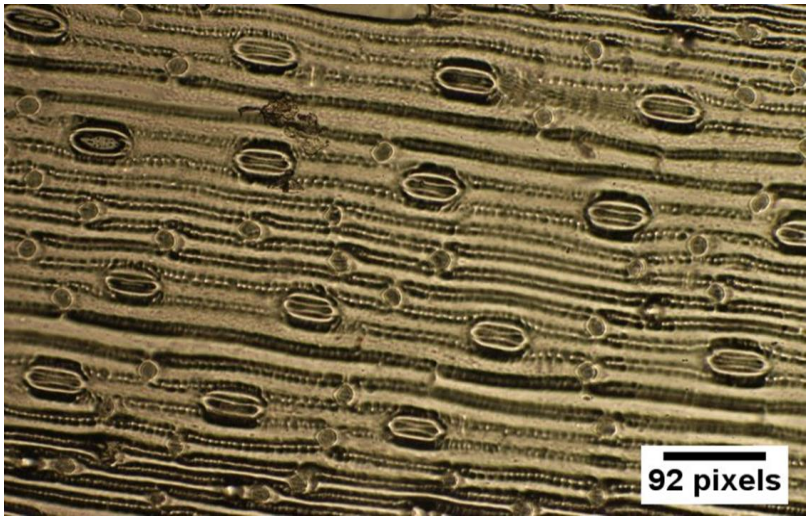


Image 5.1: An example stomata image.

All of the stomata (full shaped) in the images have been counted. In addition, three stomata per image have been examined in Image J software to measure stomatal area (stomatal size) involves the length and breadth of the pore when stomata open, and length and breadth of the guard cells when stomata closed.

5.2.2: Stomatal conductance

In order to measure the passage of water vapour leaving stomatal pore (mmol m-2 s -1), leaf stomatal conductance was examined in Experiment 3. Measurements of leaf stomatal conductance to water vapour (g_s) were carried out once in every seven days between 10:00 and 15:00 h (BST) using AP4 Leaf Porometer (Delta-T Device Ltd., Cambridge, UK). The measurements were started one week before the flowering stage and continued at weekly intervals until late maturity.

During the measurement, the sensor head of the porometer was clamped onto the leaf surface and conductance reading was taken continuously. Once the value stabilized on the monitor of the instrument, it was accepted and then recorded. The porometer was calibrated every time before it was used under a different set of environmental conditions using calibration plate which has 6 diffusion conductance settings. The data were then transferred to a computer at weekly intervals.

5.2.3: Statistical analysis

All statistical analyses were performed using GenStat software (version 17.1.0.13780, VSNI international Ltd). Relative humidity, temperature and variety were considered as categorical predictors in the control cabinet and field experiments, while relative humidity, soil moisture and variety were considered as categorical predictors for polytunnel experiment. An ANOVA was used to assess differences between treatments, a complete randomised block design was used for all experiments. Treatment effects were considered significant at $p<0.05$. When significant differences were observed, pair-wise multiple comparisons were performed using Tukey test ($p<0.05$) and T-test ($p<0.05$) to discriminate differences among the treatments and between the cultivars. Shapiro-Wilk normality test ($p<0.05$) and Bartlett`s test for homogeneity of variances ($p<0.05$) were carried out prior to each ANOVA. Data found not to satisfy these assumptions were transformed to attain homoscedasticity and normal distribution (Rasmussen, 1985; Bland and Altman, 1996; McDonald, 2009). Transformation was performed by mainly with an equation: x^3.

5.3: Results

Two Experiments (Experiment 2 and Experiment 3) were conducted over two successive growing seasons to investigate the effects of manipulated air temperature (T), relative humidity (RH) and soil moisture on stomata of two spring wheat cultivars, Blasco and Paragon. Experiments 2 and 3 represent polytunnel Experiment and field

Experiment, respectively. While soil moisture and RH treatments were applied in the Experiment 2, air T and RH treatments were applied to the cultivars in Experiment 3. Significant impacts of RH, air T and soil moisture treatments have been observed in both experiments. Summary tables showing the mean and standard deviation values, and Analysis of Variance of the stomatal parameters are shown in Appendix section of this chapter.

5.3.1: Effects of air temperature, RH and soil moisture on stomata number

We did not see any direct RH, air temperature or soil moisture effect on stomatal number at flowering stage in Blasco and Paragon in Experiment 2 and Experiment 3. However, there was a RH effect on stomatal number 12 DAA in Paragon in Experiment 3 where higher stomatal number at high RH under low air temperature conditions was found, compared to those at low RH (p=0.03, 5.1b). In Experiment 2, RH manipulation at stomatal number 12 DAA occurred at high soil moisture conditions as higher RH caused higher stomatal number (p=0.047, Fig. 5.1a). Besides this, Paragon had higher stomatal number 12 DAA at low soil moisture than those at high soil moisture under lower RH conditions (p=0.006).

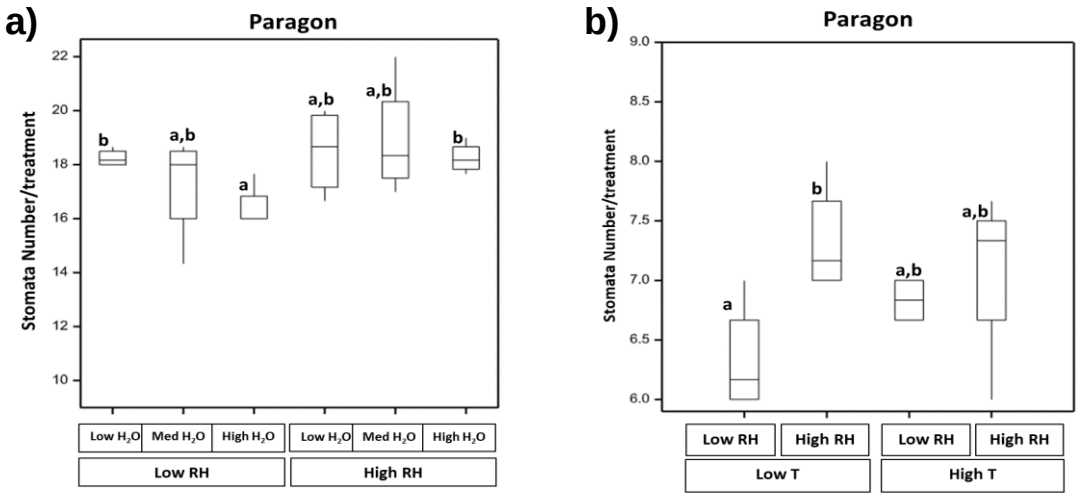


Figure 5.1: Stomatal number 12 DAA in Paragon wheat cultivar exposed to humidity and soil moisture manipulations during its life cycle (Experiment 2) (a), and Stomatal number 12 DAA in Paragon wheat variety exposed to humidity manipulation during its life cycle and temperature manipulation three consecutive days at flowering and 12 DAA (Experiment 3) (b). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

5.3.2: Effects of air temperature, RH and soil moisture on stomata area

During early grain filling in Experiment 2, stomatal area of Blasco was larger at medium soil moisture than at low soil moisture (p=0.003), while an interactive effect of RH and soil moisture has also found. Blasco had higher stomatal area at high RH/medium soil moisture than at low RH/high soil moisture (p=0.025, Fig. 5.2).

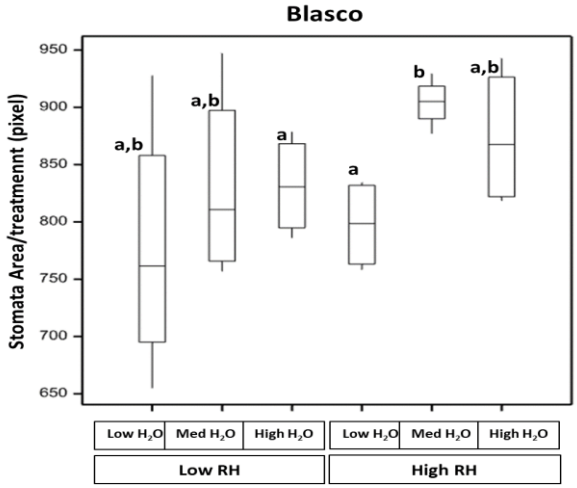


Figure 5.2: Effect of humidity and soil moisture manipulations, applied during the life cycle of the cultivars, on stomatal area in Blasco (a) and in Paragon (b) wheat varieties (Experiment 2). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at p<0.05.

We saw a combined effect of temperature and humidity on stomatal area at the flowering stage in Experiment 3. Blasco had larger stomatal area at high RH/high air temperature than those at low RH/low air temperature (p=0.024, Fig. 5.3).

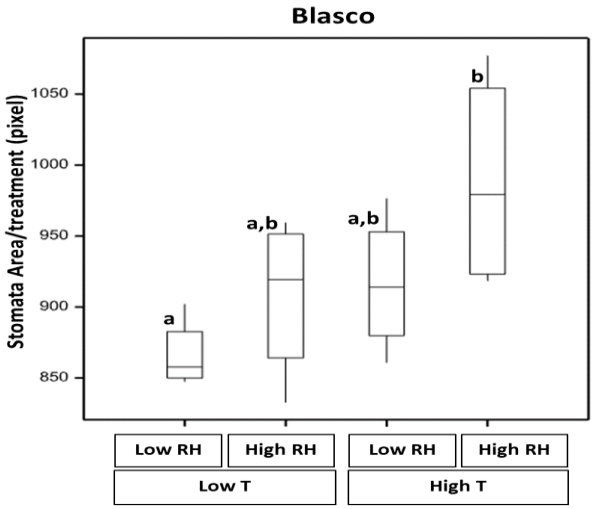


Figure 5.3: Stomatal area at the flowering stage in Blasco wheat variety after exposing to the humidity manipulation during its life time and temperature manipulation three consecutive days at the flowering and during early grain filling stages (Experiment 3). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

5.3.3: Effects of air temperature, RH and soil moisture on stomatal conductance

Even though we did not see any direct air T or RH effect on stomatal conductance per plot in the cultivars just after heat stress application at flowering stage in Experiment 3, data before the flowerings stage (without air T treatment) showed that there is direct RH effect on stomatal conductance in Blasco. At low RH, Blasco had higher stomatal conductance than those at high RH ($p= 0.021$, Fig. 5.4).

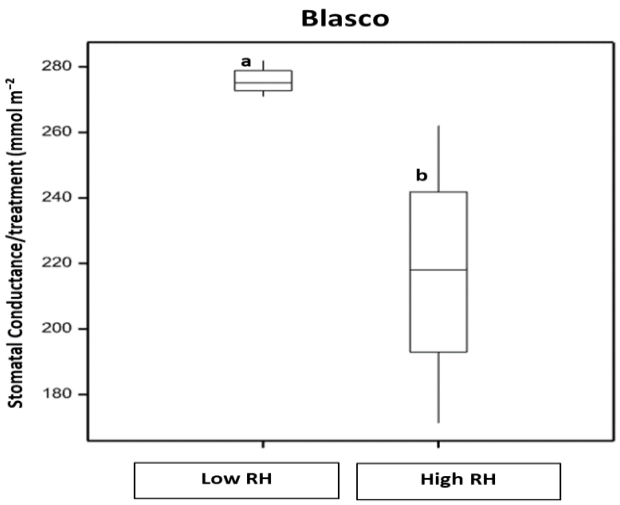


Figure 5.4: Effect of humidity manipulations, applied during the life cycle of the varieties, on stomatal conductance in Blasco (Experiment 3). Top and bottom of each box are the upper and lower quartiles, the horizontal line crossing each box is the median. Letters denote significant difference at $p<0.05$.

Alongside aforementioned analyses, we fitted a linear regression to analyse the relationship between stomatal number/area and tissue temperatures. Even though we have not observed any significant results for Blasco, we saw that stomatal number at the flowering stage is negatively correlated with the spike temperature in Paragon in Experiment 2 ($p=0.039$, Fig 5.5).

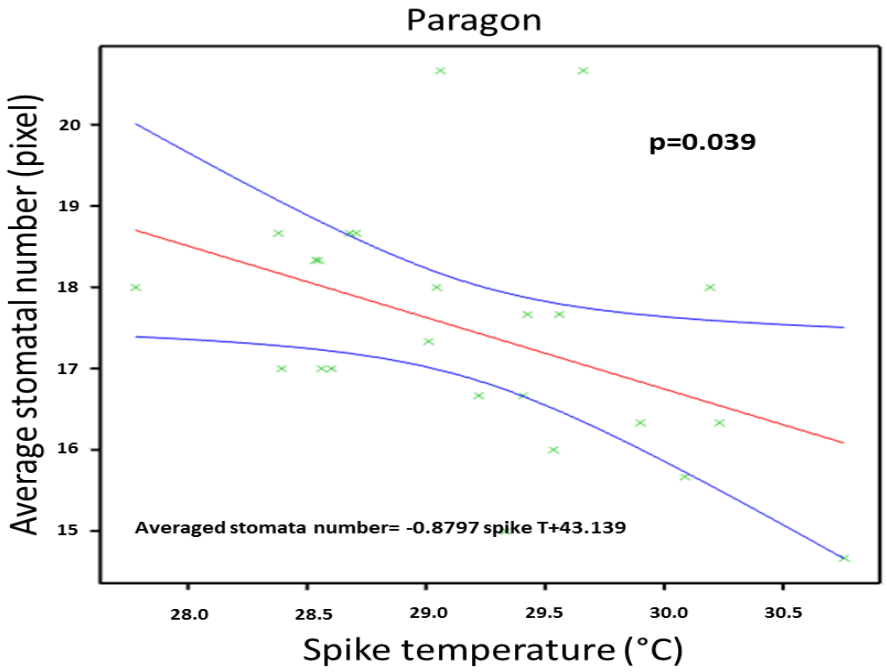


Figure 5.5 Linear regression between stomatal number at the flowering stage and spike temperatures in Paragon in Experiment 2.

5.4: Discussion

Dry atmosphere causes rise in the leaf-to-air vapor pressure deficit (VPD = vapor pressure difference between leaf and air), in other words, high gradient in water vapor through stomatal pores. Therefore, in response to decrease in atmospheric relative humidity, that is, increase in vapor pressure deficit (VPD), ABA is synthesized by roots and then stomatal close. This helps to avoid excessive water loss (El-Sharkawy *et al.*, 1985; Yong *et al.*, 1997; Bauer *et al.*, 2013; Fahad *et al.*, 2017; Pantin and Blatt, 2018). Studies in a range of trees and herbs suggested that higher VPD during development of plants causes increase in stomatal density, while the results were opposite in other

plants such as in tomato and eggplant (Aliniaeifard *et al.*, 2014; Carins Murphy *et al.*, 2014; Niglas *et al.*, 2015). There is a range of stomatal responses to elevated temperature involving stomatal closure, no significant response and stomatal opening, but the response of stomata to temperature is still not known well enough to make reliable predictions. However, it is consistent that temperature influence on stomata is generally indirect through transpiration, leaf water potential, VPD or intercellular CO_2 concentration (Schulze *et al.*, 1973; Urban *et al.*, 2017). We had a chance to examine the stomatal density and stomatal area by using samples collected at flowering and 12 DAA. In Experiment 3, we observed that high RH (lower VPD) increases stomatal density during early grain filling stage in Paragon at lower temperature, while high RH accompanied with high air T resulted in higher stomatal area at flowering stage in Blasco in compare to stomatal area at low RH/low air T.

Stomatal conductance, which is a measure of stomatal opening rate of the leaf, is an indicator of transpiration and gas exchange rate. It is a function of stomatal opening, size and density. Therefore, more open stomata result in higher conductance, that is, higher transpiration and photosynthesis rate (Gimenez *et al.*, 2005; Lawson, 2009; Pask *et al.*, 2012). Under irrigated conditions, it has been observed that grain yield of wheat is positively correlated with stomatal conductance (Reynolds *et al.*, 1994; Ayeneh *et al.*, 2002). In Experiment 3, we have examined the stomatal conductance of the cultivars. Because of technical problems, we could not analyse the combined effect of air T and RH on stomatal conductance during early grain filling stages, but data collected just after high temperature application at flowering stage showed that there is no effect of RH and air T on stomatal conductance of plants rooted in soil. Besides this, data collected before flowering stage (before high temperature application), we observed that Blasco had higher stomatal conductance at lower RH conditions.

Drought stimulates the production of plant hormone abscisic acid (ABA) causing stomatal closure and therefore decreased transpiration rate. Under drought stress, closure of stomata occurs in the efficient wheat cultivars which decrease the rate of transpiration. This stomatal closure helps to accumulate dry matter with consuming less water and increase water use efficiency (Abbate *et al.*, 2004; Fahad *et al.*, 2017). Several studies indicated that water deficit causes a decline in stomatal size and a rise in stomatal density which help plants to adapt drought (Liao *et al.*, 2005; Xu and Zhou, 2008; Kim *et al.*, 2010; Oskabe *et al.*, 2014; Mansouri and Radhouane, 2015; Fahad *et al.*, 2017). However, there are several other studies suggesting that stomatal density decreases under drought stress (Maricle *et al.*, 2009; Shabala, 2013), even though

most of the studies prove that there is a negative correlation between stomatal density and size under drought. These inconsistent results about stomatal response to drought, RH and temperature might be due to relation to species differences or not well controlled environments for experiments (Mansouri and Radhouane, 2015; Fahad *et al.*, 2017). The results of our study showed that Paragon had higher stomatal number during early grain filling at lower soil moisture under low RH conditions in Experiment 2, while Blasco had lower stomatal area during early grain filling at lower soil moisture under high RH conditions.

We can conclude that Blasco responds to the unfavourable environmental conditions with altering its stomatal area, while Paragon decreases or increases its stomatal number. High RH, high air T and low soil moisture caused increase in stomatal area in Blasco and High RH and low soil moisture resulted in higher stomatal number in Paragon. It is possible that higher stomatal conductance in Blasco at low RH that we observed is because of elevation in stomatal area, since Blasco responds to the stress factors via stomatal area. Further increase in temperature and drought stress due to climate change is likely to increase many existing pressures in stomata aperture to response the abiotic stresses.

5.5: References

Abbate, P. E., Dardanelli, J. L., Cantarero, M. G., Maturano, M., Melchiori, R. J. M. & Suero, E. E. (2004). Climatic and water availability effects on water-use efficiency in wheat. *Crop Science,* **44** (2), 474-483.

Aliniaeifard, S., Malcolm Matamoros, P. & van Meeteren, U. (2014). Stomatal malfunctioning under low VPD conditions: induced by alterations in stomatal morphology and leaf anatomy or in the ABA signaling? *Physiologia plantarum,* **152** (4), 688-699.

Assmann, S. M. (1993). Signal transduction in guard cells. *Annual review of cell biology,* **9** (1), 345-375.

Ayeneh, A., Van Ginkel, M., Reynolds, M. & Ammar, K. (2002). Comparison of leaf, spike, peduncle and canopy temperature depression in wheat under heat stress. *Field Crops Research,* **79** (2-3), 173-184.

Baldocchi, D. (1994). A comparative study of mass and energy exchange rates over a closed C3 (wheat) and an open C4 (corn) crop: II. CO2 exchange and water use efficiency. *Agricultural and Forest Meteorology,* **67** (3-4), 291-321.

Bauer, H., Ache, P., Lautner, S., Fromm, J., Hartung, W., Al-Rasheid, K. A., Sonnewald, S., Sonnewald, U., Kneitz, S. & Lachmann, N. (2013). The stomatal response to reduced relative humidity requires guard cell-autonomous ABA synthesis. *Current Biology,* **23** (1), 53-57.

Bland, J. M. & Altman, D. G. (1996). Statistics notes: Transforming data. *Bmj,* **312** (7033), 770.

Camargo, M. A. B. & Marenco, R. A. (2011). Density, size and distribution of stomata in 35 rainforest tree species in Central Amazonia. *Acta Amazonica,* **41** (2), 205-212.

Carins Murphy, M. R., Jordan, G. J. & Brodribb, T. J. (2014). Acclimation to humidity modifies the link between leaf size and the density of veins and stomata. *Plant, Cell & Environment,* **37** (1), 124-131.

Daszkowska-Golec, A. & Szarejko, I. (2013). Open or close the gate–stomata action under the control of phytohormones in drought stress conditions. *Frontiers in plant science,* **4**, 138.

El-Sharkawy, M. A., Cock, J. H. & Hernandez, A. D. P. (1985). Stomatal response to air humidity and its relation to stomatal density in a wide range of warm climate species. *Photosynthesis research,* **7** (2), 137-149.

Fahad, S., Bajwa, A. A., Nazir, U., Anjum, S. A., Farooq, A., Zohaib, A., Sadia, S., Nasim, W., Adkins, S. & Saud, S. (2017). Crop production under drought and heat stress: plant responses and management options. *Frontiers in plant science,* **8**, 1147.

Gimenez, C., Gallardo, M. & Thompson, R. B. (2005). *PLANT-WATER RELATIONS.*

Gitz, D. C. & Baker, J. T. (2009). Methods for creating stomatal impressions directly onto archivable slides. *Agronomy journal,* **101** (1), 232-236.

Hetherington, A. M. & Woodward, F. I. (2003). The role of stomata in sensing and driving environmental change. *Nature,* **424** (6951), 901.

Jarvis, A., Mansfield, T. & Davies, W. J. (1999). Stomatal behaviour, photosynthesis and transpiration under rising CO2. *Plant, Cell & Environment,* **22** (6), 639-648.

Kim, T.-H., Böhmer, M., Hu, H., Nishimura, N. & Schroeder, J. I. (2010). Guard cell signal transduction network: advances in understanding abscisic acid, CO2, and Ca2+ signaling. *Annual review of plant biology,* **61**, 561-591.

Laga, H., Shahinnia, F. & Fleury, D. (Year). Image-based plant stornata phenotyping. *In:* Control Automation Robotics & Vision (ICARCV), 2014 13th International Conference on, 2014. IEEE, 217-222.

Lawson, T. (2009). Guard cell photosynthesis and stomatal function. *New Phytologist,* **181** (1), 13-34.

Lawson, T., Oxborough, K., Morison, J. I. & Baker, N. R. (2002). Responses of photosynthetic electron transport in stomatal guard cells and mesophyll cells in intact leaves to light, CO2, and humidity. *Plant Physiology,* **128** (1), 52-62.

Liao, J.-X., Chang, J. & Wang, G.-X. (2005). Stomatal density and gas exchange in six wheat cultivars. *Cereal Research Communications,* **33** (4), 719-726.

Maghsoudi, K. & Maghsoudi, M. (2008). Analysis of the effects of stomatal frequency and size on transpiration and yield of wheat (Triticum aestivum L.). *American-Eurasian J Agric Environ Sci,* **3**, 865-72.

Mansouri, S. & Radhouane, L. (2015). Is Stomatal Density an Ideal Tool to Explain Drought

Effect on Photosynthesis in Tunisian Barley Varieties? *Journal of Chemical, Biological and Physical Sciences*.

Maricle, B. R., Koteyeva, N. K., Voznesenskaya, E. V., Thomasson, J. R. & Edwards, G. E. (2009). Diversity in leaf anatomy, and stomatal distribution and conductance, between salt marsh and freshwater species in the C4 genus Spartina (Poaceae). *New Phytologist,* **184** (1), 216-233.

McDonald, J. H. (2009). *Handbook of biological statistics.* Sparky House Publishing Baltimore, MD.

Niglas, A., Alber, M., Suur, K., Jasińska, A. K., Kupper, P. & Sellin, A. (2015). Does increased air humidity affect stomatal morphology and functioning in hybrid aspen? *Botany,* **93** (4), 243-250.

Oskabe, Y., Oskabe, K. & Shinozaki, K. T. (2014). LSP: Response of plants to water stress. *Frontiers in Plant Science*.

Pallas, J. E., Michel, B. E. & Harris, D. G. (1967). Photosynthesis, transpiration, leaf temperature, and stomatal activity of cotton plants under varying water potentials. *Plant Physiology,* **42** (1), 76-88.

Pantin, F. & Blatt, M. R. (2018). Stomatal Response to Humidity: Blurring the Boundary between Active and Passive Movement. *Plant physiology,* **176** (1), 485-488.

Pask, A., Pietragalla, J., Mullan, D. & Reynolds, M. (2012). *Physiological breeding II: a field guide to wheat phenotyping.* Cimmyt.

Raghavendra, A. S. (1981). Energy supply for stomatal opening in epidermal strips of Commelina benghalensis. *Plant physiology,* **67** (2), 385-387.

Rasmussen, J. L. (1985). Data transformation maximizing homoscedasticity and within-group normality. *Behavior Research Methods,* **17** (3), 411-412.

Reynolds, M., Balota, M., Delgado, M., Amani, I. & Fischer, R. (1994). Physiological and morphological traits associated with spring wheat yield under hot, irrigated conditions. *Functional Plant Biology,* **21** (6), 717-730.

Schulze, E.-D., Lange, O., Kappen, L., Buschbom, U. & Evenari, M. (1973). Stomatal responses to changes in temperature at increasing water stress. *Planta,* **110** (1), 29-42.

Setty, R. S. (2002). *Biotechnology-I: Including Biochemistry, Mathematics, Computer Science.* New Age International.

Shabala, S. (2013). Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. *Annals of Botany,* **112** (7), 1209-1221.

Shahinnia, F., Le Roy, J., Laborde, B., Sznajder, B., Kalambettu, P., Mahjourimajd, S., Tilbrook, J. & Fleury, D. (2016). Genetic association of stomatal traits and yield in wheat grown in low rainfall environments. *BMC plant biology,* **16** (1), 150.

Teare, I., Peterson, C. & Law, A. (1971). Size and Frequency of Leaf Stomata in Cultivars of Triticum aestivum and Other Triticum Species1. *Crop Science,* **11** (4), 496-498.

Urban, J., Ingwers, M. W., McGuire, M. A. & Teskey, R. O. (2017). Increase in leaf temperature opens stomata and decouples net photosynthesis from stomatal conductance in Pinus taeda and Populus deltoides x nigra. *Journal of experimental botany,* **68** (7), 1757-1767.

Weyers, J. D. & Paterson, N. W. (2001). Plant hormones and the control of physiological processes. *New Phytologist,* **152** (3), 375-407.

Willmer, C. & Fricker, M. (1996). *Stomata.* Springer Science & Business Media.

Xu, Z., Jiang, Y., Jia, B. & Zhou, G. (2016). Elevated-CO2 response of stomata and its dependence on environmental factors. *Frontiers in plant science,* **7**, 657.

Xu, Z. & Zhou, G. (2008). Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. *Journal of experimental botany,* **59** (12), 3317-3325.

Yong, J., Wong, S. & Farquhar, G. (1997). Stomatal responses to changes in vapour pressure difference between leaf and air. *Plant, Cell & Environment,* **20** (10), 1213-1216.

Zadoks, J. C., Chang, T. T. & Konzak, C. F. (1974). A decimal code for the growth stages of cereals. *Weed research,* **14** (6), 415-421.

Zeiger, E., Farquhar, G. D. & Cowan, I. (1987). *Stomatal function.* Stanford University Press.

5.6: Chapter 5 – Appendix

Appendix 5.1: Summary of stomatal number of Blasco and Paragon wheat cultivars prior to temperature, relative humidity and soil moisture treatments in Experiment 2 and Experiment 3.

Experiment 2

	Flowering GS Stomatal Number/ pot; mean ± SD		12 DAA Stomatal Number/ pot; mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Low	17.25±1.5	17.5±0.79	16.5±2.76	18.25±0.3 **(a)**
Dry/Med	14.33±2	17.08±1.83	18.58±1.23	17.25±2 (ab)
Dry/High	17.5±1.93	17.25±1.1	17.25±2.73	16.42±0.83 **(b)**
Wet/Low	16.08±1.26	16.92±1.6	17.42±2	18.5±1.6 (ab)
Wet/Med	18.67±4.16	17.08±1.03	17.17±1.73	18.92±1.12(ab)
Wet/High	17.83±2.62	17.08±1.03	15.67±1.9	18.25±0.57 **(a)**

Experiment 3

	Flowering GS Stomatal Number/ pot; mean ± SD		12 DAA Stomatal Number/ pot; mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Cool	8±1.12 (a)	7.17±0.3 (a)	7.25±0.8 (a)	**6.33±0.5(a)**
Dry/Warm	7.67±0.9 (a)	7.17±0.4 (a)	7.33±1 (a)	6.83±0.2 (ab)
Wet/Cool	7.33±0.8 (a)	7.33±0.5 (a)	7.25±0.3 (a)	**7.33±0.5 (b)**
Wet/Warm	7.33±0.7 (a)	7.08±0.4 (a)	7..17±1 (a)	7.08±0.7 (ab)

Appendix 5.2: Summary of stomatal area of Blasco and Paragon wheat cultivars prior to temperature, relative humidity and soil moisture treatments in Experiment 2 and Experiment 3.

Experiment 2

	Flowering GS Stomatal Area/ pot (pixel); mean ± SD		12 DAA Stomatal Area/ pot (pixel); mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Low	753±62.4 (a)	929.3±10.66 (a)	776.5±114.7 (ab)	804.1±44.8 (a)
Dry/Med	789.6±52.3 (a)	954.2±45.02 (a)	831.5±86.6 (ab)	833.4±54.1 (a)
Dry/High	819.9±74.3 (a)	972.2±86.42 (a)	831.5±43.9 **(a)**	885.7±42.5 (a)
Wet/Low	769.8±83.2 (a)	971.7±32.13 (a)	797.5±39.9 **(a)**	866.2±71. (a)
Wet/Med	754.6±36(a)	935.2±64.72 (a)	904.2±21.5 **(b)**	799.5±46(a)
Wet/High	796.6±57.2 (a)	998.3±58.27 (a)	874.2±61.8 (ab)	839.1±58.7 (a)

Experiment 3

	Flowering GS Stomatal Area/ pot (pixel); mean ± SD		12 DAA Stomatal Area/ pot (pixel); mean ± SD	
Treatment	**Blasco**	**Paragon**	**Blasco**	**Paragon**
Dry/Cool	866.3±24.78 **(a)**	1037±72.9 (a)	971±75.01 (a)	1113±72.3 (a)
Dry/Warm	916.4±48.9 (ab)	1007±48.6 (a)	967±64.77 (a)	1138±71.2 (a)
Wet/Cool	907.7±56.9 (ab)	1044±78.4 (a)	1050±108.1 (a)	1113±20.8 (a)
Wet/Warm	988.6±78.03 **(b)**	1075±61.4 (a)	974±40.43 (a)	1140±50.3 (a)

Appendix 5.3: Summary of stomatal conductance of Blasco and Paragon wheat cultivars prior to temperature, relative humidity and soil moisture treatments in Experiment 2 and Experiment 3.

Stomatal Conductance after flowering GS (mmol m^{-2} s^{-1}); mean ± SD			Stomatal Conductance before flowering GS (mmol m^{-2} s^{-1}); mean ± SD		
Treatment	**Blasco**	**Treatment**	**Treatment**	**Blasco**	**Paragon**
Dry/Cool	721.3±132.6 (a)	655.8±97.2 (a)	Dry	275.8±4.59 **(a)**	277.2±24.71 (a)
Dry/Warm	729.4±93.6 (a)	715.3±46.9 (a)	Wet	217.4±37.17 **(b)**	253.4±54.27 (a)
Wet/Cool	672.4±86.6 (a)	852.4±281.1 (a)			
Wet/Warm	738.1±73.6 (a)	830.3±138.9 (a)			

Analysis of Variance of stomatal number

Appendix 5.4: Analysis of Variance of stomatal number/pot during early grain filling stage of Paragon in Experiment 2.

Source of variation	**d.f**	**s.s**	**m.s.**	***F***	***P***
Humidity	1	9.375	9.375	4.56	0.047
Soil moisture	2	4.62	2.31	1.12	0.347
Humidity.Soil moisture	2	3.028	1.514	0.74	0.492
Residual	18	36.972	2.054		
Total	23	53.995			

Appendix 5.5: Analysis of Variance of stomatal number/plot during early grain filling stage of Paragon in Experiment 3.

Source of variation	**d.f**	**s.s**	**m.s.**	***F***	***P***
Humidity	1	1.5625	1.5625	6.08	0.03
Soil moisture	1	0.0625	0.0625	0.24	0.631
Humidity.Soil moisture	1	0.5625	0.5625	2.19	0.165
Residual	12	3.0833	0.2569		
Total	15	5.2708			

Appendix 5.6: Analysis of Variance of stomatal number/pot of Paragon between the results at low RH/high soil moisture and at low RH/low soil moisture in Experiment 2.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Soil moisture	1	6.7222	6.7222	16.88	0.006
Residual	6	2.3889	0.3981		
Total	7	9.1111			

Analysis of Variance of stomata area

Appendix 5.7: Analysis of Variance of stomatal area of Blasco between the results at low RH/high soil moisture and at high RH/medium soil moisture in Experiment 2.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	10593	10593	8.88	0.025
Residual	6	7156	1193		
Total	7	17749			

Appendix 5.8: Analysis of Variance of stomatal area of Blasco between the results at low RH/high soil moisture and at high RH/medium soil moisture in Experiment 2.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	22791	22791	22.21	0.003
Residual	6	6157	1026		
Total	7	28948			

Appendix 5.9: Analysis of Variance of stomatal conductance per plot during early grain filling stage of Blasco in Experiment 3.

Source of variation	d.f	s.s	m.s.	*F*	*P*
Humidity	1	6829.9	6829.9	9.74	0.021
Residual	6	4207	701.2		
Total	7	11036.9			

Sample preparation for stomata analysis under microscope

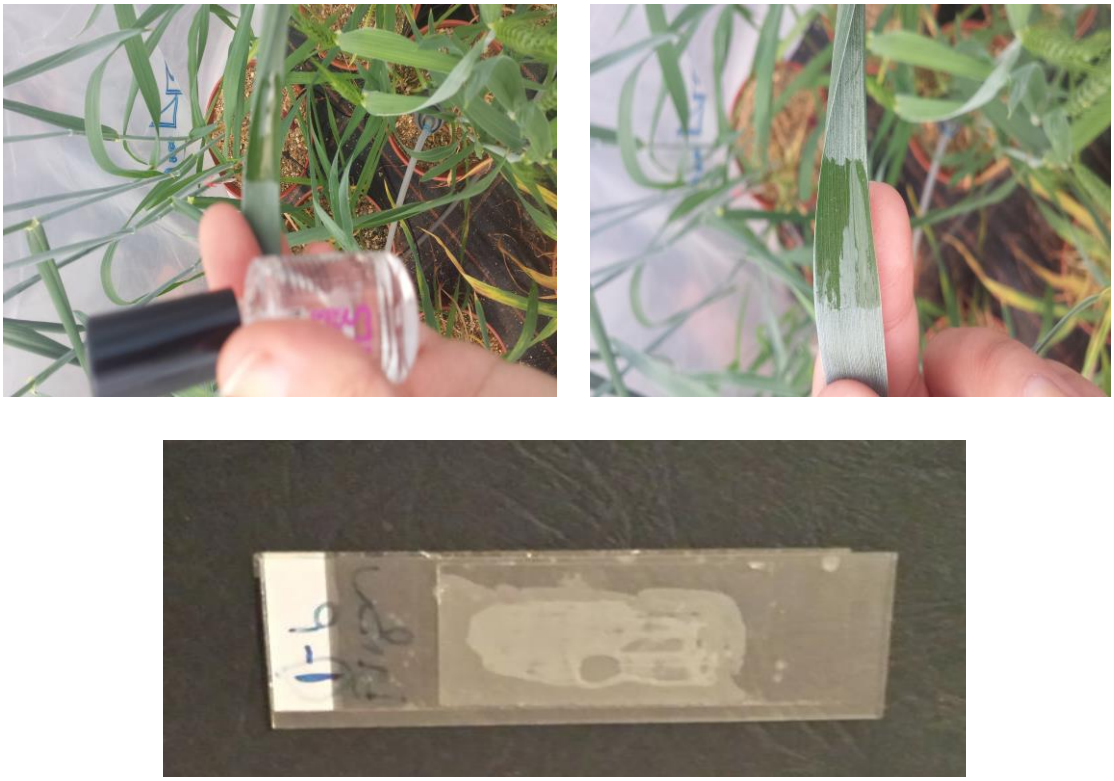


Image 5.2: Nail polish used to coat the abaxial surface of the flag leaves (a). Nail polish coated leaf surface (b). A piece of tape having epidermis from the leaf glued onto a microscope slide (c).

Chapter 6: Discussion

6.1: Thesis Overview

Studies carried out in rice indicate that organs' temperature is not only dependent on air temperature, but is also influenced by plant genotype and atmospheric relative humidity (RH). Panicle temperature at flowering, heading and during grain filling is negatively correlation with grain yield (seed setting and grain filing rate) (Yan *et al.*, 2010). Abeysiriwardena *et al.* (2002) and Yan *et al*. (2010) showed that an increase in RH at a given atmospheric temperature results in higher spikelet temperature in rice. Studies like this indicats that the impact of RH needs to be considered when evaluating effects of elevated air temperature on grain yield (Weerakoon *et al.*, 2008). To our knowledge, the role of RH in grain yield and grain quality in wheat has not been studied to date.

Paragon and Blasco, wheat varieties developed for cultivation in the UK and in the Mediterranean, respectively, and therefore likely to have been selected for different heat and humidity conditions were subjected to a manipulation of environemtnal conditions to elucidate the role of RH in their tissue temperatutre control. We conducted two control environment experiments and one field-based polytunnel experiment to identify the role of RH on the self-cooling capacity of wheat tissues at varying air temperature and soil moisture conditions. Following this, grain yield and grain quality parameters in each experiment were examined which allowed to determine how and to what extent humidity impacts on these parameters in wheat.

The four main hypotheses examined in this study were:

- H_0: Flag leaf/spike cooling capacity at flowering and early grain filling stage is positively correlated with grain yield under abiotic stress conditions.
- H_0: High RH causes an increase in alpha-amylase activity, while high air temperature results in lower α-amylase activity during grain filling.
- H_0: Elevated air temperature, high RH and low soil moisture result in higher grain protein content and alter protein composition.
- H_0: Growing conditions characterized by high RH, high air T and low soil moisture result in lower stomatal area and lower stomatal density.

Details relating to how tissue temperature, self-cooling capacity and grain yield were influenced under different environmental conditions (either in controlled environment or

in the field) can be found in Chapter 3, whilst findings about the effect of the treatments on grain quality and stomata are shown in Chapter 4 and Chapter 5, respectively. This final chapter of the thesis summarises key findings.

6.2: Tissue temperature and self-cooling capacity

The research documented in Chapter 3 showed that Blasco had higher cooling capacity (determined as the difference between air temperature and tissue temperature of the crops) than Paragon under most of the treatments in Experiment 1. We suggest that the tissue temperature difference between Blasco and Paragon might results from differences in the ear morphology between the two cultivars: Paragon is in fact awnless while Blasco has awns. Awns are needle-like structures extending from the lemma and by increasing the surface of the ear may improve the transpiration capacity of cereal spikes (Li *et al.*, 2010; Grundbacher, 1963; Rebetzke *et al.*, 2016). In wheat, awns are also photosynthetically active (Grundbacher, 1963; Li *et al.*, 2010; Hosseini *et al.*, 2012; Rebetzke *et al.*, 2016) and it has been suggested they may have higher temperature tolerance than leaf tissues. Besides this, Blum (1985) indicated that even though spikes with awns have larger surface area than the flag leaves, spikes have lower transpiration rate. This can be explained by the lower stomatal density in the spike (Teare *et al.*, 1972). Therefore, the spikes have lower temperature depression than flag leaves. Our results in Experiments 1, 2 and 3 have confirmed this, as temperature depression of the spike was lower than that of the flag leaf in both cultivars under almost all treatments. In other words, spike tissue temperatures were higher than flag leaf tissue temperatures.

The main driver of spike and flag leaf temperatures in Experiment 1 both in Blasco and Paragon at both flowering and early grain filling was the air temperature. Higher air temperature caused higher tissue temperatures in Blasco and Paragon at both high and low RH conditions in Experiment 1A and Experiment 1B. We have not observed any modification of tissue temperature by RH in Experiment 2 and Experiment 3 (Appendix 3.2), but in Experiemnt 1. This shows that tissue temperatures in both cultivars are not affected by the RH in the field conditions as mentioned in the Chapter 3.

Increase in RH at low temperature in Experiment 1 caused decrease in ear and flag leaf temperatures in Blasco at both flowering and early grain filling, whilst the same conditions applied at early grain filling was associated with higher tissue temperatures in Paragon. Since we have observed higher evapotranspiration rate in both cultivars at

low RH than at high RH under low air temperature in Experiment 1, decrease in soil moisture at low RH might have triggered Blasco to lower its stomatal opening in order to prevent excessive water loss as it has adapted to climates prone to water stress conditions. The observed decrease in stomatal area at lower RH at flowering in Experiment 3 would be in agreement with this suggestion and similar to what reported by a study carried out in rice, where stomatal opening decreased at lower RH under lower air temperatures resulting in higher tissue temperatures (Abeysiriwardena *et al.*, 2002). Besides this, it is extremely important for wheat cultivars growing under the Mediterranean climate to accumulate carbohydrates and nitrogenous compounds in the vegetative organs during or before anthesis, since the climate conditions after anthesis are generally hot and dry and so photosynthetic activity is limited (Papakosta and Gagianas, 1991). Therefore, when RH level was high under cool air temperature in Experiment 1A, Blasco might have stored more assimilates to be used during the grain filling stage. Therefore, it might have increased stomatal area on its leaves and spike to increase its photosynthetic activity as much as possible.

On the other hand, in Experiement 1, lower RH at high air temperature facilitated lower ear temperatures in both cultivars at early grain filling, similar to an effect observed in rice (Abeysiriwardena *et al.*, 2002; Yan *et al.*, 2010). Less humid and warm atmosphere allowed higher transpiration and loss of water via stomatal opening, thus helping plants to control temperature by thermal cooling (Pallas *et al.*, 1967; Hetherington and Woodward, 2003; Lawson, 2009; Laga *et al.*, 2014). The water vapour holding capacity of air increases with decreasing relative humidity while air at 100% RH cannot hold more water vapour as it is saturated (Forbes and Watson, 1992; Ramulu, 1998; Roberts *et al.*, 2000; Roberts, 1986).

6.3: Stomata

Studies carried out on a range of trees and herbs suggest that higher VPD (lower RH) during development of plants causes increase in stomatal density, while the results were opposite in other plants such as in tomato and eggplant (Aliniaeifard *et al.*, 2014; Carins Murphy *et al.*, 2014; Niglas *et al.*, 2015). In Paragon, we have found that high RH caused higher stomatal number during early grain filling at low air temperature in Experiment 3. Some of the studies have also showed that high humidity triggers stomatal opening (Lawson *et al.*, 2002; Assmann, 1993; Lawson, 2009; Weyers and Paterson, 2001). Confirming this, we found that stomatal area in Blasco at flowering was higher at high RH/high air T than those at low RH/low air T in Experiment 3.

Several studies indicated that water deficit causes a decline in stomatal size and a rise in stomatal density which help plants to adapt to drought (Liao *et al.*, 2005; Xu and Zhou, 2008; Kim *et al.*, 2010; Oskabe *et al.*, 2014; Mansouri and Radhouane, 2015; Fahad *et al.*, 2017). Our results in experiment 2 is supporting these suggestions. We found that water stress caused a decrease in stomatal area in Blasco during early grain filling at high RH, while water stress resulted in higher stomatal number during grain filling at low RH in Paragon.

6.4: Grain yield

In Experiment 1A, grain number in Paragon was affected from the combined effect of high RH and high temperature where higher tissue temperature at high RH/high air T caused decrease in grain number. Sporogenesis, pollination and fertilization might be affected negatively from high temperature on spike causing lower grain number in Paragon (Farooq *et al.*, 2011; Saini and Aspinall, 1982). But we have also observed that Paragon compensated the decrease in grain number with increasing its grain size similar to the results in the study of Rebetzke *et al.* (2016).

When RH was low in Experiment 1B, higher air temperature caused increase in ear temperature and decrease in grain yield in Blasco. At high temperature, higher RH caused increase in tissue temperatures in Paragon which resulted in lower grain yield. As previous studies suggested, high air temperature might have accelerated grain development, which might have shortened grain filling in Blasco and Paragon (Dias and Lidon, 2009; Barlow *et al.*, 2015). Therefore, starch accumulation in grains might have decreased (Barlow *et al.*, 2015) in Blasco and Paragon resulting in lower grain yield.

At low air temperature in Experiment 1B, higher RH caused decrease in the tissue temperature in Blasco and this resulted in lower grain yield. Tissue temperature at high RH/low air temperature in Blasco (ST= 15.88 °C, FLT = 12.56°C) might have been lower than the optimal temperatures for the activity of enzymes involved in starch biosynthesis. (Bettelheim *et al.*, 2012) which would explain the decrease in grain yield.

At low RH in Experiment 1B, high air temperature caused increase in tissue temperatures and increase in grain yield in Paragon. Tissue temperature in Paragon at low RH/high air temperature (ST=26.34°C, FLT= 23.91°C) might have been within the optimal range of temperatures for the enzyme activities having role in starch biosynthesis. Thus, this might have caused increase in starch biosynthesis and thus higher grain yield in Paragon.

Previous studies suggested that water stress affects grain filling duration and the activity of starch synthase negatively (Farooq *et al.*, 2011; Ahmadi and Baker, 2001). Alongside this, water stress during meiosis was suggested to prevent microsporogenesis while during anthesis would negatively affect fertilization resulting in reduced grain number (Cattivelli *et al.*, 2008; Manjarrez-Sandoval *et al.*, 1989). Confirming these observations, In Experiment 2, we found that grain yield of the cultivars was affected by soil moisture treatments where an increase in soil moisture resulted in higher grain yield in both cultivars.

The results in the field and semi-controlled (Experiment 2) experiments clearly showed that RH does not have significant impact on tissue temperature control and thus on grain yield unless it is extremely low or high. But the results in the controlled environment experiment can guide us about how RH can control tissue temperature and yield under possible extreme RH conditions in the field.

6.5: Grain quality

Previous studies suggested that increase in temperatures from about 15 °C to 30°C (Fadeel *et al.*, 1980; Reddy *et al.*, 1984), prolonged rainfall and high humidity during grain filling elevates the α-amylase amount dramatically (Thomason *et al.*, 2009; Mares and Mrva, 2014; Rakita *et al.*, 2015). But, temperatures higher than 30°C during ripening cause decrease in α-amylase activity and this might be because of higher α-amylase inactivation rate or lower enzyme synthesis or both under heat stress (Reddy *et al.*, 1984; Rakita *et al.*, 2015). In addition, Mares and Mrva, 2014 & Mares snd Mrva, 2008 suggested that high-humidity and significant decrease in temperature at harvest ripeness may give elevated levels of alpha amylase, known as the late maturity alpha amylase phenomena in susceptible cultivars. Supporting the suggestions in Reddy *et al.*, 1984 and Rakita *et al.*, 2015, we found that high tissue temperature caused lower alpha amylase activity in Paragon in Experiment 1B. This might be another reason of higher grain yield in Paragon at low RH/high air T in comparison to those at low RH/low air T in Experiment 1B, as it has higher tissue temperature at low RH/high air temperature (Chapter 3) and thus lower alpha amylase activity which probably caused less starch degradation at high RH/high air T (Reddy *et al.*, 1984). Even though it was not significant, we found that grain yield tended to be negatively correlated with alpha amylase activity in Paragon following a linear regression analyse (Appendix 4.23).

During the grain-filling stage heat stress impacts negatively on starch deposition while has little effect on protein deposition, therefore increasing grain protein concentration

since this permits more nitrogen per unit of starch (Stone and Nicolas, 1998; Farooq *et al.*, 2011). Accordingly, we observed in Experiment 1B that high RH (86%) and high temperatrue (32 °C day/24 °C night) conditions caused increase in total protein and total N content in Blasco, while high air temperature under both high and low RH resulted in higher N content in Paragon. Following the linear regression analysis, we found that N content tended to be negatively correlated with the grain yield in Blasco, even though the correlation was not statistically significant (Appendix 4.20).

Previous studies suggested that more than 80% of the total N content accumulates before flowering stage, and that this is responsible for around 50-100% of final wheat grain N content (Daigger *et al.*, 1976; Simpson *et al.*, 1983; Papakosta and Gagianas, 1991; Tahir and Nakata, 2005). Since photosynthetic process is inhibited, and leaf senescence is accelerated under heat stress from anthesis to maturity (Al-Khatib and Paulsen, 1990), pre-anthesis stores of nitrogen and carbohydrates in the stem are an important alternative nutrient supply under any kind of stress (Tahir and Nakata, 2005). However, an increase in temperature is likely to shorten the growth period of wheat, which causes a potential decrease in uptake and accumulation of N and biomass production (Al-Khatib and Paulsen, 1990; Tahir and Nakata, 2005). As it was suggested in these studies, we found that high RH and high air temperatrue caused lower grain yield in Paragon when this variable was analysed per ear instead of per circular area (0.1 m^2), and the reason might be lower N uptake and N accumulation. Even though there was not any significant effect on grain yield when this variable was analysed per area (0.1 m^2), grain yield still tended to be lower at high RH and high air temperature conditions in both cultivars (Appendix 3.4). This is showing the negative correlation between grain yield and grain protein content in the cultivars which was also observed following a linear regression analysis (Appendix 4.22).

In Experiment 2, higher soil moisture under wet condition resulted in lower nitrogen content in Paragon, while higher RH under low soil moisture condition resulted in lower gluten protein content in Blasco. Results presented in Chapter 3 show that Paragon had higher grain yield at higher soil moisture conditions, while Blasco had higher grain yield at higher RH and higher soil moisture conditions. These results show a negative correlation between grain weight and grain protein content for both cultivars which was also observed following a linear regression analysis (Appendix 4.21) broadly consistent with existing observations (Stone and Nicolas, 1998; Farooq *et al.*, 2011). Water stress might have caused lower enzyme activities for starch biosynthesis and thus this might

have permitted more nitrogen per unit of starch in Blasco and Paragon (Ahmadi and Baker, 2001; Ashraf, 2014; Farooq *et al.*, 2014) .

In Experiment 1B, high RH and high air temperature resulted in higher total gluten content and higher ω-gliadins gluten subunit in Blasco. These results are consistent with the results of the previous studies (Spiertz *et al.*, 2006; BeNCze and VeiSz, 2011; Ashraf, 2014; Koga *et al.*, 2015; Koga *et al.*, 2016). Other studies suggested that increase in ω-gliadins causes decrease in loaf height so dough strength is negatively affected (Fido *et al.*, 1997; Malalgoda *et al.*). Therefore, we can say that dough strength in Blasco was affected negatively in Experiment 1B at high RH and high air T conditions.

Water stress is reported in several published work as the reason for an increase in SDS-sedimentation volume which is positively correlated with HMW glutenin subunits (Aslani *et al.*, 2013; Li *et al.*, 2013; ZHANG *et al.*, 2014). However, Gooding *et al.* (2003) found that drought stress during grain filling stage significantly decrease the SDS-sedimentation volume. In Experiment 2, we found that Paragon had lower SDS-sedimentation volume at high soil moisture than at medium soil moisture under wet conditions. This is consistent with the results of some of the previous studies as SDS-sedimentation volume of Paragon is negatively correlation with soil moisture (Aslani *et al.*, 2013; Li *et al.*, 2013; ZHANG *et al.*, 2014).

Heat stress has beem reported as causing increase in SDS-sedimentation volume (Aslani *et al.*, 2013; Li *et al.*, 2013; ZHANG *et al.*, 2014). In Experiment 3 however, we have not observed any difference within the treatments in terms of SDS-sedimentation volume for any of the two cultivars (Appendix 4.3).

6.6: Limitations of the Study

This project has provided a novel insight into the role of atmospheric humidity on heat stress tolerance in wheat. However, there are several limitations that have to be considered.

An initial limitation of the study was the difficulty in finding published studies on the impact of atmospheric humidity variation on cereals in general, and on wheat in particular. Few studies have reported interactive effects of RH and temperature on grain yield in rice (Abeysiriwardena *et al.*, 2002; Weerakoon *et al.*, 2008; Yan *et al.*, 2010; Van Oort *et al.*, 2014; Abeysingha *et al.*, 2016), but no similar study considering the interactive effect of RH and temperature on grain yield or grain quality in wheat

were available. The lack of comparable literature is also an opprtunity for further exploratory research into the role of atmospheric humidity on both grain yield and grain quality in wheat, however this also mean our results cannot be directly compared to other published studies.

The size and the number of growth cabinets in Experiment 1 did not allow us to increase the rate of replication or to vary additional environmental conditions such as soil moisture. A larger number of growth cabinets with better control of environmental conditions would certainly increase the power of the experiment. At a later stage, we have conducted Experiment 2 by growing pot-based plants in purpose-built chambers within a large polytunnel to expose wheat to long-term ambient RH and low RH. We used dehumidifiers to decrease RH in half of the chambers. The dehumidifiers introduced an element of air circulation and a slight temperature increase (due to the electronics present) within the low RH chambers. To combat these differences, small fans were installed into ambient RH chambers and atmospheric temperature was continuously measured at 10 minute intervals. Temperatrure records were later used to normalise plant tissue temperature data between the two treatments.

There is an inherent limitation in control environment experiments as direct applicability of field conditions is limited (Anderson, 1986; Downs and Krizek, 1997; Hamasaki and Okada, 2000). Even though controlled comparison between responses to different treatments is possible in satisfactory scale especially with high level of replication, field-scale experimentation is crucial in order to develop commercially viable cultivars in breeding programmes.

Our Experiment 3 was thus carried out in field conditions, where we encountered a specific set of limitations. It is inherently difficult to investigate the effect of a single or few stress factors on crops, since the impact of compounding abiotic and biotic stresses in the field can be complicated (Petersen, 1994). Natural heterogeneity of the soil profile and microclimates within the field can make it complicated to apply abiotic stress especially drought and/or heat stresses at specific growth stages. Even though we tried to equalize the soil moisture between the humid and ambient tunnels with turning of the irrigation in humid tunnels, because of soil gradient difference and weather conditions (e.g. rainfall driven by strong winds), the control of soil humidity was imperfect. Rodent infestation caused loss of the crop in several parts of the experiment and ant infestation of one of the data logger resulted in a loss of temperature data for a short part of the experiment.

Infrared images – in order to be comparable - need to be taken under consistently cloudy or sunny conditions. Polytunnel and field observation thus take more time than in control cabinets. Even under consistent insolation, it is not possible to take all images at one time, the travel of the sun and associated duirnal fluctuation of ambient temperature introduces noise in the data despite accompanying minute temperature measurement.

The misting units used in the field experiment were custom made, it is likely that some level of variation (both in time and space) of their performance was encountered during the experimental run.

6.7: Future Work

The generality of the findings we observed in this project needs to be assessed by conducting more detailed studies. Some suggestions for future research are given below.

More control environment and field experiments, and a modelling for simulation

There are several opportunities for further investigation arising from the experiments reported in this thesis. Fundamental responses of two wheat cultivars to the interaction between temperature and RH and the interaction between soil moisture and RH have been quantified, however there is significant potential to improve our measurements. The replication in the Experiment 1 was just sufficient to support two-way ANOVA type of analysis. A growth cabinet experiment with larger rate of replication (McDonald, 2009) and including soil moisture treatment in addition to RH and temperature treatments could be conducted to develop a more detailed and accurate picture. Following this, a field-scale experiment using a stronger and better engineered RH, temperature and soil moisture treatments could uncoved any interaction between these environmetnal features (Eller *et al.*, 2005). In addition, a larger field experiment would have the potential to provide a large amount of grain yield (Johnston and Poulton, 2018) which could be used for multiple grain quality analyses.

Control environment and field experiments can provide key insights into the short-term biological responses, thus analytical models are needed for long-term predictions (Schmitz, 2000). Following the findings detailed in this thesis, simple modelling studies for simulating the impact of possible RH scenarios in the future with considering RH

interaction with temperature and soil moisture can be conducted. Data from these experiments would help construct a more precise picture of how the relationship between RH and heat stress tolerance in wheat.

Awns and awnless Blasco wheat cultivar

The research documented in Chapter 3 showed that cooling capacity of Blasco was higher than Paragon under most of the treatments in experiment Experiment 1. As mentioned past studies this might be because of ear morphological difference of the cultivars where Paragon is an awnless cultivar and Blasco has awn, which would confer different evatranspiration and photosynthetic capacity to the two cultivars under stress conditions (Li *et al.*, 2010; Grundbacher, 1963; Rebetzke *et al.*, 2016). This suggestion needs to be analysed by conducting a control environment experiment. In this experiment, Blasco wheat cultivars can be grown under different RH, air temperature and soil moisture conditions, where half of the cultivars will have awns but order half will be awnless (awns removal) (Maydup *et al.*, 2010).

Root architecture of Blasco and Paragon

Root is a crucial organ for absorption of water and mineral nutrients from the soil, and its growth is affected from soil and environments encountered by other crop plant organs (Zhang *et al.*, 2009; Wang *et al.*, 2014). For instance, (Passioura, 1983) suggested that when roots are smaller, higher assimilate is available for the shoot which resulted in higher water use efficiency and grain yield. Therefore, root architecture needs to be examined in the forthcoming field experiment, which can show if there is a root effect on the grain yield, stomatal number, stomatal area and cooling capacity.

Remobilization of the assimilates

Grain filling in wheat, especially in a Mediterranean climate where photosynthesis is limited due to dry and hot climate after anthesis, usually depends heavily on the remobilization of the pre-anthesis stored C and N assimilates in the vegetative organs (Papakosta and Gagianas, 1991; Palta *et al.*, 1994). The translocation of the assimilates depends on the genetic and environmental factors. In order to improve grain yield and N accumulation in the grain, selecting genotypes having higher assimilate translocation ability from vegetative organs to the grain would be valuable (Papakosta and Gagianas, 1991). Therefore, N and dry matter accumulation before anthesis and assimilate remobilization to grain in the cultivars could be examined in the forthcoming experiments, in order to determine their impact on grain yield and quality.

6.8: Concluding Remarks

This project contributes to our understanding of how RH impact on the perception of high air temperature and low soil moisture by the wheat plant. In showing that, based on the results of the controlled cabinet experiment, low RH in comparison to high RH may allow to increase cooling capacity at high temperature conditions, limiting heat stress in both cultivars during early grain filling and maintaining grain yield in Paragon. Low atmospheric humidity can trigger higher cooling capacity in Paragon during early grain filling at low temperatures, while it may decrease cooling capacity but increase grain yield in Blasco. However, the results in the field based polytunnel and semi-controlled pot based polytunnel experiments showing that RH may not impact tissue temperature and thus grain yield unless it is extremely low or high. What we observed in the controlled environment experiment can guide us about how RH can control tissue temperature and yield under possible extreme RH conditions in the field.

In addition, we observed that water stress may result in lower grain yield. Grain protein content and grain yield can be negatively correlated in both cultivars, while bread making quality in Blasco can be affected negatively from high RH and high air temperatrue conditions due to alteration in ω- gliadins. The results of this project in combination with the results from few more detailed studies in the future have potential to be used in wheat breeding programmes.

6.7: References

Abeysingha, N., Singh, M., Islam, A. & Sehgal, V. (2016). Climate change impacts on irrigated rice and wheat production in Gomti River basin of India: a case study. *SpringerPlus,* **5** (1), 1250.

Abeysiriwardena, D. d. Z., Ohba, K. & Maruyama, A. (2002). Influence of temperature and relative humidity on grain sterility in rice. *Journal of the National Science Foundation of Sri Lanka,* **30** (1-2), 33-41.

Ahmadi, A. & Baker, D. A. (2001). The effect of water stress on the activities of key regulatory enzymes of the sucrose to starch pathway in wheat. *Plant Growth Regulation,* **35** (1), 81-91.

Al-Khatib, K. & Paulsen, G. M. (1990). Photosynthesis and productivity during high-temperature stress of wheat genotypes from major world regions. *Crop Science,* **30** (5), 1127-1132.

Aliniaeifard, S., Malcolm Matamoros, P. & van Meeteren, U. (2014). Stomatal malfunctioning under low VPD conditions: induced by alterations in stomatal

morphology and leaf anatomy or in the ABA signaling? *Physiologia plantarum,* **152** (4), 688-699.

Anderson, E. (1986). Maize root response to N in the greenhouse and field. *Plant and soil,* **95** (2), 293-296.

Ashraf, M. (2014). Stress-induced changes in wheat grain composition and quality. *Critical reviews in food science and nutrition,* **54** (12), 1576-1583.

Aslani, F., Mehrvar, M. R., Nazeri, A. & Juraimi, A. S. (2013). Investigation of wheat grain quality characteristics under water deficit condition during post-anthesis stage. *ARPN J Agr Biol Sci,* **8** (4), 273-278.

Assmann, S. M. (1993). Signal transduction in guard cells. *Annual review of cell biology,* **9** (1), 345-375.

Barlow, K., Christy, B., O'leary, G., Riffkin, P. & Nuttall, J. (2015). Simulating the impact of extreme heat and frost events on wheat crop production: a review. *Field Crops Research,* **171**, 109-119.

BeNCze, S. & VeiSz, O. (2011). Quality of winter wheat in relation to heat and drought shock after anthesis. *Czech J. Food Sci. Vol,* **29** (2), 117-128.

Bettelheim, F., Brown, W., Campbell, M., Farrell, S. & Torres, O. (2012). *Introduction to general, organic and biochemistry.* Nelson Education.

Blum, A. (1985). Photosynthesis and transpiration in leaves and ears of wheat and barley varieties. *Journal of experimental botany,* **36** (3), 432-440.

Carins Murphy, M. R., Jordan, G. J. & Brodribb, T. J. (2014). Acclimation to humidity modifies the link between leaf size and the density of veins and stomata. *Plant, Cell & Environment,* **37** (1), 124-131.

Cattivelli, L., Rizza, F., Badeck, F.-W., Mazzucotelli, E., Mastrangelo, A. M., Francia, E., Mare, C., Tondelli, A. & Stanca, A. M. (2008). Drought tolerance improvement in crop plants: an integrated view from breeding to genomics. *Field Crops Research,* **105** (1-2), 1-14.

Daigger, L., Sander, D. & Peterson, G. (1976). Nitrogen Content of Winter Wheat During Growth and Maturation 1. *Agronomy Journal,* **68** (5), 815-818.

Dias, A. & Lidon, F. (2009). Evaluation of grain filling rate and duration in bread and durum wheat, under heat stress after anthesis. *Journal of Agronomy and Crop Science,* **195** (2), 137-147.

Downs, R. J. & Krizek, D. T. (1997). Air movement. *Plant growth chamber handbook*, 87-104.

Eller, G., Krüger, M. & Frenzel, P. (2005). Comparing field and microcosm experiments: a case study on methano-and methylo-trophic bacteria in paddy soil. *FEMS microbiology ecology,* **51** (2), 279-291.

Fadeel, A., Moll, B. A. & Jones, R. L. (1980). Effect of temperature on the synthesis and secretion of α-amylase in barley aleurone layers. *Plant physiology,* **66** (3), 466-470.

Fahad, S., Bajwa, A. A., Nazir, U., Anjum, S. A., Farooq, A., Zohaib, A., Sadia, S., Nasim, W., Adkins, S. & Saud, S. (2017). Crop production under drought and heat stress: plant responses and management options. *Frontiers in plant science,* **8**, 1147.

Farooq, M., Bramley, H., Palta, J. A. & Siddique, K. H. (2011). Heat stress in wheat during reproductive and grain-filling phases. *Critical Reviews in Plant Sciences,* **30** (6), 491-507.

Farooq, M., Hussain, M. & Siddique, K. H. (2014). Drought stress in wheat during flowering and grain-filling periods. *Critical Reviews in Plant Sciences,* **33** (4), 331-349.

Fido, R., Bekes, F., Gras, P. & Tatham, A. (1997). Effects of α-, β-, γ-and ω-gliadins on the dough mixing properties of wheat flour. *Journal of Cereal Science,* **26** (3), 271-277.

Forbes, J. C. & Watson, D. (1992). *Plants in agriculture.* Cambridge University Press.

Gooding, M., Ellis, R., Shewry, P. & Schofield, J. (2003). Effects of restricted water availability and increased temperature on the grain filling, drying and quality of winter wheat. *Journal of Cereal Science,* **37** (3), 295-309.

Grundbacher, F. (1963). The physiological function of the cereal awn. *The Botanical Review,* **29** (3), 366-381.

Hamasaki, T. & Okada, M. (2000). Thermal radiation load on temperature regimes in plant growth chambers. *Biotronics,* **29**, 57-69.

Hetherington, A. M. & Woodward, F. I. (2003). The role of stomata in sensing and driving environmental change. *Nature,* **424** (6951), 901.

Hosseini, S. M., Poustini, K., Siddique, K. H. & Palta, J. A. (2012). Photosynthesis of barley awns does not play a significant role in grain yield under terminal drought. *Crop and Pasture Science,* **63** (5), 489-499.

Johnston, A. & Poulton, P. (2018). The importance of long-term experiments in agriculture: their management to ensure continued crop production and soil fertility; the Rothamsted experience. *European Journal of Soil Science,* **69** (1), 113-125.

Kim, T.-H., Böhmer, M., Hu, H., Nishimura, N. & Schroeder, J. I. (2010). Guard cell signal transduction network: advances in understanding abscisic acid, CO2, and Ca2+ signaling. *Annual review of plant biology,* **61**, 561-591.

Koga, S., Böcker, U., Moldestad, A., Tosi, P., Shewry, P. R., Mosleth, E. F. & Uhlen, A. K. (2015). Influence of temperature on the composition and polymerization of gluten proteins during grain filling in spring wheat (Triticum aestivum L.). *Journal of Cereal Science,* **65**, 1-8.

Koga, S., Böcker, U., Moldestad, A., Tosi, P., Shewry, P. R., Mosleth, E. F. & Uhlen, A. K. (2016). Influence of temperature during grain filling on gluten viscoelastic properties and gluten protein composition. *Journal of the Science of Food and Agriculture,* **96** (1), 122-130.

Laga, H., Shahinnia, F. & Fleury, D. (Year). Image-based plant stornata phenotyping. *In:* Control Automation Robotics & Vision (ICARCV), 2014 13th International Conference on, 2014. IEEE, 217-222.

Lawson, T. (2009). Guard cell photosynthesis and stomatal function. *New Phytologist,* **181** (1), 13-34.

Lawson, T., Oxborough, K., Morison, J. I. & Baker, N. R. (2002). Responses of photosynthetic electron transport in stomatal guard cells and mesophyll cells in intact leaves to light, CO2, and humidity. *Plant Physiology,* **128** (1), 52-62.

Li, X.-f., Bin, D. & Hong-Gang, W. (2010). Awn anatomy of common wheat (Triticum aestivum L.) and its relatives. *Caryologia,* **63** (4), 391-397.

Li, Y., Wu, Y., Hernandez-Espinosa, N. & Peña, R. J. (2013). The influence of drought and heat stress on the expression of end-use quality parameters of common wheat. *Journal of Cereal Science,* **57** (1), 73-78.

Liao, J.-X., Chang, J. & Wang, G.-X. (2005). Stomatal density and gas exchange in six wheat cultivars. *Cereal Research Communications,* **33** (4), 719-726.

Malalgoda, Maneka, Ohm, J. B., Meinhardt, Steven, Simsek & Senay (2017). Association between gluten protein composition and breadmaking quality characteristics in historical and modern spring wheat. . *Cereal Chemistry*.

Manjarrez-Sandoval, P., GONZÁLEZ-HERNÁNDEZ, V. A., MENDOZA-ONOFRE, L. E. & Engleman, E. (1989). Drought stress effects on the grain yield and panicle development of sorghum. *Canadian Journal of Plant Science,* **69** (3), 631-641.

Mansouri, S. & Radhouane, L. (2015). Is Stomatal Density an Ideal Tool to Explain Drought

Effect on Photosynthesis in Tunisian Barley Varieties? *Journal of Chemical, Biological and Physical Sciences*.

Mares, D. J. & Mrva, K. (2014). Wheat grain preharvest sprouting and late maturity alpha-amylase. *Planta,* **240** (6), 1167-1178.

Maydup, M. L., Antonietta, M., Guiamet, J., Graciano, C., López, J. R. & Tambussi, E. A. (2010). The contribution of ear photosynthesis to grain filling in bread wheat (Triticum aestivum L.). *Field Crops Research,* **119** (1), 48-58.

McDonald, J. H. (2009). *Handbook of biological statistics.* Sparky House Publishing Baltimore, MD.

Niglas, A., Alber, M., Suur, K., Jasińska, A. K., Kupper, P. & Sellin, A. (2015). Does increased air humidity affect stomatal morphology and functioning in hybrid aspen? *Botany,* **93** (4), 243-250.

Oskabe, Y., Oskabe, K. & Shinozaki, K. T. (2014). LSP: Response of plants to water stress. *Frontiers in Plant Science*.

Pallas, J. E., Michel, B. E. & Harris, D. G. (1967). Photosynthesis, transpiration, leaf temperature, and stomatal activity of cotton plants under varying water potentials. *Plant Physiology,* **42** (1), 76-88.

Palta, J. A., Kobata, T., Turner, N. C. & Fillery, I. R. (1994). Remobilization of carbon and nitrogen in wheat as influenced by postanthesis water deficits. *Crop Science,* **34** (1), 118-124.

Papakosta, D. K. & Gagianas, A. (1991). Nitrogen and dry matter accumulation, remobilization, and losses for Mediterranean wheat during grain filling. *Agronomy Journal,* **83** (5), 864-870.

Passioura, J. (1983). Roots and drought resistance. *Agricultural water management,* **7** (1-3), 265-280.

Petersen, R. G. (1994). *Agricultural field experiments: design and analysis.* CRC Press.

Rakita, S. M., Torbica, A. M., Dokić, L. P., Tomić, J. M., Pojić, M. M., Hadnađev, M. S. & Hadnađev-Dapčević, T. R. (2015). Alpha-amylase activity in wheat flour and breadmaking properties in relation to different climatic conditions. *Food and Feed Research,* **42** (2), 91-99.

Ramulu, U. S. (1998). *Management of water resources in agriculture.* New Age International.

Rebetzke, G., Bonnett, D. & Reynolds, M. (2016). Awns reduce grain number to increase grain size and harvestable yield in irrigated and rainfed spring wheat. *Journal of experimental botany,* **67** (9), 2573-2586.

Reddy, L., Ching, T. & Metzger, R. (1984). Alpha-amylase activity in wheat kernels matured and germinated under different temperature conditions. *Cereal chemistry*.

Roberts, M., Reiss, M. J. & Monger, G. (2000). *Advanced biology.* Nelson Thornes.

Roberts, M. B. V. (1986). *Biology: A functional approach.* Nelson Thornes.

Saini, H. & Aspinall, D. (1982). Abnormal sporogenesis in wheat (Triticum aestivum L.) induced by short periods of high temperature. *Annals of Botany,* **49** (6), 835-846.

Schmitz, O. J. (2000). Combining field experiments and individual-based modeling to identify the dynamically relevant organizational scale in a field system. *Oikos,* **89** (3), 471-484.

Simpson, R. J., Lambers, H. & Dalling, M. J. (1983). Nitrogen redistribution during grain growth in wheat (Triticum aestivum L.): IV. Development of a quantitative model of the translocation of nitrogen to the grain. *Plant Physiology,* **71** (1), 7-14.

Spiertz, J., Hamer, R., Xu, H., Primo-Martin, C., Don, C. & Van Der Putten, P. (2006). Heat stress in wheat (Triticum aestivum L.): Effects on grain growth and quality traits. *European Journal of Agronomy,* **25** (2), 89-95.

Stone, P. & Nicolas, M. (1998). Comparison of sudden heat stress with gradual exposure to high temperature during grain filling in two wheat varieties differing in heat tolerance. II. Fractional protein accumulation. *Functional Plant Biology,* **25** (1), 1-11.

Tahir, I. & Nakata, N. (2005). Remobilization of nitrogen and carbohydrate from stems of bread wheat in response to heat stress during grain filling. *Journal of Agronomy and Crop Science,* **191** (2), 106-115.

Teare, I., Law, A. & Simmons, G. (1972). Stomatal frequency and distribution on the inflorescence of Triticum aestivum. *Canadian Journal of Plant Science,* **52** (1), 89-94.

Thomason, W. E., Hughes, K., Griffey, C. A., Parrish, D. J. & Barbeau, W. (2009). Understanding pre-harvest sprouting of wheat.

Van Oort, P., Saito, K., Zwart, S. & Shrestha, S. (2014). A simple model for simulating heat induced sterility in rice as a function of flowering time and transpirational cooling. *Field Crops Research,* **156**, 303-312.

Wang, Y., Hu, W., Zhang, X., Li, L., Kang, G., Feng, W., Zhu, Y., Wang, C. & Guo, T. (2014). Effects of cultivation patterns on winter wheat root growth parameters and grain yield. *Field Crops Research,* **156**, 208-218.

Weerakoon, W., Maruyama, A. & Ohba, K. (2008). Impact of Humidity on Temperature-Induced Grain Sterility in Rice (Oryza sativa L). *Journal of Agronomy and Crop Science,* **194** (2), 135-140.

Weyers, J. D. & Paterson, N. W. (2001). Plant hormones and the control of physiological processes. *New Phytologist,* **152** (3), 375-407.

Xu, Z. & Zhou, G. (2008). Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. *Journal of experimental botany,* **59** (12), 3317-3325.

Yan, C., Ding, Y., Wang, Q., Liu, Z., Li, G., Muhammad, I. & Wang, S. (2010). The impact of relative humidity, genotypes and fertilizer application rates on panicle, leaf temperature, fertility and seed setting of rice. *The Journal of Agricultural Science,* **148** (3), 329-339.

Zhang, X., Chen, S., Sun, H., Wang, Y. & Shao, L. (2009). Root size, distribution and soil water depletion as affected by cultivars and environmental factors. *Field Crops Research,* **114** (1), 75-83.

ZHANG, Y.-f., HUANG, X.-w., WANG, L.-l., Liu, W., WU, Z.-h., YOU, M.-s. & LI, B.-y. (2014). Proteomic analysis of wheat seed in response to drought stress. *Journal of Integrative Agriculture,* **13** (5), 919-925.

Chapter 7: Genotypic variability enhances the reproducibility of an ecological study

During the initial stages of my Ph.D. I have taken part in a multi-laboratory Experiment titled: `Do we need to introduce controlled systematic variation in ecological microcosm experiments to improve their reproducibility? Following this Experiment, a paper called `Genotypic variability enhances the reproducibility of an ecological study` presenting the results of the microcosm experiments in 14 European laboratories was published in Nature Ecology & Evolution. The paper is shown in the following pages.

I performed one of the repetitions of this experiment in the Harborne laboratory, University of Reading. The components I carried out under the supervision of Dr Martin Lukac are:

- Vernalisation of the seeds
- Setting up the pots with applying desired soil and sand patches
- Transplanting seedlings to the pots
- Ensuring the proposed environmental conditions
- Watering the pots
- Keeping the pots at proposed field capacity
- Randomising the pots within the blocks
- Measuring the height of the grasses (Brachypodium distachion)
- Inoculation of the pots having legume (Medicago truncatula)
- Measuring dry weight of shot biomass, root biomass and total biomass per species per pot
- Grinding the shot and seeds of the grasses (Brachypodium distachion) fro N conten analysis
- Examining the number and/or weight of legume nodules per pot (Medicago truncatula)
- Measuring evatotranspiration per pot by measuring weight changes
- Using tea bag index to measure litter decomposition

Genotypic variability enhances the reproducibility of an ecological study

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7.1: Abstract

Many scientific disciplines are currently experiencing a 'reproducibility crisis' because numerous scientific findings cannot be repeated consistently. A novel but controversial hypothesis postulates that stringent levels of environmental and biotic standardization in experimental studies reduce reproducibility by amplifying the impacts of laboratory-specific environmental factors not accounted for in study designs. A corollary to this hypothesis is that a deliberate introduction of controlled systematic variability (CSV) in experimental designs may lead to increased reproducibility. To test this hypothesis, we had 14 European laboratories run a simple microcosm experiment using grass (*Brachypodium distachyon* L.) monocultures and grass and legume (*Medicago truncatula* Gaertn.) mixtures. Each laboratory introduced environmental and genotypic CSV within and among replicated microcosms established in either growth chambers (with stringent control of environmental conditions) or glasshouses (with more variable environmental conditions). The introduction of genotypic CSV led to 18% lower among-laboratory variability in growth chambers, indicating increased reproducibility, but had no significant effect in glasshouses where reproducibility was generally lower. Environmental CSV had little effect on reproducibility. Although there are multiple causes for the 'reproducibility crisis', deliberately including genetic variability may be a simple solution for increasing the reproducibility of ecological studies performed under stringently controlled environmental conditions.

7.2: Introduction

Reproducibility—the ability to duplicate a study and its findings—is a defining feature of scientific research. In ecology, it is often argued that it is virtually impossible to accurately duplicate any single ecological experiment or observational study. The rationale is that the complex ecological interactions between the ever-changing

environment and the extraordinary diversity of biological systems exhibiting a wide range of plastic responses at different levels of biological organization make exact duplication unfeasible[1,2]. Although this may be true for observational and field studies, numerous ecological (and agronomic) studies are carried out with artificially assembled simplified ecosystems and controlled environmental conditions in experimental microcosms or mesocosms (henceforth, 'microcosms')[3–5]. Since biotic and environmental parameters can be tightly controlled in microcosms, the results from such studies should be easier to reproduce. Even though microcosms have frequently been used to address fundamental ecological questions[4,6,7], there has been no quantitative assessment of the reproducibility of any microcosm experiment.

Experimental standardization—the implementation of strictly defined and controlled properties of organisms and their environment—is widely thought to increase both the reproducibility and sensitivity of statistical tests[8,9] because it reduces within-treatment variability. This paradigm has recently been challenged by several studies on animal behaviour, suggesting that stringent standardization may, counterintuitively, be responsible for generating nonreproducible results[9–11] and contribute to the actual reproducibility crisis[12–15]; the results may be valid under given conditions (that is, they are local 'truths'), but are not generalizable[8,16]. Despite rigorous adherence to experimental protocols, laboratories inherently vary in many conditions that are not measured and are thus unaccounted for, such as experimenter, micro-scale environmental heterogeneity, physico-chemical properties of reagents and laboratory-ware, pre-experimental conditioning of organisms, and their genetic and epigenetic background. It has even been suggested that attempts to stringently control all sources of biological and environmental variability might inadvertently lead to amplification of the effects of these unmeasured variations among laboratories, thus reducing reproducibility[9–11].

Some studies have gone even further, hypothesizing that the introduction of controlled systematic variability (CSV) among the replicates of a treatment (for example, using different genotypes or varying the organisms' pre-experimental conditions among the experimental replicates) should lead to less variable mean response values between the laboratories that duplicate the experiments[9,11]. In short, it has been argued that reproducibility may be improved by shifting the variance from among experiments to within them[9]. If true, introducing CSV will increase researchers' ability to draw generalizable conclusions about the directions and effect sizes of experimental treatments and reduce the probability of false positives. The trade-off inherent to this approach is that increasing within-experiment variability will reduce the sensitivity (that

is, the probability of detecting true positives) of statistical tests. However, it currently remains unclear whether introducing CSV increases the reproducibility of ecological microcosm experiments and, if so, at what cost for the sensitivity of statistical tests.

To test the hypothesis that introducing CSV enhances reproducibility in an ecological context, we had 14 European laboratories simultaneously run a simple microcosm experiment using grass (*Brachypodium distachyon* L.) monocultures and grass and legume (*Medicago truncatula* Gaertn.) mixtures. As part of the reproducibility experiment, the 14 laboratories independently tested the hypothesis that the presence of the legume species *M. truncatula* in mixtures would lead to higher total plant productivity in the microcosms and enhanced growth of the non-legume *B*. *distachyon* via rhizobia-mediated nitrogen fertilization and/or nitrogen-sparing effects[17–19].

All laboratories were provided with the same experimental protocol, seed stock from the same batch and identical containers in which to establish microcosms with grass only and grass–legume mixtures. Alongside a control with no CSV and containing a homogenized soil substrate (a mixture of soil and sand) and a single genotype of each plant species, we explored the effects of five different types of within- and among-microcosm CSV on experimental reproducibility of the legume effect (Fig. 1): (1) within-microcosm environmental CSV (ENV_W) achieved by spatially varying soil resource distribution through the introduction of six sand patches into the soil; (2) among-microcosm environmental CSV (ENV_A), which varied the number of sand patches (none, three or six) among replicate microcosms; (3) within-microcosm genotypic CSV (GEN_W), which used three distinct genotypes per species planted in homogenized soil in each microcosm; (4) among-microcosm genotypic CSV (GEN_A), which varied the number of genotypes (one, two or three) planted in homogenized soil among replicate microcosms; and (5) both genotypic and environmental CSV (GEN_W +ENV_W) within microcosms, which used six sand patches and three plant genotypes per species in each microcosm. In addition, we tested whether CSV effects are modified by the level of standardization within laboratories by using two common experimental approaches (‘setups’ hereafter): growth chambers with tightly controlled environmental conditions and identical soil (eight laboratories) or glasshouses with more loosely controlled environmental conditions and different soils (six laboratories; see Supplementary Table 1 for the physico-chemical properties of the soils).

We measured 12 parameters representing a typical ensemble of response variables reported for plant-soil microcosm experiments. Six of these were measured at the microcosm level (shoot biomass, root biomass, total biomass, shoot-to-root ratio, evapotranspiration and decomposition of a common substrate using a simplified

version of the 'tea bag litter decomposition method'[20]). The other six were measured on *B. distachyon* alone (seed biomass, height and four shoot-tissue chemical variables: N%, C%, δ^{15} N and δ^{13} C). All 12 variables were used to calculate the effect of the presence of a nitrogen-fixing legume on ecosystem functions in grass–legume mixtures ('net legume effect' hereafter) (Supplementary Table 2), calculated as the difference between the values measured in the microcosms with and without legumes—an approach often used in grass–legume binary cropping systems[19,21] and biodiversity–ecosystem function experiments[17,22].

Statistically significant differences among the 14 laboratories were considered an indication of irreproducibility. In the first instance, we assessed how our experimental treatments (CSV and setup) affected the number of laboratories that produced results that could be considered to have reproduced the same finding. We then determined how experimental treatments affected the s.d. of the legume effect for each of the 12 variables both within and among laboratories (lower among-laboratory s.d. implies that the results were more similar, suggesting increased reproducibility). Finally, we explored the relationship between within- and among-laboratory s.d. and how the experimental treatments affected the statistical power of detecting the net legume effect.

7.3: Results

Although each laboratory followed the same experimental protocol, we found a remarkably high level of among-laboratory variation for most response variables (Supplementary Fig. 1) and the net legume effect on those variables (Fig. 2). For example, the net legume effect on mean total plant biomass varied among laboratories from 1.31 to 6.72 g dry weight per microcosm in growth chambers, suggesting that unmeasured laboratory-specific conditions outweighed the effects of experimental standardization. Among glasshouses, the differences were even larger: the net legume effect on mean plant biomass varied by two orders of magnitude from 0.14 to 14.57 g dry weight per microcosm (Fig. 2). Furthermore, for half of the variables (root biomass, litter decomposition, grass height, foliar C%, δ^{15}C and δ^{15}N), the direction of the net legume effect varied with the laboratory.

Mixed-effects models were used to test the effect of legume species presence, laboratory, CSV and their interactions (with experimental block—within-laboratory growth chamber or glasshouse bench—as a random factor) on the 12 response variables. The impact of the presence of legumes varied significantly with laboratory and CSV for half of the variables, as indicated by the legume ×laboratory ×CSV

threeway interaction (Table 1 and Supplementary Figs. 2 and 3). For the other half, significant two-way interactions between legume ×laboratory and CSV ×laboratory were found. The same significant interactions were found when analysing the first (PC1) and second (PC2)

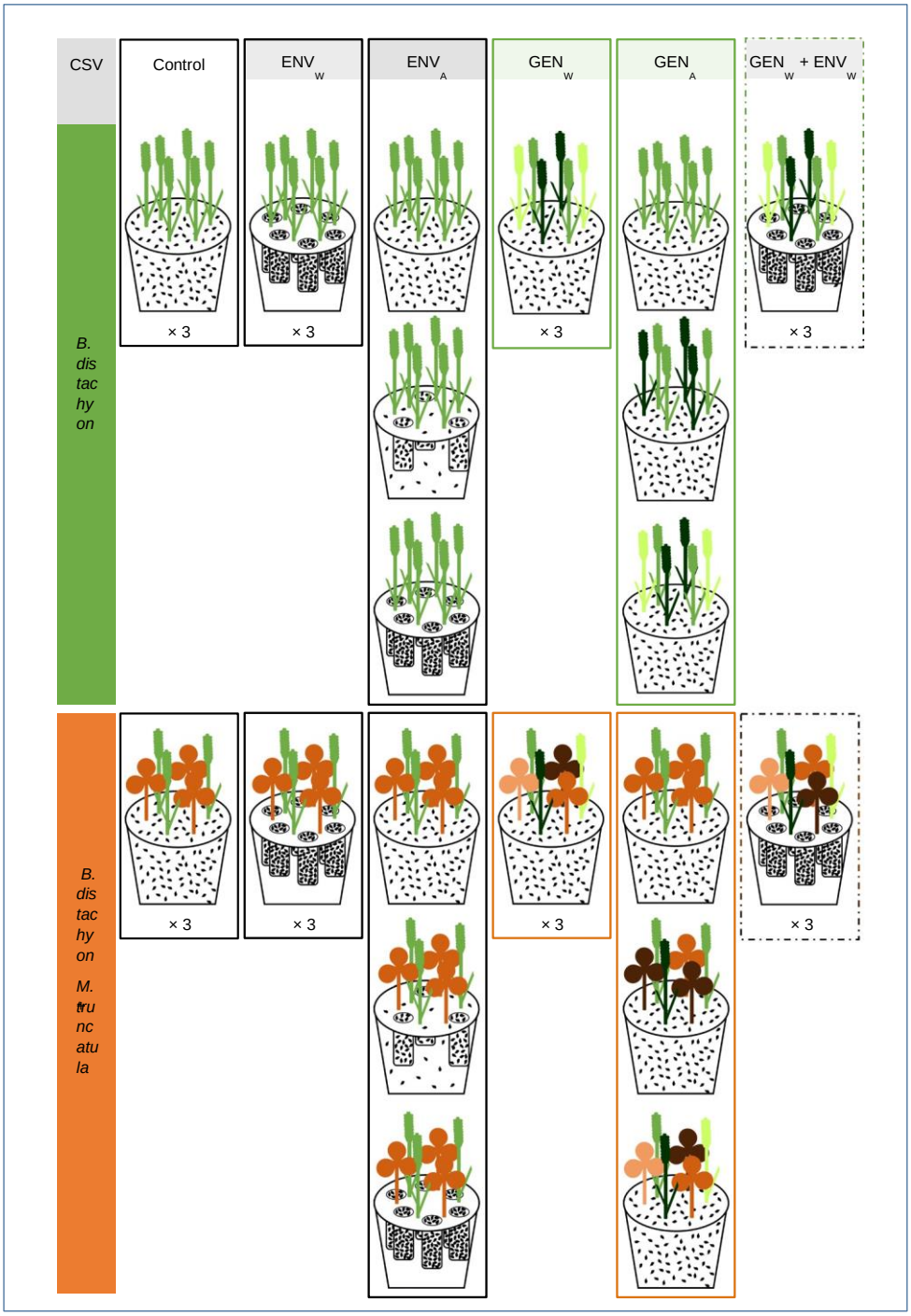
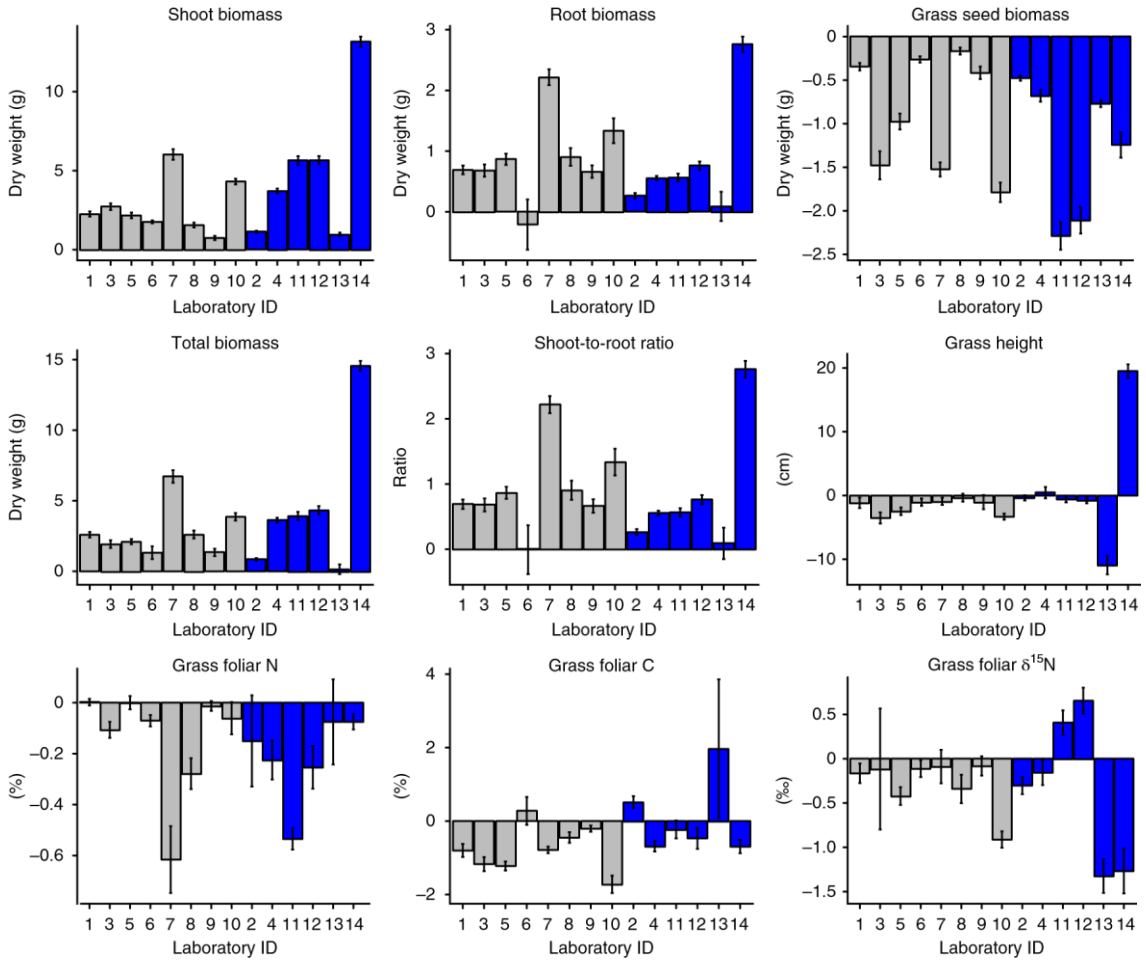


Fig. 1 | Experimental design of one block. Grass monocultures of *Brachypodium distachyon* (genotypes Bd21, Bd21-3 and Bd3-1 represented by green shades) and grass–legume mixtures with the legume *M. truncatula* (genotypes L000738, L000530 and L000174 represented by orange-brown shades) were established in 14 laboratories. Combinations of these distinct genotypes were used to establish genotypic

CSV. Plants were established in a substrate with equal proportions of sand (black spots) and soil (white), with the sand being either mixed with the soil or concentrated in sand patches to induce environmental controlled systematic variability (CSV). As indicated, for some treatments, the same genotypic and sand composition was repeated in three microcosms per block. The spatial arrangement of the microcosms in each block was re-randomized every two weeks. For the growth chamber setups, the blocks represent two distinct chambers, whereas for glasshouse setups they represent two distinct growth benches in the same glasshouse.

principal components from a principal component analysis that included all 12 response variables. PC1 and PC2 together explained 45% of the variation (Table 1 and Supplementary Fig. 4a, b). Taken together, these results suggest that the effect size or direction of the net legume effect was significantly different (that is, not reproducible) in some laboratories and that the introduced CSV treatment affected reproducibility. In a complementary analysis including the setup in the model (and accounting for the laboratory effect as a random factor), we found that the impact of the CSV treatment varied significantly with the setup (CSV ×setup or legume ×CSV ×setup interactions; Supplementary Table 3), suggesting that the reproducibility of the results differed between glasshouses and growth chambers.



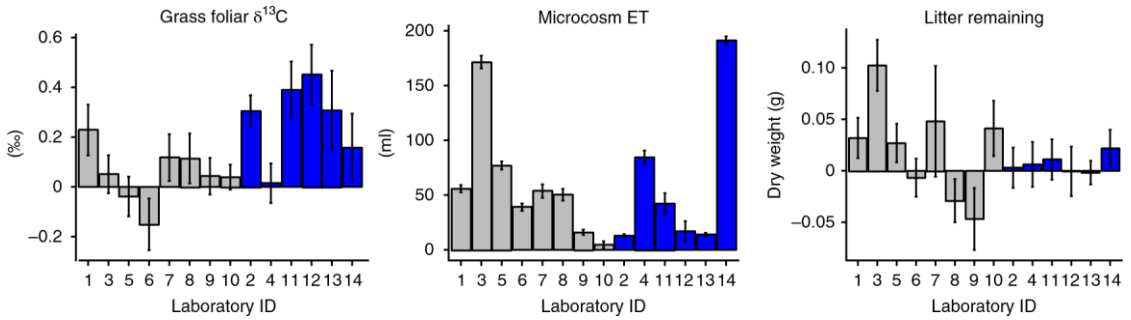


Fig. 2 | Net legume effect for the 12 response variables in 14 laboratories as affected by laboratory and setup (growth chamber versus glasshouse) treatments. The grey and blue bars represent laboratories that used growth chamber and glasshouse setups, respectively. ET, evapotranspiration. Bars show means by laboratory obtained by averaging over all CSV treatments, with error bars indicating ± 1 s.e.m. (n = 72 microcosms per laboratory).

To answer the question of how many laboratories produced results that were statistically indistinguishable from one another (that is, reproduced the same finding), we used Tukey's post-hoc honest significant difference test for the laboratory effect on PC1 and PC2 describing the net legume effect, which together explained 49% of the variation (Supplementary Fig. 4c,d). Of the 14 laboratories, 7 (PC1) and 11 (PC2) were statistically indistinguishable in controls. This value increased in the treatments with environmental or genotypic CSV for PC1 but not PC2 (Table 2). When we analysed the responses in growth chambers alone, five of eight laboratories were statistically indistinguishable in controls, but this increased to six laboratories when we considered treatments with only environmental CSV and seven in treatments with genotypic CSV (GEN_W, GEN_A and GEN_W +ENV_W). In glasshouses, introducing CSV did not affect the number of statistically indistinguishable laboratories with respect to PC1, but decreased the number of statistically indistinguishable laboratories with respect to PC2 (Table 2).

We also assessed the impact of the experimental treatments on the among- and within-laboratory s.d. Analysis of the among-laboratory s.d. of the net legume effect revealed a significant CSV ×setup interaction ($F_{5,121}$ =7.38, P <0.001; Fig. 3a, b). This interaction included significantly lower fitted coefficients (that is, lower amonglaboratory s.d.) in growth chambers for GEN_W ($t_{5,121}$ =− 3.37, P =0.001), GEN_A ($t_{5,121}$ =− 2.95, P =0.004) and ENV_W +GEN_W treatments ($t_{1,121}$ =− 3.73, P <0.001) relative to the control (see full model output for among-laboratory s.d. in the Supplementary Note). For these three treatments, the among-laboratory s.d. of the net legume effect was 18% lower with genotypic CSV than without it, indicating increased reproducibility (Fig. 3a). The same analysis performed on within-laboratory s.d. of the net legume effect only

Table 2 | impact of experimental treatments on the number of laboratories that reproduced the same finding

Source	All (*n* = 14)		Glasshouses (*n* = 8)		Growth chambers (*n* = 6)	
	PC1	PC2	PC1	PC2	PC1	PC2
Control	7	11	3	5	5	5
ENV_W	10	9	3	3	6	6
ENV_A	8	8	3	4	6	6
GEN_W	8	10	3	3	6	7
GEN_A	11	10	3	3	7	8
ENV_W + GEN_W	11	10	4	3	7	7

Numbers represent the total number of statistically indistinguishable laboratories based on a Tukey's post-hoc honest significant difference test of PC1 and PC2 of the net legume effect of the 12 response variables (see Supplementary Fig. 4c,d for the principal component analysis results).

For a detailed description of experimental treatments and abbreviations, see Fig. 1.

found a slight but significant increase of within-laboratory s.d. in the GEN_A treatment ($t_{5,121}$ =3.52, P <0.001) (see model output for within-laboratory s.d. in the Supplementary Note). We then tested whether there was a relationship between within- and among-laboratory s.d. with a statistical model for among-laboratory s.d. as a function of within-laboratory s.d., setup, CSV and their interactions. We found a significant within-laboratory s.d. ×setup ×CSV threeway interaction ($F_{5,109}$ =2.4, P <0.040) affecting among-laboratory s.d. (Supplementary Note). This interaction was the result of a more negative relationship between within- and among-laboratory s.d. in glasshouses relative to growth chambers, but with different slopes for the different CSV treatments (Fig. 4).

Introducing CSV can increase within-laboratory variation, as indicated by the positive coefficients fitted in some of the CSV treatments (see model output for within-laboratory s.d. in the Supplementary Note). Thus, for the three CSV treatments

Table 1 | impact of experimental treatments on response variables

	DF	Shoot biomass[a] (*n* = 1,005)	Root biomass (*n* = 989)	Seed biomass (*n* = 997)	Total biomass (*n* = 976)	Shoot/root (*n* = 987)	Grass height[a] (*n* = 1,008)	Shoot N%[a] (*n* = 1,008)
Legume	1	4602.95****	1131.65****	2186.64****	690.73****	1137.01****	3.33*	449.87****
CSV	5	15.57****	23.93****	58.01****	1.78 (NS)	23.98****	23.36****	0.78 (NS)
Laboratory	13	1088.67****	182.53****	364.57****	1251.96****	183.42****	317.33****	335.18****
Legume × CSV	5	23.64****	4.48****	33.62****	3.49***	4.51****	2.62**	1.34 (NS)
Legume × laboratory	13	235.99****	40.58****	78.17****	116.63****	40.38****	49.89****	14.12****
CSV × laboratory	65	6.55****	3.15****	6.93****	7.33****	3.17****	10.16****	1.98****
Legume × laboratory × CSV	65	2.22****	1.12 (NS)	2.70****	1.18 (NS)	1.12 (NS)	1.45**	1.71****

	DF	Shoot C%[a] (*n* = 1,008)	Shoot $\delta^{15}N$[a] (*n* = 963)	Shoot $\delta^{13}C$[a] (*n* = 973)	Evapotranspiration (*n* = 1,002)	Litter (*n* = 974)	PC1 (*n* = 1,008)	PC2 (*n* = 1,008)
Legume	1	110.67****	14.43****	26.62****	1269.93****	1.81 (NS)	1242.53****	988.88****
CSV	5	0.16 (NS)	8.85****	75.73****	9.37****	1.05 (NS)	12.87****	22.56****
Laboratory	13	174.50****	258.30****	888.42****	748.66****	117.34****	920.65****	513.83****
Legume × CSV	5	2.55**	6.48****	5.15****	1.24 (NS)	1.77 (NS)	7.08****	11.79****
Legume × laboratory	13	11.90****	16.78****	2.52***	172.74****	2.05**	118.12****	28.22****
CSV × laboratory	65	1.67***	4.39****	4.97****	21.69****	2.97****	7.22****	2.76****
Legume × laboratory × CSV	65	1.33**	1.84****	1.23 (NS)	1.53***	1.17 (NS)	0.93 (NS)	1.65***

Mixed-effects model outputs summarizing the *F* and *P* values (as asterisks) for the impacts of the presence of legumes, CSV and laboratory on the 12 response variables. We also present the impact of experimental treatments on PC1 and PC2 of all 12 response variables.[a]Response variables measured for the grass *B. distachyon* only. The rest of the variables were measured at the microcosm level; that is, including the contribution of both the legume and the grass species.****$P < 0.001$; ***$P < 0.01$; **$P < 0.05$; *$P < 0.1$; DF, degrees of freedom; NS, not significant ($P > 0.1$).

that produced the most consistent results (GEN_W, GEN_A and ENV_W +GEN_W), we analysed the statistical power of detecting the net legume effect within individual laboratories. In growth chambers, adding genotypic CSV led to a slight reduction in statistical power relative to the control (57% in the control versus 46% in the three treatments containing genotypic variability) that could have been compensated for by using 11 instead of 6 replicated microcosms per treatment. In glasshouses, owing to a higher effect size of legume presence on the response variables, the statistical power for detecting the legume effect in the control was slightly higher (68%) than in growth chambers, but was reduced to 51% on average for the three treatments containing

genotypic CSV—a decrease that could have been compensated for by using 16 replicated microcosms instead of 6.

7.4: Discussion

Overall, our study shows that results produced by microcosm experiments can be strongly biased by laboratory-specific factors. Based on the PC explaining most of the variation in the 12 response variables (PC1), only 7 of the 14 laboratories produced results that can be considered reproducible (Table 2) with the current standardization procedures. This result is in line with ref. [12], which reports that out of ten laboratories, only four generated similar leaf growth phenotypes of *Arabidopsis thaliana* (L.). In addition to highlighting that approximately one in two ecological studies performed in microcosms under controlled environments produce statistically different results, our study provides supporting evidence for the hypothesis that introducing genotypic CSV can increase the reproducibility of ecological studies[9–11]. However, the effectiveness of genotypic CSV for enhancing reproducibility varied with the setup; that is, it led to lower (−18 %) among-laboratory s.d. in growth chambers only, with no benefit observed in glasshouses. Lower among-laboratory s.d. in growth chambers implies that the microcosms containing genotypic CSV were less strongly affected by unaccounted-for laboratoryspecific environmental or biotic variables. Analyses performed at the level of individual variables (Table 1) showed that introducing genotypic CSV affected

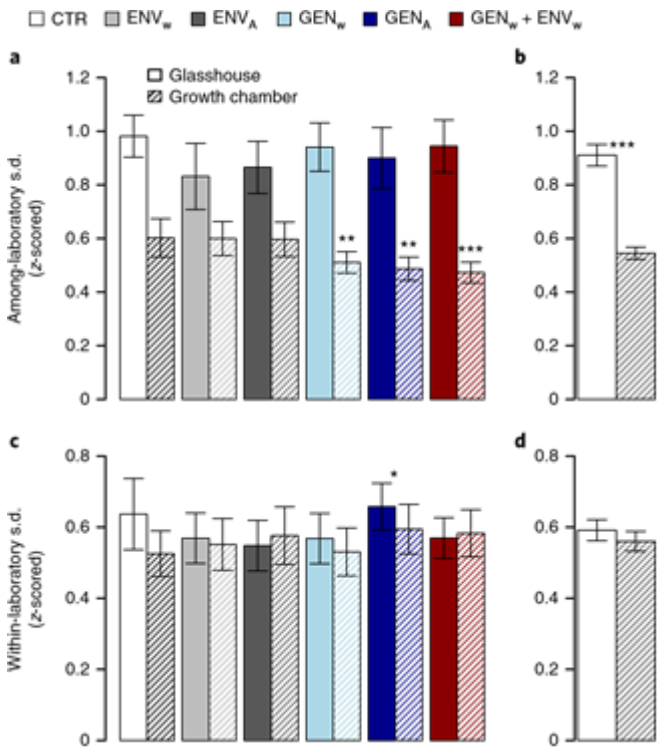


Fig. 3 | Among- and within-laboratory s.d. of the net legume effect as affected by experimental treatments. a,**b**, Among-laboratory s.d. as affected by CSV and setup (**a**) and setup only (**b**). **c**,**d**, Within-laboratory s.d. as affected by CSV and setup (**c**) and setup only (**d**). Lower amonglaboratory s.d. indicates enhanced reproducibility. Solid and striped bars represent glasshouse (n = 6) and growth chamber setups (n = 8), respectively. P values (***P < 0.001, **P < 0.01 and *P < 0.05) indicate significantly different fitted coefficients according to the mixed-effects models (see Supplementary Note for full model outputs). The asterisk in **c** indicates the significant difference between GEN_A and the control, irrespective of the type of setup.

the among-laboratory s.d. in most, but not all variables. This suggests that the relationship between genotypic CSV and reproducibility is probabilistic and results from the decreased likelihood that microcosms containing CSV will respond to unaccounted-for laboratory-specific environmental factors in the same direction and with the same magnitude. The mechanism is likely to be analogous to the stabilizing effect of biodiversity on ecosystem functions under changing environmental conditions[23–26], but additional empirical evidence is needed to confirm this conjecture.

Introducing genotypic CSV increased reproducibility in growth chambers (with stringent control of environmental conditions), but not in glasshouses (with more variable environmental conditions). Higher among-laboratory s.d. in glasshouses may indicate the existence therein of stronger laboratory-specific factors and our deliberate use of different soils in the glasshouses presumably contributed to this effect. However, the among-laboratory s.d. in glasshouses decreased with increasing within-laboratory s.d., irrespective of CSV—an effect that was less clear in growth chambers (Fig. 4). This observation appears to be in line with the hypothesis put forward in ref. [9], where it was proposed that increasing the variance within experiments can reduce the among-laboratory variability of the mean effect sizes observed in each laboratory. Yet, despite the negative correlation between within- and amonglaboratory s.d. observed in glasshouses, the among-laboratory s.d. remained higher in glasshouses than growth chambers. Therefore, we consider that the hypothesized mechanistic link between CSV-induced higher within-laboratory s.d. and increased reproducibility is poorly supported by our dataset. Nevertheless, one possible explanation for the lack of effect on reproducibility in glasshouses is that our CSV treatments did not introduce a sufficiently high level of within-laboratory variability to buffer against laboratory-specific factors for all response variables; across the 12 response variables, the average main effect (that is, without the interaction terms) of the CSV treatment contributed to a low percentage (2.6% ±1.6 s.e.m.) of the total sum of squares relative to the main effects of laboratory (43.4% ±5.2 s.e.m.) and legumes (10.9% ±3.1 s.e.m.). A similar conjecture was put forward by the other two studies that explored the role of CSV for

reproducibility in animal behaviour[9,10]. At present, we are unable to conclude that the introduction of stronger sources of controlled within-laboratory variability can increase reproducibility in glasshouses with more loosely controlled environmental conditions and different soils.

Our results indicate that genotypic CSV is more effective at increasing reproducibility than environmental CSV, irrespective of whether the CSV is introduced within or among individual replicates (that is, microcosms). However, we cannot discount the possibility that we found this result because our treatments with environmental CSV were less successful in increasing within microcosm variability. Additional experiments could test whether other types of environmental CSV, such as soil nutrients, texture or water availability, might be more effective at increasing reproducibility.

We expected higher overall productivity (that is, a net legume effect) in the grass–legume mixtures and enhanced growth of *B. distachyon* because of the presence of the nitrogen-fixing *M. truncatula*. However, these species were not selected because of their routine pairings in agronomic or ecological experiments (they are rarely used that way), but rather because they are frequently present in controlled environment experiments looking at functional genomics. In contrast with our expectation and despite the generally lower ^{15}N signature of *B. distachyon* in the presence of nitrogen-fixing *M. truncatula* (suggesting that some of the nitrogen fixed by *M. truncatula* was taken up by the grass), the biomass of *B. distachyon* was lower in the microcosms containing *M. truncatula*. The seed mass and shoot N% data of *B. distachyon* were lower in mixtures (Supplementary Fig. 1), suggesting that the two-species competed for nitrogen. The lack of a significant nitrogen fertilization effect of *M. truncatula* on *B. distachyon* could have resulted from the asynchronous phenologies of the two species: the eight- to ten-week life cycle of *B. distachyon* may have been too short to benefit from the nitrogen fixation by *M. truncatula*.

Because well-established meta-analytical approaches can account for variation caused by local factors and still detect the general trends across different types of experimental setup, environment and population, we should ask whether the additional effort required for introducing CSV in experiments is worthwhile. Considering the current reproducibility crisis in many fields of science[27], we suggest that it is, for at least three reasons. First, some studies become seminal without any attempts to reproduce them. Second, even if a seminal study that is flawed due to laboratory-specific biases is later proven wrong, it usually takes significant time and resources before its impact on the field abates. Third, the current rate of reproducibility is estimated to be as low as one-third[12–14], implying that most data entering any meta-

analysis are biased by unknown laboratoryspecific factors. The addition of genotypic CSV may enhance the reproducibility of individual experiments and eliminate potential biases in the data used in meta-analyses. Additionally, if each individual study was less affected by laboratory-specific unknown environmental and biotic factors, we would also need fewer studies to draw solid conclusions about the generality of phenomena. Therefore, we argue that investing more in making individual studies more reproducible and generalizable will be beneficial in both the short and long term. At the same time, adding CSV can reduce the statistical power to detect experimental effects, so some additional experimental replicates would be needed when using it.

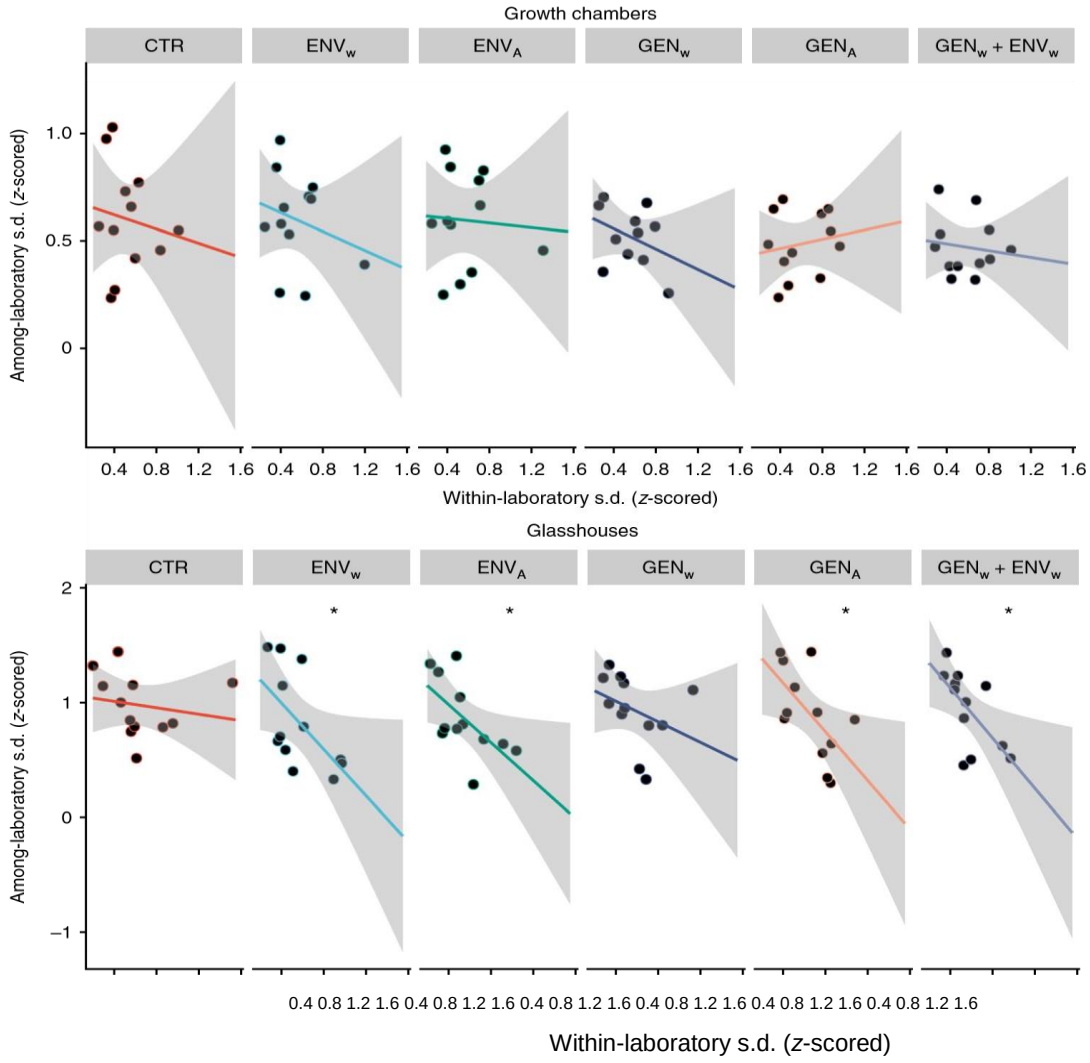


Fig. 4 | Relationship between within-laboratory s.d. and among-laboratory s.d. of the net legume effect as affected by experimental treatments. Significant within-laboratory s.d. × setup × CSV three-way interaction ($F_{5,109} = 2.4$, $P < 0.040$) affecting among-laboratory s.d. (Supplementary Note). This interaction is the result of a more negative relationship between within- and among-laboratory s.d. in glasshouses relative to growth chambers, but with different slopes for the different CSV treatments. Points represent the 12 response variables. Asterisks represent P values < 0.05 for the individual linear regressions. Note the different scale for the y axis between growth chambers and glasshouses.

Arguably, our use of statistical significance tests of effect sizes to determine reproducibility might be viewed as overly restrictive and better suited to assessing the reproducibility of parameter estimates rather than the generality of the hypothesis under test[27]. We used this approach because no generally accepted alternative framework is available to assess how close the multivariate results from multiple laboratories need to be to conclude that they reproduced the same finding. It is worth noting that although the direction of the legume effect was the same in the majority of laboratories, the differences among laboratories were very large (for example, up to two orders of magnitude for shoot biomass) and in 10% of the 168 laboratory ×variable

combinations (14 laboratories ×12 response variables) the direction of the legume effect differed from the among-laboratory consensus (Fig. 2).

7.5: Conclusion

Our study shows that the current standardization procedures used in ecological microcosm experiments are inadequate in accounting for laboratory-specific environmental factors and suggests that introducing controlled variability in experiments may buffer some of the effects of laboratory-specific factors. Although there are multiple causes for the reproducibility crisis[15,28,29], deliberately including genetic variability in the studied organisms may turn out to be a simple solution for increasing the reproducibility of ecological studies performed in controlled environments. However, as the introduced genotypic variability only increased reproducibility in experimental setups with tightly controlled environmental conditions (that is, in growth chambers using identical soil), our study indicates that the reproducibility of ecological experiments may be enhanced by a combination of rigorous standardization of environmental variables at the laboratory level as well as controlled genotypic variability.

7.6: Methods

All laboratories tried, to the best of their abilities, to carry out identical experimental protocols. While not all laboratories managed to precisely recreate all of the details of the experimental protocol, we considered this to be a realistic scenario under which ecological experiments using microcosms are performed in glasshouses and growth chambers.

Germination. The seeds from three genotypes of *B. distachyon* (Bd21, Bd21-3 and Bd3-1) and *M. truncatula* (L000738, L000530 and L000174) were first sterilized by soaking 100 seeds in 100 ml of a sodium hypochlorite solution with 2.6% active chlorine, which was stirred for 15 min using a magnet. Thereafter, the seeds were rinsed three times in 250 ml of sterile water for 10–20 s under shaking. Sterilized seeds were germinated in trays (10 cm deep) filled with vermiculite. The trays were kept at 4 °C in the dark for 3 days before being moved to light conditions (300 µ mol m^{-2} s^{-1} photosynthetically active radiation) and 20°C and 60% relative air humidity during the day and 16 °C and 70% relative air humidity at night. When the seedlings of both species reached 1 cm in height above the vermiculite, they were transplanted into the microcosms.

Preparation of microcosms. All laboratories used identical containers (2 l volume, 14.8 cm diameter and 17.4 cm height). Sand patches were created using custom-made identical 'patch makers' consisting of six rigid polyvinyl chloride tubes (2.5 cm in diameter and 25 cm long) arranged in a circular pattern with an outer diameter of 10 cm. A textile mesh was placed at the bottom of the containers to prevent the spilling of soil through drainage holes. The filling of microcosms containing sand patches started with the insertion of the empty tubes into the containers. Thereafter, in growth chambers, 2,000 g dry weight of soil, subtracting the weight of the sand patches, was added to the containers and around the 'patch maker' tubes. Because different soils were used in the glasshouses, the dry weight of the soil differed depending on the soil density and was first estimated individually in each laboratory as the amount of soil needed to fill the pots up to 2 cm from the top. After the soil was added to the containers, the tubes were filled with a mixture of 10% soil and 90% sand. When the microcosms did not contain sand patches, the amount of sand otherwise contained in the six patches was homogenized with the soil. During the filling of the microcosms, a common substrate for measuring litter decomposition was inserted at the centre of the microcosm at 8 cm depth. For simplicity, as well as for its fast decomposition rate, we used a single batch of commercially available tetrahedron-shaped synthetic tea bags (mesh size of 0.25 mm) containing 2 g of green tea (Lipton; Unilever), as proposed by the 'tea bag index' method[20]. Once filled, the microcosms were watered until water could be seen pouring out of the pot. The seedlings were then manually transplanted to pre-determined positions (Fig. 1), depending on the genotype and treatment. Each laboratory established two blocks of 36 microcosms, resulting in a total of 72 microcosms per laboratory, with blocks representing two distinct chambers in the growth chamber setups or two distinct growth benches in the same glasshouse.

Soils. All laboratories using growth chamber setups used the same soil, whereas the laboratories using glasshouses used different soils (see Supplementary Table 1 for the physico-chemical properties of the soils). The soil used in growth chambers was classified as a nutrient-poor cambisol and was collected from the top layer (0–20 cm) of a natural meadow at the Centre de Recherche en Ecologie Expérimentale et Prédictive (Saint-Pierre-lès-Nemours, France). Soils used in glasshouses originated from different locations. The soil used by laboratory 2

was a fluvisol collected from the top layer (0–40 cm) of a quarry site near Avignon in the Rhône valley, Southern France. The soil used by laboratory 4 was collected from near the La Cage field experimental system (Versailles, France) and was classified

as a luvisol. The soil used by laboratories 11 and 12 was collected from the top layer (0–20 cm) within the haugh of the river Dreisam in the East of Freiburg, Germany. This soil was classified as an umbric gleysol with high organic carbon content. The soil used by laboratory 14 was classified as a eutric fluvisol and was collected on the field site of the Jena Experiment, Germany. Before the establishment of microcosms, all soils were air-dried at room temperature for several weeks and sieved using a 2 mm mesh sieve. A common inoculum was provided to all laboratories to ensure that rhizobia specific to *M. truncatula* were present in all soils.

Abiotic environmental conditions. The set points for environmental conditions were 16 h light (at 300 μ mol m^{-2} s^{-1} photosynthetically active radiation) and 8 h dark, at 20 °C and in 60% relative air humidity during the day and 16 °C and 70% relative air humidity at night. Different soils (for glasshouses) and treatments with sand patches likely affected water drainage and evapotranspiration. The watering protocol was thus based on dry weight relative to weight at full water-holding capacity (WHC). The WHC was estimated based on the weight difference between the dry weight of the containers and the wet weight of the containers 24 h after abundant watering (until water was flowing out of the drainage holes in the bottom of each container). Soil moisture was maintained between 60 and 80% of WHC (that is, the containers were watered when the soil water dropped below 60% of WHC and water was added to reach 80% of WHC) during the first 3 weeks after seedling transplantation and between 50 and 70% of WHC for the rest of the experiment. Microcosms were watered twice a week with estimated WHC values from two microcosms per treatment. To ensure that the patch/heterogeneity treatments did not become a water availability treatment, all containers were weighed and brought to 70 or 80% of WHC every 2 weeks. This operation was synchronized with within-block randomization. All 14 experiments were performed between October 2014 and March 2015.

Sampling and analytical procedures. After 80 days, all plants were harvested. Plant shoots were cut at the soil surface, separated by species and dried at 60 °C for 3 days. Roots and any remaining litter in the tea bags were washed out of the soil using a 1 mm mesh sieve and dried at 60 °C for 3 days. The microcosm evapotranspiration rate was measured before the harvesting as the difference in weight changes from 70% of WHC after 48 h. Shoot C%, N%, δ^{13} C and δ^{15} N were measured on pooled shoot biomass (including seeds) of *B. distachyon* and analysed at the Göttingen Centre for Isotope Research and Analysis using a coupled system consisting of an Elemental

Analyzer (NA 1500; Carlo-Erba) and a gas isotope mass spectrometer (Finnigan MAT 251; Thermo Electron Corporation).

Data analysis and statistics. All analyses were done using R version 3.2.4 (ref. [30]). Before data analyses, each laboratory was screened individually for outliers. Values that were lower or higher than 1.5 ×interquartile range[31] within each laboratory, and representing less than 1.7% of the whole dataset, were considered to be outliers due to measurement errors or typos. These values were removed and subsequently treated as missing values. We then assessed whether the impact of the presence of legume varied with laboratory and the treatment of CSV. This was tested individually for each response variable (Table 1) with a mixed-effects model using the 'nlme' package[32]. Following the guidelines suggested by ref. [33], we first identified the most appropriate random structure using a restricted maximum likelihood approach and then selected the random structure with the lowest Akaike information criterion. For this model, CSV and laboratory were included as fixed factors, as well as experimental block as a random factor and a 'varIdent' weighting function to correct for heteroscedasticity resulting from more heteroscedastic data at the laboratory and legume level (R syntax: 'model =lme (response variable ~ legume*CSV*laboratory, random =~1|block, weights =varIdent (form =~1|laboratory*legume)') (Table 2). As the laboratory and setup experimental factors were not fully crossed (that is, laboratories performed the experiment only in one type of setup), the two experimental variables could not be included simultaneously as fixed effects. Therefore, to test for the setup effect, we used an additional complementary model including CSV and setup as fixed effects and laboratory as a random factor (R syntax: 'model =lme (response variable ~ legume*CSV*setup, random =~1|laboratory/ block, weights =varIdent (form =~1|laboratory*legume)') (Supplementary Table 3). To test whether the results were affected by the collinearity among the response variables, the two models were also run on PC1 and PC2 of the 12 response variables (Fig. 4a,b). PCs were estimated using the 'FactoMineR' package[34], with missing values replaced using a regularized iterative multiple correspondence analysis[35] in the 'missMDA' package[36]. The same methodology was used to compute a second principal component analysis derived from the net legume effect on the 12 response variables (Supplementary Fig. 3c,d). To assess how many laboratories produced results that were statistically indistinguishable from one another, we applied Tukey's post-hoc honest significant difference test in the 'multcomp' package to laboratory-specific estimates of PC1 and PC2 (Table 2).

To assess how the CSV treatments affected the among- and within-laboratory variability, we used the s.d. instead of the coefficient of variation, because the net

legume effect contained both positive and negative values. To calculate among- and within-laboratory s.d., we centred and scaled the raw values using the *z*-score normalization (*z*-scored variable =(raw value – mean)/s.d.) individually for each of the 12 response variables. Among-laboratory s.d. was computed from the mean of the laboratory *z*-scores for each response variable, CSV and setup treatment (*n* =144; 6 CSV levels ×2 setup levels ×12 response variables). Within-laboratory s.d. was computed from the values measured in the six replicated microcosms for each CSV and setup treatment combination, individually for each response variable, resulting in a dataset with the same structure as that for among-laboratory s.d. (*n* =144; 6 CSV levels ×2 setup levels ×12 response variables). Some of the 12 response variables were intrinsically correlated, but most had correlation coefficients <0.5 (Supplementary Fig. 5) and were therefore treated as independent variables. To analyse and visualize the relationships between the s.d. calculated from variables with different units, before the calculation of the among- and within-laboratory s.d., the raw values of the 12 response variables were centred and scaled.

The impact of experimental treatments on among- and within-laboratory s.d. was analysed using mixed-effects models following the same procedure described for the individual response variables. The model with the lowest Akaike information criterion included a random slope for the setup within each response variable, as well as a 'varIdent' weighting function to correct for heteroscedasticity at the variable level (R syntax: 'model =lme (s.d. ~ CSV*setup, random =~setup|variable, weights =varIdent (form =~1|variable)') (see also Supplementary Note). The relationship between within- and among-laboratory s.d. was also tested with a model with similar random structure but with amonglaboratory s.d. as a dependent variable and within-laboratory s.d., CSV and setup as predictors.

Because the treatments containing genotypic CSV increased reproducibility in growth chambers but slightly increased within-laboratory s.d., we also examined the effect of adding CSV on the statistical power for detecting the net legume effect in each individual laboratory. This analysis was done with the 'power.anova.test' function in the 'base' package. We computed the statistical power of detecting a significant net legume effect (if one had used a one-way analysis of variance for the legume treatment) for the control, GEN_W, GEN_A and ENV_W +GEN_W treatments for each laboratory and response variable. This allowed us to calculate the average statistical power for the aforementioned treatments and how many additional replicates would have been needed to achieve the same statistical power as we had in the control.

Life sciences reporting summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. The data that support the findings of this study are publicly available at https://doi.pangaea.de/10.1594/PANGAEA.880980.

7.7: References

1. Cassey, P. & Blackburn, T. Reproducibility and repeatability in ecology. *Bioscience* **56**, 958–959 (2006).

2. Ellison, A. M. Repeatability and transparency in ecological research. *Ecology* **91**, 2536–2539 (2010).

3. Lawton, J. H. The Ecotron facility at Silwood Park: the value of 'big bottle' experiments. *Ecology* **77**, 665–669 (1996).

4. Benton, T. G., Solan, M., Travis, J. M. & Sait, S. M. Microcosm experiments can inform global ecological problems. *Trends Ecol. Evol.* **22**, 516–521 (2007).

5. Drake, J. M. & Kramer, A. M. Mechanistic analogy: how microcosms explain nature. *Theor. Ecol.* **5**, 433–444 (2012).

6. Fraser, L. H. & Keddy, P. The role of experimental microcosms in ecological research. *Trends Ecol. Evol.* **12**, 478–481 (1997).

7. Srivastava, D. S. et al. Are natural microcosms useful model systems for ecology? *Trends Ecol. Evol.* **19**, 379–384 (2004).

8. De Boeck, H. J. et al. Global change experiments: challenges and opportunities. *BioScience* **65**, 922–931 (2015).

9. Richter, S. H. et al. Effect of population heterogenization on the reproducibility of mouse behavior: a multi-laboratory study. *PLoS ONE* **6**, e16461 (2011).

10. Richter, S. H., Garner, J. P. & Würbel, H. Environmental standardization: cure or cause of poor reproducibility in animal experiments? *Nat. Methods* **6**, 257–261 (2009).

11. Richter, S. H., Garner, J. P., Auer, C., Kunert, J. & Würbel, H. Systematic variation improves reproducibility of animal experiments. *Nat. Methods* **7**, 167–168 (2010).

12. Massonnet, C. et al Probing the reproducibility of leaf growth and molecular phenotypes: a comparison of three *Arabidopsis* accessions cultivated in ten laboratories. *Plant Physiol.* **152**, 2142–2157 (2010).

13. Begley, C. G. & Ellis, M. L. Raise standards for preclinical cancer research. *Nature* **483**, 531–533 (2012).

14. Open Science Collaboration Estimating the reproducibility of psychological science. *Science* **349**, aac4716 (2015).

15. Parker, T. H. et al. Transparency in ecology and evolution: real problems, real solutions. *Trends Ecol. Evol.* **31**, 711–719 (2016).

16. Moore, R. P. & Robinson, W. D. Artificial bird nests, external validity, and bias in ecological field studies. *Ecology* **85**, 1562–1567 (2004).

17. Temperton, V. M., Mwangi, P. N., Scherer-Lorenzen, M., Schmid, B. & Buchmann, N. Positive interactions between nitrogen-fixing legumes and four different neighbouring species in a biodiversity experiment. *Oecologia* **151**, 190–205 (2007).

18. Meng, L. et al. Arbuscular mycorrhizal fungi and rhizobium facilitate nitrogen uptake and transfer in soybean/maize intercropping system. *Front. Plant Sci.* **6**, 339 (2015).

19. Sleugh, B., Moore, K. J., George, J. R. & Brummer, E. C. Binary legume–grass mixtures improve forage yield, quality, and seasonal distribution. *Agron. J.* **92**, 24–29 (2000).

20. Keuskamp, J. A., Dingemans, B. J. J., Lehtinen, T., Sarneel, J. M. & Hefting, M. M.Tea bag index: a novel approach to collect uniform decomposition data across ecosystems. *Methods Ecol. Evol.* **4**, 1070–1075 (2013).

21. Nyfeler, D., Huguenin-Elie, O., Suter, M., Frossard, E. & Lüscher, A. Grass–legume mixtures can yield more nitrogen than legume pure stands due to mutual stimulation of nitrogen uptake from symbiotic and non-symbiotic sources. *Agric. Ecosyst. Environ.* **140**, 155–163 (2011).

22. Suter, M. et al Nitrogen yield advantage from grass–legume mixtures is robust over a wide range of legume proportions and environmental conditions. *Glob. Change Biol.* **21**, 2424–2438 (2015).

23. Loreau, M. & de Mazancourt, C. Biodiversity and ecosystem stability: a synthesis of underlying mechanisms. *Ecol. Lett.* **16**, 106–115 (2013).

24. Reusch, T. B., Ehlers, A., Hämmerli, A. & Worm, B. Ecosystem recovery after climatic extremes enhanced by genotypic diversity. *Proc. Natl Acad. Sci. USA* **102**, 2826–2831 (2005).

25. Hughes, A. R., Inouye, B. D., Johnson, M. T. J., Underwood, N. & Vellend, M. Ecological consequences of genetic diversity. *Ecol. Lett.* **11**, 609–623 (2008).

26. Prieto, I. et al. Complementary effects of species and genetic diversity on productivity and stability of sown grasslands. *Nat. Plants* **1**, 1–5 (2015).

27. Wasserstein, R. L. & Lazar, N. A. The ASA's statement on *P*-values: context, process, and purpose. *Am. Stat.* **70**, 129–133 (2016).

28. Baker, M. 1,500 scientists lift the lid on reproducibility. *Nature* **533**, 452–454 (2016).

29. Nuzzo, R. How scientists fool themselves—and how they can stop. *Nature* **526**, 182–185 (2015).

30. R Development Core Team *R: A Language and Environment for Statistical Computing* (R Foundation for Statistical Computing, Vienna, 2017).

31. Tukey, J. W. *Exploratory Data Analysis* (Addison–Wesley, Reading, USA, 1977).

32. Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & R Core Team. *nlme: Linear and Nonlinear Mixed-Effects Models* R Package Version 3.1-122 (The R Foundation, 2016); http://CRAN.R-project.org/package= nlme

33. Zuur, A. F., Ieno, E. N., Walker, N., Saveliev, A. A. & Smith, G. M. *Mixed-Effects Models and Extensions in Ecology with R* (Springer, New York, 2009).

34. Lê, S., Josse, J. & Husson, F. FactoMineR: an R package for multivariate analysis. *J. Stat. Softw.* **25**, 1–18 (2008).

35. Josse, J., Chavent, M., Liquet, B. & Husson, F. Handling missing values with regularized iterative multiple correspondence analysis. *J. Classif.* **29**, 91–116 (2010).

36. Josse, J. & Husson, F. missMDA: a package for handling missing values in multivariate data analysis. *J. Stat. Softw.* **70**, 1–31 (2016).

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Author contributions

A.M. and J.R. designed the study with input from M. Blouin, S.B., M. Bonkowski and

J.-C.L. Substantial methodological contributions were provided by S.S., T.G., L.R. and M.S.-L. Conceptual feedback on an early version was provided by G.T.F., N.E., J.R. and A.M.E. Data were analysed by A.M. with input from A.M.E. A.M. wrote the manuscript with input from all authors. All authors were involved in carrying out the experiments and/or analyses.

Competing interests

The authors declare no competing financial interests.

Additional information

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7.8: Chapter 7 – Appendix

Appendix 7.1: Pots having grass *Brachypodium distachyon* L. and the legume *Medicago truncatula Gaertn.*